Identification of sex determination gene(s) through comparative sequence analysis of X and Y chromosomes in channel catfish

by

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Abstract

One of the most fundamental and fascinating processes of life is the determination of sex. Scientists have worked for hundreds of years to understand the mechanism of sex determination in the fields of developmental and evolutionary biology. In teleost, sex determination system exhibits surprisingly diverse and plasticity during evolution.

Catfish has a XY male/XX female sex determination system, but the exact mechanism of its sex determination is unknown at present. As a first step toward the identification of sex determining gene(s) in catfish, we generated the first genome sequence assembly of the YY catfish using next-generation sequencing. This allowed the generation of the Y-chromosome sequences. QTL mapping and GWAS analysis were conducted to locate the sex determination locus to linkage group 4. Comparative analysis of X chromosome and Y chromosome sequences was performed to determine the similarity and differences of the sex chromosomes with respect to chromosome size, sequence identities, gene contents and expression patterns of their genes during early gonad development. This project would lay the foundation for further downstream analysis of genes involved in sex determination and differentiation in catfish.

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CHAPTER I. LITERATURE REVIEW: SEX DETERMINATION AND EVOLUTION OF THE Y CHROMOSOME

1.1 Sex determination mechnism

Sex determination mechanisms vary with species in nature. Generally, the sexualities can be categorized into either gonochorism, hermaphroditism or unisexual reproduction (Juchault 1999, Prevedelli et al. 2006, Wilson et al. 2015). Hermaphroditism and unisexual reproduction are rare, while, gonochorism is more common (Picq et al. 2014). In gonochoristic species, different sexes could cause individual phenotypic differences, which can be very small in just different appreances of gonads or quite significant in the external phenotypes and behaviour (Janik 2006). With aquaculture species, most species exhibit sex bimorphism in size and growth rates. In some species, females grow faster, while in others, males grow faster. For instance, male channel catfish grow 15% faster than female channel catfish (Davis et al. 2007). In contrast, female half-smooth tongue soles grows much faster than their male counterparts (Chen et al. 2009), up to 2-3 folds faster.

Depending on primary sex determining factors, sex determination systems can be categorized into two different types: genetic sex determination (GSD) and environmental sex determination (ESD) (Penman and Piferrer 2008, Piferrer et al. 2012, Matsumoto et al. 2014, Diaz and Piferrer 2015). The most extensive sex-determining mechanism is GSD. For most mammals, the sex is determined by genetics, such as human (Veitia et al. 1997), mouse (Washburn et al. 2005), and cattle (Hanset et al. 1992). However, for some animals, the question of whether the progeny will be a male or a female has little to do with the genes. In this case, external factors such as temperature, pH, behavior, hormones, physiological can influence the fate of an organism and the process of gonad differentiation. For instance, sex are mostly influenced by environmental factors in alligators and most turtle species (Woodward and Murray 1993, Baroiller et al. 2009, Wyneken 2015).

Chromosomal sex determination (CSD) is the main mode of GSD in most mammalian species (Coriat et al. 1994, Charlesworth 2002). In this case, sex is determined by a primary switch located on one or both members of a differentiated sex chromosomal pair. The other type of GSD is polygenic sex determination (PGSD), in which the genes with strong influence on sex determination and/or gonad differentiation are distributed on several chromosomes and the combination of their alleles determines the sex of an individual (Liew et al. 2012, Moore and Roberts 2013). The mechanism is more complex in some fish species that have a sex change (Chopelet et al. 2009). In many polygynous species, if the dominant female on the group dies, the most dominant male will then quickly become female and take her place. All the other males will move up one rank in the hierarchy (Kuwamura et al. 2014).

Early in the 1900s, sex chromosomes were discovered, which was considered to be the first major breakthrough in understanding sex determination (Wessel 2011). After analyses of various species over the years, scientists discovered that chromosomal differences are primarily responsible for sex determination in most animals. In human and most other mammals, sex is determined by XY sex-determination system. In this system, the sex of an individual is determined by a pair of sex chromosomes (gonosomes). Females have two of the same kind of sex chromosome (XX), and are called the homogametic sex. Males have two distinct sex chromosomes (XY), and are called the heterogametic sex (Lovellbadge 1995, Wallis et al. 2008). Most insects, like the majority of animals, have dimorphic sex chromosomes that can be distinguished cytologically (Verhulst and van de Zande 2014). However, sex determination mechanisms in insects are considerably diverse (Saccone et al. 2002). For instance, the sex determination mechanism in the fruit fly is relatively rare. It was reported that the ratio (X:A) of the number of X chromosomes to the number of sets of autosomes determined its sex (Cline 1993). Another variant is the X0 sex-determination system, which is a system that determines the sex of offspring among grasshoppers, crickets,

cockroaches, and some other insects (Nokkala and Nokkala 1984, Jaquiery et al. 2012). In this system, there is only one sex chromosome, referred to as X. Males only have one X chromosome (X0), while females have two (XX). Maternal gametes always contain an X chromosome, so the sex of the offspring depends on whether a sex chromosome is present in the male gamete. Working with birds and some reptiles, sex is determined by chromosomes known as the Z and W, and Males are the homogametic sex (ZZ), while females are the heterogametic sex (ZW) (Handley et al. 2004). Among teleost fish, both XY and ZW sex determination systems have been found (Kikuchi and Hamaguchi 2013).

1.2 Sex determination genes

Although the molecular mechanisms underlying many developmental events are conserved across vertebrate taxa, the lability at the top of the SD cascades has been evident from the fact that four master SD genes have been identified: sry (mammalian), dmrt1 (chicken), dmy (medaka), and DM-W (Xenopus laevis) (Kondo et al. 2009, Smith et al. 2009, Kikuchi and Hamaguchi 2013). Recently five novel candidates for vertebrate SD genes were reported, all of them are in fishes. These include amhy in the Patagonian pejerrey, gsdf in Oryzias luzonensis, amhr2 in fugu, sdY in rainbow trout (Salmo gairdneri) and sox3 in medaka (Oryzias dancena) (Kikuchi and Hamaguchi 2013, Takehana et al. 2014). Accumulation of knowledge on variant SD genes will allow us to distinguish conserved and diversified SD pathways among vertebrates and lead us into a deeper understanding of the vertebrate SD cascade.

The comparison of these sex determination genes indicates that vertebrate sex-determining cascades are not as conserved as once thought. In eutherian mammals, sry is a recently evolved key Y-linked testis determinant (Fechner 1996, Tanaka and Nishinakamura

2014). Sry encodes a member of a large family of nuclear proteins characterized by a DNA-binding domain, known as high mobility group (HMG) box. In mammals, the current evidence favors the idea that sry acts with the orphan nuclear receptor Sf1 to activate sox9 expression in the developing male gonad (Kozhukhar 2012). As a result, sry is turned off by sox9 which then maintains its own expression. In mammals, sox9 activates expression of the fgf9 and genes encoding prostaglandin D synthase (Jiang et al. 2013, Kikuchi and Hamaguchi 2013, Kobayashi et al. 2013). Another key role for sox9 during testicular development is the activation of Amh (Jiang et al. 2013). Amh may participate in testis determination by blocking estrogen synthesis, namely by repressing expression of the aromatase encoding gene cyp19a1 (Cutting et al. 2013). However, in the chicken embryo, dmrt1 and amh expression precedes that of sox9 by at least 2 days, and the gene is expressed in both males and females, implying that other intervening genes are involved (Lambeth et al. 2014).

In birds and lower vertebrates, there is a pervasive role for DM domain of genes in gonadal sex differentiation, and it is considered that these genes have an ancient association with sex (Gilgenkrantz 2004). In birds, testis development requires presumably the conserved Z-linked dmrt1 gene. While in Xenopus laevis (ZZ/ZW-type sex determination), sex determination is triggered by the ovary-determining gene, DM-W (Yoshimoto and Ito, 2011; Yoshimoto et al., 2008). The activator of expression of amh in the chicken and other vertebrates is unclear, but is likely to involve the orphan nuclear receptor SF1 which is expressed in male gonads compared to females suggesting that a dosed Z-linked gene such as dmrt1 may activate its expression in avians.

The key ovary determinant in mammals has not yet been defined, but the canonical β-catenin signaling pathway is required for ovarian morphogenesis (Caruso et al. 2015). The β-catenin signaling pathway appears to be conserved in the other vertebrates, including fishes, reptiles, and chickens. In these cases, R-Spo1, Wnt4, and/or β-catenin show female

upregulation (Ayers et al. 2013, Suzuki et al. 2015).This female pathway appears to be deeply conserved among vertebrates. The position of foxl2 in the ovarian pathway appears to vary among the major groups (Benayoun et al. 2009). In the chicken embryo, foxl2 expression from 5.5th embryonic day is one of the earliest known markers of ovarian development (Hudson et al. 2005). Foxl2 activates the cyp19a1 in the tilapia fish and in mammals (Li et al. 2013). A major question related to ovarian development is how the foxl2 and R-SPO1/Wnt4 pathways interact to coordinate ovarian development (Biason-Lauber 2012). The two pathways appear to be independent in goat and in chicken embryos (Kocer et al. 2008, Ayers et al. 2013, Boulanger et al. 2014).

1.3 Evolution of the Y chromosome

Two features set Y chromosomes apart from the rest of the genome: 1) a lack of recombination on the Y over some or most of its length (Charlesworth and Charlesworth 2000); 2) male-limited transmission of the non-recombining segment (Stillwell and Davidowitz 2010). Investigating Y chromosomes is challenging, because their lack of recombination prevents classical linkage mapping studies, and their high content of repetitive elements has excluded them from most genome sequencing projects (Page et al. 2010). Sex chromosomes carry the master-sex determining genes, are subject to unique evolutionary forces (Bachtrog et al. 2011, Papadopulos et al. 2015), and play a prominent role in many evolutionary processes, such as speciation (Presgraves 2008), adaptation (Zhou and Bachtrog 2012, Chen et al. 2014) and genomic conflict (Spencer et al. 2004).

The origin of sex chromosome is generally considered as ordinary autosomes and sex chromosomes have evolved independently many times in different lineages (Charlesworth 1996, Yang et al. 2014). The human X and Y chromosomes originated about 200-300 million years ago in eutherian mammals, and sex chromosomes evolved independently in birds,

reptiles, amphibians and fish (Charlesworth 1991, Chandler et al. 2009, Deshpande and Meller 2014). At the beginning, the sex is determined either by temperature-determination system or polygenic determination with multiple loci on different autosomes (Moore and Roberts 2013, Refsnider and Janzen 2015). The first step in the evolution of Y chromosomes is likely to be the acquisition of a sex-determining gene on one member of a pair of autosomes that ultimately will become the sex chromosomes, for instance, a male-determining gene forming a proto-Y chromosome (Spigler et al. 2011). In the population, this is a dynamic process via sex determination gene and other polygenic loci compete and increase or decrease in their effect.

For heteromorphic sex chromosomes to originate after the acquisition of a male-determination gene, recombination needs to become suppressed between the homomorphic proto-sex chromosomes (Charlesworth 1996, Sun et al. 2008). This allows the Y chromosome to evolve independently of its X homologue. Otherwise, genetic sex determination with homomorphic sex chromosomes is observed in many taxa in amphibians, fish, reptiles and many invertebrates (Quinn et al. 2011). There are many theory to elucidate the accumulation of the suppression of recombination. First one is due to accumulation of sexually antagonistic alleles linked to the sex determination gene. Sexually antagonistic mutations, which are beneficial to one sex, but detrimental to the other (Mank and Ellegren 2009), are thought to provide the selective force to suppress recombination between nascent sex chromosomes (Innocenti and Morrow 2010). Sexually antagonistic mutations are more likely to become established in a population if they are more often transmitted through the sex that they benefit (Grossen et al. 2012), and restriction of recombination between the nascent proto-X and proto-Y chromosomes may be a consequence of the positive selection tends to suppress the crossover between the sexually antagonistic alleles and the sex determination gene in one sex. Another source of recombination suppression could be the

chromosome rearrangement. Evidence from mammals suggests that the elimination of recombination might be achieved through chromosomal inversions on the proto-sex chromosomes (Bellott and Page 2009, Lemaitre et al. 2009, Kirkpatrick 2010, Bachtrog 2013). Inversions are known to locally suppress recombination in heterozygotes (Kirkpatrick 2010). Thus, an inversion on one of the proto-sex chromosomes can repress recombination between the proto-X and proto-Y in males, and allow them to accumulate mutations independently and differentiate, such as in mammals, birds or plants (Nicolas et al. 2005, Wang et al. 2012).

On a recombining chromosome, natural selection can act independently on each mutation. However, in the absence of recombination, selection performs on part or even entire chromosome (Rice and Chippindale 2001, Bachtrog and Gordo 2004). An entire chromosome will be fixed in the population if a beneficial mutation arises on it, or an entire chromosome will be purged if it carries a deleterious mutation (Grossen et al. 2012). Thus, Y chromosomes are expected to accumulate deleterious mutations, and incorporate fewer beneficial mutations. Consistent with these theoretical expectations, non-recombining Y chromosomes generally show lower levels of adaptation, i.e. both increased rates of accumulation of deleterious mutations and lower rates of adaptive evolution relative to recombining regions of the genome in various species (Bachtrog and Charlesworth 2002, Bachtrog 2003, Bartolome and Charlesworth 2006). When it comes to ZZ/ZW sex determination system found in birds, reptiles and fishes, those mechanism could work together. Overall, the lack of recombination of the partner chromosome results in degeneration of the Y/W chromosome (Deshpande and Meller 2014, Smeds et al. 2015).

Some species groups, such as fishes and reptiles, contain taxa at various stages with sex chromosome from morphologically identical to highly differentiated. Like the birds, reptiles have a ZW system in which the Z chromosome is similar in size across species, but

the W ranges from homomorphic to highly heteromorphic, with the hypothesis that they represent different stages of W degradation (Vicoso et al. 2013). What is worth mentioning is that many reptiles lack sex chromosomes, and that their sex will be determined by the temperature at which the eggs are incubated (Quinn et al. 2007). Evidence shows fishes are generally at the early stage of the sex chromosome evolution, thus maintain the morphological similarity between X and Y chromosome (Tanaka et al. 2007). For instance, in the medaka fish, their Y chromosome never inverted and can still swap genes with the homomorphic X chromosome (Schartl 2004). There are also studies suggest that evolution of such homomorphic sex chromosomes in fish has been attributed to the rapid turnover of sex chromosomes with acquisition of total new genes (Kitano and Peichel 2012, Yoshida et al. 2014). Such mechanism is likely to be maintained by the translocation between sex chromosome and autosome, function loss mutation of original sex determination gene or just evolution of a novel gene in an autosome (van Doorn and Kirkpatrick 2007, Kitano and Peichel 2012). Given the evidence from all the fish species mentioned, together with previous studies of other nonmammalian species, it seems reasonable to imagine that many other teleosts, reptiles and amphibians also have experienced a turnover of sex chromosomes (van Doorn and Kirkpatrick 2007, Kitano and Peichel 2012, Yoshida et al. 2014). However, the research on half-smooth tongue sole indicates the Z and W chromosome are heteromorphic, and W chromosome has already lost about two-thirds of its original protein (Chen et al. 2014). One of the major characteristic in W of tongue sole is the high percent of repetitive elements, which would suggest that amplification of simple repetitive sequences may suppress the recombination and played a major role in the evolution of Y and W chromosomes.

To date the most completely sequenced and annotated Y chromosomes are from three primates: human, chimpanzee, and rhesus macaque (Rozen et al. 2003, Skaletsky et al. 2003). Male-specific Y chromosome sequences are also available for marmoset monkey, cattle, pig,

rat, mouse, cat and dog (Zimin et al. 2009, Li et al. 2013, Prokop et al. 2013, Soh et al. 2014, Hughes et al. 2015, Skinner et al. 2015). However, Y-chromosome sequences have not been available for fishes.

1.4 Studies on the sex determination in fish

A great variety of sex determination mechanisms exist in fish among individuals within a population, contrary to the apparent conservation of the gene differentiation network in vertebrates (Graves and Peichel 2010). Generally, the sex determination mechanisms in fish include genetic and environmental regulation. In genetic systems, certain components become dominant in influencing the direction of sex determination (Devlin and Nagahama 2002). In environmental regulation, the environment the fish live in affects the embryo development crucially. In most cases, this influence is associated with the change of temperature (Diaz and Piferrer 2015). For example, normally, the sex of channel catfish is determined genetically by the XY system, but female-skewed sex ratios could be generated by applying high temperature during the critical period for sex determination (Patino et al. 1996). This observation suggests that the sex of channel catfish is a combination of both GSD and ESD, and similar phenomenon has been seen more widespread in fishes than previously believed. The sex chromosome types in fish sex determination are varied. The male heterogamety (XY) is the most common sex determination mechanism as reported in fish. For example, medaka (Oryzias latipes) (Matsuda et al. 2002), channel catfish (*Ictalurus punctatus*) (Patino et al. 1996), and rainbow trout (Oncorhynchus mykiss) (Yano et al. 2012) have a XY system. However, many species have female heterogamety (ZW). Fish such as the turbot (Scophthalmus maximus) (Martinez et al. 2009), half-smooth tongue sole (Cynoglossus semilaevis) (Chen et al. 2014) have a ZW sex- determination system.

Extensive research efforts have been made to study GSD in fish species, especially with the rapid development of modern molecular genetics and genomics techniques. Two major approaches have been used for studies of sex determining genes in teleost fish: 1) Identification of Y-specific sequences; 2) Identification of male-specific transcripts. Apparently, when the Y-chromosome is highly divergent from the X chromosome, the first approach is quite advantageous. While the X and Y chromosome are very similar, analysis of male-specific transcripts could provide more advantages. Sex-biased expressed genes in one sex were thought to drive the phenotypic differences between males and females (Ellegren and Parsch 2007, Leder et al. 2010, Assis et al. 2012). Both approaches requires genome or transcriptome sequences, as well as QTL mapping to locate the candidate sex determination region.

Six master sex-determining genes have been isolated in fish to date: DM-domain gene on the Y chromosome (dmY), the major testis-determining factor in the Japanese medaka (Oryzias latipes) (XX-XY) (Nanda et al. 2002), anti-Müllerian hormone (amhY) in the Patagonian pejerrey (Hattori et al. 2013) and anti-Müllerian hormone receptor, type II (amhr2) in fugu (Takifugu rubripes) (Kamiya et al. 2012), gonadal somatic cell derived factor (gsdf) in the Luzon ricefish (Oryzias luzonensis) (Myosho et al. 2012), sexually dimorphic on the Y-chromosome gene (sdY) in the rainbow trout (Yano et al. 2012) and finally SRY-related HMG-Box gene 3 (sox3) in the medaka (Oryzias dancena) (Takehana et al. 2014). Until 2011, all four vertebrate master SD genes were known to code for transcription factors which could have been construed as evidence that gonadal sex determination in vertebrates is always triggered by transcription factors. However, the three novel candidates for the master SD genes in the Patagonian pejerrey, Oryzias luzonensis, and fugu code for growth factors or one of their receptors. Thus, these findings suggest alternative mechanisms of genotypic sex determination, in which the main trigger is not constrained to be a transcription factor

(Graves 2013, Kikuchi and Hamaguchi 2013). Together with the sex-determining genes identified in various species, here, we provide a brief overview of the research on sex-determining genes as well as other important genes in sex determination pathway (Table 1.1)

Table 1. 1 Previously reported sex-determining genes in different species

Species	important genes in testis formation	important genes in ovary formation	Other important genes in sex determination	System	Reference
Mammals	SRY (sex-determining region of Y) Sox9 Fgf9 Dmrt1	Wnt4 Foxl2 RSPO1	Wt1 Sf1 Gata4/Fog2	XX/XY	(McElreavey et al. 1995, Koopman 2001)
Chicken *Gallus gallus*	Z-linked DMRT1 Sox9	FOXL2 WNT4 RSPO1 Aromatase Estrogen Stabilised β-catenin		ZZ/ZW	(Hudson et al. 2005, Wang et al. 2014)
Reptilian	DMRT1 SOX9 PDGFR β	Estrogen receptor α Estrogen receptor β FOXL2 Aromatase(cyp19a1) WNT4 RSPO1	Amh (MIS) Androgen receptor (AR) DAX1 FGF9	XY/XX or ZZ/ZW	(Deeming and Ferguson 1988, Ezaz et al. 2006)
African clawed frog, *X. laevis*	Dmrt1	DM-W (W-linked DM domain) cyp19 Foxl2	SF1/ad4bp ERα	ZZ/ZW	(Yoshimoto and Ito 2011)
Medaka *Oryzias latipes*	DM-domain gene on Y chromosome (Dmy/Dmrt1b(y)) dmrt1	cyp19a1a	sox9b amh amhrII foxl2 sox9	XX/XY	(Matsuda et al. 2002)

Medaka *Oryzias luzonensis*	GsdfY (gonadal soma derived growth factor on the Y chromosome)			XX/XY	(Myosho et al. 2012)
Medaka *Oryzias dancena*	Sox3			XX/XY	(Takehana et al. 2014)
Patagonian pejerrey *Odontesthes hatcheri*	amhy (anti-Mullerian hormone on the Y)			XX/XY	(Hattori et al. 2012)
Tiger Pufferfish *Takifugu rubrupes*	amhr2			XX/XY	(Kamiya et al. 2012)
Rainbow trout *Oncorhynchus mykiss*	Sdy (*sexually dimorphic on the Y-chromosome*)			XX/XY	(Yano et al. 2012)
Tilapia *O. niloticus*	dmrt1	cyp19a1a hsd17b1 foxl2	sox9 amh	XX/XY	(Ijiri et al. 2008, Baroiller et al. 2009)
Zebrafish *D. rerio*	dmrt1	cyp21a2	amh FTZ-F1 (nr5a)/ Ff1(a-d) Wt1	Polygenic Sex Determination or ZZ/ZW	(von Hofsten and Olsson 2005, Bradley et al. 2011, Liew et al. 2012, Wilson et al. 2014)
Half-Smooth Tongue Sole *Cynoglossus semilaevis*	Dmrt1a	Cyp19a Cyp19b	Sox9 Sox10	XX/XY	(Chen et al. 2014)
Fruit Fly *Drosophila melanogaster*			*Sxl* (sex lethal) *Tra* (Transformer) *Tra*-2 (Transformer-2) *Dsx* (Doublesex)	XX/XY	(Steinmannzwicky 1988, Bachtrog 2005)

CHAPTER II. OVERVIEW AND RESEARCH OBJECTIVES

Molecular mechanisms for sex determination in fish have been well studies for only a few species. Some of the best examples are medaka, fugu, and rainbow trout. Highly different from mammals, teleosts exhibit remarkable plastic and diverse sex determination mechanisms. Thus, studies using evolutionary approaches often ended with negative results (Trukhina et al. 2013).

Channel catfish (*Ictalurus punctatus*), one of the lower teleosts, is the dominant aquaculture species in the United States, accounting for more than 50% of U.S. aquaculture production (Hanson and Sites, 2014). Male channel catfish usually grow faster than females (Goudie et al. 1990, Davis et al. 2007). It normally possess a relatively simple genetic sex determination system (XX/XY male heterogametic system), but environment, especially temperature can have a major impact on phenotypic sex; high temperature applied during the sex differentiation period could cause female-skewed sex ratios (Patino et al. 1996). Thus, a better knowledge of the sex determination mechanism and its controlling genes in channel catfish may have direct applications in aquaculture. In addition, sex is considered to be one of the most fundamental features of life. Understanding sex determination and its evolution is important for evolutionary biology.

Although a few studies on the sex differentiation were conducted and sex-linked markers were identified for channel catfish (Ninwichian et al. 2012, Sun et al. 2013), little is known for its sex determining locus. This was mostly due to the low marker densities for QTL mapping of sex determination locus. In addition, the sequences of the sex chromosomes were not available. In a historical genome project, our laboratory generated the reference genome sequences for channel catfish. However, the genome was sequenced using a mitotic gynogen (XX) individual, thereby missing the Y-chromosome sequences. It was reported that no significant difference in DNA content was detected from male and female cells in channel catfish, and male and female genomes carry identical or almost identical DNA, which makes

the discovery of the Y-specific sequences difficult (Tiersch et al. 1990). It is possible that only the subtle differences of sequences located on sex-determining region are responsible for the sex determination, therefore, the study of sex determination gene of channel catfish would then be extremely difficult.

The goal of my dissertation project is to 1) generate Y-chromosome sequences; 2) perform comparative analysis between X and Y chromosomes of channel catfish; 3) identification of the sex determination region on the sex chromosomes; and 4) identify the candidate genes for sex determination in channel catfish. Understanding the mechanism of sex determination in channel catfish is not only important for understanding of evolution of sex determination, but also important for aquaculture applications.

CHAPTER III. GENOME SEQUENCING OF A YY MALE CATFISH PROVIDES INSIGHTS INTO SEX DETERMINATION GENE IN CHANNEL CATFISH

3.1 Abstract

One of the most fundamental and fascinating processes of life is the determination of sex. Scientists have worked for hundreds of years to understand the mechanism of sex determination in the fields of developmental and evolutionary biology. In teleost, sex determination system exhibits surprisingly diverse and plasticity during evolution. Catfish has a XY male/XX female sex determination system, but the exact mechanism of its sex determination is unknown at present. As a first step toward the identification of sex determining gene(s) in catfish, we generated the first genome sequence assembly of the YY catfish using next-generation sequencing. QTL mapping and GWAS analysis were conducted to locate the sex determination locus to linkage group 4. This allowed the generation of sequences of the first Y-chromosome in the aquaculture species, which size is 45.79 Mb. Comparative analysis of X chromosome and Y chromosome sequences was performed to determine the similarity and differences of the sex chromosomes with respect to chromosome size, sequence identities and gene contents. This project would lay the foundation for further downstream analysis of genes involved in sex determination and differentiation in catfish.

Keywords: sex determination, next-generation sequencing, target sequencing, QTL mapping, GWAS

3.2 Introduction

The processes of sex determination are tremendously diverse in teleosts, ranging from hermaphroditism, environmental sex determination, to genetic sex-determination (Kikuchi and Hamaguchi 2013, Shen and Wang 2014, Chalopin et al. 2015). In most cases, the mechanisms of genetic sex determination of teleost fish are quite different from those of tetrapod, even though they originated from the same lineage about 450 million years ago, and share approximately 70% of genome similarity (Le Page et al. 2010). Unlike in mammals and birds, where distinguishable sex chromosomes and common master sex-determining genes are present (Sinclair et al. 1990, Smith et al. 2009), heterogametic sex chromosomes are only observed in around 270 species (less than 1%) of teleost fish, with about 70% of species being male heterogametic (XX females and XY males) and 30% being female heterogametic (ZZ males and ZW females) (Pandian 2011). A wide variety of genetic sex-determining mechanisms have been identified in teleost fish species, with various genes as the "master sex-determining genes" such as dmrt1 in medaka (*Oryzias latipes* and *O. curvinotus*) and half-smooth tongue sole (*Cynoglossus semilaevis*) (Matsuda et al. 2002, Matsuda et al. 2003, Chen et al. 2014), Gsdf in *O. luzonensis* (Myosho et al. 2012), Sox3 in *Oryzias dancena* (Takehana et al. 2014), sdY in rainbow trout (*Oncorhynchus mykiss*) (Yano et al. 2012), and amhy in Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al. 2012). In addition to specific sex-determining genes, a trans-species missense single nucleotide polymorphism (SNP) in Amhr2 was reported to be responsible for sex determination in fugu (*Takifugu rubripes*) (Kamiya et al. 2012) .

Generation of the Y-chromosome sequences has been difficult, and to date, Y-chromosome sequences have been assembled for only a handful species including human, chimpanzee, gorilla, rhesus macaque, mouse, and pig (Skaletsky et al. 2003, Hughes et al.

2010, Hughes et al. 2012, Soh et al. 2014, Skinner et al. 2016, Tomaszkiewicz et al. 2016). The fundamental reasons behind the difficulty of sequencing the Y chromosomes are quite different, however, between the mammals and the teleosts. In the therian species including the mammals, the X and Y chromosomes had a drastically different evolutionary path in the last ~170-180 million years (Livernois et al. 2012, Cortez et al. 2014). From the original a homologous pair of autosomes (Soukup 1971), the emergent X chromosome have been subject to strong conservation across different mammalian species (Ross et al. 2005, Bellott and Page 2009), while the Y chromosome, with its acquisition of a dominant male sex-determining function and accumulation of male benefit genes, has been accompanied by genetic isolation (recombination is limited only to the PAR, the homologous pseudoautosomal region), degradation via accumulation of repeats and gene loss (Graves 2010, Bachtrog 2013). As a result, mammalian Y chromosomes are composed of PAR, and X-degenerate, ampliconic, X-transposed, and heterochromatic regions (Skaletsky et al. 2003). The ampliconic regions contain palindromic repeats that range from several kilobases to several megabases long. Sequencing and assembly of such regions are very difficult. Recently, Tomaszkiewicz et al. (2016) developed a strategy to efficiently sequence the Y chromosome through flow sorting followed by next generation sequencing, droplet digital PCR and bioinformatics.

In contrast, the sex chromosomes in most teleosts are not highly differentiated; the karyotypes of sex chromosomes are undistinguishable, and their sequences are highly homologous between the X- and Y-chromosomes. Therefore, even though genetic studies can clearly map the sex determination gene to a chromosomal region, identification of sex determination gene is still extremely difficult. In this study, we sequenced the Y chromosome of channel catfish using a YY male template where the X chromosome is entirely absent,

allowing precise generation and assembly of the whole Y-chromosome sequences. Comparative genome analysis was then conducted between the Y chromosome sequences and the X chromosome sequences we previously reported (Liu et al. 2016), allowing identification of the putative sex determination gene in channel catfish.

3.3 Materials and Methods

3.3.1 Fish sources and sampling

All procedures involving the handling and treatment of used fish during this study was approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to initiation of the project. All animal procedures were carried out according to the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the United States. YY catfish is originally from US Department of Fish and Wildlife Hatchery in Uvalde, TX (Waldbieser and Wolters 2007). The wild channel catfish used in this project were obtained from 12 populations of 7 major watersheds in Alabama (Simmons et al. 2006). The sex reversal of XY catfish was conducted as Goudie's protocol (Cheryl A. Goudie 1983). In brief, the fish were fed a 45% crude protein ration that contained 60µg testosterone/ g of feed beginning at 6 days post hatch and continued for 3 weeks. This treatment produced normal XX females and sex-reversed XY females. At two and three years of age, we spawned those females with normal XY males. Consequently, all of offspring were examined as fingerlings at 6 months for reproductive tissues indicative of male or female gender.

At two years of age, the putative YY males were selected based on microsatellite data and mated with normal, unrelated XX females. Furthermore, progeny test was conducted by checking offspring's reproductive tissues at 6 months of age.

3.3.2 DNA extraction, library preparation and sequencing

The blood sample of YY catfish was flash frozen in liquid nitrogen and shipped on dry ice then stored at -80 ºC until DNA extraction. Genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue kit (cat. 69504). The concentration of genomic DNA was measured using an Agilent 2100 Bioanalyzer. DNA integrity was checked through an easy and fast way which is to use 0.8% agarose gel, run it at 80v for 45 minutes as previously described. DNA-seq library preparation and sequencing was carried out by HudsonAlpha Genomic Services Lab (Huntsville, AL, USA). Three libraries of DNA were constructed. The DNA library that had an average insert length of 300 bp was sequenced with 100bp paired end (PE) reads on an Illumina HiSeq 2000 instrument. Other two libraries were sequenced with 150bp Nextera Mate pair (Mp) reads with insert length of 3kb and 8kb respectively on an Illumina HiSeq 2500 instrument. PacBio sequencing was performed at Interdisciplinary Center for Biotechnology Research (Gainesville, FL USA) with 16 SMRT cells.

3.3.3 Pre-assembly trimming

Illumina adapter sequences were trimmed from raw reads, then reads with average quality scores less than 30 were shredded from the 3’ ends until it achieved 30, and reads with length shorter than 30 bp were discarded using BBMap 35.10 (sourceforge.net/projects/bbmap/). The trimmed Nextera Mate Pair reads were further split to both paired end reads and long mate pair reads by splitnextera module of BBMap based on the linker orientation. The duplicated reads was removed by PRINSEQ (Schmieder and Edwards 2011). In addition, the reads were error-corrected by BFC (Li 2015). At last, the quality of trimmed reads were inspected by FastQC (Patel and Jain 2012).

3.3.4 De novo assembly of YY genome

Illumina sequence from short insert fragments (300 bp) and mate-paired reads from 3-kb and 8-kb fragments were assembled with the dominant software for genome sequencing, the *de bruijn* graph based assembler SOAPdenovo2 (Luo et al. 2012). Multiple-kmer assemblies (k-mer sizes from 51 to 95) were produced. The best assembly was evaluated according to the number of contigs generated, N50 size, average contig size, and maximum contig length. PRINSEQ (Schmieder and Edwards 2011) was used to remove assembly redundancy with the minimal overlap length and percent identity in CAP3 to 100 bp and 99%. The resulting best assembly was used in subsequent scaffolding.

3.3.5 Scaffolding and gap filling of the YY genome

The scaffolding was performed by taking advantage of not only different insert lengths of the PE (300bp) and MP libraries (3kb and 8kb) but also the long distance connectivity of PacBio reads. We built up the male genome scaffolding by using the publicly available programs PBJelly (English et al. 2012) and SSPACE software (Boetzer et al. 2011). After the scaffolding, gaps were closed by PBJelly and GapCloser (Luo et al. 2012). Finally, post assembly polishment was conducted with Pilon (Walker et al. 2014).

3.3.6 Chromosome level scaffolding

Chromosome level scaffolding was conducted by integrating previously genome assembly and high-density SNP-based genetic map of channel catfish (Li et al. 2015). The final genetic linkage map was comprised of 54,342 markers (at 29,081 unique positions)

distributed across a genetic distance of 3,505.4 cM. The 70 bp of sequence flanking each SNP locus on the genetic map was mapped to the assembly scaffolds by BLASTN alignment (E-value maximal threshold 1e-10, > 95% sequence similarity, >65bp alignment length). Scaffolds that were adjacent on a chromosome were manually joined with a string of 100 "N"'s to represent the gaps between the two adjacent scaffolds based on the high-resolution genetic map.

3.3.7 QTL Mapping and GWAS of the Sex determination region

For QTL mapping of sex determination (SD) locus of catfish, we firstly constructed a single family genetic map by genotyping 187 fish in the family 3 using the catfish 250K SNP array (Liu et al. 2014). The sex phenotype of each fish was determined by histological examination of gonads after anesthetizing the individuals and recorded as binary traits. Then, 3,418 informative SNPs on the linkage map were selected based on the pedigree information and quality value. Consequently, the linkages between each marker and SD locus were estimated using the scanone function with EM algorithm and binary model integrated in R/qtl software (Broman et al. 2003). Significant threshold of LOD was obtained by permutation tests with 1, 000 replicates. 95% bayes confidence interval was determined by using the bayesint function.

For GWAS analysis of SD locus of catfish, 199 wild catfish was genotyped using the catfish 250K SNP array and their genders are identified by screening with sex-linked markers. The analysis was performed with linear regression model with SNP & Variation Suite 7 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). Samples passing the quality control (Dish value > 0.85) and SNP call rates threshold (>95%) were retained for analysis.

SNPs with missing genotypes >5% and minor allele frequency <5% were removed. The Manhattan plot was generated by Circos (Krzywinski et al. 2009) .

3.3.8 Annotation of the YY genome assembly

A library of catfish-specific repetitive sequences was first produced using RepeatModeler v1.0.8 (http://www.repeatmasker.org/RepeatModeler.html). Next, the repeat-annotated library was used to mask the genome assembly using RepeatMasker with the option of -nolow and -s. Finally, Augustus (Stanke et al. 2004) and Fgenesh (http://www.softberry.com) were used to predict genes within the repeat-masked assembly. Gene model parameters for Augustus were trained from conserved genes from vertebrate species using CEGMA (Parra et al. 2007). The amino acids predicted from the same genomic positon shared by both algorithms were retained and integrated for the downstream homolog-based annotation. Then, the amino acid sequences of the predicted genes were retrieved and queried against the Uniprot and NCBI non-redundant (NR) databases using BLASTP to identify the homologous genes with an E-value maximum threshold of 1e-5. The names of predicted channel catfish genes were assigned based on their homologous proteins. For those predicted coding regions with no blast hits, but containing more than 100 amino acids, both Pfam A and B databases (Finn et al. 2014) were used to scan their conserved functional domain with an E-value threshold at 1e-5. The names of matching genes were assigned based on their domain families. All of the genes with names of retrotransposable element, transposon, reverse transcriptase, RNA-directed DNA polymerase from transposon, transposase, and transposable elements were treated as potential repetitive elements and removed from the gene annotation. All of the genes with names of uncharacterized, unnamed, unknown, hypothetical, predicted protein were treated as unnamed genes and searched

against Uniprot and NR database as above to return the top ten matches. Any matches with names of potential repetitive elements were excluded from the annotation. The chromosomal position of each gene was assigned based on the chromosome level assembly, and genes with adjacent locations and identical gene names were labeled. Then, the tandem duplicated genes among the labeled genes were identified using MCScanX (Wang et al. 2013), and remaining genes were treated as fragments of a same gene and sub- grouped separately based on their position and names. Only the longest protein of each subgroup was kept to represent the corresponding gene. The final YY genome annotation included genes with names derived from BLAST analysis and genes with domain names derived from Pfam analysis.

3.3.9 Comparative analysis of X and Y chromosome sequences

In order to identify the Y-specific fragments, a comparative analysis was performed with the rapidly genome aligning software MUMmer. Cross alignment was performed between Y chromosome and X chromosome with the aim to identify the Y-specific sequences, and a comparative analysis was performed between male catfish genome and the female genome by BLASTN program as well. E-value cutoff was set to 1e-10, and the identity cutoff was set more than 95%. MUMmer was employed to perform the genome-wide alignment as well.

3.4 Results and Discussion

3.4.1 Mapping of the sex determination locus

To identify the sex determination locus, our first step was to map the sex determination locus to the chromosome. This was achieved through genetic linkage mapping (Li et al. 2015). Genotyping was conducted with 187 fish of a single family, and linkage analysis indicated that there was only one sex determination locus located on linkage group 4 (LG4, Figure 3.1).

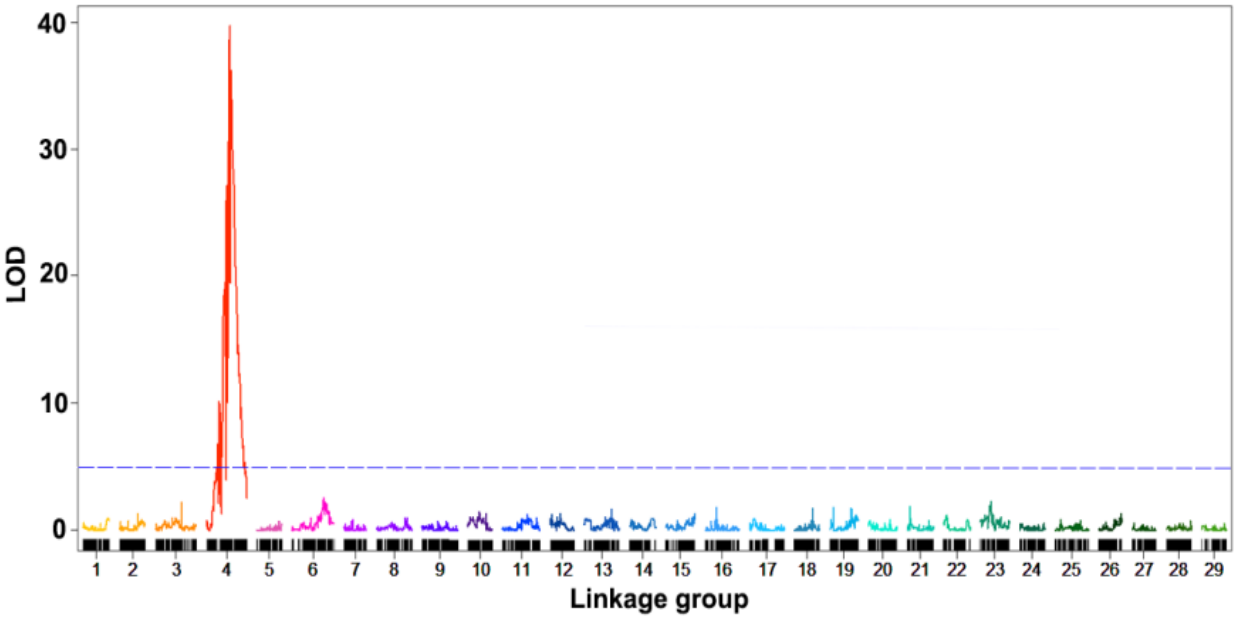

Figure 3. 1 Logarithm of odds (LOD) score versus genetic distance (cM) of 29 linkage groups of channel catfish of QTL analysis for the sex trait.

The purple dotted line indicates the threshold P-value for linkage analysis.

This linkage analysis was validated with additional 380 fish of two families (data not shown). Detailed linkage analysis indicated the sex trait was most tightly linked with 20 SNP markers spanning a genetic distance of ~1.0 cM (Figure 3.2).

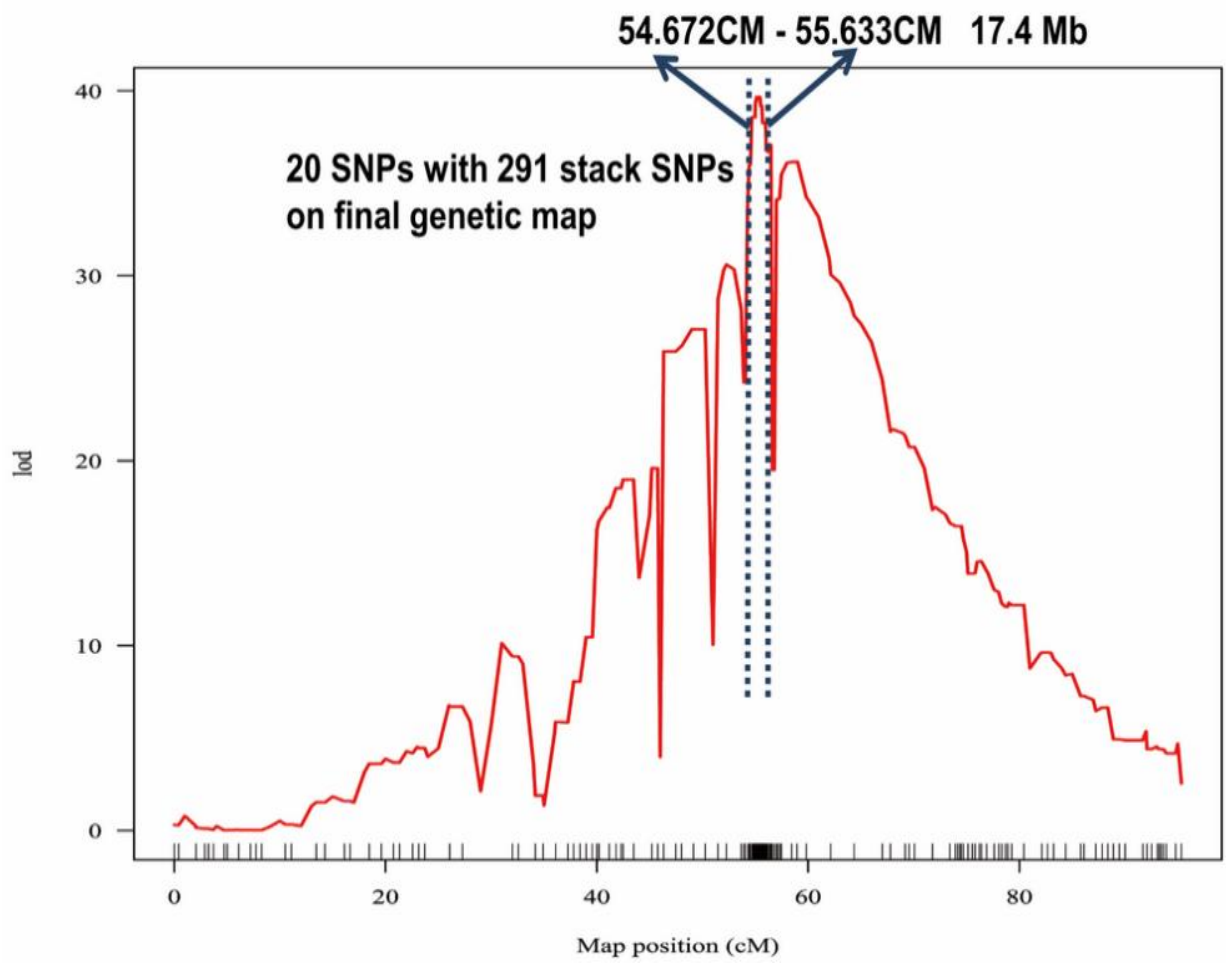

Figure 3. 2 LG4 LOD score versus genetic distance (cM).

Blue dotted lines indicate the 95% bayes confidence interval of the sex determination region of the QTL analysis.

However, placement of the linked SNP markers on the whole genome reference sequence indicated that the sex determination region spanned a physical distance of ~17.4 Mb on the sex chromosome.

Realizing the long haplotype blocks in F2 families, we attempted using GWAS analysis to take advantage of historically accumulated recombinations in the sex determination region with various unrelated fish individuals. A total of 199 wild channel catfish collected from 12 populations in seven watersheds (Simmons et al. 2006) were genotyped with the catfish 250K

SNP arrays (Liu et al. 2014). 123,199 SNPs were remained after quality control and utilized by GWAS. Consistent with the result of QTL mapping, four hundred sixty two SNPs were found significantly associated with the sex trait on LG 4, but not on any other chromosomes (Figure 3.3).

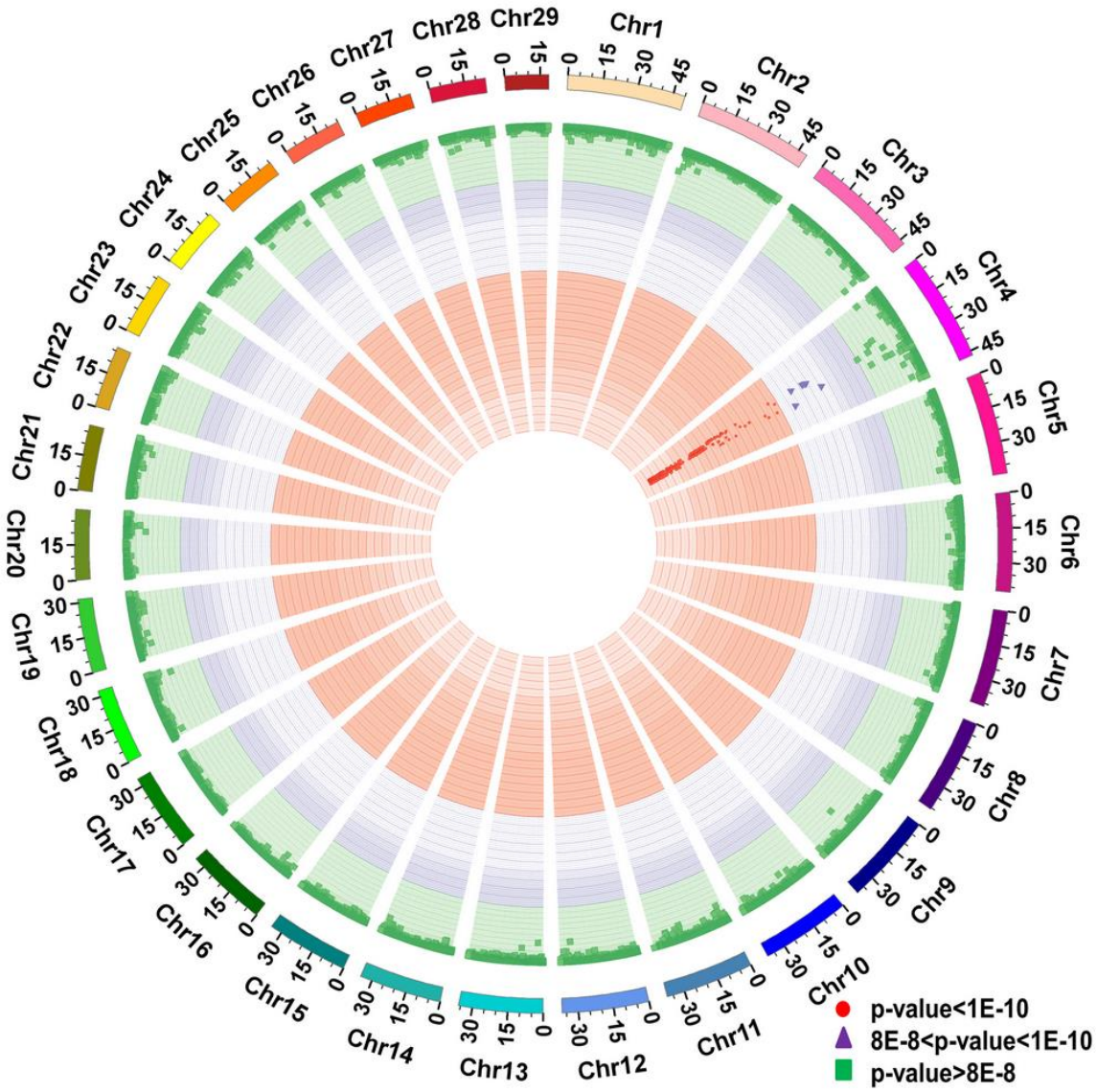

Figure 3. 3 Manhattan plot of genome-wide association analysis (GWAS) for sex trait of wild channel catfish.

Red dots indicate the threshold P-value for genome-wide significance. The purple dots indicate the threshold P-value for the significance of "suggestive association". The green dots indicate the P-value of no significance.

This allowed the sex determination region to be narrowed from 17.4 Mb as mapped using F2 population (Figure 3.2) to 8.6 Mb (Figure 3.4). Obviously, the 8.6 Mb is still a large genomic segment, but it allowed us to focus detailed analysis for sex determination of channel catfish in this limited region of sex chromosome.

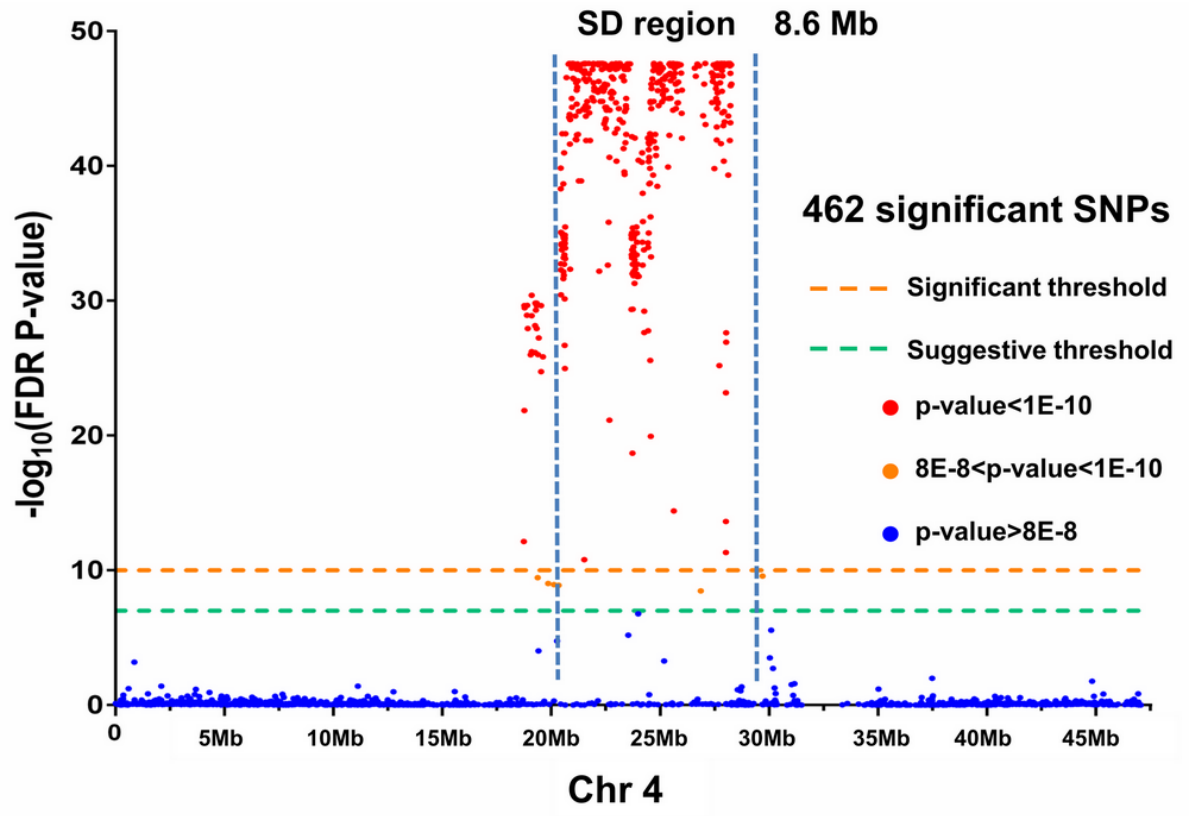

Figure 3. 4 A regional plot of the -log$_{10}$ (P-values) for 123,199 high quality SNPs versus physical genomic distances on LG 4

The Red dots indicate the threshold P-value for genome-wide significance. The orange dots indicate the threshold P-value for the significance of "suggestive association". The blue dots indicate the P-value of no significance.

3.4.2 Sequencing and assembly of the YY genome

The normal channel catfish genome contains 28 pairs of autosomes plus the sex chromosomes X and Y, with an estimated 1.0 Gb of DNA (LeGrande et al. 1984, Tiersch and Goudie 1993). We produced a reference genome sequence using an XX gynogen female template without the Y chromosome (Liu et al. 2016). Here we generated and assembled the reference genome sequence of channel catfish from a YY male without X chromosome to reduce assembly confusion between the homologous sequences from the X and Y chromosomes. Intra-scaffold gaps were filled with Illumina and Illumina-corrected PacBio sequence. Sequences of 103.3 X genome coverage were produced including 93X of Illumina sequences and 7.3X of PacBio sequences. The final YY channel catfish genome assembly included 1,032 Mb in 100,021 contigs and 18,575 scaffolds, with a contig N50 of 23,642 bp and a scaffold N50 of 1,031,626 bp (Table 3.1).

Table 3. 1 Sequencing and assembly of the YY genome of channel catfish

A. Sequences generated for the assembly of YY channel catfish genome				
Libraries	Number of reads	Average read length*	Genome coverage	Usage
300bp PE	334,232,498	88.7 bp	30X	Contig assembly
3kb mate paired	291,352,572	113.3 bp	33X	Scaffolding
8Kb mate paired	297,470,570	113.3 bp	33X	Scaffolding
Corrected PacBio reads	3,208,095	2,272 bp	7.3X	Scaffolding & gap filling
Total		-	103.3X	
B. Assembly statistics				
Total number of contigs	Total number of scaffold	Contig N50 (bp)	Scaffold N50 (bp)	Total assembled genome size in

				scaffolds (bp)
100,021	18,575	23,642	1,031,626	1,032,689,779

3.4.3 Targeted sequencing of Y chromosomes

The success of identification of the sex determination gene through comparative chromosomal analysis depends heavily on the completeness and contiguity of the sex chromosome sequencing. We used a targeted sequencing (Figure 3.5) for Y chromosome sequencing, using the XX genogen sequencing template and the YY male sequencing template, respectively, to ensure maximal levels of contiguous sequences in the sex determination region. A total of 5.9 Gb of PacBio sequences were generated from targeted sequencing for the Y chromosome. Assembly of all the available sequences for Y chromosome including all the sequences mapped to Y chromosome (discussed above) and the 3,245,015 PacBio reads from the targeted sequencing resulted in the assembly of 45.79 Mb of Y chromosome sequences in 3,405 contigs and 99 scaffold. Although gaps still existed, but the total of sequences in the gaps were estimated to be 997,081 bp, less than 2.2% of the sex chromosome sequences.

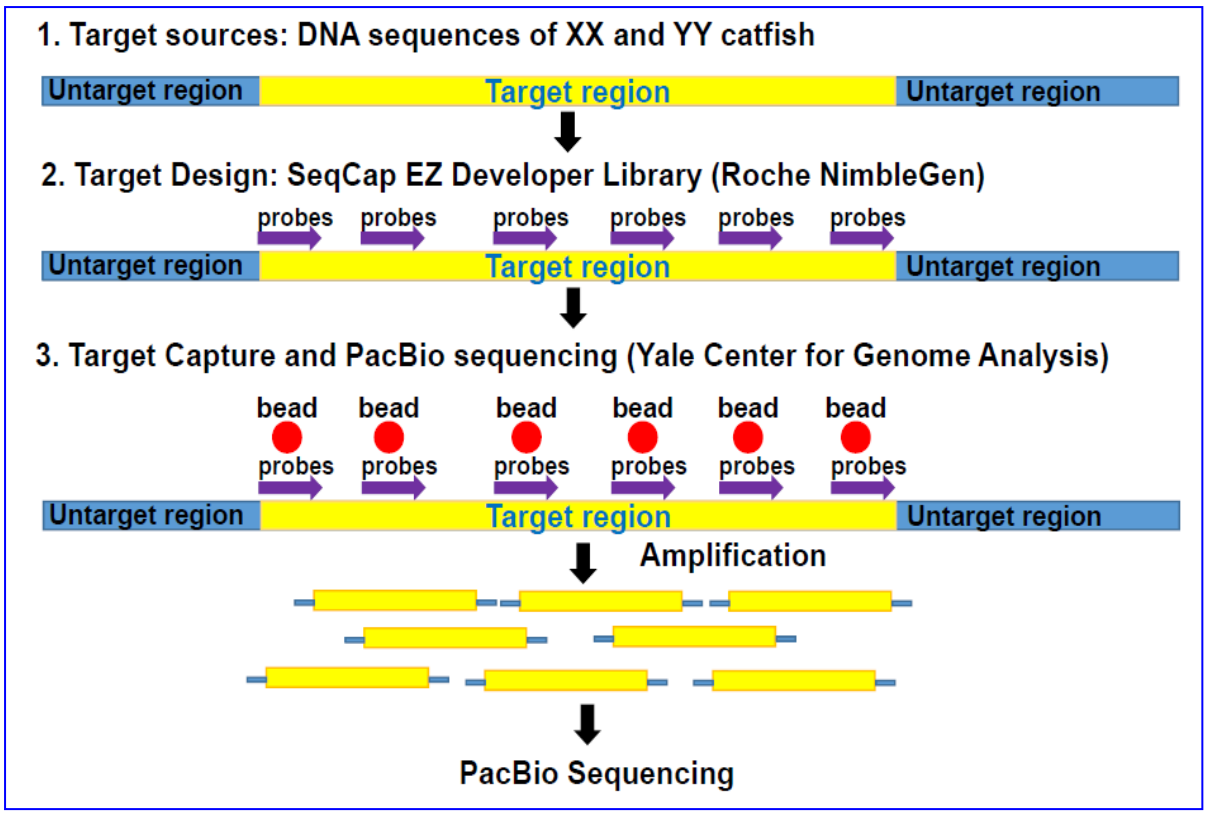

Figure 3. 5 Flow chart of target sequencing with Nimblegen probes and PacBio sequencing

After the chromosome level scaffolding and target sequencing assembly, more than 95% of scaffolds are assigned into 29 linkage groups of channel catfish (Table 3.2). Of the 18,575 scaffolds, 99 mapped to the Y-chromosome, spanning a physical size of 45.79 Mb.

Table 3. 2 Assignment of YY sequences to chromosomes

Chr4 is highlighted as sex chromosome.

Chromosome	Mapped SNPs	# of scaffolds	Physical size (bp)
1	1,843	222	49,100,713
2	2,159	273	47,970,275

3	2,049	198	46,460,332
4	2,096	99	45,787,968
5	1,903	192	42,793,815
6	2,001	142	41,460,702
7	1,914	174	40,376,802
8	2,098	205	39,783,902
9	1,783	243	37,873,879
10	2,063	246	37,476,358
11	1,501	164	36,795,683
12	1,799	125	36,109,199
13	1,666	151	35,015,328
14	1,841	113	34,311,406
15	1,785	128	34,133,473
16	1,268	115	34,033,367
17	1,390	195	32,886,635
18	1,876	118	31,851,771
19	1,638	187	30,833,328
20	1,722	147	29,255,232
21	1,577	101	27,357,732
22	1,545	73	26,149,602
23	1,635	109	25,768,931
24	1,298	169	25,202,140
25	1,464	94	25,034,826
26	1,465	186	24,726,610
27	1,618	115	23,898,114
28	1,452	120	23,629,454
29	1,079	101	18,357,885
Total	49,528	4,505	984,435,462 (95.3%)
Unmapped	4,814	14,070	48,254,317 (4.7%)

One notion we had was that we can align all the YY catfish genome sequence on the XX catfish genome sequence and vice versa, which would allow us to identify X- and Y-specific sequences. However, no chromosome-specific sequences were identified. Over 97.3% of XX catfish genome sequences were aligned on the YY genome sequence assemblies, and conversely, over 96.2% of the YY genome sequences were aligned on the XX genome sequence. In addition, over 99% of the Y chromosome sequences aligned on the X

chromosome sequence (Figure 3.6), suggesting that the catfish X chromosome and Y chromosome is extremely similar in their sequences and gene contents.

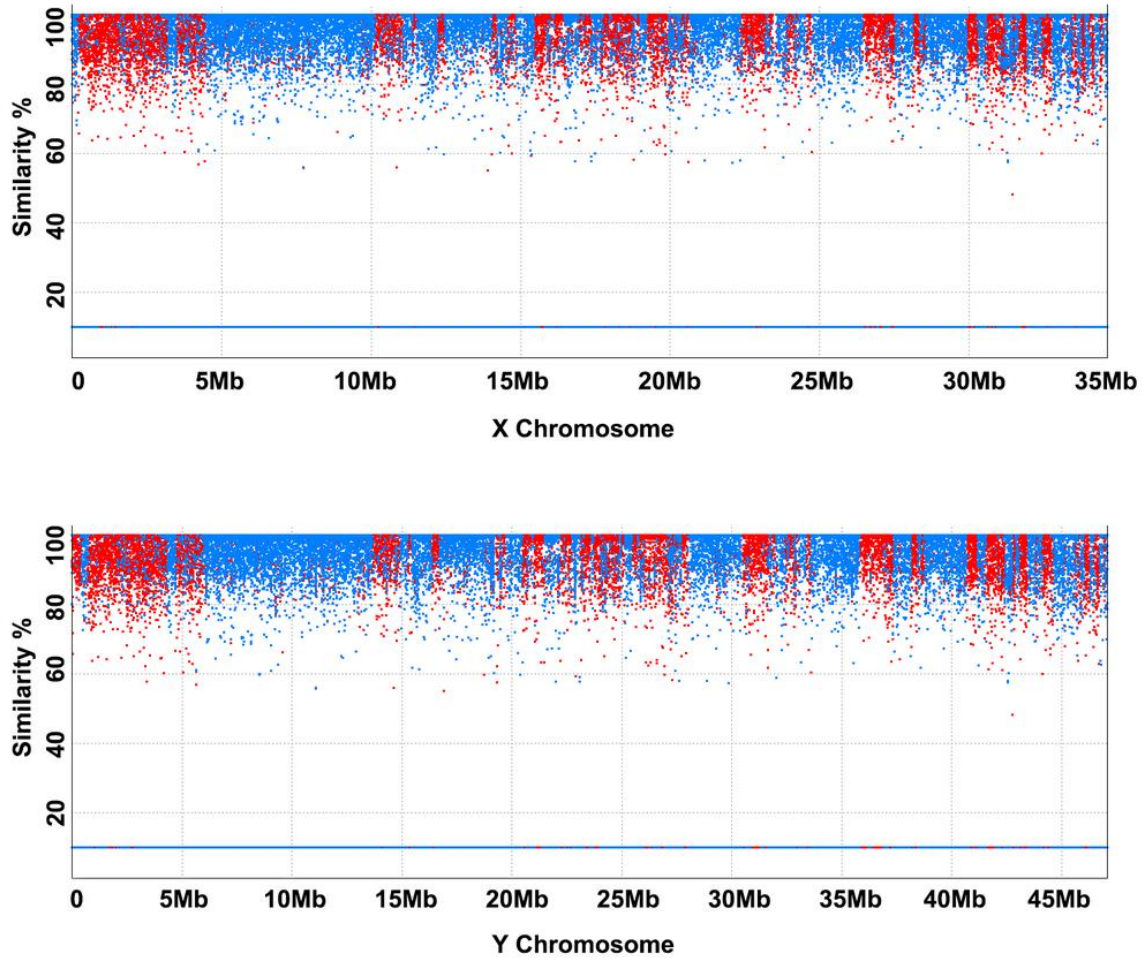

Figure 3. 6 Similarity and coverage plot of the alignment of X and Y chromosome of channel catfish

Blue dots indicate forward alignment and red dots indicate reverse alignment.

3.4.4 Gene content in the X and Y chromosomes

In total of 950 genes were annotated for both X and Y chromosome, and no sex specific genes were identified. In another way of saying, probably there is no extra gene in the catfish SD region that contains a distinct male-specific segment. Therefore, the X chromosome and Y chromosome are possibly undifferentiated. The sex of catfish could probably be controlled

by a very tiny difference in the genomic sequence. Such difference could be a duplicated copy of a specific gene, a single nucleotide polymorphism (SNP) locus or a combination of alleles, or a significantly differential expression pattern of a gene at the critical gonad developmental stage. The comparison result of X and Y chromosome in terms of sequences, SNPs and gene content are showed in Table 3.3.

Table 3. 3 Comparison of X and Y chromosomes

	Y chromosome	X chromosome
Number of scaffolds	99	39
Size (bp)	45,787,968	34,590,240 (estimated 45.8Mb with degenerates)
Size without gaps (bp)	44,790,887	34,079,759
Number of SNPs on genetic map	2,096	2,270
Genes	950	950
Genes annotated only on	0	0
Sex specific genes after reciprocal mapping	0	0

3.5 Conclusion

We generated the first assembly of YY genome sequence of channel catfish, which also provided the first Y chromosome assembly of all of the aquaculture species. For sex determination, one 8.6 Mb genome-wide significant QTL was identified on LG 4. Identification of sex determination gene was made possible in this work by direct analysis of sequences from the X chromosome and the Y chromosome. The use of the YY male, rather than the normal XY male, allowed entire avoidance of confusions or mis-assemblies of the X-chromosome sequences into Y chromosome or vice versa. This strategy should be

applicable to any system where sex reversal is possible including all teleost fish species. Technically, the sex reversal and production of YY males allowed pure templates of the Y chromosome without its homologous X chromosome, functionally equating the use of purified Y-chromosome as sequencing template (Tomaszkiewicz et al., 2016). As teleosts account for over 50% of vertebrate species, and numerous genome projects are ongoing with various fish species, the applicability of this strategy is very broad.

CHAPTER IV. IDENTIFICATION OF SEX-BIASED EXPRESSED GENES IN MALE AND FEMALE GONADS OF CHANNEL CATFISH AT THE ONSET OF OVARIAN DEVELOPMENT

4.1 Abstract

Channel catfish, Ictalurus punctatus, is the leading aquaculture species in the US. It's also a well-recognized model for study of sex determination mechanism due to its sexual plasticity. The gonadal differentiation in channel catfish starts as early as 19 day post-fertilization (dpf) in females, while the testicular differentiation in males occurs between 90 and 102 dpf. Toward the uncovering of processes involved in sex determination during sexual development, efforts are needed to profile genes that are regulated during early ovarian differentiation process. In this work, we conducted comparative transcriptome analysis of male and female gonads during sex determination stage at 10-14 and 15-19 dpf using high-throughput based RNA-Seq approach. Transcriptomic comparison between male and female gonadal tissues identified male-preferential genes such as BCAR1. The candidate genes identified in the study lay the foundation to unravel the mechanisms underlying sex determination and differentiation processes in catfish.

Keywords: fish, sex determination, RNA-Seq, ovarian differentiation, sexual development

4.2 Introduction

Sexual reproduction is a universal feature shared by multicellular animals for species propagation. Two major processes are involved in sexual development, sex determination and sexual differentiation. The sex determination process determines whether the bi-potential primordium will develop into a testis or an ovary, while the sexual differentiation process takes place after sex determination and involves the actual development of testes or ovaries from the undifferentiated gonad (Forconi et al. 2013). In vertebrate, sexual development is determined by two main factors, the genetic makeup of an individual and the environment. With genetic sex determination (GSD), genetic elements determine whether the individual is developed into a female or male. With environmental sex determination (ESD), environmental factors, such as temperature and photoperiod, control sex determination.

Gene pathways that control sexual development have been well documented in several model organisms such as *D. melanogaster*, *C. elegans*, and mammals, in which a master-switch sex-determining gene is determined. In humans, sex is determined by sex chromosomes, XX females and XY males. The sex determination depends on the presence of a single master sex-determining locus, the *Sry* gene, on Y chromosome. Expression of *Sry* early in the embryonic development initiates testis differentiation by activating male-specific developmental networks, while in its absence, the ovaries develop (Bachtrog et al. 2014). Further sexual differentiation in non-gonadal tissues and organs is initiated by the sex hormones (Ono and Harley 2013).

Sex-determining genes are mainly transiently expressed in the undifferentiated gonad, which directly determine bi-potential gonad into either a testis or an ovary. Master-switch sex-determining genes have been identified in several species and are highly conserved across taxa. For instance, the *Sry* gene determines sexual development in nearly all mammals

(Foster and Graves 1994). The *dmrt1* and its paralogs act as the primary sex determination signal in African clawed frog (*Xenopus laevis*) (Yoshimoto et al. 2008), chicken (*Gallus gallus*) (Smith et al. 2009), medaka (*Oryzias latipes*) (Matsuda et al. 2002), and tongue sole (*Cynoglossus semilaevis*) (Chen et al. 2014). In insects, the *transformer* (*tra*) gene and its paralogs act as the primary switch in sex-determination in fruit flies (*Drosophila melanogaster)* (McKeown et al. 1987), house flies (*Musca domestica*) (Hediger et al. 2010), the wasp (*Nasonia vitripennis*) (Verhulst et al. 2010) and the honeybee (*Apis mellifera*) (Beye et al. 2003). However, several cases of master-switch sex-determining genes do not have homologs found in closely related taxa, such as the *gsdfY* gene in medaka (*Orysias luzonensis*) (Myosho et al. 2012), the *amhr2* gene in pufferfish (*Takifugu rubripes*) (Kamiya et al. 2012), and the *Irf9,* an immunity-related gene in rainbow trout, (*Oncorhynchus mykiss*) (Yano et al. 2012). Polygenic sex determination mechanisms is also observed in some species. For instance, multiple sex-associated regions have been identified in the zebrafish genome (Bradley et al. 2011, Anderson et al. 2012, Liew et al. 2012). Polygenic sex determination has been also reported in the cichilid fishes (Ser et al. 2010, Parnell and Streelman 2013) and European sea bass (Vandeputte et al. 2007).

Channel catfish (*Ictalurus punctatus*) is the leading aquaculture species in the United States. It's also a well-recognized organism for sex determination studies due to its sexual plasticity. Channel catfish is a gonochoristic fish that have an XX female-XY male sex determination system (Davis et al. 1990), but does not exhibit sex chromosome polymorphism. The sex of channel catfish is mostly determined by genetic factors, and is affected by environmental factors such as temperature, photoperiod and hormonal exposure (Patiño et al. 1996). It has been reported that significantly biased sex ratios were observed after thermal and hormone treatments during early period of gonadal differentiation (Davis et al. 1990, Galvez et al. 1995, Patino et al. 1996). Relatively successful sex manipulation was

achieved in channel catfish by controlling hormonal and temporal treatments. For instance, feminization of channel catfish can be achieved by treatment of both aromatizable and nonaromatizable androgens before 21 days post-fertilization (dpf). Another study reported that a synthetic anabolic androgen, trenbolone acetate can suppress ovarian development to result in sterile masculinized females (Davis et al. 2000). In addition, high temperature (34℃) during early gonadal development also leads to increased ratio of females (Patino et al. 1996).

Genetic analyses of sex mechanisms have been conducted in channel catfish. Genomic DNA hybridization analysis of several genetic elements were conducted in the channel catfish, and identified no linkage of sex with SRY, ZFY, Bkm and Human Teleomeric Repeats that are associated with sex in mammalian sex-determining systems (Tiersch et al. 1992). In another study, according to an isozyme polymorphism, the glucosephosphate isomerase-B was reported to be linked to the sex-determining gene in the channel catfish (Liu et al. 1996). Moreover, sex-associated molecular markers have been mapped by linkage analyses (Waldbieser et al. 2001, Ninwichian et al. 2012). Further fine-scale mapping analysis resulted in the identification of one microsatellite marker (AUEST0678) that was closely associated with sex, and was effectively used for sex-typing of channel catfish from four different strains with 100% accuracy (Ninwichian et al. 2012). In order to identify the sex-determining gene in channel catfish, we assembled the testis transcriptome in a previous RNA-Seq study, and identified a pool of putative male-biased genes (Sun et al. 2013). However, only the adult testis tissue was sequenced in that study, genes involved in sexual development and differentiation were not able to be investigated.

In this work, we collected gonadal tissues starting from 10 dpf to 19 dpf to include the developmental stages involved in sexual determination. The sex-associated marker was used to sex type the individuals to obtain separate female gonadal and male gonadal tissues at

same development stages. RNA-Seq allowed direct comparison of gene expression of both female and male gonadal tissues during the early sexual differentiation stage.

4.3 Materials and Methods

4.3.1 Experimental animals and tissue collection

Three year channel catfish with good sex maturity characteristics were harvested from the ponds (E.W. Shell Fisheries Research Center, Auburn University, Alabama, USA) and held in tanks in the greenhouse. The general artificial spawning, fertilization and husbandry procedures were performed as previously described (Dunham et al. 2000, Su et al. 2013). In brief, females were injected with luteinizing hormone releasing hormone analogue (LHRHa) at 100 µg/kg body weight. Eggs were hand-stripped followed by artificial fertilization. Embryos were hatched and fry were reared in the trough (304 cm X 60 cm X 30 cm). Water temperature was maintained at 28 °C with 8~10 L/m water flow to control the ammonia to zero. The dissolved oxygen was maintained at ~6 ppm. Water quality was monitored daily. Fries were fed daily to satiation with the Aquamax Fry Starter100 (Cat#: 000-5553, Aquamax, MO, USA).

About 50 fry were collected each day starting from the 10th day post fertilization (dpf) to 19 dpf. Fry were euthanized with tricaine methanesulfonate (MS 222) at 300 mg/L before samples collection. Each fry was cut into two portions of head and body. The samples were flash-frozen and stored at -80 ˚C until DNA extraction and RNA extraction.

4.3.2 Sex genotyping

Thirty fry from each day were selected for genotyping to determine the sex with the sex-linked microsatellite marker, AUEST0678, following as previously described

(Ninwichian et al. 2012). Genomic DNA was extracted from the head sample of each catfish fry following previous protocols. Briefly, samples were digested in a 1.5 ml centrifuge tube containing digestion buffer and Proteinase K at a concentration of 100 µg/ml. DNA was isolated using the Gentra Puregene Tissue Kit (QIAGEN, USA), following manufacturer's instructions. The concentration and purity of isolated DNA were estimated using an Ultrospec 1100 Pro spectrophotometer (GE Sciences, NJ, USA) as well as electrophoresis on a 1.5% agarose gel. PCR amplification was performed in a 5-µl reaction mixture containing 1 µl of 50 ng/ µl genomic DNA, 0.5 µl of 10 PCR buffer, 0.2 µl of 50 mM $MgCl_2$, 0.4 µl of 2.5 mM dNTPs, 0.2 µl of 10 pmol/µl forward primer (5'ACATCGCTTTGAGAAGC TGC3'), 0.3 µl of 10 pmol/µl reverse primer (5'GTGAATGTGAGACTAACAGGAGG3'), 0.1 µl of 1 pmol/µl primer label IRD700 [labeled with infrared dye (IRD)-IRD700 or IRD800 from LI-COR Biosciences, Lincoln, NE] and 0.05 µl of 5 U/ µl of Platinum Taq polymerase. A touchdown PCR profile was performed as previously described (Ninwichian et al. 2012). PCR products were analyzed on a 7% polyacrylamide gel using a LICOR 4300 DNA Analyzer (LICOR Biosciences, Lincoln, NE). After gel electrophoresis, the sex of a catfish fry was determined based on the specific banding pattern that is one shared band (~212 bp) was observed in both males and females, while a 205-bp product was present only in male channel catfish (Ninwichian et al. 2012).

4.3.3 High throughput RNA sequencing

A total of 10 female fry and 10 male fry with conclusive determination of sexes were randomly selected from each day for RNA-Seq sequencing. The body portion of 10 male catfish fry collected in the same day were pooled and homogenized with mortar and pestle in the presence of liquid nitrogen. Similarly, the body portion of 10 female catfish fry that were collected in the same day were also pooled and homogenized. RNA were extracted using the

RNeasy Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. RNA concentration and integrity were measured on an Agilent 2100 Bioanalyzer. Equal amounts of RNA from every five days were pooled from 10-14 dpf and 15-19 dpf for male and female, respectively. These four pooled samples were outsourced for RNA-Seq at the HudsonAlpha Genomic Services Lab (Huntsville, AL, USA). The RNA-Seq libraries were prepared following the standard TruSeq protocols, and were sequenced on an Illumina HiSeq 2500 instrument for 100 bp paired-end reads.

4.3.4 De novo transcriptome assembly

The *de novo* assembly of short reads was performed using Trinity (2012-10-05 release) with RNA-Seq reads sequenced from all eight samples. Before assembly, raw reads were trimmed by removing adaptor sequences and ambiguous nucleotides. Reads with quality scores less than 20 and length below 30 bp were trimmed. The resulting high-quality sequences were used in the subsequent assembly. The short reads were assembled into the unique sequences of transcripts in Inchworm via greedy k-mer extension (k-mer = 25). After mapping reads to Inchworm assembled contigs, Chrysalis incorporated reads into deBruijn graphs and the Butterfly module processed the individual graphs to generate full-length transcripts. In order to reduce redundancy, the assembly was passed to the CD-Hit (version 4.5.4) for multiple alignments (Li and Godzik 2006). The threshold of sequence identity was set as 1.0, with the alignment covering >90% of the shorter sequence.

4.3.5 Transcriptome annotation

The assembled contigs were annotated by searching against the NCBI zebrafish RefSeq protein database, the UniProtKB/SwissProt database and the non-redundant (nr) protein database using the BLASTX program with E-value cutoff of 1E-5.

4.3.6 Identification of differentially expressed genes

The high quality reads from each sample were mapped onto the assembled transcript sequences using Bowtie software (Langmead and Salzberg 2012) with default parameters. The RSEM program (Li and Dewey 2011) was then used to estimate the expression abundance of the transcripts. The total number of mapped reads for each transcript was determined, and then normalized to determine FPKM (Fragments per Kilobase of transcript per Million mapped reads). Differential expression analysis was performed using edgeR package (Robinson et al. 2010). All the samples were normalized together. Normalization was performed by trimmed mean of M values (TMM) (Robinson and Oshlack 2010). TMM equates the overall expression levels of genes between samples under the assumption that the majority of them are not differentially expressed. Transcripts with absolute fold change values of greater than 1.5 and a FDR adjusted p-value < 0.05 were included in analysis as differentially expressed genes.

4.4 Results and discussion

4.4.1 Sex phenotyping

A total of 300 individuals (30 fish each day from 10 dpf to 19 dpf) were selected for genotyping to determine the sex phenotypes. The vast majority (291/300) of individuals were determined with conclusive sex phenotypes, with 139 being female and 152 being male. From each day, 10 individuals of female and male with clear sex phenotypes were pooled for RNA isolation, respectively. Equal amount of RNA from every five sequential days starting from 10 dpf to 19 dpf were pooled for female and male, respectively.

4.4.2 RNA-Seq of catfish gonads during sexual differentiation

Illumina-based RNA sequencing (RNA-Seq) generated a total of 240.0 and 252.5 million reads for the female and male gonadal tissues, respectively. Reads from each sample were retrieved through the use of multiple identifier (MID) tags (Table 4.1).

Table 4. 1 Statistics of RNA-seq data

Time-points (dpf)	Number of reads after trim		Average length (bp)		Total bases after trim (bp)	
	Female	Male	Female	Male	Female	Male
10-14	71,680,326	47,911,046	94.4	94.6	6,766,622,774	4,532,384,952
15-19	54,135,645	78,522,972	94.4	94.3	5,110,404,888	7,404,716,260
Total	125,815,971	126,434,018	-	-	11,877,027,662	11,937,101,212

At least 46 million reads were obtained for each of the eight samples. After data trimming to remove ambiguous nucleotides, low-quality sequences (quality scores < 20) and reads with too short length (< 30 bp), a total of 125.8 and 126.4 million reads with average read length greater than 94 bp were retained for female and male, respectively.

4.4.3 De novo transcriptome assembly

The *de novo* transcriptome assembly produced a total of 469,815 contigs with minimum length of 200 bp (Table 4.2). Of which, 173,308 contigs had lengths greater than 500 bp, and 94,094 contigs had length greater than 1000 bp. The largest contig had a length of 45,308 bp, and the average contig length is 832.9 bp. A total of 72% short reads were able to be mapped onto the assembly, while the rest of short reads either remain singletons or were assembled into contigs with lengths less than 200 bp.

Table 4. 2 Summary of *de novo* assembly results of Illumina sequence data from catfish using Trinity

	Trinity
Contigs(≥200bp)	469,815
Large contigs (≥500bp)	173,308
Large contigs (≥1000bp)	94,094
Maximum length (bp)	45,308
Average length (bp)	832.9
N50 (bp)	1,718
Reads mapped to final reference (%)	72.04%

4.4.4 Transcriptome annotation

The homology search-based annotation by BLASTX against various public protein databases assigned protein identities to a portion of assembled transcript sequences. A total of 138,444 transcript contigs had hits to proteins in the NCBI zebrafish RefSeq protein database, which had 20,210 unique protein hits and 2,486 of which are hypothetical proteins (Table 4.3). Similarly, a total of 116,739 transcript contigs had hits to proteins in UniProt database, with a total of 23,403 unique protein hits. By searching against NCBI NR database, 148,313 transcript contigs had hits to 23,403 unique proteins and 2,473 of which are hypothetical proteins.

Table 4. 3 Summary of gene identification and annotation of assembled catfish contigs based on BLAST homology searches against various protein databases (Zebrafish, UniProt, nr)

Putative gene matches were at E-value ≤ 1e-5. Hypothetical gene matches denote those BLAST hits with uninformative annotation. Quality unigene hits denote more stringent parameters, including score≥100, E-value ≤ 1e-20

	Contigs with putative	Annotated contigs ≥500bp	Annotated contigs ≥1000bp	Unigene matches	Hypothetical gene matches	Quality Unigene matches

	gene matches					
Zebrafish	138,444	98,091	75,229	20,210	2,486	13,244
UniProt	116,739	86,381	57,546	23,403	0	18,602
NR	148,313	101,596	64,279	33,303	2,473	24,442

4.4.5 Analysis of differentially expressed genes between male and female

1,999 differentially expressed genes between female and male gonad tissues were identified by directly comparing the expression levels at same developmental stages. At 10-14 dpf, 754 genes were expressed with higher levels in the females, while 511 genes were expressed with higher levels in the males (Table 4.4). At 15-19 dpf, 516 genes were expressed with higher levels in the females, while 800 genes were expressed with higher levels in the males.

Table 4. 4 Identification of differentially expressed genes between male and female catfish from 10 dpf to 19 dpf

Time points (dpf)	10-14		15-19	
	High in male	High in female	High in male	High in female
Number of differentially expressed contigs	589	885	946	609
Number of differentially expressed genes	511	754	800	516
Number of genes mapped on LG4	10	21	22	11

To qualify for the sex determination gene, the gene must be not only located in the sex determination region in LG4 but also be sexually differentially expressed in the males and females during early stages of sex determination, 10-19 days after fertilization (Patino et al. 1996). As shown in Figure 4.1A and Figure 4.1B, only 11 genes in the sex determination region were differentially expressed, of which only one gene that was differentially expressed in the males, but not in the females through the whole 10-19 dpf period. This gene was breast cancer anti-estrogen resistance protein 1 (BCAR1).

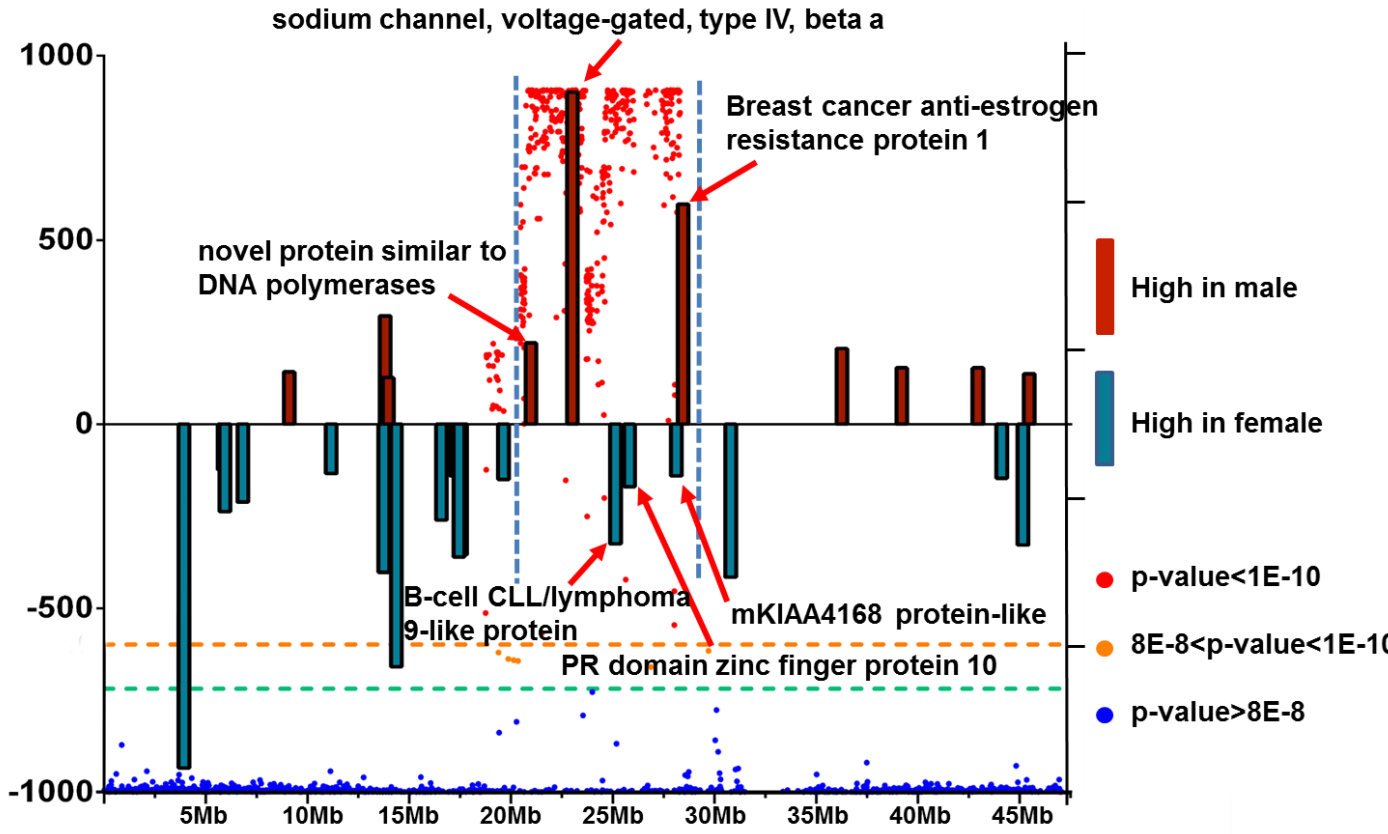

Figure 4.1 A Differentially expressed genes from 10 dpf to 14 dpf on the chromosome 4

Red bars indicate highly expressed genes in male, and blue bars indicate highly expressed genes in female.

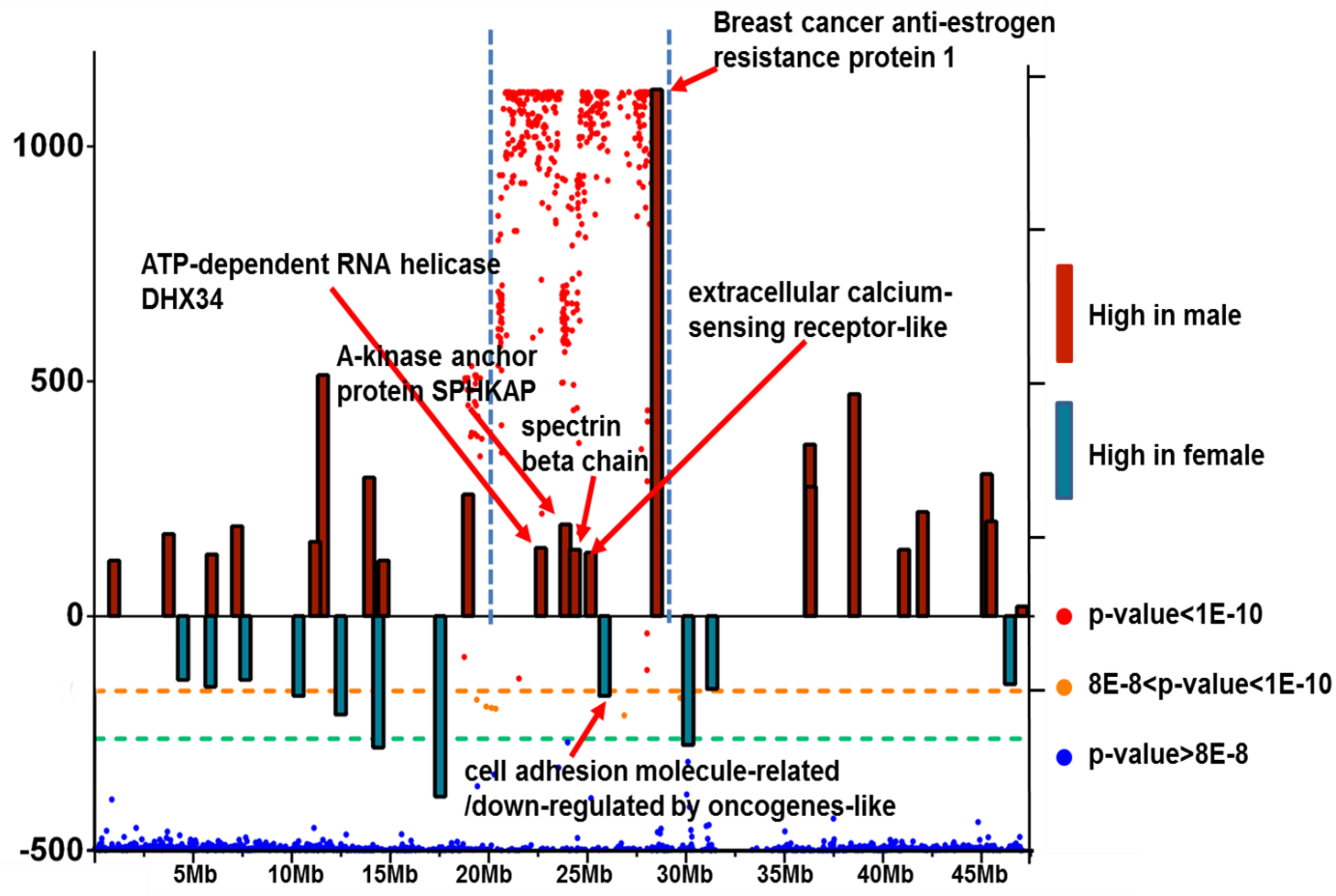

Figure 4.1 B Differentially expressed genes from 15 dpf to 19 dpf on the chromosome 4

Red bars indicate highly expressed genes in male, and blue bars indicate highly expressed genes in female.

BCAR1 was a docking protein which plays a central coordinating role for tyrosine kinase-based signaling related to cell adhesion (Cabodi et al. 2010) and interacts with estrogen receptor and modulates estrogen signaling. It was well known that estrogen is both necessary and sufficient to drive ovarian development in many nonmammalian vertebrates (Pask 2012, Trukhina et al. 2013). Also in human, overexpression of BCAR1 in estrogen-dependent breast cancer cells was sufficient to drive cell proliferation in the presence of antiestrogens (Brinkman et al. 2000). Conversely, lowering the amount of BCAR1 expression in ovarian

cancer was sufficient to suppress tumor growth and progression of cancer cells (Nick et al. 2011, Tornillo et al. 2014), which suggested BCAR1 plays an antagonistic role against the growth of the ovarian cells through stimulation of apoptotic and autophagic cell death. It was also reported that a threshold number of developing germ cells could be one possible mechanism of sex determination of ovarian fate in fish (Rodriguez-Mari and Postlethwait 2011, Kikuchi and Hamaguchi 2013). BCAR1 gene, therefore became the gene of interest for further analysis of the sex determination.

4.5 Conclusion

In this study, we conducted comparative transcriptome analysis of male and female gonads of channel catfish at 10-19 dpf that allowed expression profiling of genes expressed differentially during critical sex determination stage. In this process, 1,999 differentially expressed genes between female and male gonad tissues were identified. Among them, 11 unigenes were in the sex determination region. This RNA-Seq analysis generated a list of candidate sex determination genes for following functional analysis and provided insights into understanding the mechanism of the sex determination process of catfish.

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