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Amh and *Dmrta2* Genes Map to Tilapia (*Oreochromis* spp.) Linkage Group 23 Within Quantitative Trait Locus Regions for Sex Determination

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ABSTRACT

Recent studies have revealed that the major genes of the mammalian sex determination pathway are also involved in sex determination of fish. Several studies have reported QTL in various species and strains of tilapia, regions contributing to sex determination have been identified on linkage groups 1, 3, and 23. Genes contributing to sex-specific mortality have been detected on linkage groups 2, 6, and 23. To test whether the same genes might control sex determination in mammals and fishes, we mapped 11 genes that are considered putative master key regulators of sex determination: *Amh*, *Cyp19*, *Dax1*, *Dmrt2*, *Dmrta2*, *Fhl3l*, *Foxl2*, *Ixl*, *Lhx9*, *Sf1*, and *Sox8*. We identified polymorphisms in noncoding regions of these genes and genotyped these sites for 90 individuals of an F_2 mapping family. Mapping of *Dax1* joined LG16 and LG21 into a single linkage group. The *Amh* and *Dmrta2* genes were mapped to two distinct regions of LG23. The *Amh* gene was mapped 5 cM from UNH879 within a QTL region for sex determination and 2 cM from UNH216 within a QTL region for sex-specific mortality. *Dmrta2* was mapped 4 cM from UNH848 within another QTL region for sex determination. *Cyp19* was mapped to LG1 far from a previously reported QTL region for sex determination on this chromosome. Seven other candidate genes mapped to LG4, -11, -12, -14, and -17.

TELEOSTS display a variety of mechanisms for sex determination and sex differentiation (reviewed in Devlin and Nagahama 2002; Godwin *et al.* 2003). Primary sex determination in most species is genetic (Valenzuela *et al.* 2003). Nevertheless, sex differentiation of fishes is remarkably plastic and is determined by both genetic and environmental factors in many species (Baroiller *et al.* 1999; Baroiller and D'Cotta 2001). A few species even undergo sex change in response to behavioral cues (Devlin and Nagahama 2002). Sex differentiation can be modified by environmental factors (hormones, temperature, growth rate, and social environment) and should therefore be regarded as a complex "threshold trait" (Mittwoch 2006).

It is believed that this variety of primary signals regulates one or a few ancient molecular pathways of differentiation (Zarkower 2001). The major genes of the mammalian sex determination pathway, including *Sox9*, *Wt1*, *Dax1*, *Sf1*, *otCYP19*, *Wnt4*, *Dmrt1*, and *Amh*, have been detected in many vertebrate species (Schartl 2004; Rodriguez-Mari *et al.* 2005). The structure of these genes is conserved, but their regulation is variable (Yi and Zarkower 1999). *Sry* is considered the master key regulator (MKR) of sex determination in mammals, but this gene has not been found in most other vertebrates. *Sry*-related (*Sox*) genes have recently been studied in various fish species (*e.g.*, Galay-Burgos *et al.* 2004; Koopman *et al.* 2004; Hett and Ludwig 2005; Hett *et al.* 2005). *Sox* genes encode a family of transcription factors that are involved in sex determination and other developmental processes in vertebrates (Galay-Burgos *et al.* 2004).

No common MKR has been found in nonmammalian vertebrates. Instead, it appears that different MKRs are involved among closely related species and strains (Kallman 1984; Volff and Schartl 2001; von Hofsten and Olsson 2005). Gene expression studies in nonmammalian vertebrates have shown that the expression of *Dmrt1* and *Sox9* throughout the sex-determining period is testis specific (Shan *et al.* 2000; Baron *et al.* 2005; Yao and Capel 2005). Environmental temperature modifies the expression level of the *otCYP19* differently in males and females (Western and Sinclair 2001; Murdock and Wibbels 2003). It is therefore reasonable to study the effects of sex differentiation genes as candidates for the MKR of sex determination in fish species.

Although genetic factors probably regulate sex determination in most fishes, relatively few teleosts have

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karyotypically distinct sex chromosomes (Arkhipchuck 1995). Many other species have genetic sex determination, but the sex chromosomes are still in early stages of differentiation, before distinct differences in length or gene content have arisen. Both XY and WZ gonosomal systems have evolved repeatedly in various groups (Devlin and Nagahama 2002). In many species, additional autosomal loci contribute to sex determination (Kosswig 1964).

The sex chromosomes of tilapia, a group of African cichlid fishes widely used in aquaculture, are still at an early stage of differentiation. There are no gross morphological differences in any chromosome pair (Majumdar and McAndrew 1983; Kornfield 1984; Crosetti *et al.* 1988). A variety of evidence suggests that sex determination is principally monofactorial in tilapias (Wohlfarth and Wedekind 1991). The hypothesized sex-chromosome systems suggest that some species have the XX:XY system (*Oreochromis mossambicus*, *O. niloticus*) whereas other have the WZ:ZZ system (*O. aureus*, *O. macrochir*, *O. urolepis hornorum*). The primary support for these hypotheses comes from breeding animals that were sex reversed by hormone treatments, as discussed by Lee *et al.* (2004). Male heterogametic systems were suggested in *O. mossambicus* and *O. niloticus* because crosses of sex reversed (XX) males with normal (XX) females produce only females (Clemens and Inslee 1968; Jalabert *et al.* 1971). In *O. aureus*, mating between sex-reversed (ZZ) females and normal (ZZ) males usually results in 100% male offspring, but slight deviations have been observed (Hopkins *et al.* 1979; Mair *et al.* 1987; Lahav 1993; Rosenstein and Hulata 1994). These exceptions indicate that other genetic and environmental factors may also contribute to sex determination.

Several studies have identified genetic markers linked to sex determination in tilapia. Lee *et al.* (2003) identified an XY system on LG1 in a strain of *O. niloticus*. In a strain of *O. aureus*, epistatic interactions were observed between a WZ system on LG3 and the XY system on LG1 (Lee *et al.* 2004). Two distinct QTL for sex determination in tilapias were reported on LG23 in a hybrid cross between *O. aureus* and *O. mossambicus* (Cnaani *et al.* 2003, 2004). A variety of sex-determining systems was demonstrated for these closely related species. The pattern of epistasis among the alleles of the different systems identified on LG1 and LG3 is complex and leads to the interesting appearance of effects on other chromosomes (*e.g.*, LG23). QTL for sex-specific mortality were detected on LG2, -6, and -23 in an inbred line of *O. aureus* (Palti *et al.* 2002; Shirak *et al.* 2002). These last two studies reported an increase in the viability of a gynogenetic line through four successive generations of meiogynogenetic *O. aureus*. This improvement was likely the result of purging of deleterious alleles. However, significant distortions from expected Mendelian segregation were identified for three unlinked markers: UNH159 (LG2), UNH216 (LG23), and UNH231 (LG6). These authors suggested that the fixation of deleterious alleles in the inbred line occurred because they were closely linked to sex-determining loci. Alternatively, it may be that sex-specific mortality is part of the natural polygenic sex determination system in tilapia with some intra- and interloci allelic combinations that result in unviable individuals.

Dmrt1, *Dmo*, and *Wt1* were previously considered as candidates for MKRs, but their mapping position did not overlap with QTL regions for sex determination or sex-specific mortality in tilapia (Lee *et al.* 2005). Thus, we adopted the following criteria to select candidate genes for mapping: (1) MKRs could be Doublesex/Mab-3-related transcription factors (DMRTs) or their close downstream/upstream genes in the sex determination pathway (Matsuda *et al.* 2002; Koopman and Loffler 2003; Haag and Doty 2005); (2) MKRs could be orthologs of sex-determining mammalian genes, such as *Sry*, which regulates transcription of *Sox9* (McElreavey *et al.* 1993; Graves *et al.* 1998); and (3) MKRs could include aromatase and its upstream genes in the sex determination pathway (Desvages and Pieau 1992; Sarre *et al.* 2004; Gardner *et al.* 2005). Here we map 11 genes in tilapia that might be considered master key regulators of sex determination and compare their locations to previously identified QTL for sex determination.

MATERIALS AND METHODS

Mapping family: Breeding was performed in the laboratory of G. Hulata at the Volcani Center, Agricultural Research Organization, Bet Dagan, Israel. A red *O. niloticus* male derived from the University of Stirling stock (McAndrew and Majumdar 1983) was crossed with a normally colored *O. aureus* female from an Israeli strain (Lee *et al.* 2004). Fingerlings were shipped to the University of New Hampshire, where they were raised to sexual maturity (mean weight, 149 g) and DNA was extracted. The F_2 family consisted of 156 offspring of which 90 individuals were used for genotyping (Lee *et al.* 2005).

Orthologous sequences of human sex-determining genes: Sequences of 11 human proteins were BLASTN searched against the NCBI (http://www.ncbi.nlm.nih.gov), The Institute for Genome Research (TIGR; http://tigrblast.tigr.org), and RBEST (http://reprobio.nibb.ac.jp) databases to detect putative orthologs in tilapia, other cichlids, or other fish species (Table 1).

Primer design: The exon–intron boundaries in the detected sequences were predicted by their sequence comparison to the relevant *Homo sapiens*, *Mus musculus*, *Takifugu rubripes*, or *Danio rerio* genomic sequences. Primers were designed in adjacent exons using the "Primer3" program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). When initial primers did not provide sufficient quality of sequence for one or both grandparents, a second set of internally nested primers was designed on the basis of sequence information obtained using the initial primers (Table 2).

Amplification of genomic DNA in target genes: Using primers in adjacent exons, fragments of the target genes were amplified by high-fidelity BIO-X-ACT Long DNA polymerase

TABLE 1

Selected human proteins

Gene symbol	Annotation	Accession no.
Amh	Anti-Müllerian hormone	AAC25614
Dax1	Dosage-sensitive sex reversal gene 1	AAC13875
Dmrt2	Doublesex/Mab-3-related transcription factor 2	AAF86293
Dmrta2	Doublesex/Mab-3-related transcription factor family A2	CAI23011
Fhl2	Four and a half LIM domains 2	CAG33718
Foxl2	Forkhead box L2 transcription factor	AAY21823
Ixl	Intersex like	NP060062
Lhx9	LIM homeobox protein 9	CAH71761
otCYP19	Ovarian type cytochrome P450, family 19	NP_000094
Sf1	Steroidogenic factor 1	AAH38446
Sox8	SRY-box-containing gene 8	AAF37424

(Bioline, London) separated on agarose gels and stained with ethidium bromide. The desired DNA fragment was visualized with UV light and excised from the gel. DNA fragments were purified with the AccuPrep gel purification kit DNA (Bioneer, Life Science, Rockville, MD) and then sequenced on an ABI 377. The sequences were aligned and polymorphisms between the two parental species (red *O. niloticus* and *O. aureus*) were characterized.

Genotyping of microsatellite markers: For microsatellite DNA markers, forward or reverse primers were dye labeled, and genotypes were obtained by automated sizing of fluorescently tagged polymerase chain reaction amplification products. Markers that produced alleles distinguished in their length by two or more bases were amplified using SuperTherm Taq DNA polymerase (JMR Holding, London). Markers that produced alleles distinguished in their length by one base were amplified using High Fidelity Accuzyme DNA polymerase (Bioline). Electrophoretic analysis was conducted on a 4% acrylamide gel in an ABI-377 DNA sequencer as previously described (Palti *et al.* 2002). The DNA fragments were automatically sized by comparison with an internal standard using Genescan (version 2.1). Genotypes of individuals were determined by Genotyper (version 2.0) and automatically exported to a database.

Genotyping of SNP markers: Genotyping of SNP markers was performed using DNA MassArray technology (Jurinke *et al.* 2002). External and extension primers were designed using Sequanom's assay-design software (Table 2). This software also selected the appropriate termination mix for each of the SNPs. MALDI–TOF mass spectrometry analysis was performed using the MassArray genotype analyzer.

Gene mapping: Genotype data for the 11 genes in the F_2 mapping population were added to the published data set of 545 markers, and mapping was performed using JoinMap software (3.0) as previously described (Lee *et al.* 2005).

RESULTS

Identification of orthologous sequences: Complete *O. niloticus* mRNA sequences were obtained from the NCBI database for most of the analyzed genes (Table 3). The sequences identified by BLAST had *E*-values $< e^{-39}$. The *E*-value was relatively high only for *Amh*. When we repeated the search using the *Amh* of zebrafish (AAT77729), we identified two tilapia sequences (ONI06JC.39_H08 and ONI05ID.39_D09) with much lower *E*-values ($2e^{-15}$ and $9e^{-12}$, respectively). The two sequences were positioned in two adjacent exons of the tilapia *Amh*, and one of them was similar to the human *Amh*. In the RBEST database, an *Fhl2*-like cDNA was found with an *E*-value of e^{-104}. This cDNA showed a higher similarity to *Fhl3* ($E = e^{-109}$) in the NCBI database, which is another human gene of the FHL family. Hence, we defined the detected tilapia cDNA as part of the *Fhl3l* (*Fhl3*-like) gene.

Intron–exon boundaries and polymorphism: Reports for tilapia *Dmrta2* (CAI23011) and *otCYP19* (NP_000094) already included information on exon–intron borders. We predicted the exon–intron boundaries for the other nine genes (Table 4). Using *D. rerio* genomic structure, we predicted that the two nonoverlapping *O. niloticus* sequences for *Amh* are parts of the sixth and seventh exons of the same gene. To test this prediction, we designed primers in these exons and successfully amplified intron 6 and bridged these two cDNAs. Polymorphism was detected in introns in eight genes. In *Dmrt2*, *otCYP19*, and *Foxl2*, polymorphism was detected in translated regions (Table 4).

Grandparental genotypes: The genotypes of the red *O. niloticus* grandparent of the mapping family showed complete homozygosity for the 11 analyzed genes. *O. aureus* showed complete homozygosity for a different allele for each one of seven markers. The *O. aureus* grandparent was heterozygous for the remaining four markers (*Dmrta2*, *Lhx9*, *Dax1*, and *Fhl3l*). For each of these markers one allele was found to be common to both grandparents, possibly reflecting an impurity of the *O. aureus* stock.

Mapping position of the candidate genes: Genotypes were obtained for 76–89 individuals of the F_2 mapping population. Each gene was localized to a specific location on the tilapia linkage map (Table 5). The mapping of *Dax1* merged LG16 and LG21 into a single linkage group (Figure 1). The *Amh* and *Dmrta2* genes were mapped to two distinct regions in LG23. The *Amh* gene mapped 5 cM from UNH879 within a QTL region for

TABLE 2

Initial, second-round, and marker primers

Gene	Initial and second-round primers	Marker primers
Amh	F: AACTGAGTGCGTTCCAGGAG R: AGTTTTCTTTGCGCGTCGTA SRF: AGGTGACATGTGCAAGAACAG SRR: TCTGCCGACTTCAGAACTTTT	MF: AGGTGACATGTGCAAGAACAG MR: AAGCTGCAGCGGGATACTT
Dax1	F: CCGAAACAATAACAGCAGCA R: GCGACTGGATGTAGTGCAGA	MF: ACGTTGGATGGAAGTAGTTAGGTAAAGTGGC MR: ACGTTGGATGATCCTAAACTTCCACCCCTG EP: GTAAAGTGGCATGTCTGTAC
Dmrta2	F: GAGGTGTTTGGTTCCGTCAG R: CTCCGTCTTTCAAAGGCTTG	MF: ACGTTGGATGAAGGCCATCAAAAGCGCATC MR: ACGTTGGATGGTCTGTCGCTTTACTGTCTC EP: GCATCAGCACAGAATCATAAT
Dmrt2	F: GCCAAAAGCATCCTCGAA R: GTTTCCAGCTCCTTGTCTGC SRF: AATTCCAAACACACTCGATGC SRR: GAGGGAAACATCGACATCGT	MF: TGCATCAGAAGATAGACCAGTTT MR: CACAAATAAAGTTATTCAAACATGC
Fhl3l	F: CGATTGGCTCAAAGTCCTTC R: TTCTTGGCGTACAGGTTTCC	MF: TTGCACACGCTGTAAAAAGG MR: TCAAAGGTCATGTGGAAATCTG
Foxl2	F: CGCAATTTGGAGGACAGTTT R: CCTTTTTCGGTGCAGCTTAC	MF: ACGTTGGATGGCGCACACACCAAAACAAAC MR: ACGTTGGATGTTCACCGATCGAGACAGAAG EP: CACCAAAACAAACAAGTCC
Ixl	F: AGAATCGCCTCCCTGAATTT R: TGTCGATGCTCTGTGAGAGG	MF: AGAATCGCCTCCCTGAATTT MR: CTGAAGTGTCGCTGCTTTTG
Lhx9	F: GCGGTGGACAAGCAGTGG R: TAACGGGCTGACGCTGACTA	MF: ACGTTGGATGTAGCGTTTCAGTTTCACGGG MR: ACGTTGGATGACTGGGTCAGTGGATTTAGG EP: ACGGGCTTGCTCCAAA
otCYP19	F: TTTCAGCCGTTCGGTTCAG R: TTTCAGTGTTAGCAGGTTTAAATG	MF: TCTCCACATGAGGTTCTTACCC MR: TTCACATCATGCATACAGTTTGAG
Sf1	F: GCGGAAAACCAGGAGTGTAA R: AACTTGAAACCGTTGGATCG	MF: GCGGAAAACCAGGAGTGTAA MR: CAGGCAAAATGTCCCTGTTT
Sox8	F: GACTACAAGTACCAGCCTCGG R: CTGAGCTCGGAGATGTCCAC SRF: CTGGAGCAGAACTGGCTCAT SRR: CCTGCTGCTGTCAACAAGTC	MF: GGACTGGGAGGAATGAGTGA MR: CCTGCTGCTGTCAACAAGTC

F and R, initial forward and reverse primers; SRF and SRR, second-round forward and reverse primers; MF and MR, marker forward and reverse primers; EP, extension primers for SNP markers.

sex determination, and 2 cM from UNH216 within a QTL region for sex-specific mortality. *Dmrta2* was mapped 4 cM from UNH848 within a QTL region for sex determination. *Cyp19* was mapped to the start of LG1 but this region is not contained within the previously reported QTL region for sex determination. Seven other candidate genes mapped to LG4, -11, -12, -14, and -17.

DISCUSSION

In this study we focused on analysis and mapping of 11 genes for which there is a broad consensus in the literature about their role in mammalian sex determination. Due to their high level of conservation, we were able to use the sequences of human proteins in a BLAST analysis to recover the sequences of cichlid homologs for 9 of the 11 targeted genes. Primers based on sequences of more distantly related fish species such as *O. latipes*, *D. rerio*, *T. rubripes*, and *S. salar* were also successfully used to amplify specific regions in tilapia. We used comparative sequence information to predict exon–intron boundaries and design primers and to amplify parts of the tilapia *Lhx9* and *Sox8* genes, even without any preliminary sequence information from tilapia.

TABLE 3

Identifying sequences of tilapia, cichlids, and other fish species in public databases (human proteins *vs.* translated teleost sequences)

Gene	Accession nos.	Species	Database	*E*-value	Identity (%)	Similarity (%)
Amh	ONI06JC.39_H08	*O. niloticus*	RBEST	3.0*e*-06	29 (35/117)	41 (48/117)
Dax1	AY135397	*O. niloticus*	NCBI	4.0*e*-54	46 (126/269)	63 (170/269)
Dmrt2	AY149606	*O. niloticus*	NCBI	9.0*e*-46	78 (85/108)	86 (93/108)
Dmrta2	AY149605	*O. niloticus*	NCBI	2.0*e*-39	72 (78/108)	77 (84/108)
Fhl2(Fhl3l)	ONI05DB.39_H04	*O. niloticus*	RBEST	1.0*e*-104	54 (164/299)	68 (204/299)
Foxl2	AY554172	*O. niloticus*	NCBI	9.0*e*-70	62 (178/286)	67 (192/286)
Ixl	TC1294	Haplochromis	TIGR	1.1*e*-50	73 (99/135)	84 (114/135)
Lhx9	AY534647	*O. latipes*	NCBI	1.0*e*-110	90 (208/229)	95 (219/229)
	BC093258	*D. rerio*		4.0*e*-161	90 (287/317)	95 (302/317)
otCYP19	AF472620	*O. niloticus*	NCBI	2.0*e*-147	52 (243/466)	73 (341/466)
Sf1	AB060814	*O. niloticus*	NCBI	9.0*e*-133	58 (277/474)	70 (335/474)
Sox8	AY935980	*T. rubripes*	NCBI	2.0*e*-76	61 (217/352)	71 (252/352)
	DQ294028	*S. salar*		3.0*e*-66	59 (203/339)	67 (229/339)

Map positions of 2 of the 11 genes overlapped with two of the four previously reported QTL for sex determination in tilapia species and their hybrids (Cnaani *et al.* 2003, 2004; Lee *et al.* 2003, 2004). *Amh* and *Dmrta2* mapped 5 and 4 cM from UNH879 and UNH848, respectively (Figure 2). These markers were highly associated with two different QTL for sex determination on LG23 (Cnaani *et al.* 2003, 2004). Moreover, *Amh* is located 2 cM from UNH216, which marks a QTL region for sex-specific mortality (Shirak *et al.* 2002). The overlap in position on LG23 of two QTL for sex determination and sex-specific mortality may be a result of accumulation of deleterious alleles near sex-determining genes as predicted by Palti *et al.* (2002). Thus, we propose *Amh* and *Dmrta2* as candidate genes for master key regulators for sex determination in tilapia. *Amh* was differentially expressed in adult human testis germ cells among 79 tissues tested in GeneAtlas (http://symatlas.gnf.org/SymAtlas/). Likewise, *Dmrta2* had the highest expression in adult ovary but expression was also high in most other tissues. Nevertheless, it is worthwhile to explore expression for both *Amh* and *Dmrta2* during the critical stages of sex determination in tilapia at the embryo level. In mammals, *Amh* expression is induced by *Sox9* (Vidal *et al.* 2001). Knockout of *Amh* in mice suggests that normal primary male sex determination still occurs in spite of the absence of *Amh*. In birds and fish, unlike in mammals, expression of *Amh* in the undifferentiated gonads of both sexes occurs in the absence of *Sox9* (Oreal *et al.* 2002; Rodriguez-Mari *et al.* 2005). Furthermore, in birds, *Amh* is able to alter primary sex determination (Elbrecht and Smith 1992). In zebrafish, *Amh* expression peaked at 20 days postfertilization at a period when the oocytes undergo apoptosis in

TABLE 4

Accession numbers of sequenced regions, marker positions, and polymorphism

Gene	Genomic fragment used for prediction[a]	No. of exons predicted	Position of marker	Accession nos.		Polymorphism	
				O. niloticus	*O. aureus*	SNP	Microsatellites (bp)
Amh	BX005098 (DR)	7	Sixth intron	AM232733	AM232734		107/108
Dax1	CAAB01000386 (TR)	2	Intron	AM232753	AM232754	C/G	
Dmrt2	AJ295039 (TR)	3	3′-UTR of third exon	AM232741	AM232742		104/105
Dmrta2	—	2	Intron	AM232736	AM232735	A/G	
Fhl3l	BX530064 (DR)	6	Fourth intron	AM232752	AM232751		162/163
Foxl2	AC120148 (MM)	1	Exon	AM232737	AM232738	C/A	
Ixl	NC000019 (HS) NC007124 (DR)	4	Third intron	AM232748	AM232747		165/167
Lhx9	AJ277917 AJ277918 (HS)	4	Third intron	AM232745	AM232746	A/G	
OtCYP19	—	9	3′UTR of ninth exon	AM232740	AM232739		185/187
Sf1	CAAB01010380 (TR)	4	Third intron	AM232744	AM232743		169/183
Sox8	AY688943(TR)	3	Second intron	AM232749	AM232750		254/256

[a] TR, *T. rubripes*; HS, *H. sapiens*; MM, *M. musculus*; and DR, *D. rerio*.

TABLE 5

Positions of the genes on the second-generation linkage map of tilapia (Lee *et al.* 2005)

Gene	No. of F_2 genotypes	Linkage group	Mapping position (cM)	Overlap with a QTL region[a]	Comments and references
Amh	82	23	22	SD, SSM	Shirak *et al.* (2002); Cnaani *et al.* (2003)
Dax1	86	16 and 21	—	—	Connecting LG16 and LG21
Dmrt2	85	12	43	—	Located near *Dmrt1*
Dmrta2	84	23	5	SD	Cnaani *et al.* (2004)
Fhl3l	89	11	69	—	
Foxl2	86	14	17	—	
Ixl	83	14	41	—	
Lhx9	89	17	13	—	
OtCYP19	76	1	5	—	
Sf1	80	12	6	—	
Sox8	83	4	43	—	

[a] SD, sex determination; SSM, sex-specific mortality.

presumptive males, leading to the development of testes (Uchida *et al.* 2002). Furthermore, *Amh* is a strong candidate for a direct regulator of aromatase, which has been shown to inhibit aromatase biosynthesis in the rat (di Clemente *et al.* 1992). This hormone may play a central role in sex determination and the response to temperature changes (D'Cotta *et al.* 2001). In addition to *Sox9*, other Sox family genes, *e.g.*, *Sox8*, were shown to regulate *Amh* for testis differentiation in mice by interaction with the *Sox*-binding element of *Amh* promoter (Schepers *et al.* 2003). The transcriptional regulation of teleost *Amh* has so far not been elucidated, but the *Amh* gene promoter sequence contains putative binding sites for the same transcription factors that regulate mammalian *Amh* (von Hofsten and Olsson 2005). Comparison of the *O. aureus* and *O. niloticus* sequences predicts a single amino acid difference in the coding region of *Amh* between these two tilapia species. Recently, two alternatively spliced mRNA variants were reported in *O. aureus* for *Amh* (ABB69056/7), but no gender differences were noted. Our linkage results provide additional

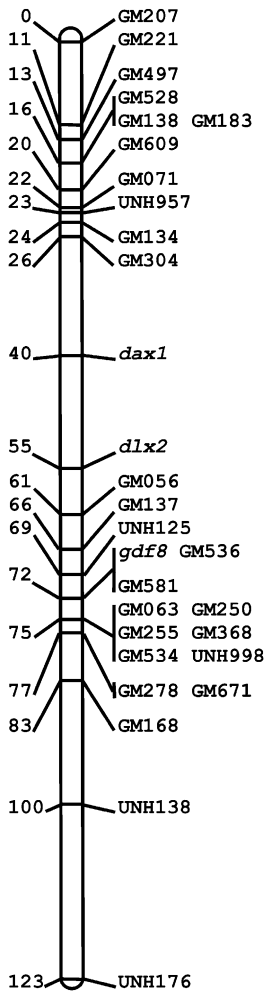

Figure 1.—The joint linkage group of LG16 and LG21.

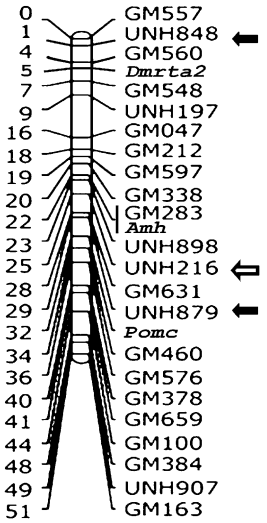

Figure 2.—The tilapia linkage group 23. Mapping positions of QTL for sex determination and sex-specific mortality are denoted by solid and open arrows, respectively.

incentive for examining the functional significance of these various forms.

The doublesex/mab3 (DM) gene family was originally described on the basis of similarities of the Drosophila (*dsx*) and *Caenorhabditis elegans* (*mab3*) homologs. Apart from the analysis of DM genes in these two species, the functional role of DM-domain-containing genes has not been extensively studied (Yi and Zarkower 1999; Lei and Heckert 2002). It was therefore a surprise to find that a DM gene was responsible for sex determination in medaka (*O. latipes*) (Matsuda *et al.* 2002; Nanda *et al.* 2002). *Dmy* is a recent duplication of the *Dmrt1* gene, which created a new sex chromosome in medaka ~10 MYA (Zhang 2004). It has been suggested that other DM genes may regulate *Dmrt1* in a dosage-dependent fashion in the sex-determining pathway (Nanda *et al.* 1999).

Guan *et al.* (2000) isolated two DM-containing sequences from tilapia gonads. These cDNAs are encoded by two different genes, *Dmrt1* and *Dmo*, which are predominantly expressed in the testis and the ovary, respectively. Moreover, they showed that *Dmrt1*, but not *Dmo*, has a *Sry* consensus site. The map positions of these genes do not overlap with any of the defined QTL regions for sex determination (Lee *et al.* 2005). *Dmrta2* and *Dmo* (*Dmrta1*) belong to the same DMRTA subfamily of *Dmrt* genes. It will be interesting to analyze *Dmrta2*-binding sites for Sry-related transcription factors and also to learn if *Dmrta2* has regulatory effects on the expression of other DM-domain genes in tilapia. Sex-specific alternative splicing of *Dmrt1* was reported in zebrafish and rice field eel (Guo *et al.* 2005; Huang *et al.* 2005). Alternatively spliced products of DM genes were also observed in human *Dmrt2* (Ottolenghi *et al.* 2000). However, we have no such information for tilapia *Dmrt* genes.

Lee *et al.* (2005) constructed a linkage map of tilapia containing 525 microsatellite and 20 type I (gene) markers. The markers were positioned into 22 large and two small linkage groups. They predicted that the two small linkage groups (8 and 24) would eventually merge with other linkage groups to correspond with the 22 chromosomal pairs of tilapia (Lee *et al.* 2005). Unexpectedly, *Dax1* merged two relatively large linkage groups, LG16 and LG21, and simultaneously extended the tilapia linkage map by 29 cM. It remains unlikely that LG1, LG3, and LG23, which contain the QTL for sex determination, will eventually coalesce into a single linkage group corresponding to a single chromosome.

A major problem in the maintenance of laboratory and commercial purebred stocks of tilapia is the high risk of hybridization and dilution of purebred stocks. The morphology of such hybrids is frequently indistinguishable from that of the parental species (Taniguchi *et al.* 1985; Macaranas *et al.* 1986; Mair and Little 1991). The high number of common alleles (36%) between the two lines studied here suggests the possibility that *O. niloticus* alleles may have introgressed into the *O. aureus* stock. Alternatively, this finding may also reflect common ancestral polymorphism for these closely related species (Agnese *et al.* 1997; Rao and Majumdar 1998).

New QTL for sex determination may emerge in hybrids due to the interactions of alleles from different species, such as those predicted by the autosomal theory (Hammerman and Avtalion 1979). The multiple QTL phenomenon may also be due to different MKRs controlling sex determination among closely related tilapia species. Although investigation of sex determination in tilapia hybrids, rather than in purebred lines, introduces significant complications (Mair *et al.* 1991), it also provides a unique model for the study of interactions between multiple QTL in the sex determination pathway.

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