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APPROACH TO THE IDENTIFICATION OF SEX-DETERMINING GENES IN THE TILAPIA GENOME BY GENETIC MAPPING AND COMPARATIVE POSITIONAL CLONING

BY

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B.S., Chungbuk National University, Korea, 1997
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DISSERTATION

Submitted to the University of New Hampshire
In Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
In
Genetics

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ABSTRACT

APPROACH TO THE IDENTIFICATION OF SEX-DETERMINING GENES IN THE TILAPIA GENOME BY GENETIC MAPPING AND COMPARATIVE POSITIONAL CLONING

by

BO-YOUNG LEE

UNIVERSITY OF NEW HAMPSHIRE, DECEMBER 2004

Tilapia (*Oreochromis* species) are one of the most dominant species in the aquaculture market. Genomic approaches may contribute to tilapia culture in the near future by identifying genes controlling traits valuable at the market. One of the most important traits for farming of tilapia is sex because monosex culture shows the best efficiency of culture. In tilapia, however, the mechanisms of sex-determination have been poorly understood because of the variation in the genetic basis of sex-determination and the lack of morphological differences between sex chromosomes.

To facilitate QTL analysis of these traits, a genetic linkage map was constructed from hybrids between *Oreochromis aureus* and *O. niloticus* using CRIMAP. The linkage map consists of 25 linkage groups with about 500 genetic markers spanning 2345 cM. Using the information of the linkage map, two

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different chromosomal regions (LG1 and LG3) responsible for sex determination were identified in these species (*O. niloticus* and *O. aureus*). BAC clones containing genetic markers in the sex-determining regions were isolated. Shotgun and end-sequences from these BACs identified syntenic regions among *Fugu*, tilapia, and human. A few genes such as a DEAD box protein, Sox family, and a LIM/homeobox, seem to be good candidates for sex-determining genes, and will need further study. AFLP (amplified fragment length polymorphism)/BSA (Bulked segregant analysis) technique was performed to add more markers in the sex-determining region in *O. niloticus*. This method appeared to be not so efficient in this study, because, although 3 of 128 selective primer pairs were informative, none of them were closer to sex than the markers that were already identified in the sex-determining region. So, more markers should be developed to further fine map the sex-determining genes within the region.

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CHAPTER 1.

INTRODUCTION AND BACKGROUND ON SEX DETERMINATION IN VERTEBRATES, WITH SPECIAL ATTENTION TO TILAPIA

The mechanisms of animal sex determination are diverse and labile (Bull, 1983). Sex-determining systems in vertebrates have been largely divided into two groups: genetic sex determination (GSD) and environmental sex determination (ESD). In the GSD systems, sex chromosomes can contain a single dominant regulator of sexual development, such as the *Sry* (sex-determining region gene) on the mammalian Y chromosome (Sinclair, et al., 1990). In mammals and birds, the genetic constitution established at the time of fertilization determines the type of gonad that develops. In other vertebrates, environmental factors such as temperature, social environment or hormones affect sex determination. The different sex determining mechanisms in vertebrates are summarized in Table 1-1 (Zarkower, 2001).

A single species can have more than one mechanism at once, as in the case of the wood lemming *Myopus schisticolor* (Schutt, et al., 2000). In marsupial mammals, separate mechanisms can operate in different tissues of the same individual. Their gonadal sex is determined by the presence of a Y

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chromosome, but the choice of female pouch versus male scrotum depends on X chromosome dosage (Marshall Graves, 1996). In teleost fishes that show the most remarkable variety of sex determination and differentiation patterns, different species in a single genus show different sex mechanisms. In the genus *Xiphophorus* and *Oreochromis*, some species have female heterogamety and others have male heterogamety (Kallman, 1984; Mair, et al., 1991a; Mair, et al., 1991b).

Chromosomal Mechanisms of Sex Determination in Vertebrates

Sex chromosomes play a causal role in sex determination. Major chromosomal mechanisms of sex determination are male heterogamety (XX female: XY male system) and female heterogamety (ZW female: ZZ male). So the genotype of a female mouse is XX and that of a female chicken is ZW.

Sex chromosomes usually pair up at meiosis and form a bivalent. Crossing over and chiasma formation may occur between homologous regions. In humans, the X and Y chromosomes only recombine at a pseudoautosomal region (PAR) at the ends of both chromosomes, which are needed to guide correct pairing and segregation of the sex chromosomes during male meiosis (Haqq and Donahoe, 1998). The sex chromosomes form a synaptonemal complex at their tips rather than at the usual position near the centromeres.

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The morphology of sex chromosomes varies among species. The sex chromosomes of mammals and birds are heteromorphic, but the shape of sex chromosome in teleosts is almost indistinguishable from that of autosomal chromosomes. In heteromorphic sex chromosomes, differentiated chromosomes pairs in diverse species display certain common characteristics, normally comprising one largely heterochromatic, genetically inactive chromosome and one euchromatic genetically active chromosome (e.g. the mammalian Y and X respectively). It is widely accepted that dimorphic sex chromosomes evolved from homologous pairs of autosomes. Marshall Graves et al (2001) suggest that XY male heterogamety in mammals, and ZW female heterogamety in birds and some reptiles, evolved independently.

Environmental Sex Determination in Vertebrates.

While sex of some vertebrates is determined by sex chromosomes at the time of fertilization, sex of other vertebrates is determined by the environment after fertilization. The effect of incubation temperature on sex determination has been well investigated in reptiles, amphibians, and teleosts (Pieau, et al., 1999; Belaid, et al., 2001; Francis, 1992). Higher temperature at a particular period of incubation produces one sex (males or females) and lower temperature produces the other. In some cases, females develop at extreme temperatures and males

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at intermediate temperature and vice versa. In these cases, the temperature of the eggs during a certain period of development determines the sex of the embryo. Another environmental factor that determines sex in vertebrates is hormones. The administration of steroid hormones such as androgen and estrogen has been applied in studies of sex differentiation and showed successful masculinization and feminization in many fish species (Francis, 1992). An enzyme involved in environment-dependent sex determination is aromatase, which can convert testosterone into estrogen. In the European pond turtle, *Emys obicularis*, aromatase activity is very low at the male-promoting temperature of 25°C and increases dramatically at the female promoting temperature of 30°C (Belaid, et al., 2001).

Molecular Mechanisms of Sex Determination

Sex determination and differentiation require a complex set of events in the appropriate tissues at appropriate time of development. In mammals, the sex-determining process can be divided into several steps; 1) formation of the sexually indifferent gonad from intermediate mesoderm; 2) commitment of the gonad to testis or ovary development; 3) and differentiation into a testis or an ovary (Gilbert, 2000). There are many genes involved in the sex determination cascade. These have mostly been discovered in sex-reversed patients and

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confirmed by knockout experiments in mice (Table 1-2, and Figure 1-1). The primary sex-determining gene in mammals is *Sry*, the Y-linked testis-determining gene. Several other genes are also known to be important for sex determination in mammals, such as *Sox9*, *Amh*, *Wt1*, *Sf1*, *Dax1*, and *Dmrt1* (Swain and Lovell-Badge, 1999; Koopman, 2001; Zarkower, et al., 2001; Cotinot, et al., 2002; Morrish and Sinclair, 2002). Analyses of these genes in humans with gonadal dysgenesis and mouse models have revealed that sex determination results from a complex interplay between the genes in this network. Although these genes are conserved in other vertebrates, such as chickens and alligators, and show gonad-specific expression in these species during the period of sex determination, the sequence, sex specificity and timing of expression of these genes during sex determination show intriguing difference among species (Morrish, et al., 2002).

Formation of the Bipotential Gonad

Prior to sexual differentiation, the ovaries and testes cannot be distinguished and therefore are called bipotential or indifferent gonads. These bipotential gonads arise from the genital ridge, a region adjacent to the mesonephros that ultimately contributes cell lineages to the adrenal cortex, gonads and kidney (Gilbert, 2000). Several genes are crucial for early gonadal

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development, such as *Lim1*, *Lhx9*, *Emx2*, *Wt1*, and *Sf1*. These are related to genital ridge development and the formation of other primordial having a common intermediated mesoderm origin such as kidneys and adrenals (Table 1-2). *Lim1* encodes a member of the LIM class of homeobox protein. Mice homozygous for a deletion in *Lim1* have no kidneys or gonads (Shawlot and Behringer, 1995). It is clear that *Lim1* is involved in maturation of the genital ridges, but the precise role of *Lim1* in early gonad development has not been studied in any detail. *Emx2* is a gene that encodes a transcription factor containing a homeobox domain. Mice deficient for this gene show impaired gonadal and kidney development (Miyamoto, et al., 1997). *Emx2* is expressed in the genital ridge as well as the Wolffian duct, mesonephric tubule, and coelomic epithelia. In mice lacking *Lhx9* function, germ cells migrate normally, but somatic cells of the genital ridge fail to proliferate and a discrete gonad fails to form (Cotinot, et al., 2002). Unlike other genes, *Lhx9* mutants do not exhibit additional major developmental defects. *Wt1* and *Sf1* arise at several levels of in sexual differentiation. These are necessary to make the bipotential gonad. The gonad of embryos lacking *Sf1* cease to develop and degenerate via apoptosis. The adrenal glands also fail to form, but genital ridges begin to form and are colonized by the germ cells. *Sf1* gene expression is specifically associated with the gonad and the adrenal as they arise. *Wt1* knockout mice die in uterus with a complete absence of kidneys and gonads (Kreidberg, et al., 1993). Gonadal

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development is initiated but is then arrested at a very early stage, indicating a role for *Wt1* in early establishment of the genital ridges.

Testis-Determining Pathway

Sry (Sex-determining Region of the Y chromosome). *Sry* is known to be the testis-determining factor (TDF) that is located on the short arm of the Y chromosome in mammals. It is an intronless gene that encodes a 204 amino acid protein encompassing a conserved DNA-binding region of 79 amino acids, a HMG (high mobility groups) box protein, likely to act as a transcription factor (Cotinot, et al., 2002). It's an essential trigger of male gonad differentiation. Mutations in this gene give rise to XY females with gonadal dysgenesis (Swyer syndrome); translocation of part of the Y chromosome containing this gene to the X chromosome causes XX male syndrome. Since the discovery of *Sry*, extensive efforts have failed to find its orthologue in other vertebrates, indicating evolutionary plasticity in the genes that cause sex determination. There is no homologue of *Sry* gene outside mammals, which indicates that *Sry* became involved in sex determination recently. The best candidate for an Sry target is *Sox9*.

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Sox9 (Sry-related HMG box). *Sox9* is one of the autosomal genes involved in sex determination. *Sox9* encodes a putative transcription factor that also contains an HMG box. The HMG box region of SOX9 has been shown to bind to the sequences AACAAT and AACAAAG, and in some cases it has been found to bind to the variant sequences ATGAAT and CACAAT (Koopman, 1999). *Sox9* is expressed just slightly after *Sry* expression. It acts during chondrocyte differentiation and, with *Sf1*, regulates transcription of the anti-Muellerian hormone (AMH) gene, providing a critical link in the pathway toward a male phenotype. High expression of *Sox9* is always correlated with testis differentiation, independent of the presence of *Sry*, and abnormal up-regulation of *Sox9* or an extra dose by a chromosomal duplication in XX individuals is associated with female-to-male sex reversal in human and mice (Huang, et al., 1999; Vidal, et al., 2001). Deficiencies lead to the skeletal malformation syndrome called campomelic dysplasia, frequently with sex reversal (Foster, et al., 1994; Wagner, et al., 1994). It is clear that *Sox9* lies at a crucial step in testis formation. While *Sry* is found specifically in mammals, *Sox9* appears to be highly conserved among mammals as well as in other vertebrate species, such as birds and reptiles.

Sf1 (Steroidogenic factor 1). *Sf1* plays a critical role in testis hormone production. *Sf1* appears to be active in masculinizing both the Leydig and the Sertoli cells. In the Sertoli cells, Sf1, working in collaboration with Sox9, is

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needed to elevate the levels of AMH transcription (de Santa Barbara, et al., 1998; Nachtigal, et al., 1998). In the Leydig cells, SF1 activates the genes encoding the enzymes that make testosterone. The importance of SF1 for testis development and AMH regulation in humans is demonstrated by an XY patient who is heterozygous for *Sf1* (Gilbert, 2000). It is thought that *Sry* directly or indirectly activates the *Sf1* gene, and the SF1 protein then activates both components (Sertoli AMH and Leydig testosterone) of the male sexual differentiation pathway. Knockout mice carrying homozygous deletions of *Sf1* lack gonads and adrenals and die shortly after birth. In these mice, the gonads begin to form, are colonized by germ cells, but cease development at day 11.0-11.5 after mating and then undergo apoptosis (Luo, et al., 1994)

Wt1 (Wilms tumor 1). *Wt1* is an autosomal gene that plays important roles in kidney and gonadal development. As a stimulating cofactor with SF1, WT1 induces transcription of the *Amh* gene by interactions with an AGGTCA promoter element. In addition, the *Wt1* gene plays roles in regulating sex-specific gene expression. *Wt1* mutation has been shown to cause Wilms' tumor and several human conditions that involve developmental abnormalities of the kidneys and gonads (Kredberg, et al., 1993). *Wt1* is a complex gene encoding at least 24 different isoforms by use of alternative start sites, splicing and RNA editing. One of these isoforms, the –KTS can bind and transactivate the *Sry* and *Dax1* promoters in vitro (Kim, et al., 1999; Hossain and Sanders, 2001). Although the

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–KTS isoforms cannot bind to the *Amh* promoter, it has been shown to synergize with SF1 to activate *Amh* transcription in vitro (Nachtigal, et al., 1998). In XX individuals, Dax1 is thought to interfere with the interaction of SF1 and WT1, thus repressing male-specific development (Nachtigal, et al., 1998). Another isoform, +KTS, seems to be required to allow enough expression of *Sry* in the testis determination pathway.

M33 (CBX2: chromobox homolog 2). M33 has been shown to play a role in testis differentiation, probably by interfering with steps upstream of Sry. Mice carrying a disrupted *M33* gene show a significant delay in gonad development in both sexes (Katoh-Fukui, et al., 1998). These retarded gonads give rise to adult organs although they are not completely normal and the phenotype is variable (Swain and Lovell-Badge, 1999). The XY animals showed different degrees of sex reversal. This retardation of gonad formation leads to the development of small ovaries in XX null mice and to partial or complete male-to-female sex reversal in XY null mice (Cotinot, et al., 2002). M33-deficient mice show homeotic transformations in the structure of their skeleton (Core, et al., 1997; Bel, et al., 1998; Katoh-Fukui, et al., 1998).

Dmrt1 (Doublesex and mab-3 related transcription factor 1). *Dmrt1* is a member of a family of genes that share the highly conserved DM-domain involved in male sexual development. This gene is related to genes encoding

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the transcriptional regulator *mab-3* in *Caenorhabditis elegans* and *doublesex* in *Drosophila* (Raymond, et al., 1998).

The evidence that *Dmrt1* is a male sexual regulator in vertebrates includes embryonic expression, chromosomal position and mutational analysis. *Dmrt1* is expressed very early in the genital ridge. In all species except the mouse, *Dmrt1* is expressed at a higher level in future male than in future female genital ridges before gonad differentiation. These expression patterns indicate that *Dmrt1* is likely to have a conserved role in testis development (Smith, et al., 1999; De Grandi, et al., 2000; Smith, et al., 2002). Chromosomal position also implicates Dmrt1 in sexual development in mammals and birds. In humans, *Dmrt1* maps to a short interval of chromosome 9 that is required in two copies for testis differentiation. In birds, which have ZZ/ZW sex determination (and lack *Sry*), *Dmrt1* maps to the Z chromosome, which is very similar in sequence and organization to human chromosome 9. In both cases, therefore, the presence of two copies of *Dmrt1* correlates with testis differentiation. The *Dmrt1* gene has been functionally analyzed only in the mouse. It is required in testis differentiation, affecting both Sertoli cells and germ cells. This phenotype is similar to that caused by some human 9p deletions, indicating that the sex reversal caused by human 9p deletions might involved *Dmrt1*, either alone or with nearby genes (Zarkower, 2001).

In medaka fish (*Oryzias latipes*), *DMY* (*Dmrt1bY*) is a strong candidate for the male sex-determination gene on the Y chromosome (Matsuda, et al., 2002;

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Kondo, et al., 2003). This gene appears to be a duplication of an autosomal segment containing *dmrt1*, which was inserted into another chromosome that then became the Y chromosome. In the Y-specific region, the *DMY* (*Dmrt1bY*) gene is the only functional gene. *DMY* (*Dmrt1bY*) is expressed only in male embryos and mutation in this gene cause XY-sex-reversed females.

Amh (Anti-Mullerian hormone). AMH induces the regression of the Mullerian ducts, which in the female give rise to the oviducts and uterus. *Amh* gene is expressed at 12 dpc in the developing Sertoli cells of the mouse testis in a pattern that closely follows the up-regulation of Sox9 (Swain and Lovell-Badge, 1999). *Amh* gene has a consensus binding site for SF1 in the promoter region so that SF1 can bind and activate the *Amh* promoter (Shen, et al., 1994). The *Amh* promoter region also contains a consensus binding site for proteins containing an HMG box domain (Haqq, et al., 1993). SOX9 is a better candidate to bind to this site because it is present in the male genital ridge at the time that *Amh* is activated, and continues to be expressed in Sertoli cells throughout development and adulthood (de Santa Barbara, et al., 1998). SOX9 will bind to the HMG box consensus site in the *Amh* gene. Also, SOX9 can synergize SF1 activation of the *Amh* promoter, suggesting that both factors act in concert to bring about tissue specific expression of *Amh*. WT1 has also been implicated in the regulation of *Amh* expression. Mice carrying a homozygous deletion in the *Amh* coding region have normally developed testis, but are infertile due to

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persistence of Mullerian-derived structures that interfere with sperm transfer (Behringer, et al., 1994).

Dhh (Desert hedgehog). *Dhh* is a candidate factor involved in cell-cell interactions between Sertoli and germ cells. Male mice deficient for *Dhh* are infertile owing to absence of sperm. Testis weights were reduced in mutant mice, and in later stages histological analysis showed a germ cell deficiency and a block in spermatogenesis. However, it is unclear whether the reduction in size is due to a block in proliferation of germ cells, or Sertoli cells or both. It is possible that DHH could contribute to the signal that makes germ cells enter into mitotic arrest in the testis, whereas in its absence, cells go into meiotic arrest (Swain and Lovell-Badge, 1999). Expression studies have shown that *Dhh* is present in male gonads at 11.5 dpc and it is associated with Sertoli cells at later stage of testis development. Early expression patterns suggest that *Dhh* is a direct target of SOX9 or SRY.

Ovary-Determining Pathway

Wnt4 (wingless-related MMTV integration site 4). The *Wnt* gene family consists of structurally related genes that encode secreted signaling proteins. These proteins have been implicated in oncogenesis and in several

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developmental processes, including regulation of cell fate and patterning during embryogenesis. *Wnt4* is a member of the *Wnt* gene family, and is the first signaling molecule shown to influence the sex-determination cascade in humans. *Wnt4* is a potential ovary-determining gene on an autosome. This gene is expressed in the mouse genital ridge while it is still in its bipotential stage. *Wnt4* expression then becomes undetectable in XY gonads, whereas it is maintained in XX gonads as they begin to form ovaries (Gilbert, 2002). The human *Wnt4* gene encodes a protein that shows 98% amino acid identity to the Wnt4 protein of mouse and rat. In transgenic XX mice that lack the *Wnt4* genes, the ovary fails to form properly, and its cells express testis-specific markers, including AMH-and testosterone-producing enzyme (Vainio, et al., 1999). This gene and *Dax1* play a concerted role in both the control of female development and the prevention of testes formation.

Dax1 (nuclear receptor subfamily 0, group B, member 1). *Dax1* is a potential ovary-determining gene on the X chromosome. This encodes an unusual member of the nuclear hormone receptor superfamily. Duplication of *Dax1* in XY individual shows a sex reversal phenotype in humans (Bardoni, et al., 1994). Deletions involving Dax1 do not disrupt testis differentiation, and Dax1 expression in mice is down-regulated with testis differentiation, but persists in the developing ovary (Swain, et al., 1996). Transgenic mice overexpressing Dax1 have been shown to undergo male-to-female sex reversal, suggesting that *Dax1*

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appears to antagonize the function of *Sry* (Swain, et al., 1998). However, targeted inactivation of Dax1 in mice does not affect ovaria development, instead blocking spermatogenesis in males (Yu, et al., 1998). It is concluded that Dax1 is a spermatogenesis and antimaleness gene rather than an ovarian-determining gene (Swain, et al., 1996).

Importance of Sex Determination in Tilapia.

Maternal mouth brooding tilapia from the genus *Oreochromis* are an important species in the aquaculture market (Mair, 2001). Control of reproduction is often one of the biggest challenges in commercial tilapia production, because the fish mature early and can begin to reproduce before reaching market size (Phelps, 2001). Male tilapia grow faster than female tilapia and monosex culture of male tilapia has resulted in the economically best production. Studies of sex determination in tilapia can provide an important technical basis for the improvement of the cultural yields of this species. The lack of genetic markers for sex is one of the problems that make the elucidation of sex determination in this species difficult. Genetic markers identified by studying sex determination will be useful in tilapia breeding programs.

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What is Known about Sex-Determination in Tilapia

Most basic information on the sex determining system in tilapia has been observed from studies of sex ratio in the progenies from interspecific, intraspecific, or sex-reversed fish hybrids (Mair, et al., 1991a; Mair, et al., 1991). By these methods, the tilapia genus, *Oreochromis*, has been shown to have both female and male heterogametic systems. *Oreochromis niloticus* (Nile tilapia), and *O. mossambicus* (Mozambique tilapia), have a male heterogametic (XX/XY) sex determining system, while *O. aureus* (blue tilapia), *O. hornorum,* and *O. macrochir* have a female heterogametic system (ZZ/ZW). However, temperature and secondary genetic factors can also influence sex determination.

There are several cases in which sex ratios do not fit perfectly with the expectations of a heterogametic system, suggesting the influence of either minor sex determining genes or environmental influences on the process of sex differentiation. There have been several studies on the influence of temperature (Wang and Tsai, 2000; Baroiller, et al., 1996) and hormone treatments on sex determination in tilapia (Francis, 1992; Guiguen, et al., 1999). Progenies can be functionally masculinized when reared at elevated temperatures (32-36°C) if applied before and during gonadal sex differentiation (Baroiller, et al., 1995; Baras, et al., 2001). The use of aromatase inhibitors, which block estrogen synthesis, caused partial or complete inhibition of ovarian differentiation and

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resulted in a masculinization in a number of fish (Kwon, et al., 2000; Bertollaafonso, et al., 2001).

The sex chromosomes of tilapia are morphologically indistinguishable from autosomal chromosomes. As there is no morphological difference between sex chromosomes, several cytogenetic studies have been performed to identify the sex chromosomes in different species of *Oreochromis* by observing the synaptonemal complex (SC) in meiotic chromosomes, because there is the region of restricted chromosome pairing in heterogametic fish (Campos-Ramos, et al., 2001; Carrasco, et al., 1999). The longest chromosome is considered to be the sex chromosome in the species with XX/XY mechanisms such as *O. niloticus* and *O. mossambicus*. In *O. aureus*, two chromosomes are thought to be related to sex. One is the same largest bivalent as in XX/XY system and the other is one of smaller bivalents (Campos-Ramos, et al., 2001).

Not many genes related to sex-determination have been characterized in this species. A few genes such as *aromatase*, *vas*, *wt1*, *dax1*, and *dmrt1*, have been cloned and their expression pattern examined in *O. niloticus* (Table 1-3; Kwon, et al., 2001; Kobayashi, et al., 2000; Wang, et al., 2002; Guan, et al., 2000). The *dmrt1* gene has been cloned in *O. niloticus* and a *Sry*-consensus site was found in the 5' upstream regions of this locus suggesting that one of the upstream regulatory genes of DMRT1 in tilapia could be a *Sry*-like gene from the Y chromosome (Guan, et al., 2000). In tilapia, DMRT1 homologues (tDMRT1) possess the male-specific motif and expression appears to be testis (Sertoli

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cells) specific, whereas another DM homologue (tDMO) lacked this motif and was found expressed only in ovary (Guan, et al., 2000). Interestingly, examination of upstream sequences reveals a SRY binding site within tDMRT1 but not tDMO, suggesting a close linkage between Sox and DMRT1 gene products in sex determination pathways. Tilapia *Dax1* is highly expressed in gonads. Unlike other vertebrates, tilapia DAX1 mRNA is also expressed in the intestine, muscle, and gill. It is also expressed in the liver, but at a relatively low level, especially in females, compared with other non-mammalian vertebrates. It is interesting that in tilapia, sex difference in *Dax1* expression was found in several tissues, but not in gonads, with higher expression levels seen in males (Wang, et al., 2002). *Vas* is a *Drosophila vasa* homologue gene which has two isoforms, *vas-s* and *vas*. Prior to meiosis, no differences in expression pattern are observed in male and female germ cells. In ovary, compared with *vas* expression *vas-s* expression predominated throughout oogenesis. In testis, *vas* expression was predominant compared with *vas-s* during spermatogenesis (Kobaysahi, et al., 2000; Kobayashi, et al., 2002).

Overall Goals of the Dissertation

The objectives of this dissertation were to map or identify genes involved in sex determination and sex differentiation in tilapia genomes. The first step in

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these studies was to construct a genetic linkage map well-saturated with genetic markers (Chapter 2). As a framework, the linkage map will also be useful for QTL analysis of interesting genes other than sex. This genetic map was used to scan the tilapia genome to localize the regions associated with sex determination in the most important tilapia species (Chapter 3 and Chapter 4). Once the chromosomal region of sex determination and markers flanking the regions were identified, a comparative positional cloning approach was taken to identify candidate genes (Chapter 5).

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Species	Primary sex-determining system	Sexes	
		Male	Female
Most eutherian mammals	GSD: Dominant Y	XY	XX
Mole voles (*Ellobius*)			
E. fuscocapillus	GSD:Dominant Y	XY	XX
E. lutescens	Unknown	XO	XO
E. tancrei	Unknown	XX	XX
Wood lemming (*Myopus schisticolor*)	GSD:Dominant Y; Dominant X*	XY	XX, X*X, or X*Y
Marsupial mammals	GSD:Dominant Y (testis) X dosage (2X:pouch; 1X;scrotum)	XY	XX
Birds	GSD: Ratio?	ZZ	ZW
Turtles (*Trachemys scripta*)	ESD:Temperature	Cool	Warm
Alligators	ESD:Temperature	Warm	Cool
Fish			
Trout (*Oncorhynchus mykiss*)	GSD: Dominant Y	XY	XX
Jewel lyretail anthias (*Psedanthias squmipinns*)	ESD:social;protogyny	Sequential hermaphrodite female, then male	
Dusky anemonefish (*Amphiprion malanopus*)	ESD:social;protandry	Sequenctial hermaphrodite male, then female	
Tilapia (*Oreochromis*)			
O. niloticus	GSD	XY	XX
O. aureus	GSD	ZZ	ZW

Table 1-1. Sex-determining systems in vertebrates (Zarkower, 2001). (GSD: Genetic Sex Determination, ESD: Environmental Sex Determination).

Gene	Product	Human Locus	Phenotype of Loss-of-Function Mutation
Bipotential gonad formation			
Lhx9	Transcription factor	1q31-32	Blockage in genital ridge development (KO)
Lim1	Transcription factor	11p12-13	Absence of kidneys and genital ridges (KO)
Emx2	Transcription factor	10q26	Blockage in genital ridge and kidney development (KO)
Sf1	Transcription factor	9q33	blockage in genital ridge and adrenal gland development (KO)
Wt1	Transcription factor	11p13	Blockage in kidney, spleen and adrenal gland development, heart failure, and absence of gonads (KO)
Testis-determing pathway			
Sry	Transcription factor	Yp11.3	XY Male-to-female sex reversal (human and murine mutations)
Sox9	Transcription factor	17q24	XY male-to-female sex reversal and skeletal dysmorphology (human mutation)
Sf1	Transcription factor	9q33	XY-Male-to-female sex reversal and adrenal failure (KO and human mutation)
Wt1	Transcription factor	11p13	XY-Male-to-female sex reversal and kidney defects (human mutations)
M33	Transcription factor	17q25	XY-Male-to-female sex reversal (KO)
Dmrt1	Transcription factor	9p24.3	XY-Male-to-female sex reversal (multigene deletion in humans) Loss of Sertoli and germ cells in postnatal tesis (KO)
Fgf9	Signaling molecule	13q11-13	XY-Male-to female sex reversal/gonadal dysgenesis, lung defects (KO)
Amh	Signaling molecule	19p13	No XY sex reversal, persistence of Mullerian duct derivatives in XY individuals (human mutations, KO)
ATRX	Helicase	Xq13	XY Male-to-female sex reversal, mental retardation, alpha-thalassemia (human mutation)
Dhh	Signalling molecule	16q24	Loss of germ cells (KO)
Ovary-determing pathway			
Wnt4	Signaling molecule	1p35	Testosterone synthesis and male duct development in XX mice (KO)
Dax1	Neclear receptor	Xp21	No XX sex reversal, progressive degeneration of the testicular germinal epithelium in XY individuals (KO)
FoxL2	Transcription factor	3q23	premature ovarian failure and eyelid defects (BPES in humans) XX-Female-to-male sex reversal (polled mutation in goats)
Gdf9	Signaling molecule	5p11	Failure of ovarian follicular development (KO)

KO : Knonck out experiments in mice

Table 1-2. Genes involved in sex determination in mammals.

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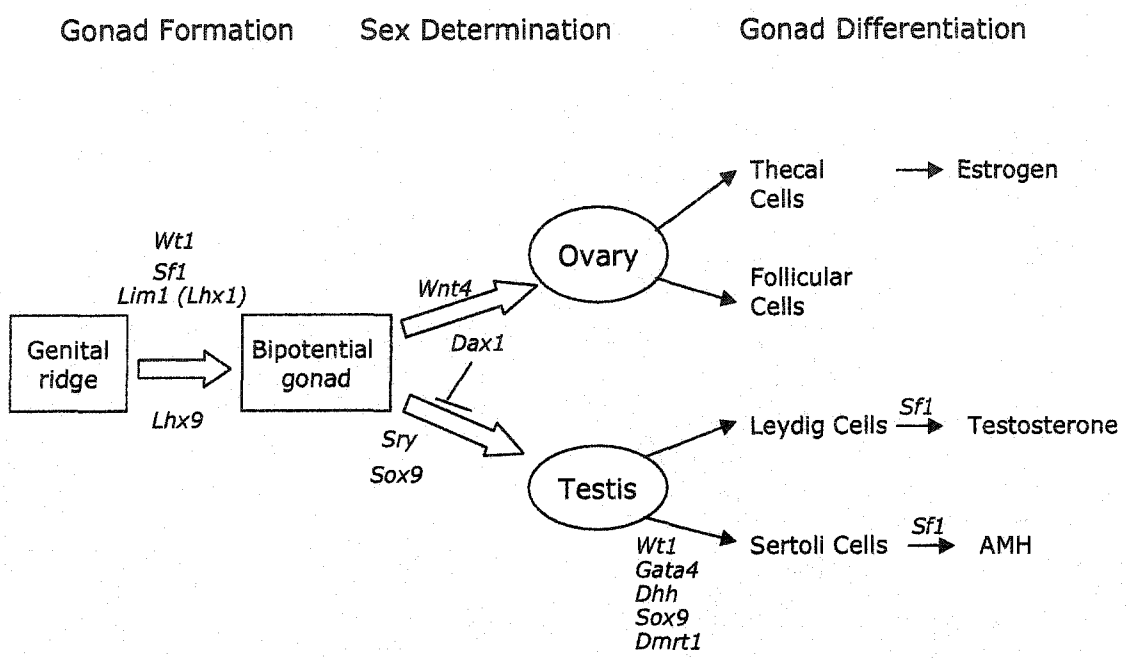

Figure 1-1. Postulated cascade of sex-determining genes in mammals.

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Gene	Expression	Cloned in tilapia	Mapping in tilapia
aromatase	ovary	AF135851	FISH (Harvey, *et al.* 2002)
vasa	PGC, ovary	AB032467	no
vas-s	PGC, testes	AB051835	no
dax1	gonad, intestine, muscle, gill	AY135379	no
dmt	testes	AF203489	Linkage group 12 (Lee *et al.* in prep)
dmo	ovary	AF203490	Linkage group 3 (Lee *et al.* in prep)
wt1	gonad	AF534550/1	Linkage group 7 (Lee *et al.* in prep)

Table 1-3. Expression and mapping information of sex-determining candidate genes in tilapia.

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CHAPTER2.

CONSTRUCTION OF A GENETIC LINKAGE MAP OF TILAPIA (*OREOCHROMIS SPP*)

Abstract

A genetic map for tilapia (*Oreochromis aureus* x *O. niloticus*) was constructed by analyzing the segregation of 479 microsatellites and 14 protein encoding genes in an F_2 hybrid cross between *O. aureus* and *O. niloticus*. Using a threshold LOD score of 3.0, a total of 25 linkage groups were identified. The linkage map spans 2345 cM with an average marker spacing of 4.76 cM. Overall, the female map is a little shorter than the male map (2.3%). Syntenies identified in previous tilapia maps are largely confirmed, but some differences in gene order among species may exist. The map was used to identify markers linked to sex using the non-parametric methods of MapQTL. Six markers significantly associated with sex were detected ($p < 0.005$). All six markers show linkage with other markers on LG3.

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Introduction

Tilapia has become an important species in aquaculture and has received increasing scientific interest over the past few decades. A major part of research for the genetic improvement of tilapias is focused on the identification of quantitative trait loci (QTL) responsible for the commercially important traits such as color, temperature tolerance, and salinity tolerance in order to use the genetic information for marker-assisted selection.

Genetic linkage maps provide a framework for QTL analysis. A genetic linkage map also provides good information for manipulation of interesting genes or for identification of the chromosomal location of cloned genes. Genetic maps have been constructed for a number of fish species including zebrafish, *Danio rerio* (Postlethwait, et al., 1994; Johnson, et al., 1996; Hukriede, et al., 1999; Kelly, et al., 2000); rainbow trout, *Oncorhynchus mykiss* (Young, et al., 1998), and medaka, *Oryzias latipes* (Ohtsuka, et al., 1999; Naruse, et al., 2000; Kondo, et al., 2001). In zebrafish, studies of gene mapping are well advanced at the highest level and a large amount of raw sequence data exists in public databases.

A genetic linkage map gives information on recombination rate difference between sexes as well as the total genome size based on recombination rate. There is a scarcity of information on sex specific recombination difference in

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teleost species. According to Haldane's rule (1922), gonochoristic species are expected to show sex-specific recombination differences, with the heterogametic sex exhibiting lower recombination levels. Current mapping studies in fish support the rule (Sakamoto, et al., 2000). There are sex-specific maps for comparing recombination rate between male and female in medaka (Kondo, et al., 2001), rainbow trout (Sakamoto, et al., 2000), and zebrafish (Singer, et al., 2002).

In tilapia, a few linkage maps have been published. Kocher et al (1998) constructed the first tilapia genetic map of *Oreochromis niloticus* using haploid larvae. For this map, 62 microsatellite and 112 AFLP markers were used and map length was estimated to be about 1200cM in 30 linkage groups covering 22 tilapia chromosomes. Agresti et al (2000) created a genetic map in a three-way interspecific tilapia mapping population using microsatellite and AFLP markers. McConnell et al (2000) constructed a genetic map with an interspecific backcross between *O. niloticus* and *O. aureus* using 82 microsatellite including many of published UNH markers, but this was a partial map covering only 10 linkage groups. They identified a possible chromosomal inversion between the two species. They also showed the possibility that the female map is smaller than the male map.

The objective of this study was to develop a well-saturated linkage map which can be used to identify the chromosomal regions of interesting genes or QTLs, responsible for commercially or evolutionarily important traits. I identified

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the region associated with the sex trait on the linkage maps. In addition, sex-specific maps made with CRIMAP were compared to examine the difference in the recombination rate between sexes.

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Materials and Methods

Mapping Panel

For construction of the linkage map, an interspecific cross of a female blue tilapia, *Oreochromis aureus* and a male Nile tilapia, *O. niloticus* was produced by Dr. Gideon Hulata at the Agricultural Research Organization (ARO), Israel. A population of 156 F_2 was obtained by crossing one pair of F_1 full-sibs. Seventy-two individuals including the parents were used for linkage analysis and map construction.

Isolating Microsatellite Markers

Markers labeled 'GM' were developed by a private company, GenoMar, in Norway. For UNH markers, microsatellite libraries were made with *O. niloticus* genome using the hybrid capture methods (Carleton, et al., 2001). From the CA enriched genomic libraries, each clone was sequenced in one direction on an ABI377. After sequence analysis, a small fraction of the clones were sequenced from the opposite strand to resolve ambiguities in the sequences. Sequences that contained perfect repeats of at least 12 microsatellite units were selected.

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Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were designed to generate PCR products between 100bp and 250bp in length.

Type1 Markers

Fourteen protein encoding genes, including *cski*, blue-opsin (*bluops*), UV-opsin (*uvops*), *pax9*, *rasgrf2*, *clcn5*, IGF2 (*ifg2/th*), *wt1*, insulin (*ins*), *mhc1*, prolactin (*prl*), transferrin (*tf*), myostatin (*gdf8*), and *dlx2*, were typed in the F2 (Table 2-1). Some genes (*cski*, *bluops*, *uvops*, *rasgrf2*, *clcn5*, *ifg2/th*, *wt1*, *ins*, and *prl*) have a microsatellite in an intron sequence. For other genes (*pax9* and *dlx2*), SNPs were identified in their sequences and primers were designed in the flanking regions to amplify the variable region. RFLP was performed to map those genes in this cross. Myostatin (*gdf8*) was genotyped by GenoMar, Norway and *tf* and *mhc1* were genotyped at ARO, Israel (Cnaani, et al., 2002; Cnaani, et al., 2003).

Microsatellite Genotyping

All 'GM" markers were genotyped by GenoMar, Norway. The genotyping data were provided to University of New Hampshire (UNH) for mapping. For

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UNH markers, the forward primer in each pair was fluorescently labeled with either TET, 6-FAM, or HEX (Operon Technologies Inc., Alameda, CA, USA). Each marker was typed in a 20 ul PCR reaction containing 250 nM of each primer, 30-50 ng DNA, 2 mM $MgCl_2$, 0.2 mM of each dNTP and 0.3 U *Taq* polymerase in the cycling conditions as follows; 94°C for 2 min; 27-28 cycles of 94°C for 20s, 50-55°C for 30s, 72°C for 1 min; 72°C for 5 min. PCR products were loaded on 4% acrylamide gels using an ABI 377 automated DNA sequencer and some of the markers were multiplexed based on difference in the fragment size and in fluorescent dye color. Fragment sizes were analyzed with GeneScan (ver. 3.1.2). New markers were first tested in parents and four randomly chosen F2 individuals to test for segregation in the mapping family. Informative markers were then genotyped in the whole mapping population.

Linkage Analysis and Mapping.

Linkage analysis was performed using CRIMAP (ver. 2.4) (Green, et al., 1990), software to construct the linkage map, hosted at the UK MRC Human Genome Mapping Project Resource Centre (HGMPRC). A two-point linkage identification by which all 551 markers were analyzed against each other was performed using a CRIMAP option TWOPOINT. From the results of the two-point analysis, markers belonging to the same linkage group were assembled

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and the marker order was identified using the ALL and BUILD options. Finally, the order of the markers was statistically confirmed using the FLIPS function.

Mapping Sex Trait

Sex traits were analyzed with MapQTL (ver. 4.0) (van Ooijen, et al., 2002) using the nonparametric mapping method. This program employs a Kruskal-Wallis rank sum test for handling the categorical phenotypic data. In this single marker analysis, all individuals are concurrently ranked according to the phenotype and genotypic classes. A QTL of large effect linked closely to a given marker will result in a large difference in the mean ranks of the genotypic classes. The test ranks all individuals according to the sex trait and marker genotypes. A segregating QTL linked closely to the tested markers will result in large differences in average rank of the marker genotype classes. Under the null-hypothesis that there is no segregating sex QTL, the Kruskal-Wallis test statistic is distributed approximately as a chi-square distribution with the number of genotype classes minus one as degrees of freedom. Since the test is performed on many linked and unlinked loci, I report here markers with association at a p-value less than 0.005.

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Results

Genotyping

A total of 551 markers consisted of 182 UNH and 355 GM microsatellites, and 14 genes (*cski*, *bluops*, *uvops*, *pax9*, *rasgrf2*, *clcn5*, *ifg2/th*, *wt1*, *insulin*, *mhc1*, *prl*, *tf*, *gdf8*, and *dlx2*) were informative in this cross. In addition to UNH markers used in the previous tilapia haploid map, 156 new UNH markers were newly designed for this mapping. One hundred fourteen (73%) were informative and of the remaining 42 markers, 17 markers were homozygous. The other 25 markers were difficult to score because of multiple, complex peaks. The allele size in the F2 and PCR annealing temperatures for the informative markers are listed in Table 2-2.

Linkage Map

Five hundred forty (98%) of 551 markers showed detectable linkage to another marker by LOD score of 3.0 or higher. Eleven markers appeared to be unlinked to any other markers. Forty-seven of 540 failed to be placed in this map. The linkage map consists of 25 linkage groups spanning 2345 cM with

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average spacing of 4.77 cM (Figure 2-1). The size of the linkage groups, excluding unlinked markers and unplaced markers, range from 6 to 264 cM (mean: 94 cM). The number of markers per linkage group varies from 5 to 59. The largest interval in the linkage map is 36.6 cM in LG10. These results are summarized in Table 2-3.

Sex-Specific Maps

Sex-specific maps were made with CRIMAP and the two maps were compared (Figure 2-2). The sizes for the sex-specific maps and the difference between the two maps are shown in Table 2-3. The male map is a little (2.3%) longer than the female map. Although the average difference between female and male was small, some linkage groups show bigger size differences. For instance, the female map is 139.3 cM in LG21 while the male map is only 35 cM, which is a 291% difference between sexes (Figure 2-3).

Mapping of Sex Trait

Single marker analysis using the Kruskal-Wallis test detected 6 markers (GM271, GM354, UNH168, GM204, UNH131, and GM139) significantly

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associated with the sex trait (Table 2-4). GM271, GM354, and GM139 are placed in LG3 and the rest (UNH168, GM204, and UNH131) failed to be mapped, even though those were linked to certain markers in LG3 by two-point analysis. Only LG3 contains a QTL region for sex in the whole genome analysis of this cross (Figure 2-4).

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Discussion

The current tilapia map represents a significant increase in resolution that will, I believe, enhance the detection of quantitative trait loci (QTL). In addition, it provides good information for comparative linkage maps between tilapia and other cichlid species. However, there are a couple of things to be considered here. First, the number of linkage groups is greater than the number of tilapia chromosomes. One of the possibilities for this phenomenon is that the number of markers (or individuals) used for the linkage mapping is still not enough. Additional genotyping may link clusters of a few markers with the larger linkage groups. Another possibility is that, because of translocation between *O. niloticus* and *O. aureus,* the linkage map of hybrids may be disrupted. Second, in the previous haploid map (Kocher, et al., 1998), map length was estimated about 1200 cM. However, the estimated map length here is nearly twice as long as the haploid map. The haploid map length could be underestimated, if the AFLP markers were more highly clustered in narrow regions of chromosomes than microsatellite markers were. Variation between two sex maps may contribute to the estimation because the first map was only female meiotic map. In tilapia, female map length is shorter than male map (Agresti, et al., 2000). This overestimated map length may also be related to the inter-specific cross or possibly to remaining genotyping errors.

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Most published tilapia linkage maps have some UNH markers in common, allowing a comparison among linkage maps. In all of those maps, although the number of common markers is still not enough to compare complete linkage groups, markers in the same linkage groups are placed together in most cases. It seems that the synteny of markers in some regions of chromosomes were fairly conservative. Chromosomal reorganization or actual difference in chromosome structure could exist among different species or hybrids. Chromosomal inversion was reported by comparing the *On* haploid map (Kocher, et al., 1998) to other maps (Agresti, et al., 2000; McConnell, et al., 2000). I extended this observation to the interspecific map (LG9). The comparison shows that markers in LG9 are consistent with M-2, AXR-1, and McConnell-1 (Figure 2-5). The existence of chromosomal inversions may become apparent when different species are crossed. Agresti et al (2000) suggested that different species have been shown to have the same chromosome number, but the number of arms may be different because of the chromosomal rearrangements, such as Robertsonian translocation or fusions, which can cause different loci to be linked in the different maps.

In many organisms, the rate of genetic recombination is not uniform along the chromosomes or between sexes. When meiotic recombination rate varies between the two sexes, it is usually the heterogametic sex that has suppressed recombination (Haldane, 1922). This rule holds for most mammals such as human (Dib, et al., 1996), dog (Neff, et al., 1999), pig (Marklund, et al., 1996),

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and mouse (Dietrich, et al., 1996). Although many fish species do not have heteromorphic sex chromosomes, some of them showed different recombination rates between sexes. In zebrafish, nearly all linkage groups showed that recombination is dramatically suppressed in male meiosis compared to the female meiosis. Most of the decrease in male recombination relative to female recombination takes place around the centromere and near the telomeres. This trend partially explains why the male map is suppressed relative to the female map in zebrafish (Singer, et al., 2002). The tilapia sex-specific maps show that the female map is a little shorter than the male map (2.3%). The recombination rate between sexes, however, varies among linkage groups, unlike in zebrafish where the length of male maps is definitely shorter than that of female maps in all linkage groups. Of 25 tilapia linkage groups, the male map is longer in 13 linkage groups and the female map is longer in 12 linkage groups (Figure 2-4). The magnitude of sex-specific differences in recombination rate is variable in every linkage group. For LG21, the female map is 291% longer than male map and for LG18, the male map is 70.3% longer than female map.

A well-saturated linkage map is a very useful tool for QTL analysis. I used the linkage map to identify a QTL for sex. MapQTL (ver. 4.0) successfully localized the sex trait near 6 markers in LG3. However, three of those markers could not be placed in the map by the CRIMAP algorithm. The sex-associated markers in LG3 shows unusual linkage pattern between sexes. In the female sex-specific map of LG3, GM271 is closely linked to GM354 and GM139 (8.2 cM

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and 10.7 cM) but in the male map GM271 is located 100 cM far away from these markers (Figure 2-2). Two-point analysis data provided by CRIMAP showed that the recombination fraction between GM271 and UNH115 was 0.34 in female and 0.05 in male, while that between GM271 and GM354 was 0.04 in female and 0.00 in male. Based only on the data for these 3 markers, one might conclude that the recombination rate is very different between sexes. However, the fact that three other markers could not be mapped raises the possibility that there might be a difference in gene order between sex chromosomes.

CRIMAP provides sex-specific map based on the difference in recombination rate in the male and female parents, but cannot detect the difference in gene order between the sex chromosomes. CRIMAP put GM271 100 cM (not linked) away from the GM139 and GM354 in the male map, because it could not figure out the suitable distance in the male map with the marker order given by the female map. This is likely the reason that the other 3 markers could not be placed by CRIMAP.

Sex chromosomes in other species are known to differ by chromosomal inversions. Among fish species, the spiny eel (*Mastacembelus aculeatus*) has heteromorphic sex chromosomes which differ by a pericentic inversion (Liu, et al., 2002). This tilapia family used for the map was the cross of two species with different sex mechanisms between female *O. aureus* (ZW) and male *O. niloticus* (XY). If there is an inversion between sex chromosomes, the further study will be

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required to understand that this phenomenon is specific to sex chromosomes in all tilapia species, to XX/XY system species, or to ZW/ZZ system species.

However, since I know where the chromosomal region controlling sex lies on the genome, I can identify it through positional cloning. Gaps between markers can be filled by comparative mapping using many ESTs within the region to identify synteny between vertebrate or teleost genomes.

Future work should also include the addition of highly conserved type I markers to the map so that the tilapia genome can be compared to other organisms with homologous markers. The markers linked to the detected QTL could be used for a marker-assisted selection (MAS) to maximize productivity in tilapia aquaculture.

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Gene	GenBank Accession No.	Pirmer		Polymorhism
		Forward	Reverse	
bluops	AF247120	TGCATGCAAGATTGAAGGAT	TGCAATGATATTGGCACCAG	(GAA)7
clc5	AF182216	AGGGTGAAGGATCCAGGAGT	AGGACAGCGCTGCATAGTTC	(CA)13
cski	AJ012011	GTCAGTCACATTCCTGGCTG	TTCTATGCTCCTGCGGTTTT	(GA)29
dlX2	AF534538/9	GGGTCACCGTTTGGACAG	CAGGCTACTTTGTGGATCGG	*SacI*
ifg2/th	AH006117	GGGTACAGCCCAGACAACAT	TGGTGGTGAAGCGACAGTAA	(CT)15
insulin	AF038123	GATCAGATCATGTCGGCTCA	ACAGCCCTGTGAAGAGATGG	(CA)17
mhc1	AJ577831-6	TGTCCYGAGTGGGTGAAGAAGTAT	GGRAGWCTTCTGRAGGAGAGACA	Length
gdf8	AF197193	GGAAATGACTTAGCTGTGACCTC	TGAAATCTTCACCTCCATGAAC	Length
pax9	AF534548/9	TCCGAAACAAGATTGGGAAT	GAGTGGGCACTTTGGACG	*MnlI*
prolactin	X92380	AGTTTTCGTGTCTTGTGGGG	TTTGAATGGATGCAACAGGA	(CA)34
rasgrf2	U63663	CTTGATCACCCCACCAAACC	TGGGTCTCCAAACATTCACA	(CA)11
transferrin	AJ312311	CCATTAGGTGGTGTGCTGTG	GAACCAGACCACACTTTCCAG	Length
uvops	AF191221	AGCTGCTGGGTGCTCTGA	CTGCAACCTGCAGAGGAAAC	(TCAA)12
wt1	AF534550/1	CAGTGCCGTCTTTAAAGTTTTG	CACACACATTCAGCACGAGTT	*NlaIII*

Table 2-1. Accession numbers, primer sequences and polymorphisms of Type1 markers placed in the linkage map.

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Marker	Allele size	PCR Temp	Marker	Allele size	PCR Temp	Marker	Allele size	PCR Temp
UNH719	124, 126	50C	UNH890	244, 254, 270	52C	UNH951	195, 206	55C
UNH735	168, 178, 182	50C	UNH891	167, 172, 159	55C	UNH952	191, 193	55C
UNH738	157, 171	50C	UNH896	157, 227, 234	55C	UNH954	135, 185	55C
UNH773	207, 211, 253	50C	UNH898	274,280	55C	UNH957	161, 174, 176	55C
UNH817	99, 119	50C	UNH899	146, 166, 172	55C	UNH958	143, 153, 155	55C
UNH832	189, 193, 195	50C	UNH901	145, 159, 161	55C	UNH960	130, 177	55C
UNH840	131, 138, 140	55C	UNH904	136, 146, 164, 134	55C	UNH961	191, 195	55C
UNH841	114, 156	52C	UNH905	146, 151, 198	55C	UNH965	179, 192, 174	55C
UNH843	109, 120, 124	55C	UNH906	152, 154, 156	55C	UNH967	116, 217, 161	55C
UNH844	98, 104, 116	55C	UNH907	122, 139	55C	UNH968	196, 201, 209	55C
UNH845	175, 181	55C	UNH908	107, 163, 178	55C	UNH970	89, 94, 114, 152	55C
UNH846	173, 203	55C	UNH909	238, 246, 286	50C	UNH971	201, 214, 234	55C
UNH848	180, 192, 205	55C	UNH910	93, 107	55C	UNH973	127, 132, 147, 136	55C
UNH849	155, 191	52C	UNH911	140, 144, 147	55C	UNH974	177, 222, 185	55C
UNH851	117, 122, 111	55C	UNH913	98, 109, 115	55C	UNH977	132, 152, 143	55C
UNH853	172, 174, 186	55C	UNH914	162, 183, 146	50C	UNH979	239, 251, 269	55C
UNH854	225, 229	52C	UNH915	145, 147, 153	55C	UNH980	212, 221, 223	55C
UNH855	152, 162	55C	UNH916	135, 163, 142	55C	UNH982	106, 120, 130	55C
UNH856	189, 197	55C	UNH917	197, 222	55C	UNH985	133,155	55C
UNH857	147, 179	52C	UNH918	105, 134	55C	UNH986	193, 204, 266	55C
UNH858	257, 280	50C	UNH919	182, 188, 192	53C	UNH988	204, 210, 207	55C
UNH860	191, 229, 233, 241	55C	UNH920	150, 260	50C	UNH989	142, 150, 152	55C
UNH863	140, 164, 169	55C	UNH921	199, 211, 215	53C	UNH990	150, 155, 162	55C
UNH865	218, 228	50C	UNH923	131, 151	55C	UNH991	164, 166	55C
UNH866	153, 162	55C	UNH925	220, 242	55C	UNH993	161, 186, 190	55C
UNH868	216, 220	50C	UNH927	196, 204, 229	53C	UNH994	204, 215, 223	55C
UNH869	149, 151	55C	UNH929	134, 172	53C	UNH995	219, 236	55C
UNH871	219, 328	50C	UNH931	199, 203, 240	53C	UNH996	194, 204, 206	55C
UNH874	209, 219, 236, 225	55C	UNH932	122, 124, 143	53C	UNH997	125, 130	55C
UNH875	135, 150	52C	UNH933	241, 243, 245	53C	UNH998	112, 116, 120	55C
UNH876	217, 247, 249	52C	UNH934	221, 241	53C	UNH999	108, 110, 105	55C
UNH878	155, 166, 171	55C	UNH937	187, 193	53C	UNH1000	136, 143	55C
UNH879	195, 203	55C	UNH940	153, 157, 189	53C	UNH1003	167, 189, 160	55C
UNH880	146, 204, 182	55C	UNH942	133, 135	53C	UNH1004	166, 177, 185	55C
UNH884	105, 127, 145	55C	UNH943	137, 146	53C	UNH1005	144, 157	55C
UNH886	163, 172, 174	55C	UNH946	153, 178	55C	UNH1007	155, 247	55C
UNH887	155, 161, 172, 178	55C	UNH948	180, 198	55C	UNH1008	95, 99	55C
UNH888	212, 319	55C	UNH949	154, 158	55C	UNH1009	148, 179, 189	55C

Table 2-2. Allele sizes and PCR annealing temperature for *Oreochromis* markers.

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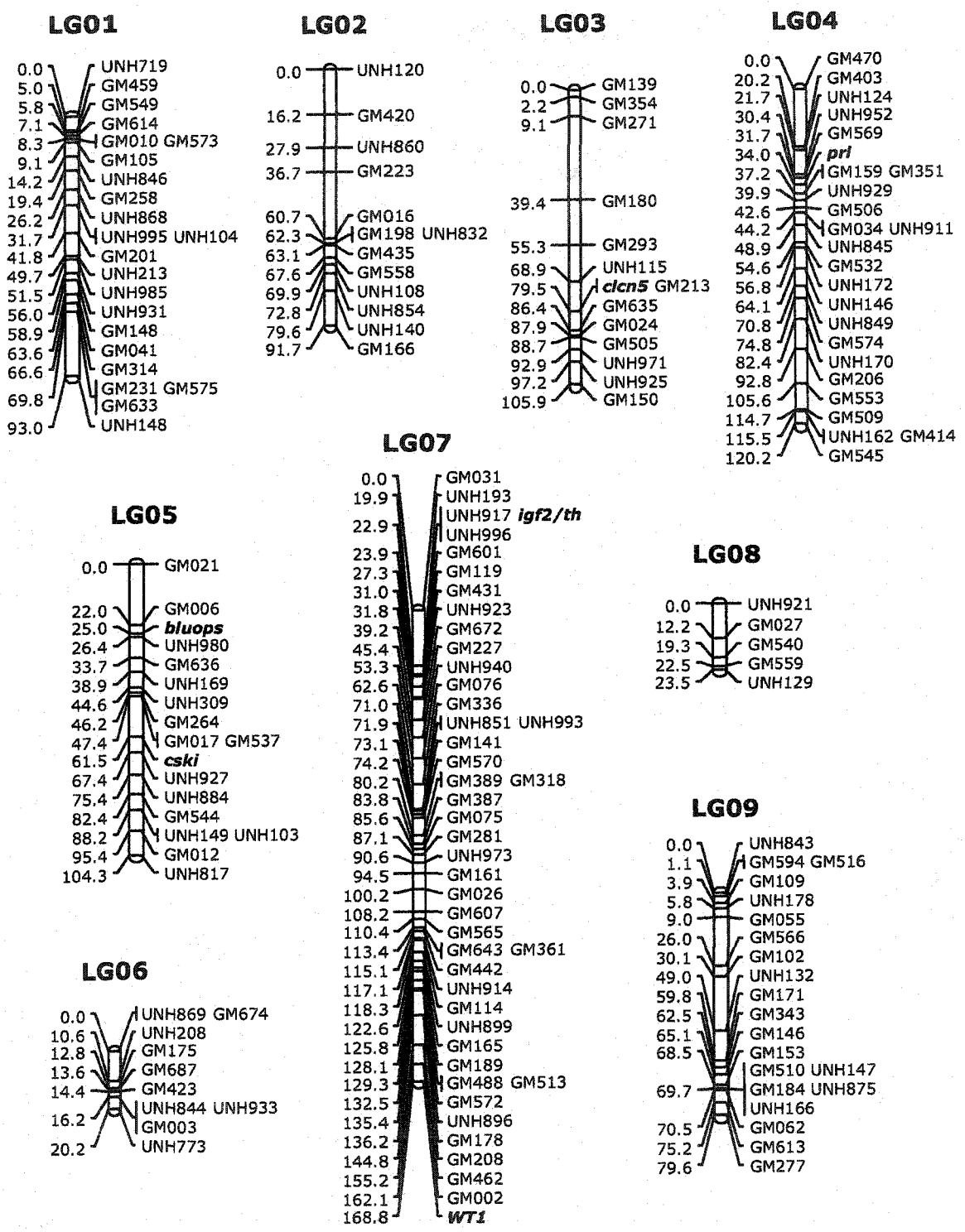

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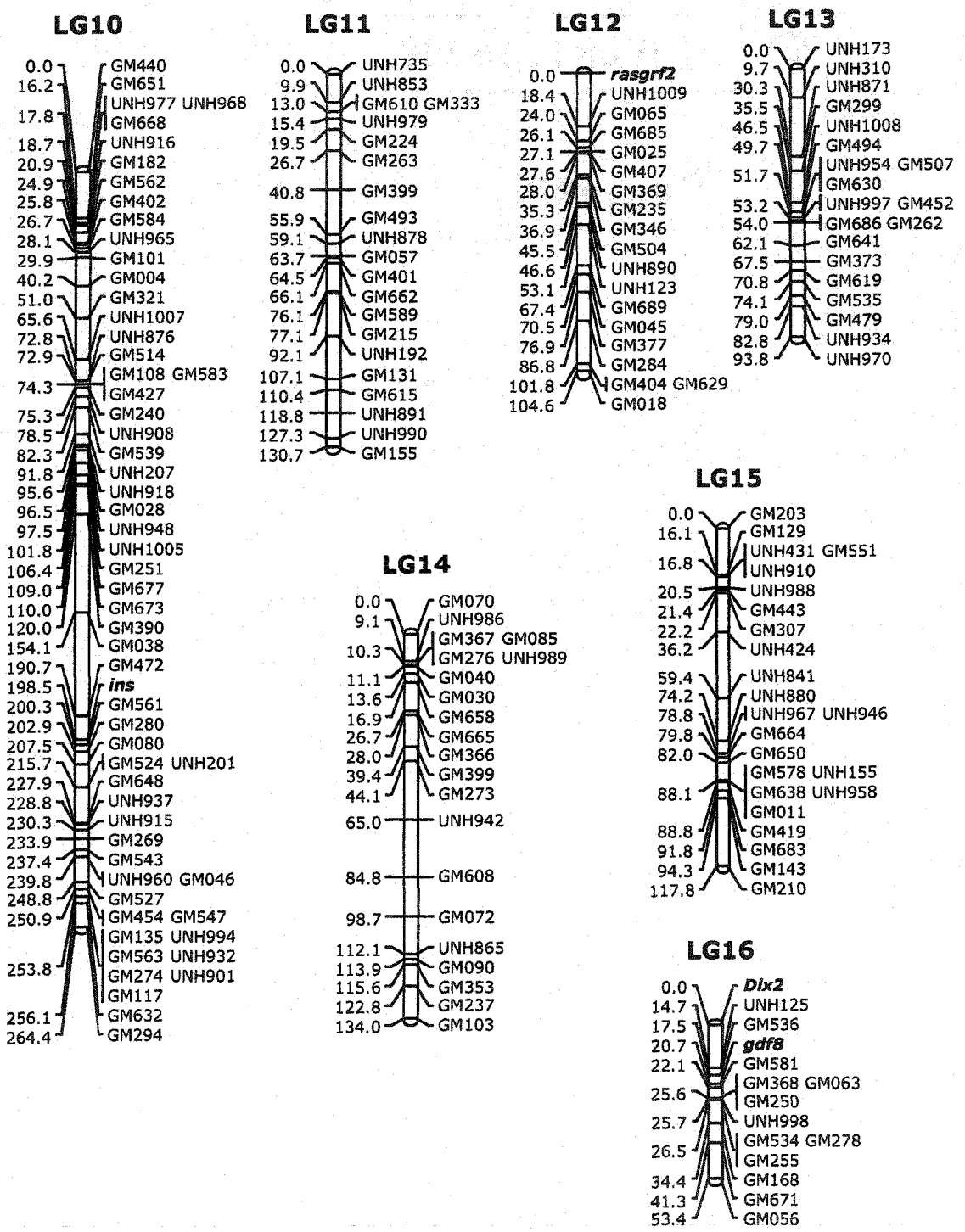

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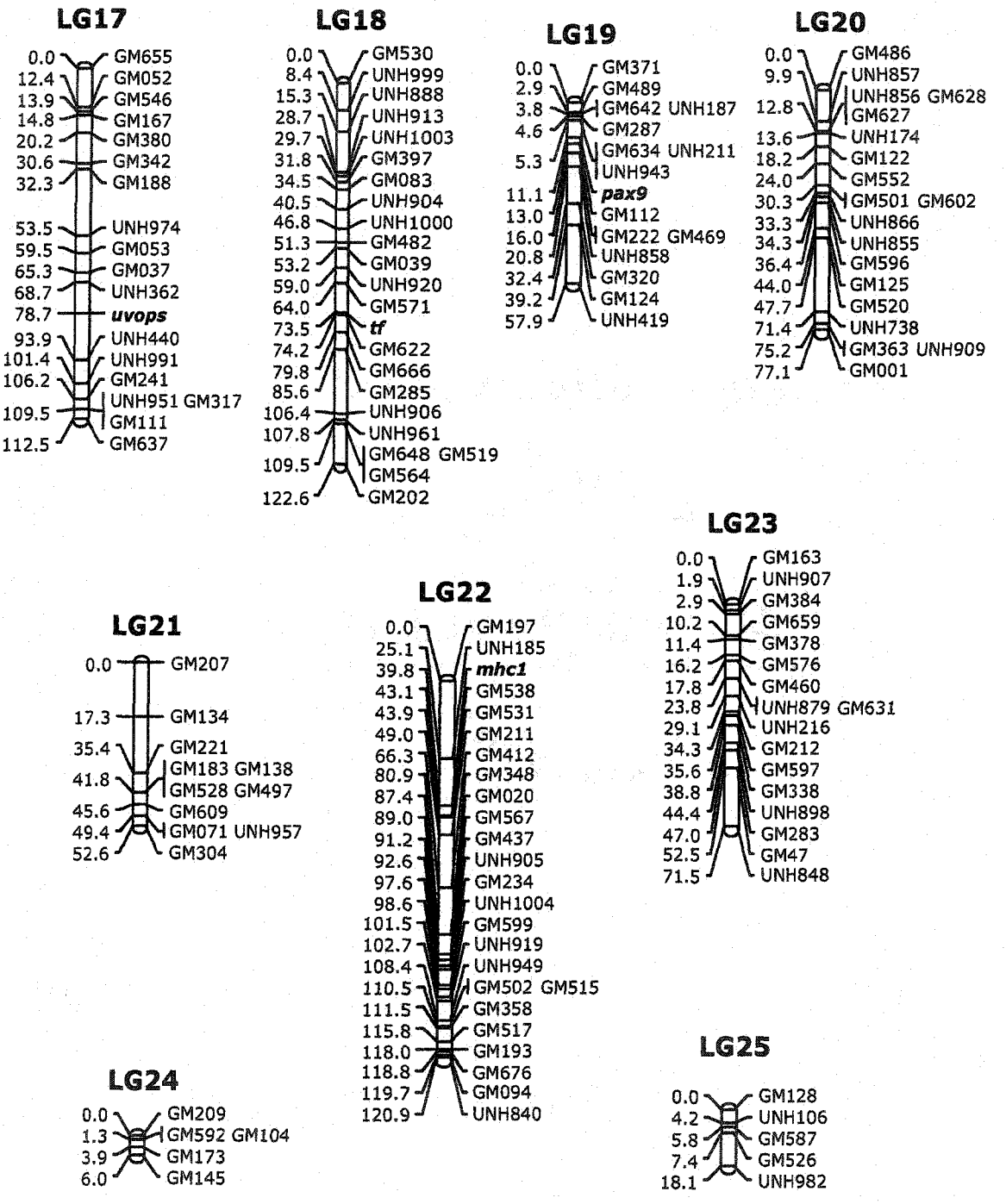

Figure 2-1. Sex-averaged genetic linkage map of tilapia species (*Oreochromis aureus* X *O. niloticus*).

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LG	No. of Markers			sex averaged	Sex-Sepecific (cM)		% difference
	UNH	GM	Genes	(cM)	Female	Male	female vs male
1	9	14		93	80	104.9	-23.7
2	6	7		91.7	52.5	130.7	-59.8
3	3	10	*clcn5*	105.9	212.6	143.7	47.9
4	10	14	*prolactin*	120.2	105.7	112.4	-6.0
5	8	8	*cski, bluops*	104.3	92.6	116.5	-20.5
6	5	5		20.2	23.4	14.9	57.0
7	11	32	*igf2/th, wt1*	168.8	134.1	201.9	-33.6
8	2	3		23.5	20.9	25.7	-18.7
9	6	15		79.6	99.9	53.6	86.4
10	18	40	*insulin*	264.4	270.1	263.2	2.6
11	7	14		130.7	138.2	125.5	10.1
12	3	15	*rasgrf2*	104.6	104.9	101.5	3.3
13	8	12		93.8	80.9	105.4	-23.2
14	4	17		134	163.6	109.8	49.0
15	10	14		117.8	118.1	117.6	0.4
16	2	11	*dlx2, gdf8*	53.4	50.3	37.7	33.4
17	5	13	*uvops*	112.5	121.9	106.5	14.5
18	9	13	*tf*	122.6	56.3	189.7	-70.3
19	5	10	*pax9*	57.9	44	50.4	-12.7
20	7	12		77.1	61.4	94.6	-35.1
21	1	10		52.6	139.3	35.6	291.3
22	6	18	*mhc1*	120.9	111.7	130.5	-14.4
23	5	12		71.5	90.3	52.5	72.0
24	0	5		6	3.7	8.2	-54.9
25	2	3		18.1	17.9	18.3	-2.2
Total	152	327	14	2345.1	2394.3	2451.3	-2.3

% difference is calculated as((female size-male size)/male size)x100

Table 2-3. Summary of *Oreochromis* spp. genetic linkage map.

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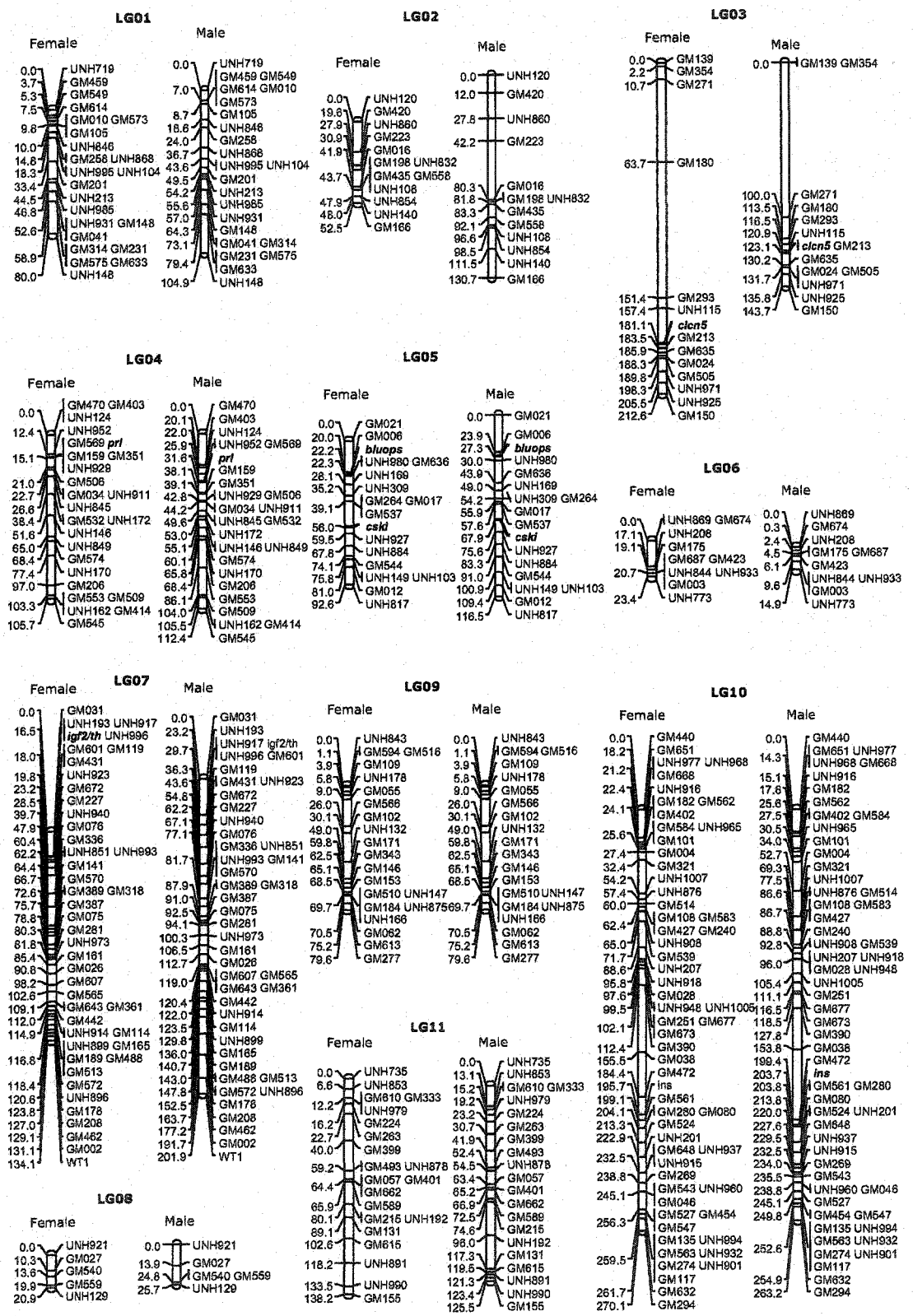

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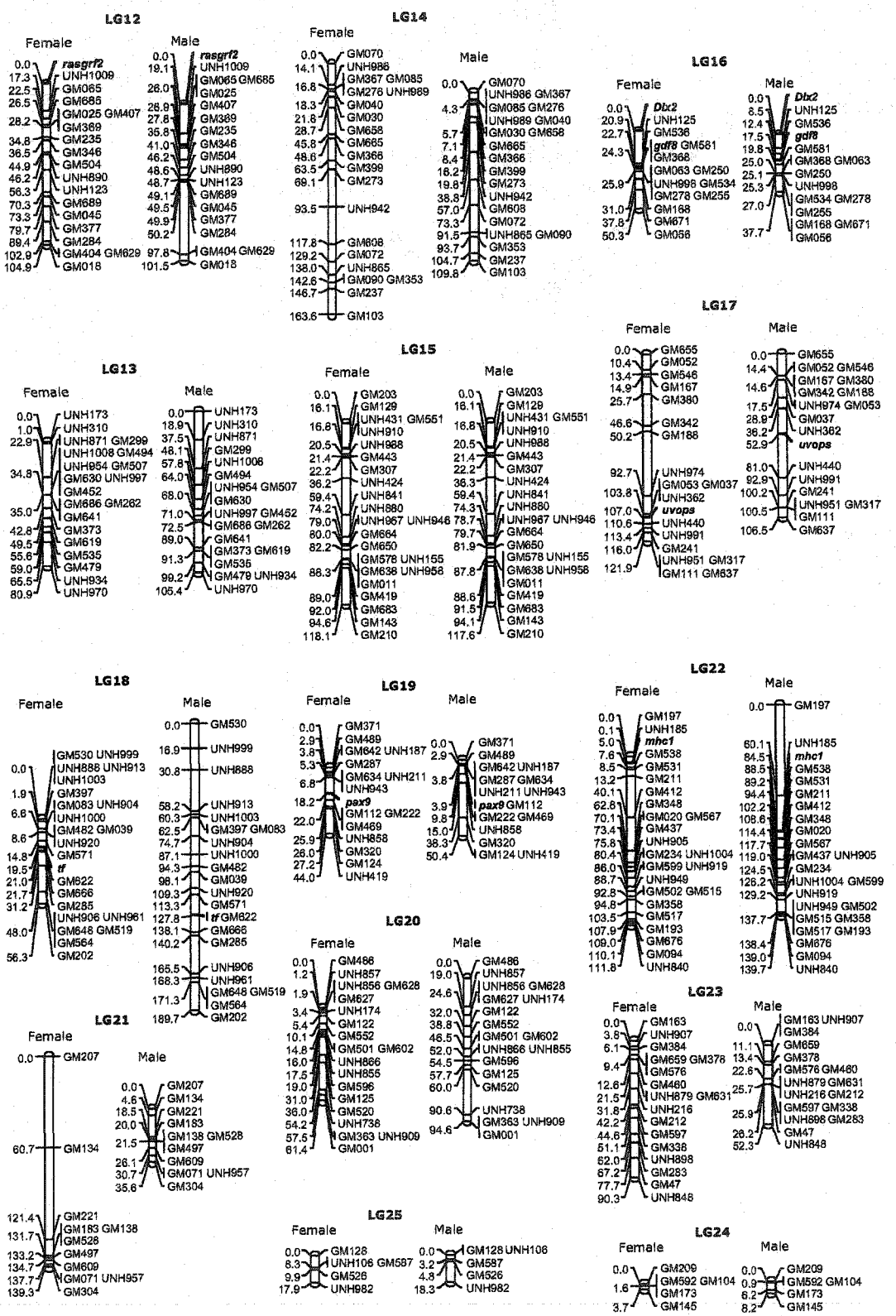

Figure 2-2. Sex specific genetic linkage maps of tilapia species (*Oreochromis aureus* X *O. niloticus*)

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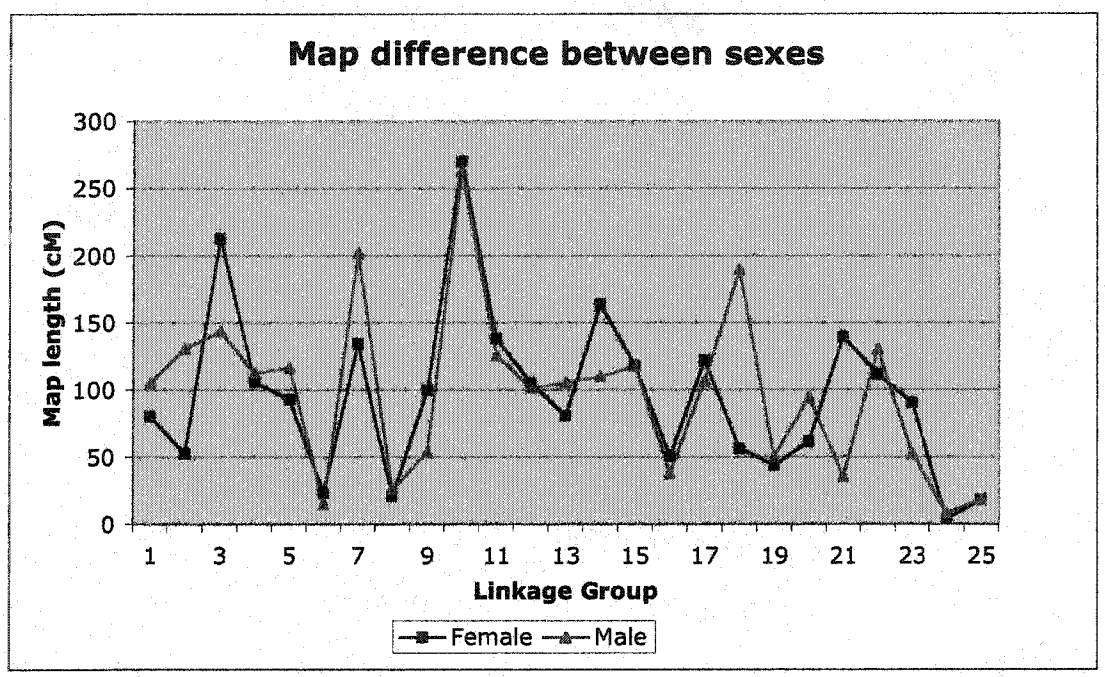

Figure 2-3. Map differences between sexes by linkage map. Green triangles represent female maps and blue squares show male map.

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Marker	K*	df	p-value	Map
GM271	33.689	2	p<0.0001	yes
GM354	28.875	1	p<0.0001	yes
UNH168	20.009	2	p<0.0001	no
GM204	19.108	2	p<0.0001	no
UNH131	15.298	1	p<0.0001	no
GM139	10.599	1	p<0.005	yes

Table 2-4. Sex associated markers detected by the Kruskal-Wallis test at $p < 0.005$.

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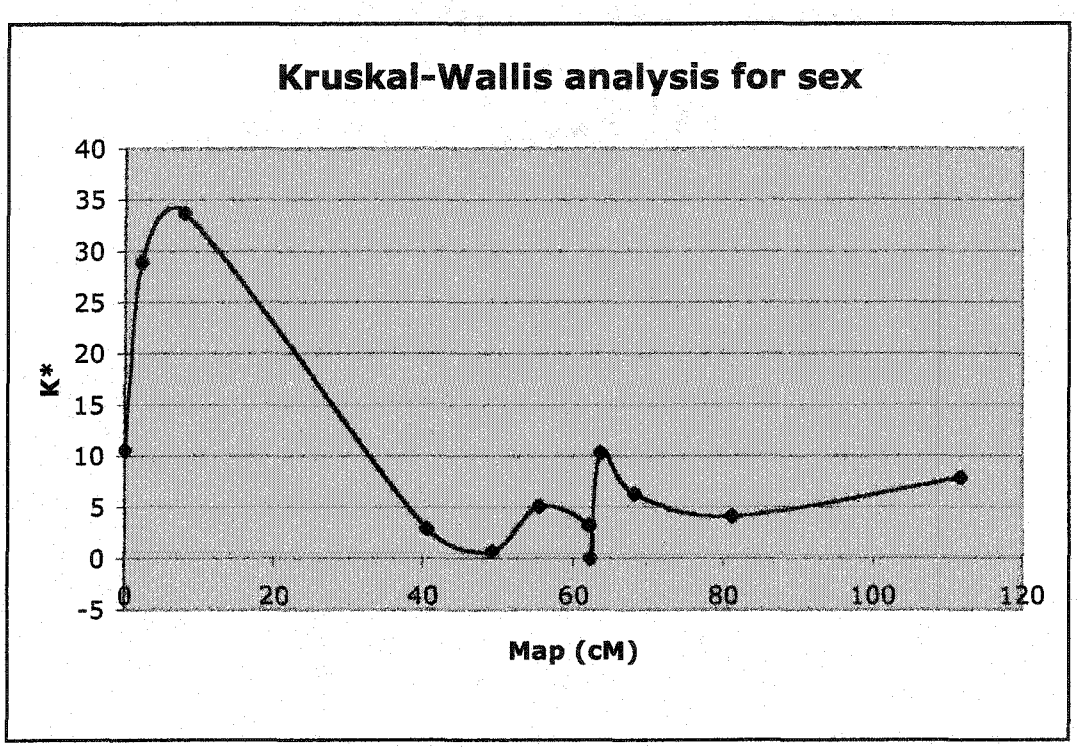

Figure 2-4. The result of the Kruskal-Wallis test for sex trait on LG3.

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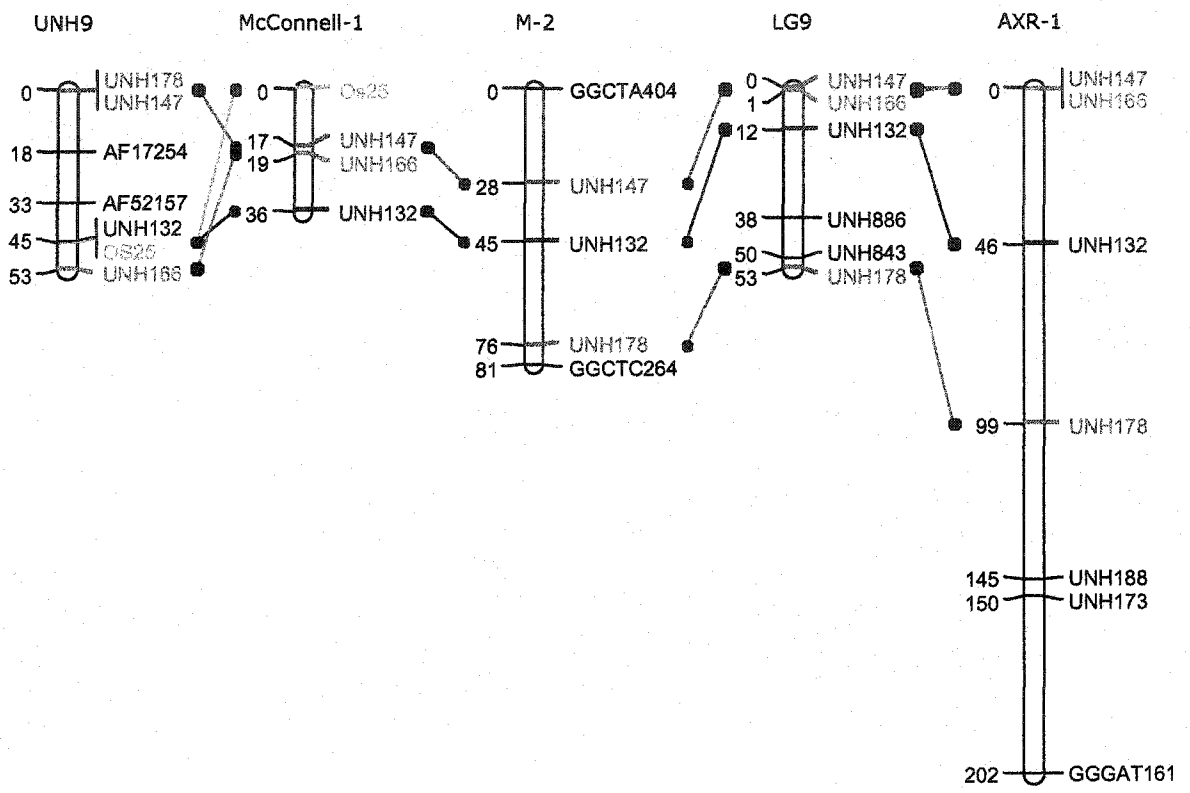

Figure 2-5. Comparison of a linkage group from different tilapia linkage maps. UNH9 - female *On* (Kocher, et al., 1998); AXR-1 and M-2 - male *Oa* X R*On* and female *Om* (Agresti, et al., 2000); McConnell-1 - male *Oa* (McConnell, et al., 2000); and LG9 – *Oa* X *On*.

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CHAPTER 3.

IDENTIFICATION OF A SEX-DETERMINING REGION IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*) USING BULKED SEGREGANT ANALYSIS

Abstract

Sex determination in the Nile tilapia (*Oreochromis niloticus*) is thought to be an XX-XY (male heterogametic) system controlled by a major gene. I searched for DNA markers linked to this major locus using bulked segregant analysis. Twelve microsatellite markers belonging to linkage group 1 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. The 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

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of the Y haplotype. This chapter has been published as "Lee, Bo-Young, David J. Penman, and Thomas D. Kocher, 2003. Identification of a sex-determining region in Nile tilapia (*Oreochromis niloticus*) using bulked segregant analysis. Animal Genetics 34(5): 379-383".

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Introduction

Unwanted spawning by tilapia in aquaculture ponds results in overcrowding, and eventually the harvest of stunted and unmarketable fish. In most culture systems, monosex male tilapia produce greater harvested yields than mixed-sex populations. In mixed-sex populations, there is usually a size differential between the sexes at harvest, with males up to 50% larger, depending on the system (Mair, 2001). Commercial production of tilapia therefore relies on the stocking of all-male fingerlings. There are several ways to produce all-male fingerlings: manual sexing, interspecific hybridization, hormonal sex reversal, and genetic breeding. The sorting of sexes by hand is considered inefficient due to the difficulty of accurately sexing fish at small sizes. So, manual sexing is now rarely applied to produce monosex progeny. Monosex hybrids result from the differences in the sex mechanism between tilapia species. The hybrid crosses of most commercial interest are those using *O. niloticus* females (XX) crossed to males of either *O. aureus* (ZZ) or *O. hornorum* (ZZ). The major disadvantage of hybridization is that hybrids normally exhibit characteristics between those of the two parents. Although *O. niloticus* is considered the best species for the tilapia production, the culture performance of *O. niloticus* hybrids is usually inferior to that of the pure species. Hormonal sex reversal is presently the most widespread technology in use for production of monosex or near-

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monosex male tilapia, but the issue of legislative approval of the procedure and consumer acceptance of hormone-treated fish is a major challenge. Mair et al. (1997) developed a genetic breeding program based on a combination of hormonal feminization and progeny testing to produce YY supermale (Figure 3-1), which can be bred with normal XX females to produce all-male fingerlings. However, this method is not entirely reliable, in part because of its technical complexity, but also because the sex of tilapia is affected by high temperatures (Baroiller, 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; Karayucel, et al., 2004), which can cause deviations from the sex ratios predicted by simple gonosomal models.

Gynogenesis has 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 meiogynote offspring of some females (Mair, et al., 1991; Hussain, et al., 1994; Muller-Belecke and Horstgen-Schwark, 1995; Sarder, et al., 1999).

To this point, no other genetic markers for the major sex-determining locus in *O. niloticus* have been described. We have recently constructed a linkage map for an interspecific cross of tilapia (*Oreochromis aureus* X *O. niloticus*) using about 500 microsatellite markers (Chpter 2.). A putative sex-determining region was discovered on LG3 in this cross. However, it turned out that these markers

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were not associated with sex in pure *O. niloticus* families (Table 3-1). In order to identify a major sex-determining locus in *O. niloticus*, I scanned the *O. niloticus* genome using bulked segregant analysis (BSA) and microsatellite markers from each linkage group.

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Materials and Methods

Fish families and DNA extraction

Three families of tilapia (*Oreochromis niloticus*) produced at the University of Stirling were used for this study. After sexing by inspection of gonads at Stirling, fin-clips from each fish were sent to the University of New Hampshire for genotyping. Sample sizes 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). DNA was extracted from each fin-clip using a standard phenol/chloroform method (Kocher, et al., 1989).

Marker Selection

One hundred and five microsatellite markers were selected 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.; Chapter 2). Those markers consisted of 102 microsatellite markers and 3 genes (*clcn5*, *rasgrf2*, and *bluops*).

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Bulked Segregant Analysis

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 a DyNA Quant2000 (Amersham Pharmacia Biotech, Piscataway NJ) and each DNA was diluted to a final concentration of ~ 10 ng/ul. This allowed us to equalize the contribution of each individual to the pool. 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. PCR was performed in a total volume of 20 ul 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). PCR products were run on 4% gels using an ABI377 and fragment sizes were analyzed using ABI GeneScan software (ver. 3.1.2).

Genotyping of Individuals

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For those markers that showed a quantitative difference in allelic composition in the pooled DNA, I repeated the genotyping on individual DNA samples using the same PCR conditions described above. I also genotyped individuals for additional markers on the same linkage group. Goodness-of-fit (G-tests) were used to assess whether there were significant differences in the genotypic distributions between males and females (Sokal and Rohlf, 1995).

Mapping of Sex-linked Markers

All genotypes for Family 5 and Family 7 were used to make a linkage map. Linkage analysis was performed by CRIMAP (ver. 2.4) using TWOPOINT command with a LOD of 3.0. Map orders were decided by ALL and confirmed by FLIPS. Sex average map and sex-specific map were made by BUILD.

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Results

Identification of Sex-linked Markers in Pooled DNA.

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. Figure 3-2 shows the chromatograms demonstrating the differences in allelic distribution between pools of male and female DNA for marker UNH985. The 133 bp allele of UNH985 was found at high frequency only in the male pools. This result encouraged me to repeat the genotyping on individual animals for these and other linked markers. Figure 3-3 shows sex-specific maps of this linkage group. The female map is 32 cM longer than the male map.

Analysis of Sex-associated Markers on Linkage Group1.

The genotypic proportions in males and females, and the associated G-tests, are shown in Table 3-2. All of the markers on linkage group 1 (LG1) showed significant differences in genotypic frequency between males and females in Families 5 and 7. I analyzed the inheritance of multilocus haplotypes

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in Families 5 and 7, and scored the proportion of individuals whose phenotypic sex was consistent with the hypothesized Y chromosome (Figure 3-4). The graph shows a peak around 50 cM in females and 60 cM in males, which corresponds to markers GM201, UNH995 and UNH104. Flanking markers show a decreasing correspondence with phenotypic sex, as recombination breaks up the sex-linked haplotype. However, significant associations are seen with markers as much as 30 cM from the peak.

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Discussion

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 Y-haplotype, for 12 microsatellite markers on LG1 (Figure 3-5). The sex-determining locus appears to lie near markers GM201, UNH995 and UNH104. The microsatellite genotype in this region predicts phenotypic sex with 95% accuracy in Families 5 and 7. The remaining 5% of individuals whose sex is 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 1 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.

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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; Figure 3-1). 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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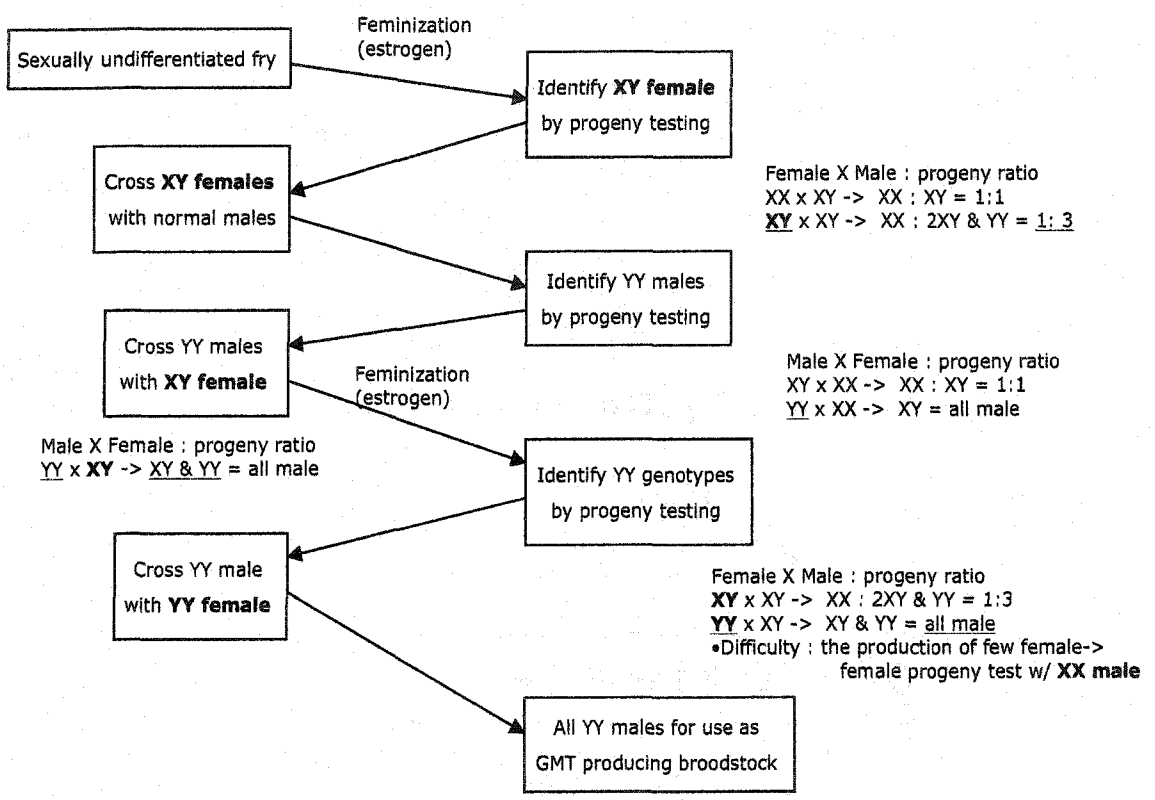

Figure 3-1. Schematic diagram of the genetic breeding program for the large-scale production of YY male broodstock.; Bold type represents hormonally sex-reversed fishes.

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	Population size	Allele frequencies			Genotype frequencies		
Family	(M/F)	GM271	GM354	GM204	GM271	GM354	GM204
F2	26 (14/12)	0.375	0.009	0.009	0.267	0.018	0.018
F5	56 (23/33)	0.11	0.58	0.28	0.25	0.7	0.56
F7	49 (23/26)	0.33	NI	NI	0.33	NI	NI

- NI : Noninformative
- $P > 0.05$

Table 3-1. G-test of 3 markers in pure *O. niloticus* families by allele and genotype frequency. None of them were sex-associated in these families

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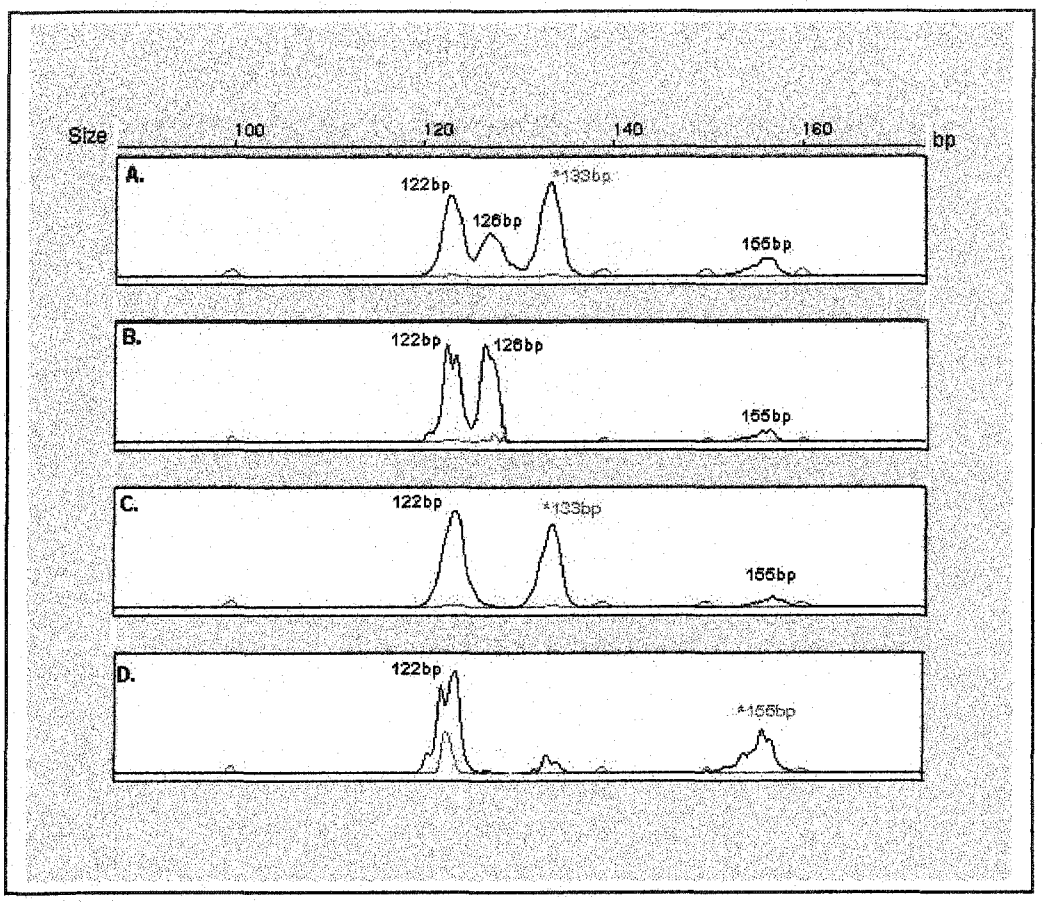

Figure 3-2. Identification of allele frequency differences at marker UNH985 by bulked segregant analysis. The chromatograms show that a 133 bp allele is present in males (panel A) but not females (panel B) from Family 5. Panels C and D show the pattern in males and females from Family 7.

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	Family2				Family5				Family7			
Marker	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	
					192/186	3	4					
					192/226	3	5					
UNH985	127/127	5	9	0.82	123/127	11	2	18.04***	123/133	2	17	26.17***
	127/125	5	4		123/133	2	10		123/156	22	4	
					156/127	8	4					
					156/134	1	7					
UNH931	199/199	6	7	0.16	205/199	9	2	18.58***	199/218	5	1	19.35***
	199/203	6	5		205/240	2	10		199/240	1	8	
					218/199	10	3		205/218	13	3	
					218/240	1	7		205/240	2	9	
UNH148	150/150	6	9	0.51	152/150	18	3	25.01***	152/152	22	5	21.47***
	150/152	6	5		152/161	3	20		152/161	3	17	
UNH213	201/201	5	7	0.16	187/170	1	11	32.42***	190/170	4	17	20.31***
	201/197	6	6		187/201	10	0		190/187	21	4	
					191/170	2	10					
					191/201	9	2					
GM201	189/174	4	7	0.13	190/188	1	22	45.82***	190/190	25	4	38.75***
	189/208	8	7		190/208	22	1		190/188	0	17	
UNH995	206/171	8	7	0.7	165/169	1	11	35.91***	165/169	24	2	41.38***
	206/173	4	7		165/173	7	0		165/219	1	19	
					169/169	2	11					
					169/173	11	1					
UNH104	169/135	5	6	0.36	129/132	2	11	39.36***	129/132	23	2	38.98***
	169/137	7	5		129/137	8	0		129/179	1	19	
					132/132	1	10					
					132/137	11	0					
UNH846	205/175	3	8	2.7	185/185	6	0	28.8***	185/173	5	12	7.32**
	205/197	8	5		185/197	14	3		185/175	18	7	
					185/179	1	11					
					179/197	2	9					
GM258	147/128	8	6	1.41	113/113	6	0	28.8***	113/132	19	7	7.57**
	147/132	4	8		113/128	1	11		113/139	6	13	
					139/113	14	3					
					139/128	2	9					
UNH719	124/101	8	7	0.7	116/109	11	5	15.21**	125/109	8	11	3.93*
	124/109	4	7		116/124	1	7		125/123	16	6	
					124/109	3	0					
					124/124	2	10					

*P<.05
**P<.01
***P<.001

Table 3-2. Genotypic proportions for sex-linked markers on LG1 in male and female *Oreochromis niloticus*.

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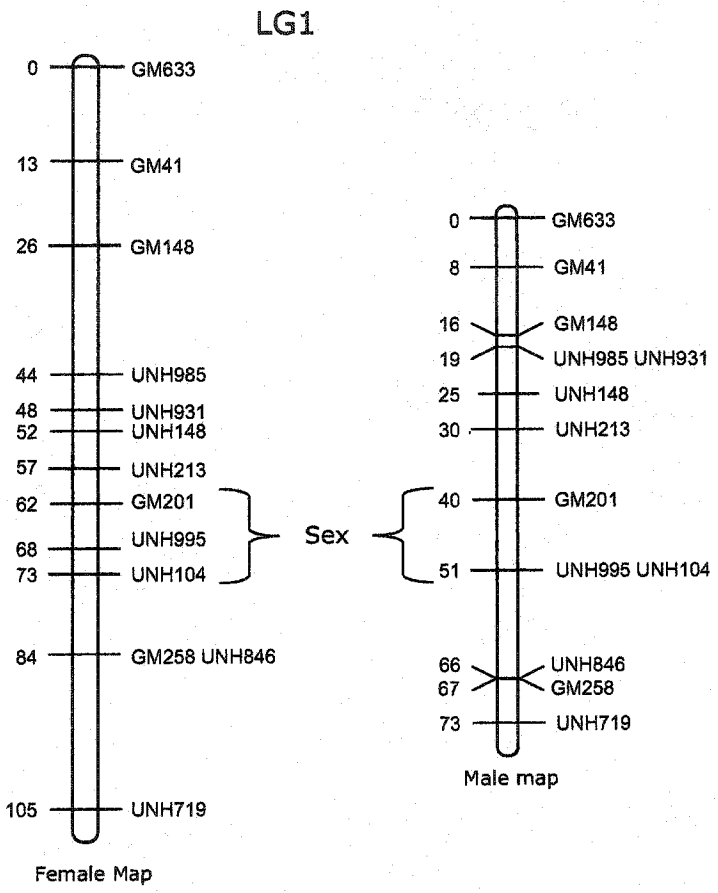

Figure 3-3. Sex-specific linkage maps for LG1 in *Oreochromis niloticus*.

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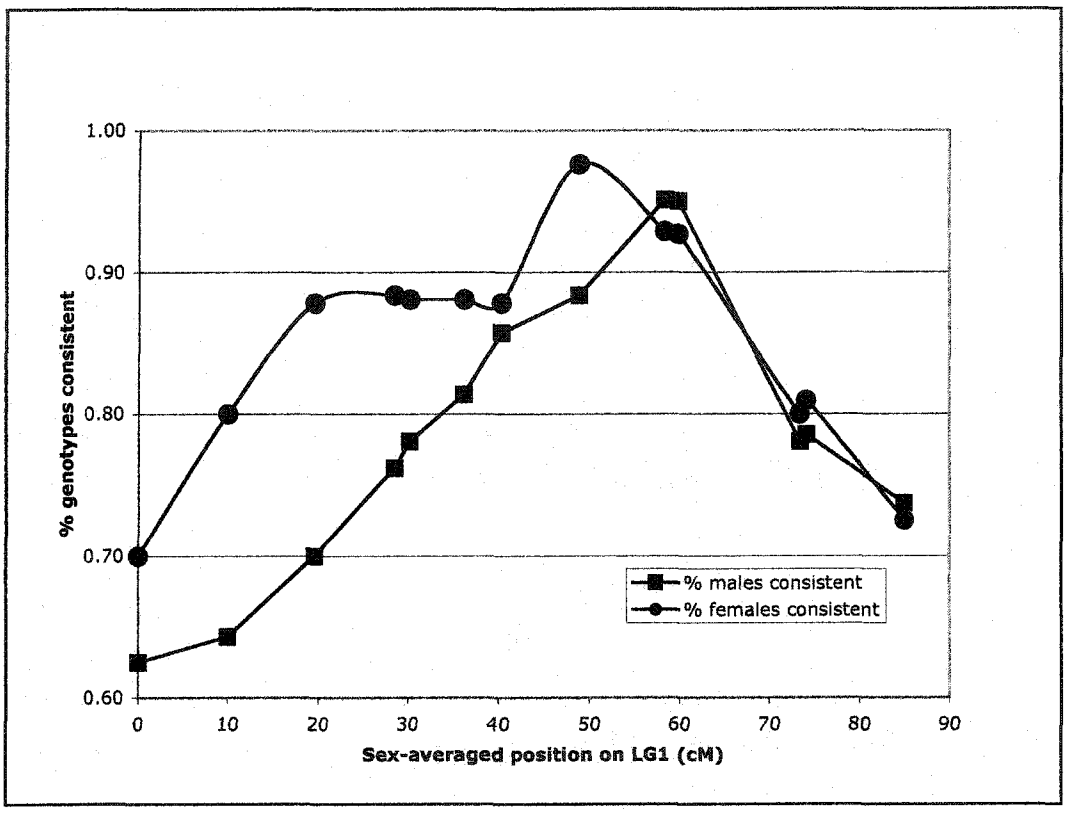

Figure 3-4. Proportion of males (■) and females (●) in Families 5 and 7 whose phenotypic sex is consistent with the hypothesized Y haplotype on LG1

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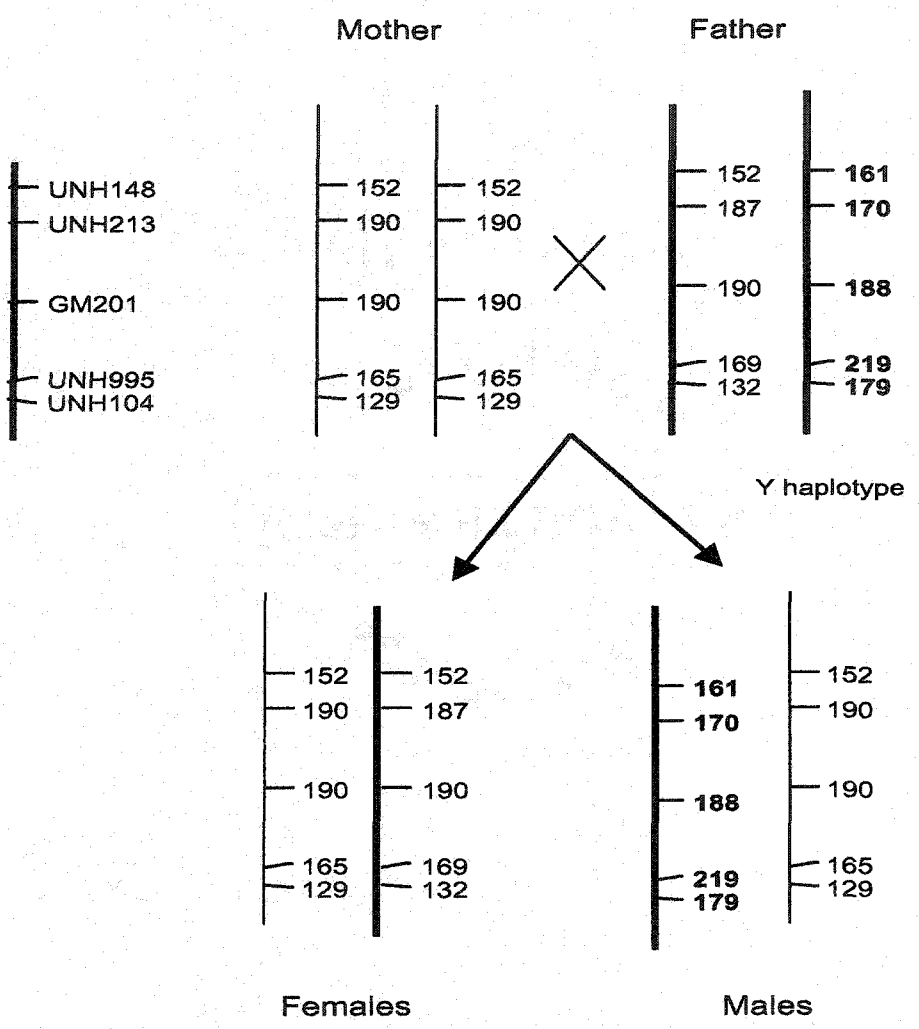

Figure 3-5. Schema of distribution of parental genotype to male and female individuals. Suggestive Y haplotype (bold number) is segregated from father to male offsprings. Meiotic recombination was not considered in this diagram.

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CHAPTER 4.

TWO UNLINKED LOCI CONTROLLING THE SEX OF BLUE TILAPIA (*OREOCHROMIS AUREUS*)

Abstract

Sex determination in the blue tilapia (*Oreochromis aureus*) is thought to be a WZ-ZZ (female heterogametic) system controlled by a major gene. I searched for DNA markers linked to this major gene using the technique of bulked segregant analysis. I 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. The 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 suggest 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. This chapter has been published

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as "Lee, Bo-Young, Gideon Hulata, and Thomas D. Kocher, 2004. Two unlinked loci controlling the sex of blue tilapia (*Oreochromis aureus*). Heredity 92(6): 543-549."

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Introduction

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. Although it is commonly considered that *Oreochromis niloticus* is the best tilapia species for tropical freshwater culture, *O. aureus* or more often its monosex hybrid with *O. niloticus*, is grown in some regions because of the cold tolerance properties of *O. aureus*.

Oreochromis aureus 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 1 female (WW): 1 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

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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 2 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 MacAndrew, 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

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this point, no DNA sequence markers for the major sex determining locus in *O. aureus* have been described. A linkage map for tilapia has been constructed using more than 550 microsatellite markers (Lee, et al., in prep.; Chapter 2.). Here I used markers selected from this map to rapidly scan the genome for sex-linked markers in bulked segregants. I then studied genotypes of individual fish to localize the sex-determining regions and study epistatic interactions among loci.

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Materials and Methods

Fish source and DNA extraction

A single family of tilapia (*Oreochromis 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 ~4 months (at a mean size of ~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

I selected 119 microsatellite markers, at intervals of approximately 20 cM, based on a linkage map produced from an F_2 population from the interspecies

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cross of *O. aureus* with *O. niloticus* (Lee, et al., in prep.; Chapter 2; http://hcgs.unh.edu/comp). Those markers consisted of 92 UNH markers, 24 GM markers, and 3 genes (*clcn5*, *rasgrf2*, and *uvops*), all of which are deposited in GenBank.

Bulked Segregant Analysis

To accelerate the identification of sex-linked markers, I used the technique of bulked segregant analysis (Michelmore, et al., 1991). I 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) and each DNA was diluted to a final concentration of ~ 10 ng/ul. This allowed equalization of the contribution of each individual to the pool. PCR was performed in a total volume of 20 ul for 2 min at 94°C followed by 28 cycles of 30s at 94°C, 30s at 55-60°C, 60s 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). PCR products were separated on an ABI377 DNA sequencer and fragment sizes were analyzed using ABI GeneScan (ver. 3.1.2) software (Applied Biosystems, Foster City CA).

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Genotyping and Statistics

For those markers that showed a qualitative difference in allelic composition in the pooled DNA, I repeated the genotyping on individual DNA samples from 48 females and 45 males using the same PCR conditions described above. I genotyped these individuals for all available markers on LG1 (9 markers) and LG3 (11 markers) (Table 4-1). Goodness-of-fit (G-tests) were used to assess whether there were significant differences in genotypic distributions between males and females (Sokal and Rohlf, 1995). 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 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.

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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 9 markers (*clcn5*, 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 (Figure 4-1). Alleles unique to the male pool were found at GM271 (121 bp) and GM354 (129 bp). UNH971 had 230 bp 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. I tested another marker (UNH424) located only 5 cM from GM210, but

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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 me 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 4-1. The strongest associations were with a female-determining haplotype on LG3. All individuals with the 187 bp allele at UNH131 were females. Figure 4-2 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

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on LG1. All 193/193 (UNH131) individuals with an 189 bp allele at UNH104 were males (Table 4-2). Ten 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 4-3). 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.

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Discussion

I detected two unlinked loci which interact to determine sex in this family of *O. aureus*. The interpretation of the data, and the 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 187bp 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 189bp 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.

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These 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 1970's 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.

A dilocus genotype, however, must be considered here. As the allelic state for the LG3 WZ locus in *O. niloticus*, or the allelic state for the LG1 XY locus in *O. aureus* was not known, the *O. niloticus* female was considered 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 52-100% 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 (e.g. 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

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putative ZZXX genotypic class of our family is consistent with the influence of additional 'autosomal' loci affecting sex ratio.

It is now commonly accepted [e.g. 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 this 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*.

The results of this study 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. Because the homozygous ZZXX animals in our family are of mixed sex (67% male), additional genetic factors may be affecting determining the sex of these homozygotes. It is worth noting that no effect of the putative WZ locus was observed in three families of *O. niloticus*

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(Lee, et al., 2003; Chapter 3). 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. A similar sex-specific pattern of recombination was observed in this study, and a centromere will be predicted to be 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). The results of tilapia are also consistent with these patterns, suggesting they may be general for teleost fishes.

Theses studies begin to explain the variety of sex ratios which 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 which give a higher percentage of males for commercial production.

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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<.05
**p<.01
***p<.001

Table 4-1. Genotypic proportions for sex-linked markers in male and female *O. aureus*. Asterisks (*) indicate the Bonferroni-corrected p-values for each test.

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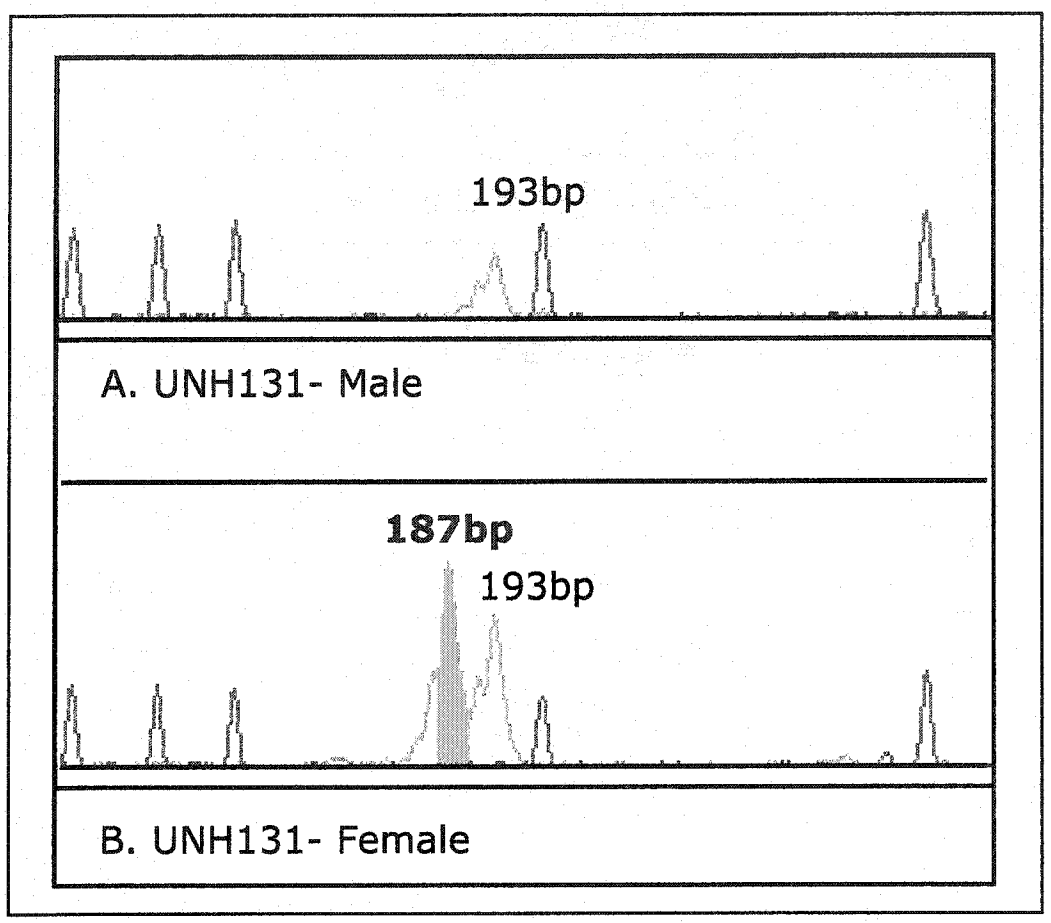

Figure 4-1. Identification of allele frequency differences at marker UNH131 by bulked segregant analysis. The chromatograms show that a 187 bp allele is present in females but not males.

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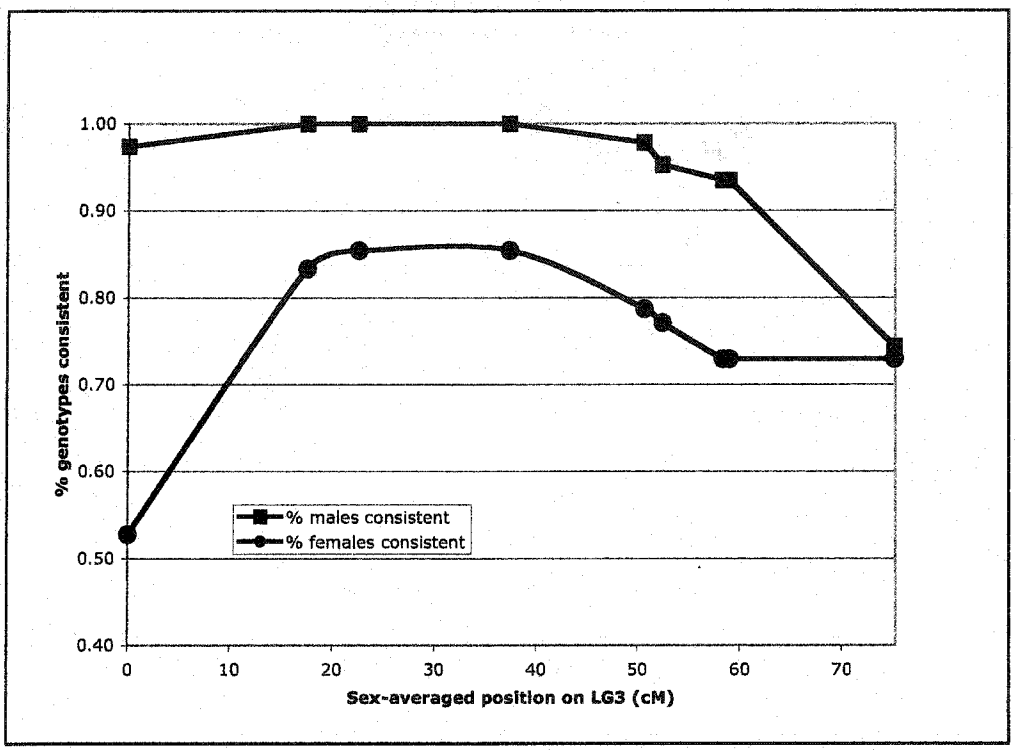

Figure 4-2. Proportion of individuals whose phenotypic sex is consistent with the hypothesized female haplotype on LG3. The map spans markers GM139 to GM150; markers UNH168 and GM271 both map to 22.6 cM. The parents were not informative for marker GM635 (88 cM).

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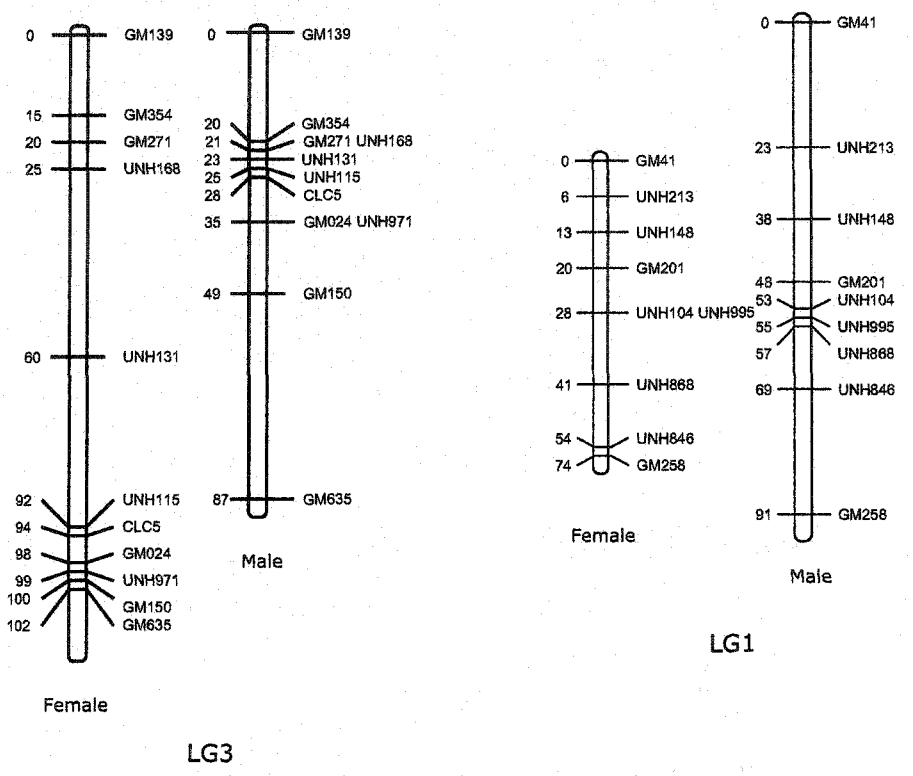

Figure 4-3. Sex-specific linkage maps for LG3 and LG1 in *Oreochromis aureus*.

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

Table 4-2. Epistatic interactions of the sex determining loci on LG1 and LG3 in a family of *O. aureus*. 'A' stands for alleles other than 189 bp at locus UNH104 (e.g. 137, 181 or 185 bp).

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CHAPTER 5.

COMPARATIVE POSITIONAL CLONING OF CANDIDATE GENES IN THE SEX-DETERMINING REGIONS IN TILAPIA

Abstract

A comparative positional cloning strategy, using information from the complete human genome and pufferfish sequences, was used to predict the candidate genes involved in sex determination of tilapia. BAC clones containing genetic markers in the sex-determining regions of LG1 and LG3 were isolated. BLAST analyses of shotgun and end sequences of these BACs identified syntenic regions among pufferfish, tilapia, and human. A few candidate genes include a DEAD box protein, Sox family, and a Lim/homeobox. AFLP/BSA analysis identified a few selective primer pairs that appeared to be sex-specific, but these polymorphisms did not map in the sex-determining region of LG1 in *Oreochromis niloticus*.

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Introduction

Comparative genomics has played an important role in identifying genes (Lander, et al., 2001; Venter, et al., 2001, and Aparicio, et al., 2002). The pufferfish (*Fugu rubripes*) has been considered as a model organism for whole genome sequencing because the *Fugu* genome has approximately the same number of genes as human, even though it has the smallest vertebrate genome (400 Mb) (Brenner, et al., 1993). Short introns and a scarcity of repetitive DNA in this compact genome simplifies comparative analysis of sequence and the identification of genes. Comparative analysis between *Fugu* and other vertebrates has also been considered useful for identifying conserved regulatory elements of important genes (Aparicio, 1995). There are several patterns of conservation of synteny within genomic sequence between *Fugu* and human (Clark, et al., 2001): complete conservation of gene order (Brunner, et al., 1999), conservation of synteny, but not gene order (Gellner and Brenner, 1999), and extensive differences in gene order within regions of conserved synteny (Gilley and Fried, 1999).

Although tilapia has a genome size 2.5 times bigger than *Fugu*, it is closely related to the pufferfish. The *Fugu* genome sequence is therefore useful for identifying candidate genes by comparative mapping. To identify candidate genes responsible for sex determination in tilapia, I took a strategy of

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comparative positional cloning using the results from QTL analyses. This approach basically starts with genetic linkage analysis to locate the map position of sex determining genes. I have discovered the chromosomal regions on LG1 and LG3 that are involved in the sex determination in tilapia species, as I described in chapter 3 and 4 (Lee, et al., 2003; Lee, et al., 2004). Based on this information, I identified the BAC clones corresponding to the genetic markers in the sex-determining regions on both LG1 and LG3. I then shotgun-sequenced these BACs and performed BLAST analyses to identify syntenic region between *Fugu* and tilapia.

However, the scarcity of markers in the sex-determining region is a major obstacle to this strategy. The distances between markers surrounding the region are about 10 cM in *O. niloticus* and 16 cM in *O. aureus*. So, I also attempted to identify amplified fragment length polymorphisms (AFLPs) in the sex-determining region by bulked segregant analysis (BSA) (Vos, et al., 1994; Michelmore, et al., 1991). I also performed BAC end sequencing to facilitate chromosome walking of this region. The efforts are focused on *O. niloticus*, because of the relatively smaller gap between flanking markers on LG1.

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Materials and Methods

Pooling of BAC Libraries for PCR Screening

Bacterial Artificial Chromosome (BAC) libraries for Nile tilapia, *Oreochromis niloticus*, were constructed at Tokyo University of Fisheries in Japan (Katagiri, et al., 2001). Replicates of the libraries were transferred to University of New Hampshire (UNH). A total of 24,000 clones from Library 3 and Library 4 were pooled to facilitate PCR screening. Average insert sizes of Library 3 and Library 4 are 134 kb and 191 kb respectively. These BAC pools provide more than 3X coverage of the genome and the probability of finding a given gene is expected to be about 95%. The 250 plates have been organized into 10 groups of 25 plates. Pooling proceeded in five steps using a Biomek 2000 robot (Beckman Coulter): 96 well plate →row pool→ plate pool → superrow and supercolumn → superpool. In order to improve PCR efficiency in screening BAC pools, DNA was extracted from superpools and superrow and supercolumn pools by phenol/chloroform extraction.

PCR Screening the BAC Libraries for Sex Markers

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Each PCR was performed in 50 ul reaction containing 1X PCR buffer (50 ml Kcl; 10 mM Tris-HCl, pH 9.0; 0.1% Triton-X; 2 mM $MgCl_2$), 0.8 mM dNTPs, 0.2 uM each forward and reverse primer, and 1 U of *Taq* DNA polymerase in the cycling condition: 94°C for 3 min; 35 cycles of 94°C for 20 sec, 50-60°C for 30 sec, 72°C for 1 min; 72°C for 5 min. Genomic DNA was amplified as a positive control. All PCR products were run on 2% agarose gels.

Shotgun Library Construction and Sequencing

High quality BAC DNA was isolated using the Qiagen Large-Construct Kit or Psi Big Cloning Kit (Princeton Separations). Isolated BAC DNA was resuspended in TE buffer (pH 8.0) and sheared with a Hydroshear (GeneMachines) using a #025 orifice, a speed code of 5-6, and 20 cycles of shearing. Sheared DNA was end-repaired using the End-It, DNA end-repairing kit (Epicentre), incubating at room temperature for 45 min. Phenol/chloroform extraction was performed to remove the enzymes that inhibit the next steps. End-repaired DNA was A-tailed by incubating at 70-72°C for 15-30 min in 10 ul reaction with Promega *Taq* buffer, $MgCl_2$, 1 mM dATP, 5 unit Promega *Taq*. The A-tailed inserts were ligated into the ampicillin (Amp) resistant pGEM-T cloning vector (Promega) at 4°C for overnight (~15h). The ligation reaction was desalted by ethanol precipitation before electroporation into DH10B competent bacterial

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cells (Invitrogen). Transformed cells were grown on X-gal/IPTG/ Amp agar plate for 16 hours and white colonies were picked for overnight culture. Inserts were then amplified by PCR using the universal primer T7 and M13R. For cycle sequencing, PCR products were purified using solid phase reversible immobilization (SPRI) (Hawkins, et al., 1994). Cycle sequencing was carried out in 10 ul reactions containing 4ul of cleaned PCR products, 0.25 ul T7 primer (10 uM), 4 ul DYEnamic ET Terminator Cycle Sequencing chemistry (Amersham) and 1.75 ul sdH_2O. Sequence reactions were cleaned up using Sephadex G-50 (Amersham) and were run on an ABI377 automated DNA sequencer.

BLAST Analysis

Sequences from shotgun libraries were edited and trimmed to remove vector and ambiguous sequences using Sequencher™4.1 (Gene Codes Corporation, MI). BLAST (Basic Local Alignment Search Tool) analyses were performed at the NCBI (http://www.ncbi.nlm.nih.gov) and JGI (*Fugu* version 3.0) (http://genome.jgi-psf.org/fugu6.home.html). BLASTn (nucleotide sequence comparisons) and/or BLASTx (translated nucleotide sequence compared to protein database) were done against both databases. BLAST hits with e-scores of 1e-05 or smaller were retained for further analysis.

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AFLP/BSA Analysis

From each of two informative Nile tilapia families used in Chapter 3 to identify the sex-determining regions, I constructed two phenotypic pools of 10 male or 10 female fishes based on genotypes at flanking markers. The final concentration of DNA from each individual in the pool was 10 ng/ul. DNA were digested with both *EcoR1* and *MseI* and ligated with adaptor pairs (*EcoRI* F: 5' CTCGTAGACTGCGTACC 3' and R: 5' AATTGGTACGCAGTCTAC 3'; *MseI* F: 5' GACGATGAGTCCTGAG 3' and R: 5' TACTCAGGACTCAT 3') simultaneously in one reaction that contained 1X T4 DNA ligase buffer (30 mM Tris-HCl, pH=7.8; 10 mM $MgCl_2$; 10 mM DTT; 1 mM ATP), 50 mM NaCl, 50 ng/ul BSA, 1 unit *MseI*, 5 units *EcoR1*, 1 unit T4 DNA ligase, 50 pmol *MseI* adaptor pair, 50 pmol *MseI* adaptor pair and 5 pmol *EcoRI* adaptor pair. This reaction was incubated at 37°C for 2 hours and was then diluted 1:20 with TE/10. The diluted restriction-ligation reaction was then PCR amplified using pre-selective primers to create a quantity of partially selected DNA fragments. Preselective primers contained one additional selective base beyond the adaptor sequence (*EcoR* I-5' GACTGCGTACCAATTC[A] 3', *MseI* 5' GATGAGTCCTGAGTAA[C] 3'), which reduced the fragment complexity 16-fold. The cycling conditions were as follows: 72°C for 2 min; 20 cycles of 94°C for 20 sec, 56°C for 30 sec, and 72°C for 2

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min; and finally a hold at 60°C for 30 min. Preselective reactions were diluted 1: 20 with TE/10 and used for selective PCR with selective primers (1 pmol *EcoRI* primer and 5 pmol *MseI* primer). The selective primers contained two additional selective bases beyond that of the preselective primers, which further reduced the fragment complexity a factor of 256. The cycling conditions for the selective amplification were: 94°C for 2 min; 10 cycles of 94°C for 2 min, 66°C 30 sec, 72°C for 2 min decreasing 1°C after each cycle; 20 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min. All selective reactions were run on an ABI377 automated sequencer and gels were analyzed with GeneScan (ver. 3.1.2). (Applied Biosystems). AFLP markers that were specific to one phenotypic pool were then tested on each individual in the family. The genotypes were coded as dominant markers and then mapped in the sex-determining region by CRIMAP.

BAC End Sequencing

For each sequencing reaction, the BAC clone of interest was cultured in 1.5 ml LB with chloramphenicol at 37°C for 20h. DNA was isolated using QIAgen R.E.A.L. prep kit and isopropanol precipitation. The pellet was resuspended in 12 ul water. DNA was preheated at 96°C for 5 min, prior to adding sequencing premix. Cycling sequencing was carried out in 20 ul reactions containing 8 ul DYEnamic ET Terminator Cycle Sequencing chemistry (Amersham), 2 ul

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universal sequencing primer (10 uM), and 10 ul of preheat DNA in the cycling condition as follows; 100 cycles of 95°C for 20s, 50°C for 15s, and 60°C for 60s. Sequence reactions were cleaned up using Sephadex G-50 (Amersham) and were run on an ABI 377 automated DNA sequencer.

Mapping Putative Sex Locus

The putative sex locus was mapped in the sex-determining region using CRIMAP. Males were considered affected individuals (heterozygotes) and females were scored as unaffected individuals (homozygotes).

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Results

Shotgun Sequencing Results of Sex-associated Markers on LG3

Nine markers in the sex-determining region on LG3 were screened to identify corresponding BAC clones. BAC clones were identified for six markers (GM354, GM204, GM271, CLCN5, GM180, and UNH115) but not for 3 markers (UNH131, UNH135, and UNH168). For the 6 markers, each marker was located in 2–4 clones that were used to detect contigs in the FPC database. Each marker had 1-3 contigs including the BAC clones identified by PCR at a tolerance of 5 and e-value of 1e-7. One of the BACs in each contig was chosen for making a shotgun library. Table 5-1 summarizes the results, including BLAST analysis of the sequences. Based on the sequencing results, many repetitive DNAs seem to be accumulated in this region. Many shotgun sequences hit repetitive elements such as SINE, LINE, reverse-transcriptase, and non-LTR retrotransposable element. Especially, sequences from BACs containing GM204 and GM271 hit nothing but repetitive DNAs. From the other libraries, a few sequences blasted to the same *Fugu* scaffold. Three sequences from BAC clone b04TI056AD04 (GM180) hit *Fugu* scaffold 4353 that includes the region of diacylglycerol kinase (DGK). Three sequences from b03TI064CB12 and five from b04TI076CC06 hit *Fugu* scaffold 733 containing Tumor necrosis factor family (TNF) genes. Six

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sequences, from both b03TI064CB12 and b04TI076CC06, hit chloride channel protein 5 (clcn5, *Fugu* scaffold 5668). Three sequences from b04TI056AD04 hit scaffold 4353 and two of them hit the gene diacyglycerol kinase, delta (DGKD). Four sequences from b04TI059DG05 hit unannotated portions of *Fugu* scaffold 2144. Four hundred fifty sequences were obtained from b03TI066DH01 (GM354). Nineteen of them aligned with almost the first half area of the scaffold 1833 that contains annotated genes of phosphoinositide 3-kinase (PIK3) and diacylglycerol acyltransferase (DGAT) (Figure 5-1). The other half of this *Fugu* scaffold is annotated as containing HMG group genes such as sry or sox family.

Sequence Analysis of Sex-Determining Region in LG1

More than 35,000 clones of tilapia (*O. niloticus*) BAC libraries were restriction fingerprinted and FPC analysis was performed to construct contigs (Katagiri et al., in prep). Since two markers (UNH104 and UNH995) showed a strong association with sex determination with 95% accuracy in Nile tilapia, I screened pools of BAC libraries in order to identify BAC clones containing those markers. According to the result of PCR screening, the two markers were very closely linked to each other in the genome because both markers detected the same 4 BAC clones (b03TI094AA08, b03TI094DB09, b03TI094DF07, and b04TI071CD06). Using FPC analysis, I was able to identify a relatively reliable

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contig containing 22 BAC clones including the 4 clones isolated by PCR under the value of t5 1e-7 (Figure 5-2). However, I couldn't find the BAC clones for another strong sex-specific flanking marker (GM201) with PCR screening.

In order to obtain the sequences from the contig containing BACs for the markers (UNH995 and UNH104), I chose 4 BAC clones (b04TI073CB11, b04TI079DH06, B04TI071CD06, and b03TI094DB09) that cover the whole contig and made shotgun libraries. By the shotgun sequencing, a total of 581 sequences were obtained and were subjected to BLAST analysis. The BLAST results are summarized in Table 5-2. An average of 6.1% of the sequences represent repetitive DNAs (SINE, LINE, Rex6 retrotransposon, transposase, reverse transcriptase) and an average of 4.9% of the sequences showed strong similarity to zebrafish repetitive DNAs. Of 4 BAC clones sequenced, b03TI094DB09 showed more than 10% of sequences hit repetitive DNAs. There were a few *Fugu* scaffolds that more than 2 sequences are blasted to, but unfortunately, the scaffolds are not annotated by genes. Of 581 sequences, 44 sequences from three adjoining clones (b04TI071CD06, b04TI079DH06, and b04TI073CB11) and 22 sequences from 2 clones (b04TI071CD06 and b04TI079DH06) in this contig hit scaffold 670 and 1924 respectively. Three BAC clones (b04TI071CD06, b04TI079DH06, and b04TI073CB11), seem to be in a syntenic region with the *Fugu* scaffold 670 (130 kb) and 2 clones (b04TI071CD06 and b04TI079DH06) with the scaffold 1294. Based on information provided by Greg Elgar, (UK Human Genome Mapping Resource Centre), the *Fugu* scaffolds

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670 and 1924 made a supercontig with other scaffolds, 3165, 2342, 1832, 10868, and 1946. Also, 2 sequences from b04TI071CD06 and b03TI094DB09 hit another scaffold 2342, which is one of the scaffolds consisting of the *Fugu* supercontig (Figure 5-3). The 44 sequences that hit scaffold 670 are distributed along the whole scaffold, including intergenic regions and predicted genes (Figure 5-4). The gene models aligned along the *Fugu* scaffold 670 are hypothetical protein FLJ21918, nuclear factor of activated T-cells 3 isoform 3 (NFATc3), DEAD-box family, talins, tropomyosin, and F-box protein (Figure 5-4). Table 5-4 shows the relationship between the shotgun sequences and the *Fugu* gene models annotated on the scaffolds. Of the 44 sequences, 3 showed similarity to NFATc3, 10 to FLJ21918, 9 to Talin, and 1 to DEAD box polypeptide 28 (Ddx28). Of 22 sequences from scaffold 1924, 10 showed similarity to autocrine motility factor receptor (AMFR), 2 to glucosamine-phosphate N-acetyltransferase, and 1 to leukotriene B4 receptor (LTB4R). Most of the genes hit by the sequences appeared to be located in human chromosome 15 and 16. FLJ21918, NFATc3, Ddx28, AMFR are in chromosome 16 and Talin2 in chromosome 15. A BAC end sequence (M13F) of b04TI025AC01 in this contig hit ISL2 transcription factor containing LIM/homeodomain that also resides on human chromosome 15. Therefore, the BLAST result showed that the part of the sex-determining region in tilapia shares synteny with *Fugu* scaffold 670 and human chromosome 15 and 16.

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Mapping of Sex locus and AFLP Markers

The putative sex locus was placed in the middle of the sex-determining region (6 cM away from GM201 and 5.1 cM from UNH995). Out of 128 selective primer pairs, 2 markers (MCTT/EACG -382 and MCAC/EAAC-391) showed sex-specific patterns in male and female BSA pools in both Families 5 and 7 (Figure 5-5). However, neither marker fell in the sex-determining region, and instead mapped 2-3 cM away from the region (Figure 5-6). Another marker (MCTA /EAAG-425) was informative in Family 7 but not in Family 5. Without any information from Family 5, mapping analysis put this marker to the same place as UNH995. Comparing sex specific maps, recombination appears restricted in males (Figure 5-6).

Identification of Candidate Genes

Based on BLAST analyses, one of 169 sequences from BAC b04TI079DH06 hit DEAD box gene family in the scaffold 670. The shotgun sequence was an open reading frame (ORF) of 558 bp from which an extended sequence was obtained by primer walking on the BAC. The total length of this ORF is 1686 bp containing 8 motifs of a DEAD box family (Figure 5-7). The

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BLASTx result of the sequence shows that the gene is significantly similar to DDX28 (DEAD box polypeptide 28) of the mouse, rat, and human with e-value of e-116, e-113, and e-112 respectively. This might be another novel DEAD box gene in tilapia. In addition to shotgun sequences, a BAC end sequence of b04TI025AC01 (M13F) showed a significant hit to potential LIM/homeodomain gene.

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Discussion

Comparative positional cloning is a powerful and economical strategy to identify candidate genes underlying phenotypic traits. Genes for sex determination have been intensively studied in mammals and other vertebrates, but have not been as well investigated in teleosts. Some of the candidate genes were cloned in tilapia and a few of them were mapped in tilapia linkage map (Chapter 1). None of them are located in the sex-determining region that I found (Lee, et al., 2003; Lee, et al., 2004).

Analyzing the shotgun sequences from tilapia BAC clones in the sex-determining region provides lots of information to identify candidate genes that might be involved in sex determination. A BAC contig containing microsatellite markers (UNH995 and UNH994) in the tilapia sex-determining region (LG1) seemed to share a syntenic region with a *Fugu* contig containing sequence scaffolds 670, 1924, and 2342, which is about 190 kb (Figure 5-3). This region appears to be homologous to portions of human chromosome 16 and 15. It is difficult to say how well the gene order is conserved between tilapia and *Fugu*, because mapping information for genes in the region of synteny is not available in tilapia. By scanning the genes annotated in the *Fugu* scaffold, potential candidate genes for sex determination can be found, such as the DEAD box gene in *Fugu* scaffold 670. The DEAD box gene is a candidate gene for sex

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determination because some members of the DEAD box protein family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division. I was able to obtain the whole sequence of this gene and it will be further characterized at the sequence level and in its pattern of expression. An end sequence of a BAC in the same contig showed similarity to ISL2 (*Islet-2*) transcription factor, a LIM/homeodomain protein that is also located in human chromosome 15. This gene is also a candidate because some homeobox proteins of the LIM class, such as *Lim1 (Lhx1)* and *Lhx9,* are related to sex determination, as I described in Chapter 1. So, obtaining the whole sequence of the gene will be useful for further study. The HMG gene on *Fugu* scaffold 1833 (Figure 5-1) can also be considered a candidate, because it is likely that *Fugu* and tilapia share synteny at this scale. It would be worth designing degenerate primers and mapping this gene in tilapia.

Chromosome walking by BAC contigs and shotgun sequencing across the region would be a big assist for comparative positional cloning. Although we don't have information on the precise relationship between physical distance and linkage distance, the distance between markers previously identified in the region of *O. niloticus* is about 10 cM, which is probably not small enough for chromosome walking by BAC clones. Using the AFLP/BSA technique, I tried to identify more markers that are closer to the sex locus. However only 2.3% of AFLP selective primer sets (3/128) produced informative markers and none of

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them were tightly linked to the sex. Developing more markers by this technique does not seem so promising.

There are several obstacles to identify sex-determining genes in tilapia. It is known that fish genomes have numerous copies of repetitive DNAs accumulated in the sex-determining region. In tilapia, CiLINE2 (cichlid LINE2) and Ron-1 (SINE-like repetitive for *O. niloticus*) are enriched in the long arm of chromosome 1 (Oliveira, et al., 1999; Harvey, et al., 2002). As shown by the sequencing results (Table 5-1 and Table 5-2), repetitive DNA sequences from each BAC were about 20% in LG3 and 12% in LG1.

A shortage of genetic markers in the region and furthermore a lack of BAC clones covering the gene region are a major hurdle in getting more genetic markers. Thus it seems obvious that obtaining genetic markers from the sex-determining region is a much less efficient way to approach the sex gene than typing more recombinants. Although AFLP put more markers around the region, it is difficult to identify more tightly linked markers within the gene region. Anyhow it is more sensible that a bigger mapping family is required to dissect the tilapia sex-determining region. Meanwhile, BAC-based chromosome walking could be a useful strategy. However, I have failed to identify BAC clones harboring several sex-associated markers, including GM201 that is one of the boundary markers surrounding the sex-determining region. So, high-density BAC filters prepared may need to be screened to isolate the BACs for more sex-associated markers.

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Comparative mapping by sequencing analysis and BLAST search as described in this study can help find candidate genes involved in sex determination. However, if the genes of sex-determination in tilapia are totally novel and do not have similarity to the genes identified in other teleosts, it would be difficult to find the genes by comparative mapping. Strategies using cDNA or expression difference in developing gonads might be useful for identifying genes involved in sex determination.

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Marker	No. of clones by PCR screening	No. of Contigs	BAC clones representing the contig	No. clones in the Contig	No.of sequences	Repetitive DNA (%)	Zebrafish DNA (%)	Fugu scaffold (No. of seqeunces)	Genes hit
GM354	3	3	b03TI062CG03	2					
			b03TI066DH01	4	450	3.3	2	1833 (19)	*PI3K, DGAT*
			b04TI004DB08	singleton					
GM204	2	2	b03TI070BF03	4					
			b04TI071BH02	5	96	19.8	6.3	-	
GM271	4	2	b04TI074AA04	4	179	17.9	5	-	
			b04TI056BC05	singleton	77	19.5	7.8	-	
CLCN5	2	1	b03TI064CB12	20	144	11.8	3.5	733 (3)	*TNF, CLCN5*
			b04TI076CC06		126	6.3	3.2	733 (5)	*TNF*
								5668 (3)	*CLCN5*
GM180	4	1	b04TI056AD04	6	72	11.1	1.4	4353 (3)	*DGKD*
UNH115	3	1	b04TI059DG05	9	63	17.5	12.7	2144 (4)	

Table 5-1. The results of screening, fingerprinting, and shotgun sequencing of BACs for markers in the sex-determining region of LG3. Genes hit were based on the combined result from BLASTn and BLASTx against JGI *Fugu* and NCBI data bases.

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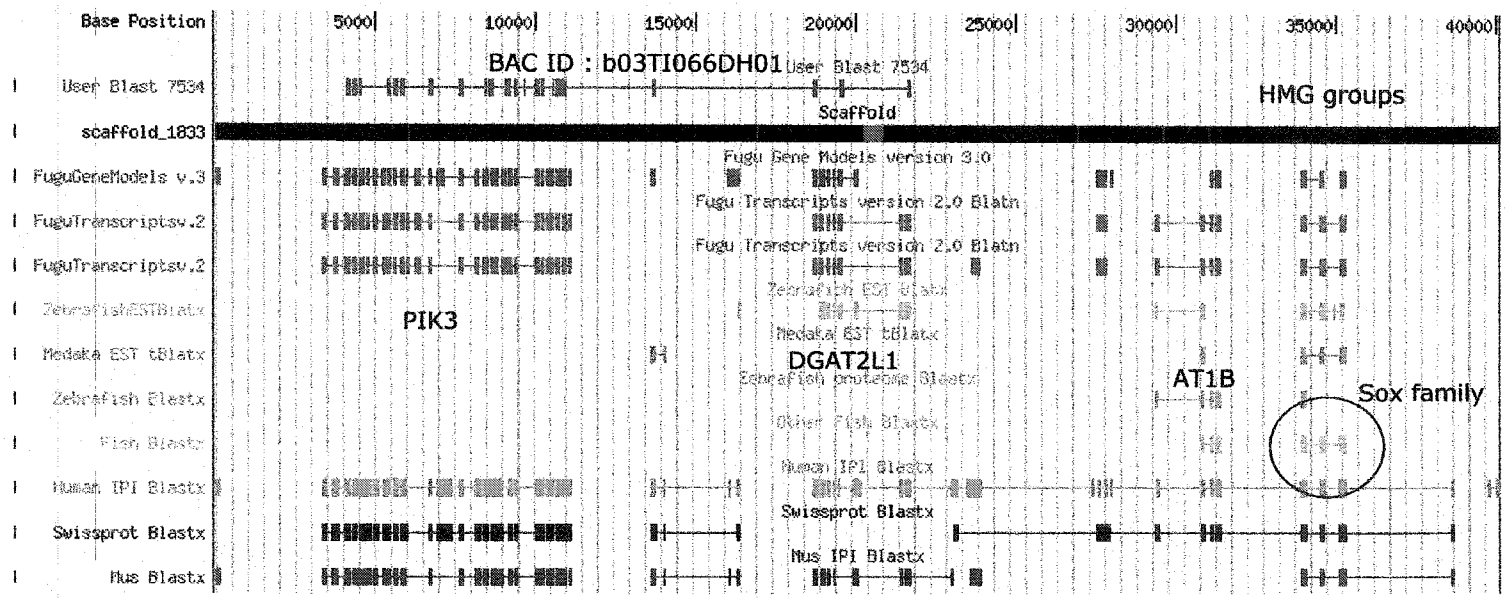

Figure 5-1. *Fugu* scaffold 1833 with viewer of *Fugu* assembly (ver. 3.0). Many data sets have been aligned to the sequence assembly to aid comparative analyses. User Blast represents all pieces of shotgun sequences that hit scaffold 1833 from shotgun library of BAC clone b03TI066DH01.

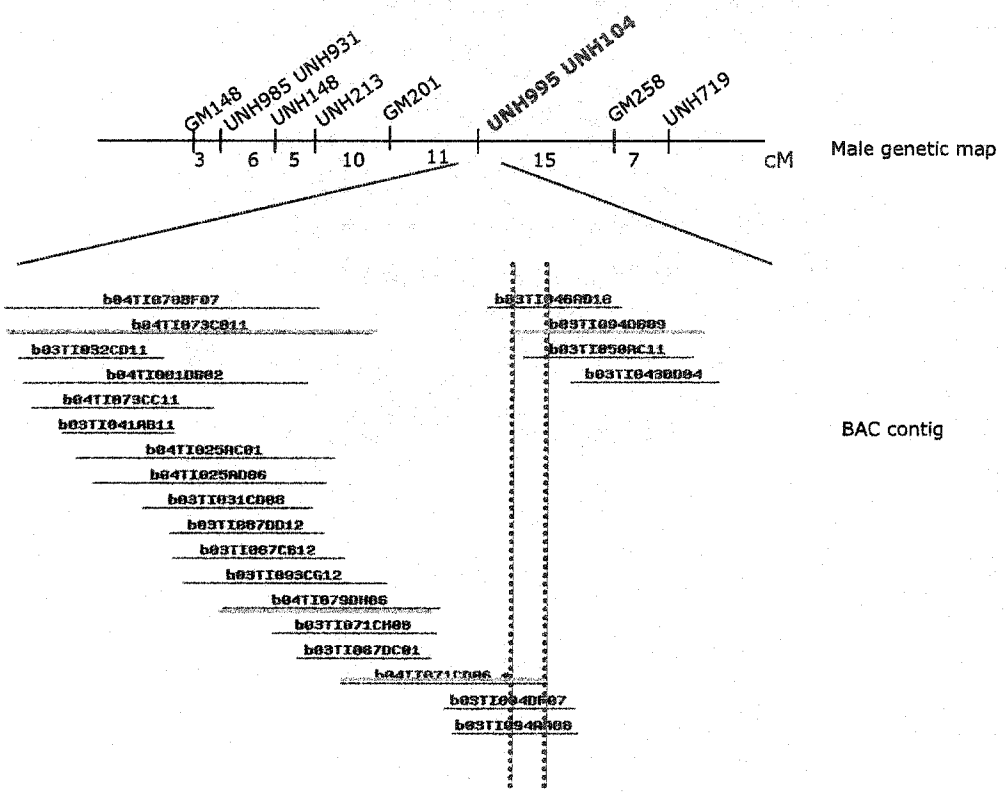

Figure 5-2. A BAC contig identified by screening markers UNH995 and UNH104 in LG1 and FPC fingerprinting analysis (t5, e-7). Those two loci might be located in the area between the dotted lines. 4 BACs highlighted with green were used for shotgun sequencing. The orientation of the contig is unknown.

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BAC ID	NO. of sequences	Repetitive DNA (%)	Zebrafish DNA (%)	Scaffolds (No. of hit)	Gene (human chromosomal position)
b03TI094DB09	168	10.7%	2.9%	4730 (2)	
				4666 (2)	
				4390 (3)	
				4071 (2)	
b04TI071CD06	129	7.8%	8.5%	670 (6)	hypothetical protein FLJ21918 (16q22.1)
				4666 (2)	
				1924(7)	AMFR (16q21), STYX*
b04TI079DH06	172	4.1%	2.9%	670 (21)	hypothetical protein FLJ21918 (16q22.1)
					NFATc3 (16q22.2)
					DEAD box helicase;ddx28 (16q22.1)
				2082 (5)	
				1924(14)	AMFR (16q21)
b04TI73CB11	115	1.7%	5.2%	670 (17)	Talin 2 (15q21)
				2159 (2)	hypothetical protein MGC15619

Table 5-2. The results of BAC shotgun sequencing and BLAST analysis in the sex-determining region of LG1. Asterisk (*) represents the gene not annotated in the corresponding *Fugu* scaffold.

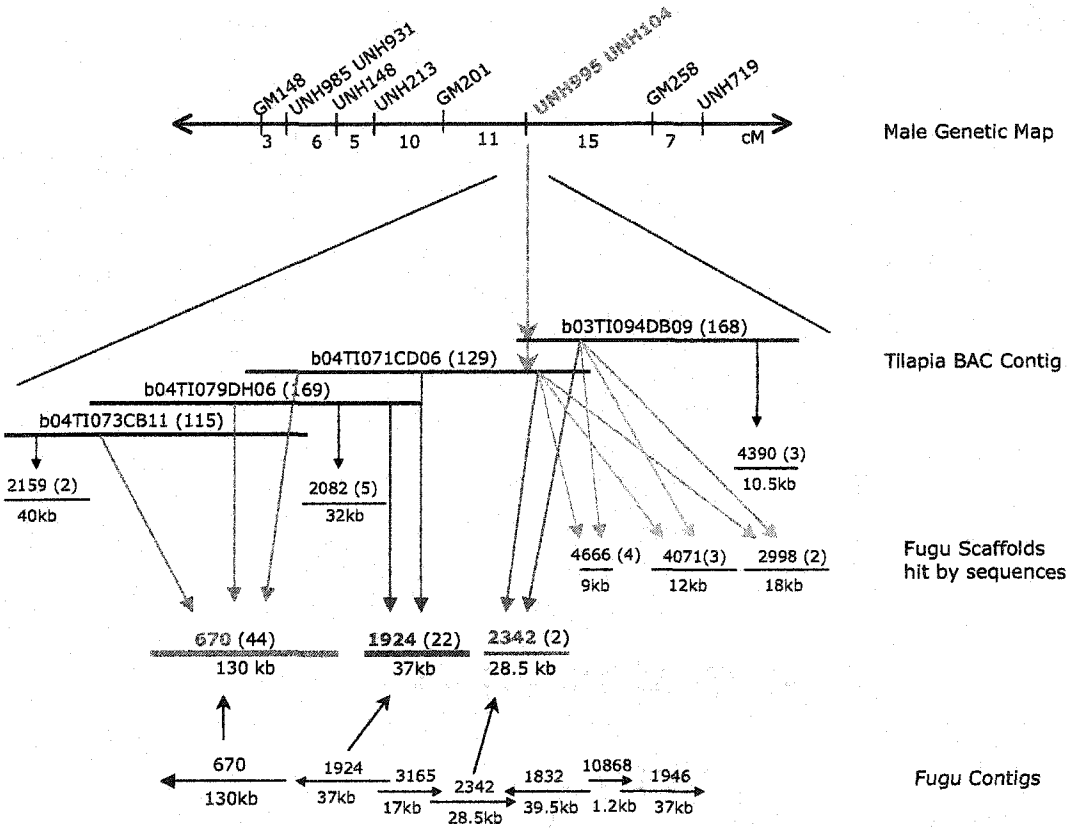

Figure 5-3. Diagram showing a putative synteny of *Fugu* scaffolds and tilapia sex-determining region of LG1 by BLAST analyses. Numbers in the parenthesis indicate number of sequences.

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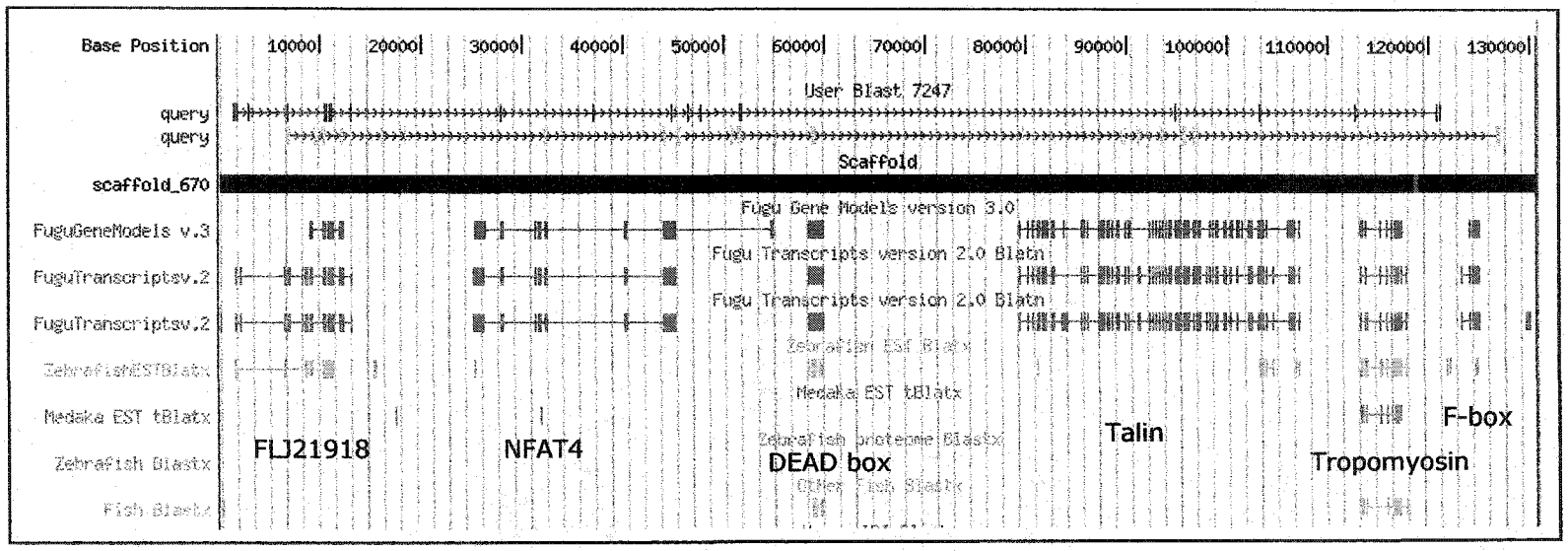

Figure 5-4. View of the *Fugu* scaffold 670 that 44 sequences from 3 BAC clones aligned. Sequences hit are distributed overall scaffold. Queries represent shotgun sequences, which are separated in two parts along the scaffold.

Fugu Scaffold (No. of hit)	Gene model annotated	Human Chromososme
670 (44)	hypothetical protein FLJ21918 *	16q22.1
	NFATc3*	16q22.2
	DEAD box helicases;ddx28*	16q22.1
	Talin*	15q15-q21
	tropomyosin	
	F-box only protein 22	15q23
1924(22)	3',5'-cyclic AMP phosphodiesterase	
	ebiP2441 [Anopheles gambiae str. PEST]	10q21.1
	glucosamine-phosphate N-acetyltransferase*	
	AMFR*	16q21
	leukotriene B4 receptor (LTB4R)*	14q11.2-q12
2082 (5)	non-LTR retrotransposable element	
	gag polyprotein/similar to gag-protease	
4666 (4)	no annotation	
4071 (3)	no annotation	
2159 (2)	FLJ10581/CGI-150 protein	17p13.3
	FLJ12614 (similar to red-1-gene)	17p13.3
	hypothetical protein MGC15619*	12q24.13
2998 (2)	Kinesin-like protein KIF3A	5q31
2342 (2)	SH3 domain-binding protein	Xq21.1
	reverse transcriptase-like protein	
4390 (3)	no annotation	

Table 5-3. Summary of *Fugu* scaffolds blasted by shotgun sequences, gene models annotated along the scaffolds, and the most likely homology with the human genome. The scaffold numbers were based on the result of shotgun sequences in LG1. Asterisk (*) represents the gene that the sequences actually hit.

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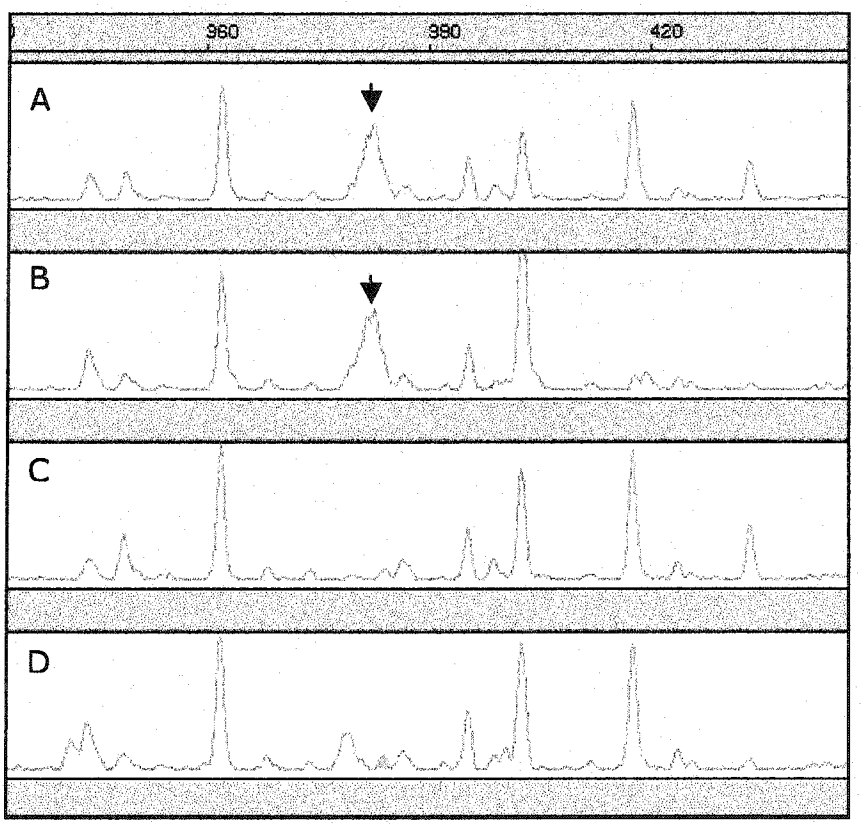

Figure 5-5. Identification of sex-specific AFLP marker (MCTT/EACG-382) in *O. niloticus* sex-determining region using BSA analysis. A. Family 5 male pool; B. Family 7 male pool; C. Family 5 female pool; D. Family 7 male pool. Only male pools show the 382 bp peak.

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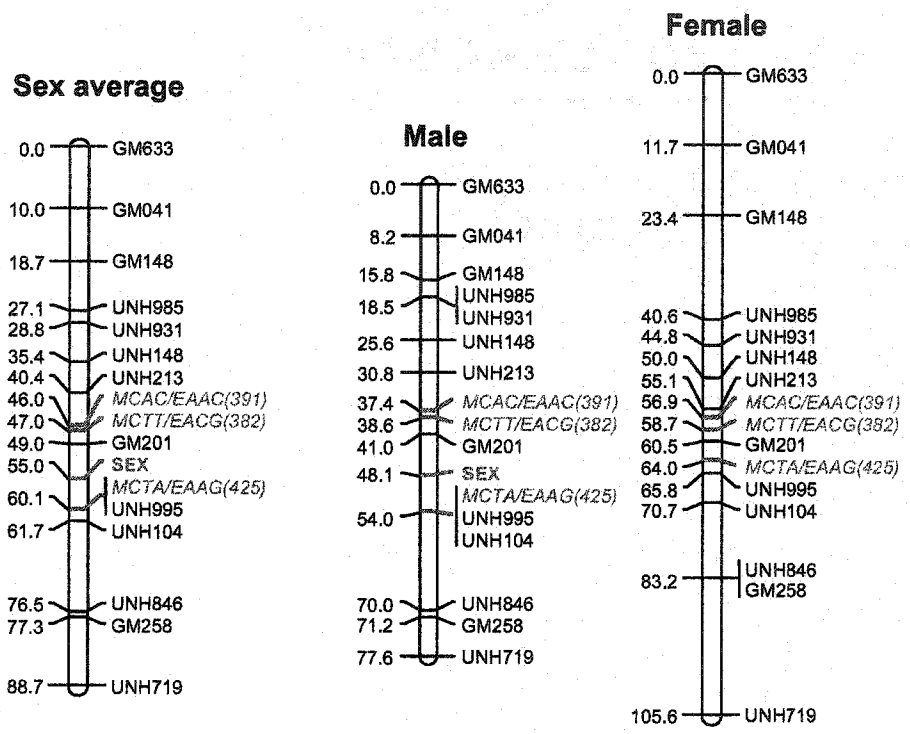

Figure 5-6. Mapping of AFLP markers (green) and the putative sex locus (red) in LG1 by CRIMAP (ver. 2.4). The sex locus was mapped as in disease loci; male was considered to be affected individuals (heterozygotes) and female as unaffected individuals (homozygotes).

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```
-264  ACA AAA CAC ATG AGT TGA ATC GGG ATT CGG CTT TTC TGT ACA CCT CGT CAC GCC ACG CCC
       T   K   H   M   S   .   I   G   I   R   L   F   C   T   P   R   H   A   T   P
-204  ACT GAC ATC ATC AGC ACT CGC CGG GAA AGC ATG GCG ACA TGA CTG TCT AGT ATA TAA TTT
       T   D   I   I   S   T   R   R   E   S   M   A   T   .   L   S   S   I   .   F
-144  CTG CGC CTT TTT GGA CTT TTA TAT TGT CTT TTG TGG AAT TAC AGT TAA TTT AAT TAG CTT
       L   R   L   F   G   L   L   Y   C   L   L   W   N   Y   S   .   F   N   .   L
-84   TAT TTC CAA ATT TTT ATG GTT CGG TTT TGC TGC TTT TTC TCG TCA GCT GAC CTT TGG ACA
       Y   F   Q   I   F   M   V   R   F   C   C   F   F   S   S   A   D   L   W   T
-24   CTT GCG AGC GTT TAA GTA TTT AAA ATG CAG GCT GTG AAG GTC GCC CAT CTG GCT TTG GTA
       L   A   S   V   .   V   F   K   M   Q   A   V   K   V   A   H   L   A   L   V
37    GCG TCC AGA GCT CTC GGA TCA AGA AGA TTG TGT TGC TGT GAG CTT TTT AAA GCG TCG GCT
       A   S   R   A   L   G   S   R   R   L   C   C   C   E   L   F   K   A   S   A
97    TGC CTT CGG TCT CTC GGT CAG AGT CGC TTC TGT CAG ACT GGA GCA GAG ACC GCG GTC ATT
       C   L   R   S   L   G   Q   S   R   F   C   Q   T   G   A   E   T   A   V   I
157   CGT ATT CCC CGG TAC CTG CAG AGA CGC GTT GAA AAC GTG AAG GAA ATT CGA AGC AAA AGC
       R   I   P   R   Y   L   Q   R   R   V   E   N   V   K   E   I   R   S   K   S
217   AAG ATC AAC ACC ATC AAA GCT GGA AAG CTC CTC ATC CAG AGC AAG AAC CCA GCT CTG AAC
       K   I   N   T   I   K   A   G   K   L   L   I   Q   S   K   N   P   A   L   N
277   CAG TCT GCC GGA TAC ATA CTG GGA AAA TTC GAG CAG CCT TCT CTT TGC TCC AAA GGA TGG
       Q   S   A   G   Y   I   L   G   K   F   E   Q   P   S   L   C   S   K   G   W
337   AAA CAT AGC AAA TCA TTC GGT GAC TAT TTC AGC ATC AAC AAC GTC AAG GCT GTT GCA CCT
       K   H   S   K   S   F   G   D   Y   F   S   I   N   N   V   K   A   V   A   P
397   TAT GTT GCT GAA AAC TGG AAT GAG GAC GGT GGA CAG AAG CCG CTA GCC ACT TTT AAT AAT
       Y   V   A   E   N   W   N   E   D   G   G   Q   K   P   L   A   T   F   N   N
457   CTC CAC ATC TGC AAG GAG CTA GTG GAG ACT TTA GAG ACT CTC AGT ATT AAA CAT CCC ACC
       L   H   I   C   K   E   L   V   E   T   L   E   T   L   S   I   K   H   P   T
517   ACT GTG CAG CTT CAG ACC ATC CCC AAA GTC ATG AGA GGT CAC AAT GTA CTC TGT GCT GCT
       T   V   Q   L   Q   T   I   P   K   V   M   R   G   H   N   V   L   C   A   A
577   GAG ACT GGC AGT GGG AAG ACG CTG AGT TAT CTC CTA CCT GTT ATT CAC AGA CTG CAG GCT
       E   T   G   S   G   K   T   L   S   Y   L   L   P   V   I   H   R   L   Q   A
637   GAT AAG GAG TCT GAA AGT TAC TCT GAG AGT GCA CAC AAG ATA TGC ACT GTG GTA CTC GTG
       D   K   E   S   E   S   Y   S   E   S   A   H   K   I   C   T   V   V   L   V
697   CCT TCA AGA GAG CTG GCG GAG CAA GTG GCA GCT GTG TCC AGG ACT CTG TGT GCG CCA TTT
       P   S   R   E   L   A   E   Q   V   A   A   V   S   R   T   L   C   A   P   F
757   GGT TTC GTT ACA AGG ACC GTT GGA GGA GGA CGA GGT GTG GGA CAC ATC AAG ACA GTC TTC
       G   F   V   T   R   T   V   G   G   G   R   G   V   G   H   I   K   T   V   F
817   AGG AGG GAT CAT CCG GAT ATT TTA GTG GCT ACG CCA GGT GCT CTG GTC AAG GCC CTG CGG
       R   R   D   H   P   D   I   L   V   A   T   P   G   A   L   V   K   A   L   R
877   AGG CGT TGT CTG GAT TTG AGT GAG CTG AGG TTC TTT GTG GTC GAT GAG GCT GAC ACG ATG
       R   R   C   L   D   L   S   E   L   R   F   F   V   V   D   E   A   D   T   M
```


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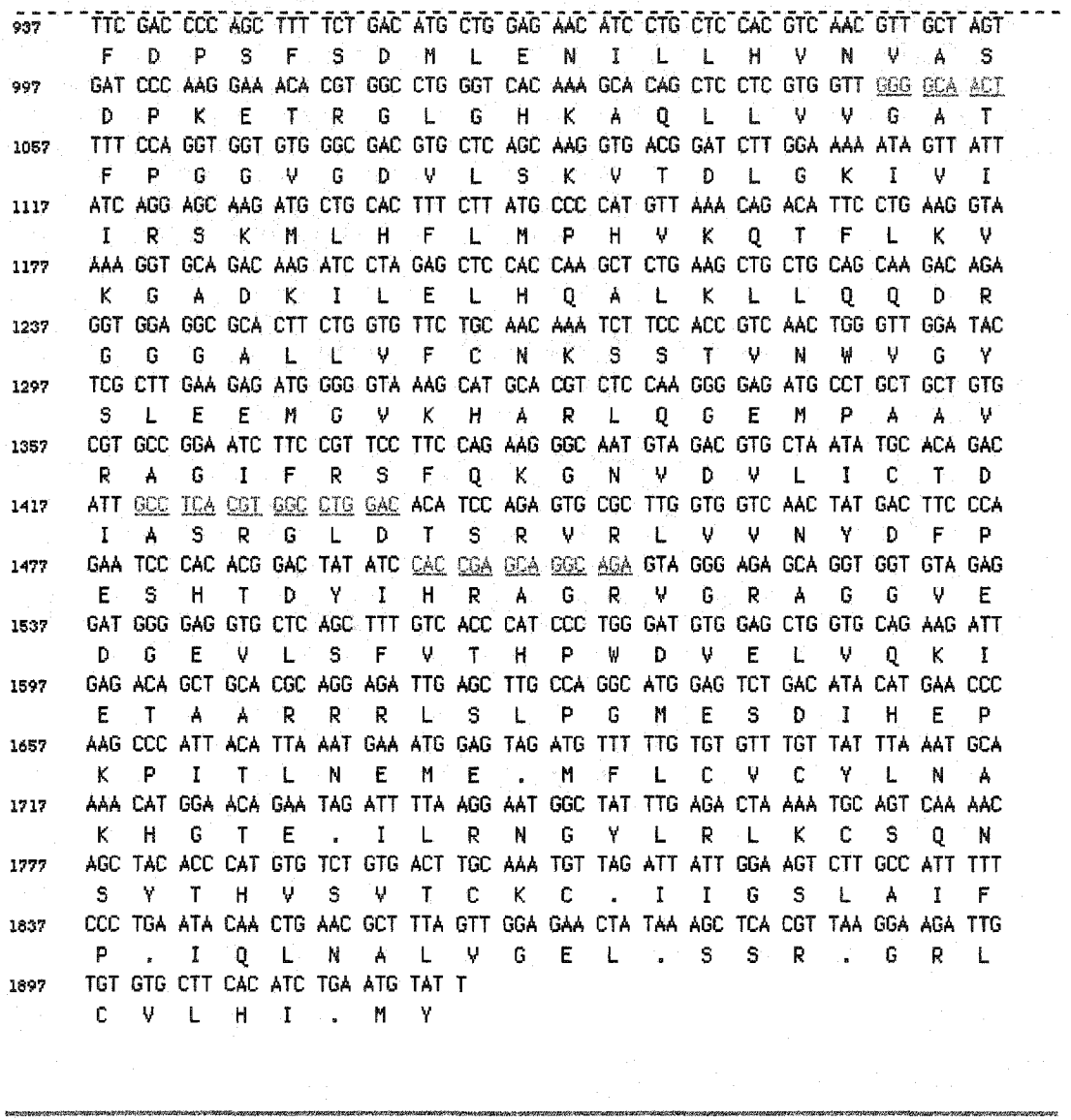

Figure 5-7. Sequence of a novel DEAD box gene in tilapia. Green letters underlined represent motifs of DEAD box gene family.

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