

Identification of a sex-determining region in Nile tilapia (*Oreochromis niloticus*) using bulked segregant analysis

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Summary

Sex determination in the Nile tilapia (*Oreochromis niloticus*) is thought to be an XX-XY (male heterogametic) system controlled by a major gene. We searched for DNA markers linked to this major locus using bulked segregant analysis. Ten microsatellite markers belonging to linkage group 8 were found to be linked to phenotypic sex. The putative Y-chromosome alleles correctly predict the sex of 95% of male and female individuals in two families. Our results suggest a major sex-determining locus within a few centimorgans of markers UNH995 and UNH104. A third family from the same population showed no evidence for linkage of this region with phenotypic sex, indicating that additional genetic and/or environmental factors regulate sex determination in some families. These markers have immediate utility for studying the strength of different Y chromosome alleles, and for identifying broodstock carrying one or more copies of the Y haplotype.

Keywords aquaculture, *Oreochromis*, sex chromosomes, sex linkage, tilapia.

Unwanted spawning by tilapia in aquaculture ponds results in overcrowding, and eventually the harvest of stunted and unmarketable fish. Commercial production of tilapia therefore relies on the stocking of all-male fingerlings. Beginning with the work of Hickling (1960), a variety of methods have been used to produce unisex fingerlings, including interspecific hybridization (Hulata *et al.* 1983), hormone treatment (Phelps & Popma 2000) and YY supermales (Mair *et al.* 1997). These methods are not entirely reliable, in part because of their technical complexity, but also because the sex of tilapia is affected by high temperatures (Baroillier *et al.* 1995; Abucay *et al.* 1999) and may also be influenced by additional genes (Mair *et al.* 1991; Hussain *et al.* 1994; Sarder *et al.* 1999; I. Karayucel, M.T. Ezaz, S. Karayucel, B.J. McAndrew & D.J. Penman in preparation), which can cause deviations from the sex ratios predicted by simple gonosomal models.

Breeding of hormonally sex-reversed fish has demonstrated a primarily male-heterogametic (XY) system of sex determination in *Oreochromis niloticus*. Crosses of sex-reversed XX males with normal XX females produce progenies which are nearly 100% female (Jalabert *et al.* 1974;

Calhoun & Shelton 1983). Gynogenesis has also been used to study sex determination: if female *O. niloticus* are homogametic (XX), then their gynogenetic offspring should all be female. Experimental results generally support this (Penman *et al.* 1987), but some studies have found a small percentage of males in the meigynote offspring of some females (Mair *et al.* 1991; Hussain *et al.* 1994; Muller-Belecke & Horstgen-Schwark 1995; Sarder *et al.* 1999).


The sex chromosomes of tilapia are apparently still at an early stage of differentiation. There are no gross morphological differences in any chromosome pair that would identify the X and Y chromosomes (Kornfield 1984; Majumdar & McAndrew 1986; Crosetti *et al.* 1988). Carrasco *et al.* (1999) visualized the synaptonemal complex and observed an incompletely paired segment in the longest bivalent, which they suggested could be the sex-determining region. Harvey *et al.* (2002) developed DOP-PCR paint probes from microdissected chromosomes and showed a quantitative difference in hybridization between XX, XY and YY genotypes. To this point, however, no other genetic markers for the major sex determining locus in *O. niloticus* have been described. We have recently constructed a linkage map for tilapia which contains more than 550 microsatellite markers (unpublished data). Here we use these markers to identify a major sex-determining locus in *O. niloticus*.

Three families of tilapia (*O. niloticus*) produced at the University of Stirling were used for this study. After sexing at Stirling, fin-clips from each fish were sent to the University of New Hampshire for genotyping. Sample sizes

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for each family were: Family 2 (12 females and 14 males), Family 5 (23 females and 23 males), and Family 7 (25 females and 22 males). The DNA was extracted from each fin-clip using a standard phenol/chloroform method (Kocher *et al.* 1989).

One hundred and five microsatellite markers were selected at intervals of approximately 20 cM, based on a linkage map produced from an F₂ population from the interspecies cross of *O. aureus* with *O. niloticus* (Lee *et al.* unpublished data). Those markers consisted of 102 microsatellite markers, and three genes (*CLCN5*, *RAS-GRF2*, *blue-opsin*). To accelerate the identification of sex-linked markers, we used bulked segregant analysis (Michelmore *et al.* 1991). For each family, we made separate pools of male and female DNA. Before pooling, the concentration of each DNA was quantified using DyNA Quant2000 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and each DNA was diluted to a final concentration of ~10 ng/μl. For Family 2, all 12 female and 14 male DNAs were used to construct the pools. For Family 5, 15 males and 15 females were used, and for Family 7, 22 males and 23 females were pooled. Polymerase chain reaction (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 50–55 °C, 60 s at 72 °C, with a final elongation step of 5 min at 72 °C. One member of each pair of PCR primers was synthesized with a HEX, TET, or FAM fluorescent label (Operon Technologies, Alameda CA, USA). The PCR products were run on 4% gels using an ABI377 and fragment sizes were analysed using ABI GeneScan software (vers. 3.1.2).

Eighty of the 105 markers were successfully amplified from all three families. Two of these (*UNH985* and *UNH995*) showed segregation differences between males and females in Family 5 and Family 7. The 133 bp allele of *UNH985* was found at high frequency only in the male pools. This result encouraged us to repeat the genotyping on individual animals for these and other linked markers. Goodness-of-fit (G-tests) were used to assess whether there were significant differences in the genotypic distributions between males and females (Sokal & Rohlf 1981). We also used Crimap (vers. 2.4) to analyse the linkage of markers in Family 5 and Family 7. Figure 1 shows sex-specific maps of this linkage group. The female map is 32 cM longer than the male map because of greater recombination in two intervals.

The genotypic proportions in males and females, and the associated G-tests, are shown in Table 1. All of the markers on linkage group 8 (LG8) showed significant differences in genotypic frequency between males and females in Families 5 and 7. We analysed the inheritance of multilocus haplotypes in Families 5 and 7, and scored the proportion of individuals whose phenotypic sex was consistent with the hypothesized Y chromosome (Fig. 2). The graph shows a peak around 50 cM in female and 60 cM in male, which corresponds to markers *GM201*, *UNH995* and *UNH104*.

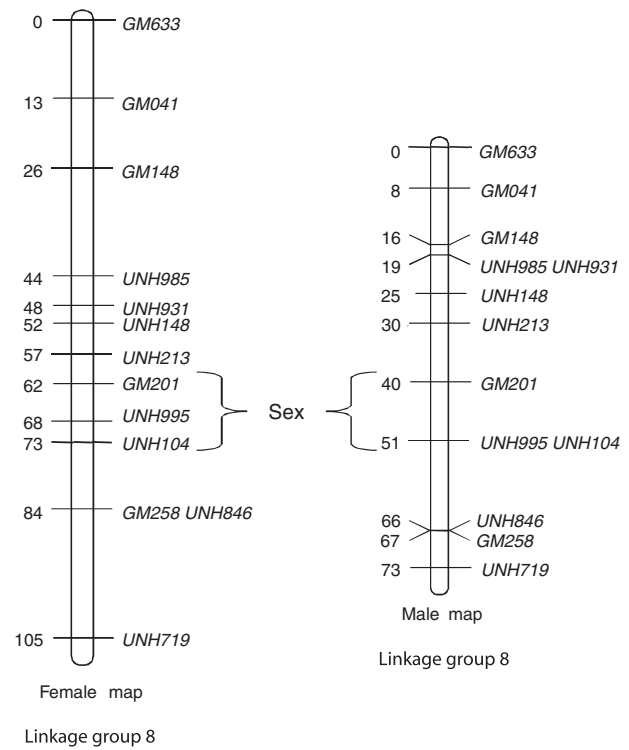


Figure 1 Sex-specific linkage maps for linkage group 8 in *Oreochromis niloticus*.

Flanking markers show a decreasing correspondence with phenotypic sex, as recombination breaks up the sex-linked haplotype. However, significant associations were seen with markers as much as 30 cM from the peak. The microsatellite genotype in this region predicted phenotypic sex with 95% accuracy in Families 5 and 7. The remaining 5% of individuals whose sex was opposite to expectation can be attributed to errors in phenotyping, recombination, or the action of additional genetic or environmental factors affecting sex determination. If genetic, these factors may be identified in larger samples where we can genotype large numbers of the exceptional individuals.

Linkage group 8 had no influence on the sex of animals in Family 2, despite the fact that all three families came from the same *O. niloticus* stock. Family 2 did not appear to be segregating for the same Y-haplotype identified in Families 5 and 7. This result suggests that additional sex-determining factors are segregating in this stock, and further reinforces the impression that sex-determining systems are highly polymorphic in this group of fishes. Complete enumeration of the genes controlling sex in *O. niloticus* will require analysis of many more families and strains from various sources under carefully controlled environmental conditions.

To our knowledge, this is the first report of DNA sequence markers linked to sex in tilapia. We detected significant differences in genotypic proportions, suggestive of a

Table 1 Genotypic proportions for sex-linked markers on linkage group 8 in male and female *Oreochromis niloticus*.

Marker	Family 2				Family 5				Family 7			
	Genotypes	Female	Male	G-test	Genotypes	Female	Male	G-test	Genotypes	Female	Male	G-test
GM633	193/195	6	8	0.13	193/193	8	4	5.90	193/202	7	2	6.86
	193/205	6	6		193/200	10	7		193/204	4	5	
					193/204	1	6		202/202	8	2	
					200/204	4	6		202/204	5	9	
GM041	252/240	7	7	0.17	252/252	17	10	4.33*	252/263	19	5	14.75***
	252/263	5	7		252/267	6	13		252/267	4	15	
GM148	186/186	5	8	0.59	190/186	14	4	17.7***	190/192	20	4	23.09***
	186/180	7	6		190/226	0	9		190/226	2	16	
UNH985	127/127	5	9	0.82	192/186	3	4	18.04***	123/134	2	17	26.17***
	127/125	5	4		192/226	3	5		123/134	2	17	
					123/134	2	10		123/156	22	4	
					156/127	8	4					
UNH931	199/199	6	7	0.16	156/134	1	7	18.58***	199/218	5	1	19.35***
	199/203	6	5		205/199	9	2		199/218	5	1	
					205/240	2	10		199/140	1	8	
					218/199	10	3		205/218	13	3	
UNH148	150/150	6	9	0.51	218/240	1	7	25.01***	205/240	2	9	21.47***
	150/152	6	5		152/150	18	3		152/152	22	5	
UNH213	201/201	5	7	0.16	152/161	3	20	32.42***	152/161	3	17	20.31***
	201/197	6	6		187/170	1	11		190/170	4	17	
GM201	189/174	4	7	0.13	187/201	10	0	45.82***	190/187	21	4	38.75***
	189/208	8	7		191/170	2	10		190/188	25	4	
UNH995	206/171	8	7	0.7	191/201	9	2	35.91***	190/190	0	17	41.38***
	206/173	4	7		190/208	22	1		165/169	24	2	
UNH104				0.36	165/173	7	0	39.36***	165/219	1	19	38.98***
	169/135	5	6		169/169	2	11		129/132	23	2	
	169/137	7	5		169/173	11	1		129/137	8	0	
					129/132	1	10		132/132	1	10	
UNH846	205/175	3	8	2.7	132/137	11	0	28.8***	185/173	5	12	7.32**
	205/197	8	5		185/185	6	0		185/175	18	7	
GM258	147/128	8	6	1.41	179/197	2	9	28.8***	113/132	19	7	7.57**
	147/132	4	8		113/113	6	0		113/132	19	7	
					113/128	1	11		113/139	6	13	
					139/113	14	3					
UNH719	124/101	8	7	0.7	139/128	2	9	15.21**	125/109	8	11	3.93*
	124/109	4	7		116/109	11	5		125/109	8	11	
					116/124	1	7		125/123	16	6	
					124/109	3	0					
			124/124	2	10							

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

Y-haplotype, for 12 microsatellite markers on LG8. The sex determining locus appears to lie near markers GM201, UNH995 and UNH104. We also have identified AFLP

markers linked to sex in the same strain of *O. niloticus* (M.T. Ezaz, S.C. Harvey, C. Boonphakdee, A.J. Teale, B.J. McAndrew & D.J. Penman in preparation), but is not yet clear

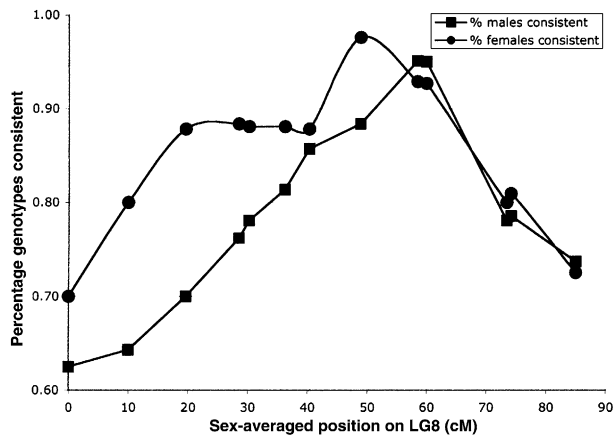


Figure 2 Proportion of males (■) and females (●) in Families 5 and 7 whose phenotypic sex is consistent with the hypothesized Y haplotype on linkage group 8.

whether these markers are linked to the microsatellite markers on LG8.

These microsatellite markers have immediate utility for tracking sex-linked haplotypes in breeding programs aimed at controlling the sex of fingerlings for commercial production. For example, they could eliminate the tedious process of progeny-testing males during the production of YY-supermales (Mair *et al.* 1997). They can also be used in experiments aimed at quantifying the strength of different Y-chromosome alleles from different individuals, strains and species.

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