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Sex-linked markers and microsatellite locus duplication in the cichlid species *Oreochromis tanganicae*

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Cichlid species of the genus *Oreochromis* vary in their genetic sex-determination systems. In this study, we used microsatellite DNA markers to characterize the sex-determination system in *Oreochromis tanganicae*. Markers on linkage group 3 were associated with phenotypic sex, with an inheritance pattern typical of a female heterogametic species (WZ–ZZ). Further, locus duplication was observed for two separate microsatellite markers on the sex chromosome. These results further advance our understanding of the rapidly evolving sex-determination systems among these closely related tilapia species.

Keywords: sex determination; sex chromosomes; tilapia

1. INTRODUCTION

The tribe Tilapiini is a group of more than 70 species (family *Cichlidae*, order Perciformes) native to Africa and the Middle East, among which are some of the most important species in African fisheries and the world aquaculture industry. Variation in the genetic mechanism of sex determination among tilapia species has been recognized for over 40 years, but the sex-determination system has been characterized in only a few species (Wohlfarth & Wedekind 1991). In a comparative study of the genetic sex determination in six tilapiine species, we found that there are two separate sex chromosomes with different sex-determination mechanisms (Cnaani *et al.* 2008). The early stages in the evolution of sex chromosomes are of great interest for developing and testing hypotheses on the chromosomal origin of sex determination and the evolution of heteromorphic sex chromosomes (Charlesworth *et al.* 2005). It is estimated that the mouthbrooding tilapias shared a common ancestor less than 5 Myr ago (Nagl *et al.* 2001). The variation among tilapia species therefore represents contemporary evolution of sex-determination systems. Study of sex chromosomes of these species may shed light on the mechanisms of sex chromosome differentiation and evolution.

2. MATERIAL AND METHODS

Oreochromis tanganicae (Gunther) is a maternal mouthbrooding tilapia species native to Lake Tanganyika. A breeding group was set up in a 200 l tank with one male and six females. After 3 days, spawning eggs were removed from the female buccal cavity and transferred to a hatching jar to complete incubation. Fry were reared to a size at which gonads could be dissected and visually identified as testis or ovary. In total, 40 fish were killed for the phenotypic characterization, 22 females and 18 males.

DNA was isolated from fin samples by the ‘salting out’ procedure (Zilberman *et al.* 2006). All individuals were genotyped with the microsatellite markers *CLCN5*, *GM139*, *GM271*, *GM354* and *UNH168*, located on tilapia linkage group 3 (LG3), and with *GM201* and *UNH104*, located on tilapia linkage group 1 (LG1). These markers were previously found to be associated with sex in several tilapia species (Cnaani *et al.* 2004, 2008; Lee *et al.* 2003, 2004). Genotyping was conducted using an ABI-377 DNA sequencer, as previously described (Cnaani *et al.* 2004; Lee *et al.* 2005).

A Pearson χ^2 analysis was conducted to test for linkage between the phenotypic sex and the genotypes of microsatellite markers. Maternally and paternally inherited alleles were analysed separately, to determine whether the mode of sex determination is male or female heterogametic. Parental haplotypes were reconstructed from offspring genotypes by examining linked alleles in adjacent markers, and assuming that the parental phase are the common haplotypes and that rare haplotypes represent recombinants (Cnaani *et al.* 2008).

3. RESULTS

Each locus was analysed separately for association with phenotypic sex (table 1). Four markers located on LG3 (*CLCN5*, *GM139*, *GM271* and *GM354*) were highly associated ($p<0.0001$) with the phenotypic sex. This analysis demonstrated that the maternally inherited alleles segregate in male and female offspring, respectively, while the paternally inherited alleles segregated randomly among the sexes. We therefore conclude that this species has a maternal heterogametic sex-determination system (WZ–ZZ). The haplotype of the female-determining chromosome (W) is marked in table 1. Our analysis also determined that, while paternal recombinants were found, there was no recombination between sex-linked markers on the maternal chromosomes (figure 1). The two markers located on LG1 (*GM201* and *UNH104*) were not associated with sex (table 1).

Out of the five analysed markers that are located on LG3, two markers had a pattern of multiple PCR products (figure 2). By analysing the inheritance pattern of these PCR products, we were able to distinguish between alleles of two different loci amplified by each of the microsatellite primer pairs. Alleles of sizes 155, 159, 161, 181 and 185 bp were observed for *GM139* (figure 2*a*) and alleles of sizes 96, 98, 104 and 110 bp were observed for *GM271* (figure 2*b*), with the inheritance pattern for this marker implying that there is also a null allele at one of its loci. The duplicated loci of these markers are marked as *GM139a* or *GM139b* and *GM271a* or *GM271b*.

Three loci on LG3 (*GM139a*, *GM271a* and *UNH168*) were not associated with sex. However, for these loci the mother was homozygous and therefore not informative.

4. DISCUSSION

In a previous study comparing six other tilapia species, we demonstrated that there are two major sex chromosome systems in tilapias; a male heterogametic system (XX–XY) on LG1 and a female heterogametic system (WZ–ZZ) on LG3 (Cnaani *et al.* 2008). Phylogenetic analysis could not determine which one of them was the ancestral sex-determination

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Table 1. Proportions of genotypes for markers on LG3 and LG1 in male and female *O. tanganicae*. The sex-linked alleles are marked in bold on the maternal genotype and characterize the W chromosome.

marker	maternal genotype	paternal genotype	offspring genotype	females	males	Pearson χ^2-test
GM139b	159/**185**	181/181	159/181	0	17	$\chi^2=36.1$
LG3			181/185	22	1	$p<10^{-4}$
GM139a	161/161	155/161	155/161	11	12	$\chi^2=1.1$
LG3			161/161	11	6	$p>0.05$
GM354	**115**/133	125/135	115/125	12	1	$\chi^2=36.3$
LG3			115/135	10	0	$p<10^{-4}$
			125/133	0	11	
			133/135	0	6	
UNH168	123/123	123/142	123/123	11	11	$\chi^2=0.5$
LG3			123/142	11	7	$p>0.05$
GM271b	104/**null**	104/null	104/_[a]	11	18	$\chi^2=12.4$
LG3			null/null	11	0	$p<10^{-3}$
GM271a	110/110	96/98	96/110	11	6	$\chi^2=1.1$
LG3			98/110	11	12	$p>0.05$
CLCN5	**205**/223	213/245	205/213	10	2	$\chi^2=26.1$
LG3			205/245	10	0	$p<10^{-4}$
			213/223	1	9	
			223/245	1	7	
GM201	182/184	184/184	182/184	12	8	$\chi^2=0.2$
LG1			184/184	10	9	$p>0.05$
UNH104	183/187	183/187	183/183	5	4	$\chi^2=0.4$
LG1			183/187	13	12	$p>0.05$
			187/187	4	2	

[a] The two genotypes, 104/104 and 104/null, could not be differentiated.

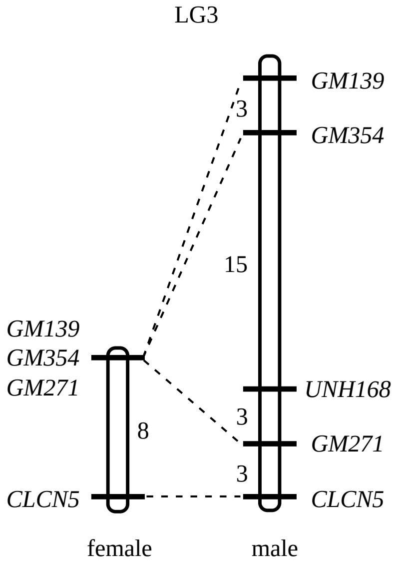

Figure 1. Sex-specific linkage map for LG3 in *O. tanganicae*.

system; however, there is evidence that suggests that LG3 is the ancestral sex chromosome, and that control of sex determination has shifted to one or more loci on LG1 several times independently (Cnaani *et al.* 2008). *Oreochromis tanganicae* is closely related to *Oreochromis mossambicus* and *Oreochromis karongae* (Nagl *et al.* 2001; Klett & Meyer 2002), and from a phylogenetic point it cannot support any hypothesis of original sex chromosome. However, this is another tilapia species having a female heterogametic sex-determination system in which LG3 has characteristics of a relatively old sex chromosome, including locus duplications that were not found in the other species.

There was a complete linkage between the duplicated loci, implying that they are the result of tandem duplications within the sex chromosome. Duplication of microsatellite loci was previously found in the tilapia diploid genome (Palti *et al.* 2001), but the association of such loci with sex determination is interesting. Models for the evolution of sex chromosomes suggest that, once an autosomal locus acquires the role of primary sex determiner, gradual changes occur on this new sex chromosome. These include recombination suppression that enables duplications and deletions of loci and the accumulation of repetitive elements (Charlesworth *et al.* 2005). Sequence analyses of nascent sex chromosomes in two other fish species, threespine stickleback (*Gasterosteus aculeatus*) and medaka (*Oryzias latipes*), demonstrated an abundance of duplications and accumulation of repetitive elements on the sex chromosome (Peichel *et al.* 2004; Kondo *et al.* 2006), and different kinds of genomic rearrangements were found in the unstable sex-determining region of the platyfish (*Xiphophorus maculatus*) (Volff & Schartl 2002). In the tilapia WZ sex chromosome (LG3), a large region of recombination suppression was detected around the sex-determination region (Cnaani *et al.* 2008) and an abundance of repetitive elements are observed on this chromosome (Martins *et al.* 2004). As in other female heterogametic tilapia species, recombination is suppressed in the sex-determining region of LG3 in *O. tanganicae*. The microsatellites locus duplications and the existence of null alleles are additional characteristics that are typical of a developing sex chromosome.

This study was carried out in compliance with the current laws governing genetic experimentation in the USA.

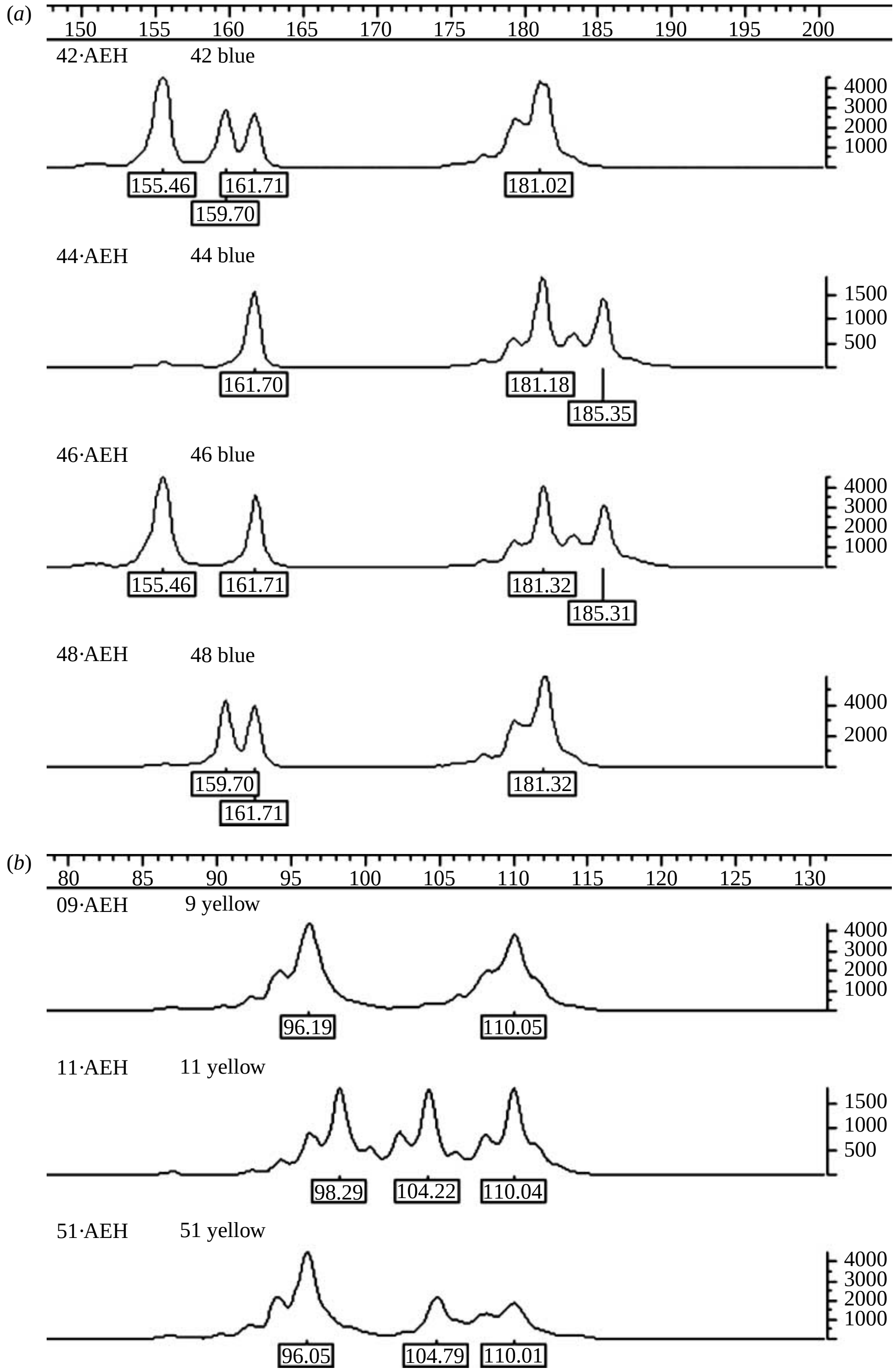

Figure 2. Loci duplication on the sex chromosome (LG3) of *O. tanganicae* as reflected in patterns of multiple PCR products. Each line represents one individual fish, genotyped for the microsatellite markers (*a*) *GM139* or (*b*) *GM271*.

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