A Syntenic Region Conserved from Fish to Mammalian X Chromosome

Guijun Guan, 1,2 Meisheng Yi, 2,3 Tohru Kobayashi, 2,4 Yunhan Hong, 5 and Yoshitaka Nagahama 2,6

1 Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, College of Fisheries and Life Sciences, Shanghai Ocean University and Laboratory of Reproductive Biology, Ministry of Education, Huchenghuan Road 999, Shanghai 201306, China
2 Laboratory of Reproduction, National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan
3 Laboratory of Molecular Reproductive Biology, School of Marine Sciences, Sun Yat-sen University, 135 Xingang West Road, Guangzhou, China
4 Lab of Molecular Developmental Biology, Institute for Environmental Sciences, University of Shizuoka, Yada, Shizuoka 422-8526, Japan
5 Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543
6 South Ehime Fisheries Research Center, Institution for Collaborative Relations, Ehime University, 3 Bunkyo-cho, Matsuyama 790-8577, Japan

Correspondence should be addressed to Guijun Guan; gjguan@shou.edu.cn and Yoshitaka Nagahama; nagahama.yoshitaka.mh@ehime-u.ac.jp

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1. Introduction

Understanding the evolution of sex is an on-going challenge for biologists. In spite of the common features of sexual reproduction and associated processes of cell differentiation present in vertebrates, the sex determination systems and sex chromosomes are highly divergent between species and have rapidly evolved [1]. Sex can be determined genetically (genetic sex determination, GSD) and environmentally (environmental sex determination, ESD) in response to stimulation from temperature, other environmental factors, or social cues or a combination of GSD and ESD [2]. Even with the GSD system, different species have adapted diverse GSD factors as a trigger to initiate the onset of sex determination. In mammals, the heterogametic male XY system uses SRY, a sex-determining gene on the Y chromosome (SRY) to initiate the male pathway. In birds and reptiles, a homogametic male or a ZW system uses dosage compensation to initiate the sex differentiation cascade. Thus the initiation factors and the sex chromosome sets are highly variable across species, although some of the downstream regulatory factors driving differentiation may be highly conserved, such as Dmrt1 [3]. In teleosts...
fish, there are a variety of sex determination mechanisms ranging from hermaphroditism to gonochorism and from ESD to GSD [4]. Therefore, teleost species are especially suitable for studying sex determination from the evolutionary point of view. Different factors initiate the sex-determining pathway among numerous teleost species displaying GSD. For example, a DM-domain transcription factor on the Y chromosome (DMY) has been identified as the master gene in medaka fish (Oryzias latipes) [5, 6]; Y-linked anti-Mullerian hormone (amh) initiates the onset of sex differentiation in Odontesthes hatcheri [7]; and a sex-linked polymorphism of amh receptor (amhr2) is responsible for male development in Takifugu rubripes [8]. Other sex-determining genes include gsd3, sox3, and sdY in Oryzias luzonensis, Oryzias dancena, and Oncorhynchus mykiss, respectively [9–11]. These genetic elements are scattered among various sex chromosomes, with a lack of homology among fish [12] and are not shared by birds or mammals. In tilapia, a group of cichlid fishes that are one of the most important food fishes in the world, few morphological differences exist and few sex-linked molecular markers are available for the cytogenetic identification of X or Y homomorphic sex chromosomes, which are apparently poorly differentiated [13]. Sex can also be easily reversed by temperature, social factors, or hormone treatment in this fish, and most hybrids are fertile [14]. All these features contribute to the reasons why the sex-determining factor and sex chromosome are still poorly understood in this species. Nevertheless, from breeding test [15] in the Nile tilapia (Oreochromis niloticus), sex is thought to be determined primarily by an XY system, in which the heterozygous sex is the male (XY). The largest chromosome pair (chr1) has been proposed as the sex chromosome, based on the presence of an unpaired segment in the terminal region in males visualized by synaptonemal complex (SC) analysis [16] and a quantitative sequence difference existing between X and Y detected with DOP PCR probes from chr1 microdissection [17, 18]. Several AFLP markers mapped to chr1 have been reported to be tightly linked with phenotypic sex in certain families but absent in other families, indicating a distance between these AFLP markers and sex-determining locus [19, 20]. Recently, a single nucleotide polymorphism (SNP) within amh was reported to associate to phenotypic male in certain tilapia line [21]. A gene involved in a testicular differentiation, tilapia Dmrtl (DM related transcriptional factor 1), is not a Y-linked gene as it was largely induced in sex-reversed XX-males where the expression correlates with testicular differentiation [22]. In medaka, phylogenetic analysis revealed that the duplication of autosomal Dmrtl and translocation of that copy (dmt1lB/Y or dmy) onto the Y chromosome occurred only in certain Oryzias species relatively late in evolutionary terms [23]. It is not present in tilapia [24]. So far, seven DM-related genes have been cloned in tilapia, but none of these DM genes are associated with the Y chromosome Guan et al. [22]. On the other hand, estrogen biosynthesis is an evolutionary ancient process and estrogens are indispensable for ovarian differentiation in female fish [25]. The expression of cytochrome P450 (aromatase) which is responsible for production of estrogens from androgens occurs prior to gonad differentiation in female, indicating its potential roles in ovarian differentiation [26]. Aromatase expression was reduced in XX-males produced by hormone treatment or temperature induction [27, 28]. Two types of aromatase genes have been identified in tilapia [29], but neither the brain type nor the ovarian type is located on chr1 [30].

We employed cytogenetic tools and molecular analysis to identify any DNA differences between X and Y chromosomes. Both comparative genomic hybridization (CGH) and random amplified polymorphic DNA (RAPD) assay are powerful methods commonly used to detect molecular differences between normal and cancer cell genomes at the cytogenetic level [31] and to identify sex-linked elements in plants and animals [32, 33], respectively. CGH assay has also been applied in the identification of Y chromosomes in Drosophila, the W chromosome in bird, and the Y chromosome in mammals including human [34].

2. Materials and Methods

2.1. Experimental Animals. Two fish stocks were used in this study. One originated from Egypt and has been inbred for seven generations, termed XeXe for females and XeYe for males. The other is YfYf supermales (Fishgen strain), a commercial strain purchased from the Aquaculture and Aquatic Resources Management program., Asian Institute of Technology in Thailand. All females were produced by artificial fertilization of genetic female (XeXe) eggs with sperm of neomales (XeYf) produced by hormonal treatment described previously [28]. All males (XeYf) were produced by crossing genetic females (XeXe) to YfYf supermales. The XeYf males were mated to XeXe females to make an outbred stock. Fish were kept in tanks supplied with recirculated fresh water at 26°C until use. It was confirmed that no sex reversal was observed under these rearing conditions, as phenotypic sex was consistent with gonadal sex, as phenotypic sex was consistent with gonadal sex based on results of sacrificing individuals randomly selected from each experimental batch. Genetic XeYf males utilized in this work consist of five F1 generation individuals (XeXe female crossed with YfYf supermale) and two F4 individuals after three successive generations of XeXe and XeYf (sibling-mating).

2.2. Chromosome Preparations and Comparative Genomic Hybridization. Metaphase spreads were prepared from head kidney leucocytes of O. niloticus according to a previous description with some modification [35]. Leucocytes were collected and mitotically arrested by colchicine (1 μg/mL), prior to suspension in hypotonic 0.075N KCl. The suspension was then centrifuged and resuspended in Carnoy fixative solution (3:1 methanol:acetic acid solution) and then dropped onto glass slides. Slides were kept under −20°C refrigerator until use.

Probes for CGH were derived from genomic DNA extracted from XeXe sex-reversed males and YfYf supermales with phenol chloroform treatment and ethanol precipitation. X-derived and Y-derived probes were labeled with digoxigenin (DIG) or biotin by random priming using “High

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with FITC-anti-biotin antibody (in green) and should identify signals derived from X∗Xe specific DNA contents (Figure 1(a)). In contrast, DIG-labeled probes derived from YfYf genomic DNA prehybridized to X∗Xe samples were detected by rhodamine-conjugated antibody against DIG (in red). Therefore, using these X∗Xe-green and YfYf-red probes, we expected to identify sex chromosome with small DNA content differences resulting from recombination suppression. Results of dual-color CGH are entirely consistent with our expectations. The difference of DNA contents between the largest chromosomes in XY samples was obvious in XY spreads compared to X∗Xe spreads shown in Figures 1(b) and 1(c). Signals from the Y-derived probe hybridized preferentially at the long arm of chromosome 1 (chr 1), the largest sex chromosome, in contrast to the X-derived signals which were evenly distributed over the whole region of this chromosome. Thus, a difference was clearly visualized by this dual-color CGH in XY spreads. In addition to chr 1, the DNA heterogeneity extends to one small pair of chromosomes identified by asterisks in Figures 1(b) and 1(c), indicating some difference between X∗Xe and YfYf strains, which may be linked to phenotypic male and female sex.

3.3. Comparative Genome Analysis of R52. The tilapia genome has recently been sequenced and partially annotated. Thirty microliters of pretreated probe mixture were hybridized to chromosome slides from X∗Xe females and YfYf males in a moist chamber. After 72 hours of incubation at 50 °C and subsequent reaction with rhodamine-anti-DIG and FITC-anti-biotin antibodies (Roche Diagnostics, Basel, Switzerland), fluorescence signals were captured by CCD image analysis system (Leica) and documented with Openlab software (Improvision).

3.2. Isolation of a Putative X-Linked Marker. A total of 298 primer combinations from 24 arbitrary 10-mer oligonucleotides were tested using DNA pools from eight X∗Xe pseudomales, seven X∗Yf males (five F1 X∗Yf male and two F4 X∗Yf males after three successive generations of X∗Xe female mating to X∗Yf male), and two YfYf supermales, respectively, as shown in Figure 2(a). Products from sixteen primer sets displayed a specific fragment derived from X∗Xe and/or YfYf, with an example shown in Figure 2(b). Polymorphisms from ten primer combinations were replicated by PCR using DNA from each individual fish. Eleven fragments were finally excised, gel purified, and subcloned for further analysis. Five clones were sequenced for each excised fragment. Clones present more than twice among each five sequences were used as a probe for Southern analysis and further confirmed by a more reliable and reproducible STS-PCR analysis. Two X-linked fragments were detected from RFLP analysis with probe for R52, indicating that R52 was associated with the X chromosome (Figure 3(a)). Putative sex-linked bands were also found with probes of R17 and R102 (data not shown). Primers were designed from both ends of putative sex-associates fragments for STS-PCR. The R17 probe revealed a polymorphism unrelated to genotypic sex (Figure 3(b) and Table 2). The R102 probe revealed an additional band derived from YfYf individuals; this polymorphic fragment was inherited in all X∗Yf (n = 87) specimens but was not seen in F4 X∗Yf males (n = 10) after three successive sibling matings, suggesting that R102 is not associated with the Y chromosome. Primers derived from R52 amplified a fragment in X∗Xe females and X∗Yf and X∗Yf males but not YfYf (n = 6) supermales, indicating that R52 is X-linked (Figure 3(b)).
Figure 1: Chromosomal heterogeneity in two pairs of Nile tilapia. (a) Schematic illustration of chromosome heterogeneity detected by two-color CGH analysis. (b) XX metaphase from a female tilapia. (c) XY metaphase from a male tilapia. Signals for XX-derived probe (green) and YY-derived probe (red) are seen on the largest pair of chr 1 (arrows) and a pair of small chromosomes (asterisks).

4. Discussion

We employed CGH to visualize the DNA difference on chr 1, the poorly differentiated sex chromosome in Nile tilapia. We further isolated an X-linked DNA marker R52 that is localized in the putative sex-determining region on chr 1. Sex chromosomes are thought to be derived from chromosome pairs which were originally homologous. Differences between X-Y and Z-W result from the suppression of meiotic recombination in a region flanking the sex-determining genes. Due to the accumulation of genetic changes, sexual selection, and genetic drift, these differences increased over time and led sex chromosomes to vary in morphology and in DNA contents. It has been demonstrated that CGH is capable of identifying the differentiated sex chromosomes in both the XY and ZW systems of mammals and insects, respectively [34]. Highly sensitive dual-color CGH enables us to successfully identify differences spanning a large region on the long arm of chr 1 in XY males, corresponding to the findings from SG analysis and chromosome painting above. Using...
Figure 2: Strategy of RAPD sex-linked marker selection. (a) DNA pools are derived from XX, XY, and YY individuals. (b) RAPD PCR was performed with random primer sets. Fragments derived from X-linked or Y-linked are in dashed box.

dual-color CGH, we confirmed differences of DNA contents in the putative sex chromosomes in Nile tilapia, in which Carrasco et al. [16] reported an incompletely paired segment present in the largest chromosome pair (chr 1) of the heterogametic genotype during the process of meiotic synapsis, with nucleotide diversity occurring in this unpaired region. This nucleotide diversity was also detected by chromosome painting with probes derived from chr 1 microdissection [18]. In addition to chr1, a small pair of chromosomes also displayed diversity in XY male mitotic metaphase in our CGH analysis, indicating that the nucleotide diversity between male and female also exists on this small chromosome pair, although strain-dependent sequence heterogeneity should not be excluded. This raises the possibility of multiple chromosome sets controlling sex determination in Nile tilapia. Multiple loci and multiple sex chromosome sets involved in directing testicular differentiation in tilapia were proposed from bulked segregant analysis [36] and sex ratio departures of Y\textsuperscript{f}Y\textsuperscript{f} male progenies [19]. Two sets of incompletely paired chromosomes (both the biggest chromosome and a small one) were observed by SC analysis in O. aureus, the species closely related to Oreochromis niloticus [40]. Although O. niloticus is proposed to display male heterogamy, in contrast to female heterogamy in O. aureus, they belong to the same genus. They share several similarities as both have similar mitotic karyotypes in chromosome number (2n = 44) and morphology. Probes developed from chr 1 in O. niloticus also cross-hybridized to the chr 1 of O. aureus [41], indicating the existence of homology between these chromosomes. The restriction of meiotic recombination to the small chromosome of O. niloticus may be undetectable due to the limited resolution of SC analysis but captured by the more sensitive dual-color CGH analysis.

RAPD is a sensitive method to resolve differences between male and female genomes. We isolated one putative sex-associated DNA markers in tilapia with RAPD method.
Figure 3: Characterization of RAPD markers. (a) Southern analysis revealed two X-linked fragments present in XX females and XY males but absent in YY males. (b) Results of STS PCR with primers derived from R17, R52, and R102 are listed in Table 1. An X-linked 1.3 kb band was only amplified with R52 primer1 and 2 with DNA from XX female and XY males, in contrast to a maternal 1.1 kb and a paternal 1.8 kb fragment in R102 and a 2.2 kb fragment of R17 from all XY, XX, and YY autosome. NC: negative control with H2O instead of DNA as a PCR template.

Figure 4: Processes of syntenic segment addition or transmission during sex chromosome evolution. (blue) ■, (green) □ Relic of protosex chromosomes; (red) ■ male specific regions on the Y (MSY). Divergence times were from the literature [58, 59].
Table 1: Sequences of RAPD clones and primer sets for STS PCR.

| Name | Size (kb) | Accession | Primers |
|------|----------|-----------|---------|
| R17  | 2.2      | DQ323031  | R17F: GATCCAGTACCCACGACGACGAC<br>R17R: GATCCAGTACCTCAGGACGACGAC |
| R52  | 1.3      | DQ351276  | R52F: GGAACATCCAGGTAAAGG<br>R52R: GATCAAGTCCATCAGTGGAAG |
| R102 | 1.8      | DQ334865  | R102F: GGAACAAATCTTTGCAACACAG<br>R102R: GGAACAAATCAGGACAGCAGTT |

STS PCR using primers derived from R52 identified a putative X-linked allele in two experimental stocks that originated from different genetic backgrounds. From a total of 298 combinations of primer sets, only one RAPD marker was isolated to be putatively sex-linked, reflecting a very slight difference between male and female sex chromosomes, which was corresponding to the homomorphic and poorly differentiated sex chromosome sets in tilapia. The X-linked R52 marker is assembled and colocalized in a highly conserved region of LG3 containing UNH115, clcn5 gene, and GM180 [42]. Both UNH115 and clcn5 were physically mapped to chr 1 within a putative sex-determining region [13, 43]. Notably, they are annotated on LG6-21 according to the genome database rather than LG3 demonstrated by genetic mapping and physical mapping with FISH. The result of a LG6-21 location derived from Oreni.11 blast could be considered either from errors in Oreni.1 database assembly or strain variation in chromosome structure among strains (personnel communication from T. Kocher).

Comparative genomics of conserved synteny across phyla facilitates tracing the origins of protochromosomes prior to their reshaping and reconstitution. It also enables the discovery of events and mechanisms occurring in vertebrate genomes across large time scales during evolution. Sex chromosomes have been assumed to have evolved independently during the evolution [39, 44], with different species adapting different ancestral autosomes as their protosex chromosomes. For instance, few signs of ortholog are found among sex chromosomes of human (X or Y) and birds (Z or W) [45]. Some particular chromosomes are repeatedly used as sex chromosomes in several teleost fish [46]. In fact, the synteny segment of clcn5 within the putative SD region in tilapia is also found in the sex chromosome of rainbow trout, where the Y-linked sdY localizes [10] and in stickleback, revealing a common segment shared among these sex chromosomes, indicating that they are derived from a common ancestor. This synteny segment is further well conserved in X1, one of multiple sex chromosomes in platypus. Homology of platypus X1 and X5 with the therian (eutherians and marsupials) XY PAR and bird ZW-system, respectively, is consistent with this species’ position in the phylogenetic tree between birds, reptiles, and therians [47]. Evidence of the presence of this segment in eutherians and human X chromosome's pseudoautosomal regions (PARs) [48] supports a hypothesis of a common ancestor of certain teleost fish evolutionarily linked to mammal lineage. Interestingly, medaka sex chr 1 is related to bird ZW and platypus X5Y5 and contains the Dmy/Dmrtb4 gene [6, 49], consistent with the idea that protosex chromosomes arose independently in medaka and mammals [12, 47, 50]. Nevertheless, sex chromosome sets of the monotreme show homology with the therian XY system at one end and to the bird ZW system at the other end, thus providing an evolutionary link between XY and ZW systems that were previously thought to be independent and unrelated [51, 52]. Evidence from DNA markers from mammalian X and bird Z syntenic in a salamander (Ambystoma) [53], suggests that these regions are evolutionarily related. We do not know whether this homology is a relic of shared ancestry or merely that a specific transposable element is favored for sex chromosome acquisition. However, our striking findings of this link in X chromosomes from fish to mammals should provide insights for understanding the process of sex chromosome evolution in vertebrates.

5. Conclusions

Production of genetic males is a common way to improve the production efficiency in tilapia aquaculture. This can be achieved via several approaches such as interspecific hybridization [54], hormone treatment [55], and YY supermales [15]. Production requires several generations to establish the YY supermale stocks. Our RAPD markers together with other sex-linked DNA markers [56, 57] will help to simplify the process of YY supermale production and screening of individuals as males and reduce the costs in commercial aquaculture of tilapia. These sex-linked markers are also indispensable for the study of sex determination loci in tilapia and will be useful in understanding the mechanisms of sex determination in low vertebrates, as well as sex chromosome evolution in vertebrates.

Accession Numbers. Sequence data from this paper have been deposited in the GenBank ([http://www.ncbi.nlm.nih.gov/Genbank](http://www.ncbi.nlm.nih.gov/Genbank)) under accession numbers R17: DQ323031; R102: DQ334865; R52: DQ351276.

Conflict of Interests

The authors declare that they have no competing interests

Authors’ Contribution

Guijun Guan, Tohru Kobayashi, and Yoshitaka Nagahama designed the experiment. Guijun Guan, Meisheng Yi, and Tohru Kobayashi carried out the experimental work. Guijun Guan performed the data analyses and wrote the paper. Guijun Guan and Yunhan Hong finalized the paper. All authors read and approved the final paper.

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