Acta Oceanol. Sin., 2018, Vol. 37, No. 4, P. 34–39
DOI: 10.1007/s13131-018-1152-1
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Cytogenetic characterization and description of an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in *Collichthys lucidus* (Richardson, 1844)

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Received 2 March 2017; accepted 2 June 2017

Abstract

The chromosomes of spinyhead croaker *Collichthys lucidus* (Richardson, 1844) were characterized for the first time by fluorescence staining, self genomic *in situ* hybridization (self-GISH), and multicolor fluorescence *in situ* hybridization (FISH) with 18S rDNA, 5S rDNA and telomeric sequence probes. The female karyotype has exclusively 24 pairs of acrocentric chromosomes (2n=48a, NF=48), while the male one consists of 22 pairs of acrocentric chromosomes, 2 monosomic acrocentric chromosomes and a metacentric chromosome (2n=1m+46a, NF=48). The difference between female and male karyotypes indicates the presence of a sex chromosome of $X_1X_1X_2X_2/X_1X_2Y$ type, where Y is the unique metacentric chromosome in the male karyotype. As revealed by FISH, 5S rDNA and 18S rDNA sites were mapped at syntenic position of the largest acrocentric chromosome (X_1), and the short arms of the Y chromosome as well. An X_1-chromosome specific interstitial telomeric signal (ITS) was detected overlapping the 5S rDNA sites. In addition, self-GISH revealed that the repetitive DNAs accumulated on all the putative sex chromosome. Chromosome fusion accompanied by a partial deletion in the ancestral karyotype (2n=48a) is hypothesized for the origin of such multiple sex chromosome system. The present study, as the first description of differentiated sex chromosome in family Sciaenidae, will give clues to the studies on the sex chromosome of other Sciaenids.

Key words: *Collichthys lucidus*, karyotype, sex chromosome, rDNA, fluorescence *in situ* hybridization, interstitial telomeric signal (ITS)

Citation: Zhang Shoukang, Zheng Jiao, Zhang Jing, Wang Zhiyong, Wang Yilei, Cai Mingyi. 2018. Cytogenetic characterization and description of an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in *Collichthys lucidus* (Richardson, 1844). Acta Oceanologica Sinica, 37(4): 34–39, doi: 10.1007/s13131-018-1152-1

1 Introduction

Most fish species do not have morphologically differentiated sex chromosomes given the wide variety of sex determination systems and the recent origin of most of them. The heteromorphic sex chromosomes were observed in only about 10% of fish species karyotyped (Devlin and Nagahama, 2002). However, a variety of sex chromosomes have been described in fish, including both the simple sex chromosome systems (XX/XY and WZ/ZZ) and the multiple sex chromosome systems ($X_1X_1X_2X_2/X_1X_2Y$, XX/XY_1Y_2, W_1W_2Z/ZZ and $W_1W_2Z_1Z_2/Z_1Z_1Z_2Z_2$). In addition, sex chromosome turnover is relatively rapid and popular in fish (Kitano and Peichel, 2012). The first multiple sex determination system was described in a Mexican cyprinodontid species (Uyeno and Miller, 1971). Since then, the multiple sex chromosome systems have been identified in approximately 40 fish species across diverse families, suggesting that they have evolved independently in multiple lineages (Kitano and Peichel, 2012; Ferreira et al., 2016; Bitencourt et al., 2017).

The family Sciaenidae (croaker or drum) consists of approximately 67 genera and 283 fish species, mainly distributing along the Pacific, Atlantic and Indian Ocean, and south of East Africa (Nelson et al., 2016). Fish of the family are highly important commercial fishery resources. In this family, cytogenetic data have been reported in about 40 species, but only two species have been analyzed with FISH (Zheng et al., 2016; Liao et al., 2017). The available cytogenetic data show a remarkable chromosomal stability among Sciaenids, as most of them have a similar karyotype as 2n=48a and no one has morphologically differentiated sex chromosome (Accioly and Molina, 2008; Arai, 2011). However, this general karyotype conservation among Sciaenids has not been well established yet, for the stability might be resulted from the limited number of analyzed species, or from the absence of high-resolution cytogenetic analyses.

The spinyhead croaker *Collichthys lucidus* (Richardson 1844) is an economically important sciaenid species distributing in coastal waters of northwestern Pacific, ranging from Kyushu, Japan to the South China Sea (Cheng et al., 2012). So far, the karyotype data of *C. lucidus* are not available yet. Therefore, the aims of this study were to characterize *C. lucidus* chromosomally, and reveal the presence of a multiple sex chromosome system of the $X_1X_1X_2X_2/X_1X_2Y$ in spinyhead croaker as well, providing an interesting exception out of the croakers that were characterized with

Foundation item: The National Natural Science Foundation of China under contract Nos 31272653 and 41706157; the Natural Science Foundation of Fujian Province under contract No. 2017J01449.
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conservative karyotype.

2 Materials and methods

2.1 *Specimen collection and chromosome preparations*

Specimens were collected from the Sansha Bay, close to Fujian Province of China (26°42′33″N, 119°46′49″E) (Fig. 1). The specimens were identified and deposited in the ichthyological collection at Jimei University. All procedures performed in studies involving in animals were in accordance with the ethical standards of Jimei University. Twelve specimens (6 males and 6 females) with length range from 8 cm to 15 cm were analyzed with classical and molecular cytogenetic methods. The chromosome preparations were obtained from the head kidney cells according to Gold et al. (1990). The chromosomes were stained with 1 μg/mL DAPI in phosphate buffered saline (PBS, pH 7.0) for 10 min. Fin tissues of *C. lucidus* were fixed in ethanol to extract DNA.

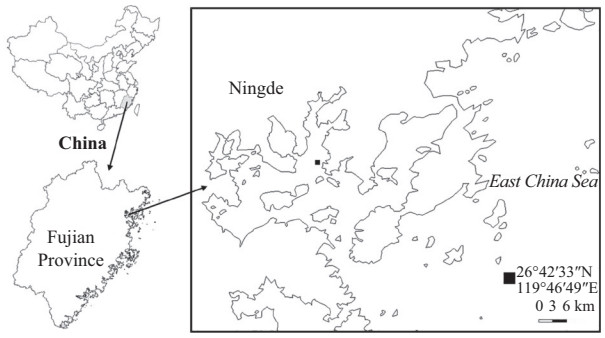

Fig. 1. The collection sites of *Collichthys lucidus* in the Sansha Bay, Fujian Province, China.

2.2 *Self-GISH*

Self-GISH was performed as described previously (Zheng et al., 2016). Genomic DNA was extracted from the fixed fin tissue of *C. lucidus* using a Genomic DNA Kit (Generay, Shanghai, China). The genomic DNAs of spinyhead croaker were labeled with biotin-11-dUTP with a nick translation kit (Roche, Basel, Switzerland). Chromosomes slides were denatured for 2 min in 70°C 70% formamide in 2× SSC, and then dehydrated in an ethanol series. A hybridization mixture, containing 2 ng/μL denatured probes, 50% deionized formamide, 10% dextran sulfate, 2× SSC, and double-deionized, was denatured at 75°C, and then added onto the chromosome slides. Hybridization was performed at 37°C for 8–16 h in a moist chamber. Post-hybridization washes were performed in 50% formamide in 2× SSC at 37°C for 20 min, 2× SSC and 1× SSC at room temperature for 20 min each, and 4× SSC at room temperature for 5 min. After stringent washing, the biotinylated probes were detected with avidin-Alexa fluor-488 (Invitrogen, Carlsbad, CA, USA).

2.3 *Multi-FISH*

Multi-FISH were performed allowing a simultaneous hybridization of three probes, 18S rDNA, and 5S rDNA and the telomeric sequence. All of the three probes were obtained with PCR and labeled with nick translation kits (Roche, Basel, Switzerland). For 18S rDNA probes, a partial coding region was amplified with a universal primer pair (F: 5′-CGCGCAAATTACCCACTCCC-3′, R: 5′-CTGAACGCCACTTGTCCCT-3′), and then labeled with biotin-11-dUTP (Roche, Basel, Switzerland). For 5S rDNA probes, the whole coding and non-transcribed region of the 5S rDNA was obtained by PCR amplification with the primers F (5′-GTCAGGCCTGGTTAGTACTTGGAT-3′) and R (5′-GGGCGCATTCAGGGTGGTAT-3′), and then labeled with Digoxigenin-11-dUTP. For Telomeric repeats, (TTAGGG)*n* were obtained by PCR without a template using $(TTAGGG)_5$ and $(TAACCC)_5$ primers according to Ijdo et al. (1991), and then labeled with Cyanine 5-dUTP (Perkin-Elmer, Boston, MA, USA). The obtained PCR product was cloned into the pEASY-T1 vector (TransGen Biotech, Beijing, China) and sequenced for verification by a custom service (Shenggong, Shanghai, China). The manipulations of chromosome slides denaturation, hybridization and post-hybridization washing were similar to those in the self-GISH protocol as described above. The biotinylated and digoxigenated probes were detected with anti-digoxigenin-rhodamine (Roche, Basel, Switzerland) and avidin-Alexa fluor-488 (Invitrogen, Carlsbad, CA, USA), respectively. The cyaninated probes were observed directly.

2.4 *Microscopy and image analyses*

Metaphases were examined and photographed using an epifluorescence microscope (Olympus BX53) coupling with a digital image capture system (Olympus DP 80), and analyzed with cellSens Standard 1.7 (Olympus Corporation, Japan) and Adobe Photoshop software. The chromosomes were classified according to Levan et al. (1964).

3 Results

Chromosomes from 272 metaphases of *C. lucidus* were counted in total. The modal chromosome numbers were 48 for the females and 47 for the males, respectively (Fig. 2). The karyotype of females has exclusively 24 pairs of acrocentric chromosomes (2n=48a, FN=48; Figs 3a and c), while the karyotype of males consists of 22 pairs of acrocentric chromosomes, 2 monosomic acrocentric chromosomes and a metacentric chromosome (2n=1m+46a, FN=48; Figs 3b and d). Therefore, an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system is clearly evidenced, where Y is the unique metacentric chromosome in the male's karyotype. One of the monosomic acrocentric chromosomes in males was identified easily for it presented a distinguished negative band after DAPI staining, and thereby was designated as the X_1 chromosome (Fig. 3a).

After self-GISH, strong fluorescence signals present at the centromeric of all autosomes. On the Y and the X_1, the signal ex-

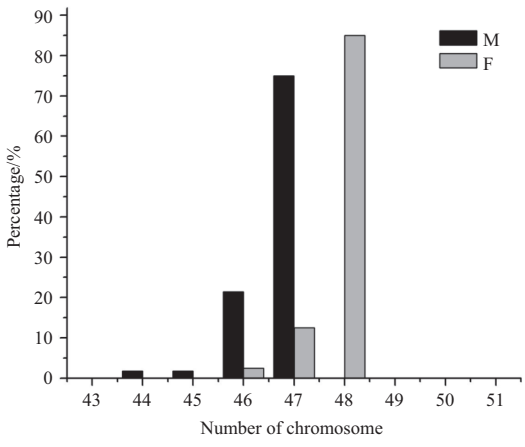

Fig. 2. Chromosome count in the males (M) and the females (F) in *C. lucidus*.

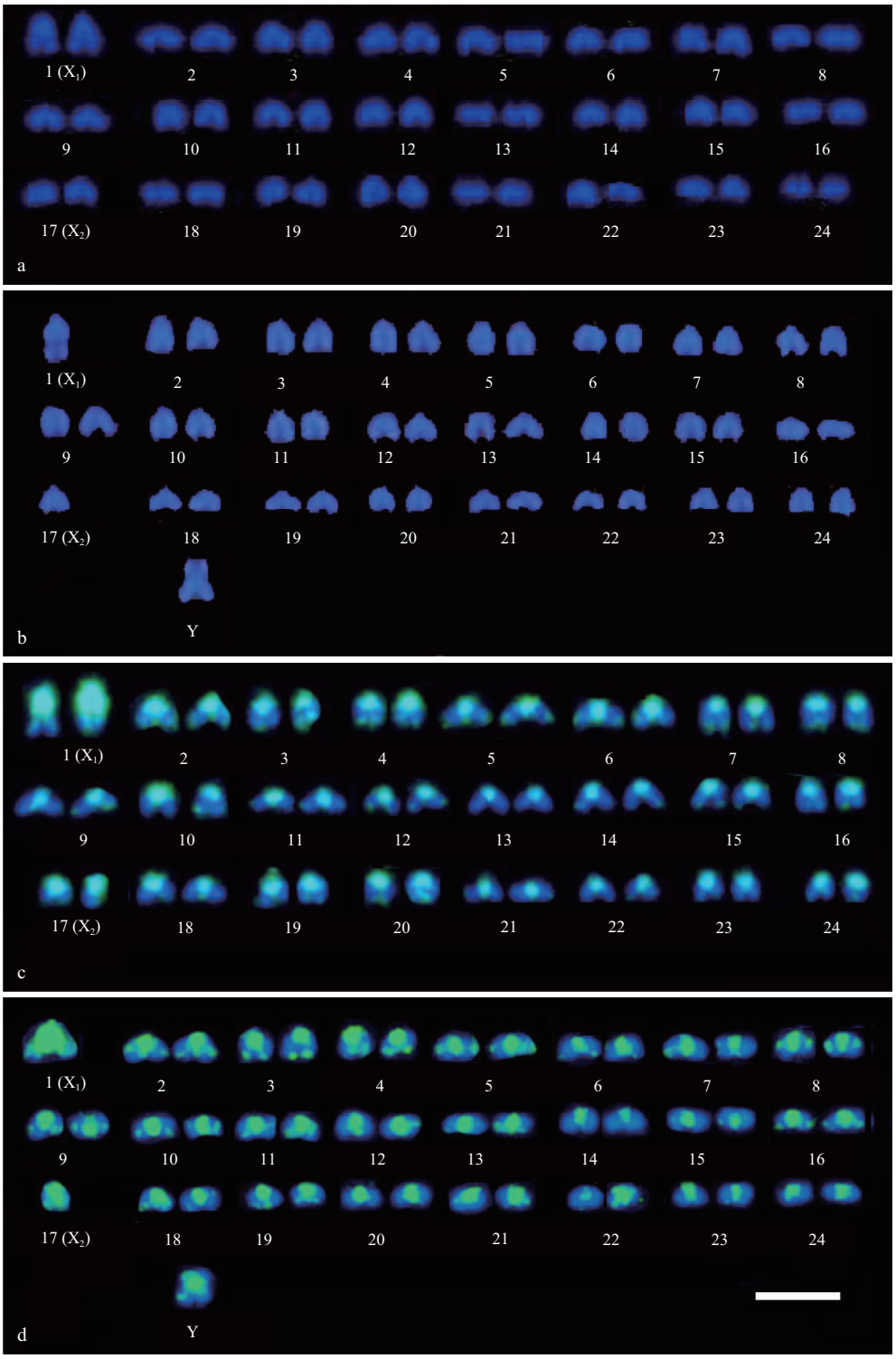

Fig. 3. Karyotype of *C. lucidus* after DAPI staining and self-GISH. a and c. Female, and b and d. male. Scale bars represent 5 μm.

tends from the centromere to the interstitial region. Another chromosome being of extended self-GISH signals was Chromosome 17, which was monosomic in the male's karyotype. Thus, Chromosome 17 was the strong candidate of X_2 chromosome (Figs 3c and d).

Multi-color FISH detected 18S rDNA and 5S rDNA sites aligned at the interstitial region of the largest acrocentric chromosome (X_1). In the males, both rDNAs sites were mapped on the short arm of the Y chromosome besides the X_1 chromosome (Figs 4a, b, e and f). In both sexes, the 18S sites were distal to the centromere in comparison with the 5S rDNA sites. In addition, the size of rDNA signals on the Y chromosome was obviously smaller than that on the X_1 chromosome. Telomeric sequence probes detected a rare X_1 chromosome specific interstitial telomeric signals (ITS) overlapping the 5S rDNA sites (Figs 4c, d, g and h).

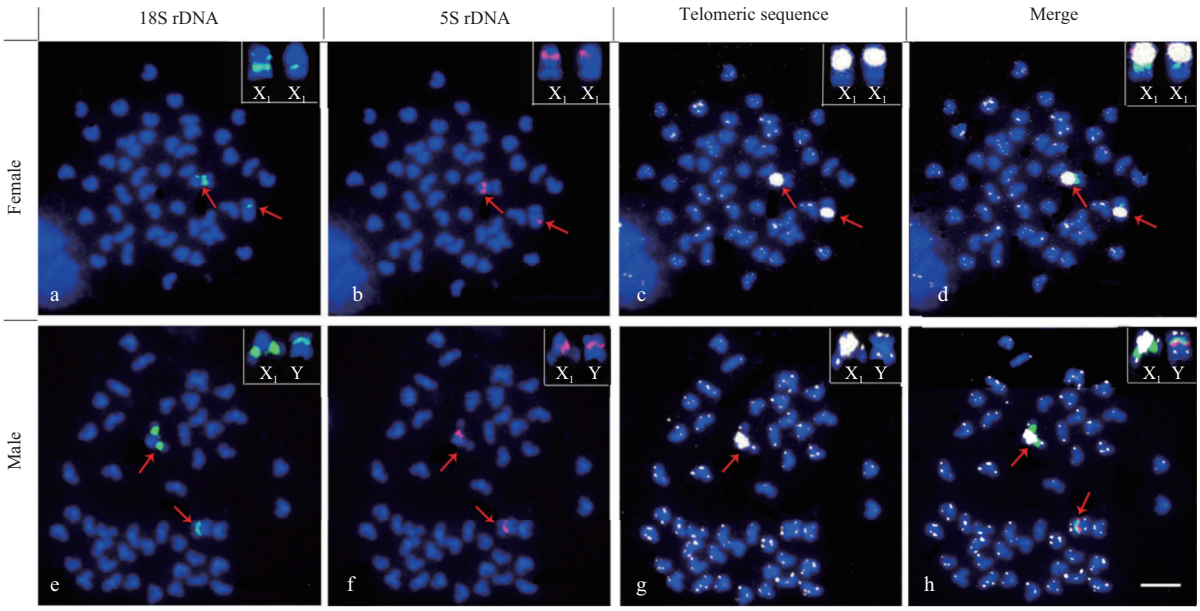

Fig. 4. Multi-color FISH using 18S rDNA, 5S rDNA and telomeric probes on *C. lucidus* chromosomes. With FISH, 18S rDNA and 5S rDNA sites were mapped adjacently on X_1 chromosome and the short arm of Y chromosome (red arrow), and a cluster of strong interstitial telomeric signal was detected at the similar position of 5S rDNA sites on the X_1 chromosome (red arrow) specifically. Scale bars represent 5 μm.

4 Discussion

The comparative analysis between the karyotypes of males and females of *C. lucidus* shows heteromorphism between sexes, which corresponds to a multiple sex chromosome system of $X_1X_1X_2X_2/X_1X_2Y$ type. According to Kitano et al. (2009), $X_1X_1X_2X_2/X_1X_2Y$ systems may arise through three mechanismes: (1) fusions between an autosome and a Y chromosome, (2) centric fission of the X chromosome in species with an XY system, or (3) reciprocal translocations between the X chromosome and an autosome in species with an ancestral XX female/XO male sex chromosome system. In *C. lucidus*, chromosome fusion would contribute to the origin of the $X_1X_1X_2X_2/X_1X_2Y$ systems, for the males had one less chromosome than the sister taxas.

Both 18S rDNA and 5S rDNA sites were visualized as a single pair with FISH, locating on the syntenic position of the interstitial region of the largest acrocentric chromosomes (X_1) and the corresponding position of the short arm of the Y chromosome (Fig. 4). The correspondence reinforced that the chromosome fusion involving the proto-X_1 chromosome had occurred. The rDNA sites were previously reported on the sex chromosomes in many fish species, such as *Fundulus diaphanus* (Howell and Black, 1979), *Salvelinus alpinus* (Reed and Phillips, 1995), *Hoplias malabaricus* (Born and Bertollo, 2000), *Triportheus guentheri* (Artoni and Bertollo, 2002; Diniz et al., 2008), and *Harttia punctata* (Blanco et al., 2014). The rDNA sites on the sex chromosome may play some role in the synapsis process of the opposite sex chromosomes during meiosis (Ren et al., 1997; Stitou et al., 1997), or contribute to limiting the opportunity for additional recombination near the major sex-determining locus (Reed and Phillips, 1997). However, it is not common that both kinds of rDNA locate on syntenic position, especially on the sex chromosome. In fact, this is the second case that both rDNA involved in a multiple sex chromosome besides *H. punctata* (Blanco et al., 2014).

FISH with telomeric sequence probes revealed a rare X_1 chromosome-specific ITS (Fig. 4), suggesting that chromosome rearrangement had occurred on the proto-X_1 chromosomes prior to the chromosome fusion for the origin of the Y chromosome. Furthermore, chromosome deletion should have occurred when (or after) the chromosome fusion, for the Y chromosome lacks the ITS. In addition, the fact that the p arm of the Y chromosome is about 50% smaller than the expected length of proto-X_1, as well as that both rDNA sites in the Y chromosome are smaller than their homologues in the X_1 chromosome, reinforces this inference. Therefore, it can be deduced that the metacentric neo-Y of *C. lucidus* derives from a fusion between pro-X_1 and proto-X_2 chromosomes following by at least a chromosome deletion involving ITS and part of both rDNA sites (Fig. 5). Similar pattern of Y chromosome formation, chromosome fusion plus fragment deletions, were also observed in other fish species with X_1X_2Y system, such as *Gasterosteus aculeatus* (Ross and Peichel, 2008), *G. wheatlandi* (Ross et al., 2009), *Harttia punctata* (Blanco et al., 2014), and *Achirus achirus* (Bitencourt et al., 2017).

Self-GISH, a modified procedure of GISH, has been used to survey the distribution of the repetitive DNAs in chromosome complements for the signal pattern of self-GISH was demonstrated to be in accordance with that obtained from FISH with *Cot*-1 DNA (She et al., 2007). With self-GISH, the distribution of repetitive DNAs of *C. lucidus* was roughly visualized at centromeric and telomeric regions of all autosomes, whereas expanding to the interstitial region of the putative sex chromosomes, not only on Y, but also on X_1 and X_2 (Fig. 3). The accumulation of repetitive DNAs on the sex chromosome was popularly observed in other fish species (Yano et al., 2014; Poltronieri et al., 2013), other vertebrates (Graves, 2006), invertebrates (Palacios-Gimenez et al., 2013), and plants (Liu et al., 2004), and was considered as one of the basic steps for sex chromosomes evolution (Charlesworth et al., 2005). It can be expected that accumulation of repetitive DNA on the heterogametic sex chromosome (Y or W) contributes for the suppressed recombination. Intriguingly, however, the accumulation also presents on the homogametic sex chromosome (X or Z) at high level, which is exemplified by many fish

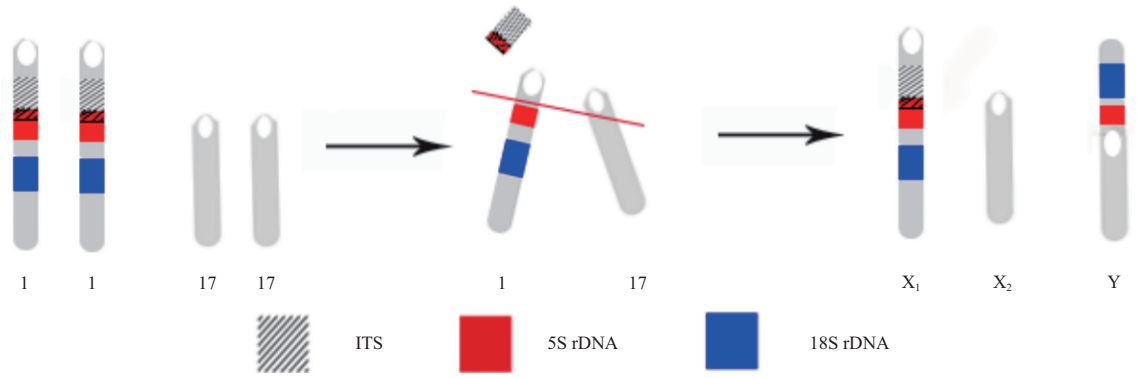

Fig. 5. Scheme of the probable origin of the sex system $X_1X_1X_2X_2/X_1X_2Y$ in *C. lucidus*.

species with cytogenetic or DNA data, such as *T. trifurcates* (Yano et al., 2014), *Cynoglossus semilaevis* (Chen et al., 2014), and *Xiphophorus Maculatu* (Chalopin et al., 2015). This phenomenon brings to a question whether the repetitive DNAs were pre-existing on a pro-sex chromosome, making it more prone to chromosomal fusion or to harbor the nascent sex determination (SD) gene. The fact that repetitive DNAs accumulate on the sex chromosomes or at the SD regions highlights the important role of the repetitive DNA in sex chromosome evolution, although this is not well understood yet.

Most of reported Sciaenid species have the same karyotype as 2n=48a, similar to the hypothetical ancestral karyotype of fish (2n=48a), showing a remarkable conservatism in their karyotypic macrostructure (Accioly and Molina, 2008; Arai, 2011). Mapping of the major rDNA (or NORs) has been carried out in 13 Sciaenids, revealing a basal condition for this family as single pair of 18S rDNA sites, consistent with the conserved single-site pattern in Perciformes (Gornung, 2013). For the 5S rDNA sites, an additional pair of small 5S rDNA sites was located on the other chromosome beside the major one adjacent to the 18S rDNA site in *N. albiflora*. Thus, it is believable that more structure variation would be exhibited when more species were analyzed with more cytogenetic markers.

5 Conclusions

The present study described the cytogenetic characterization of *C. lucidus* for the first time, and revealed an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in this species as the first case of differentiated sex chromosome in the family Sciaenidae. Based on the signal pattern of multi-FISH and self-GISH, an event of fusion between Chromosome 1 and Chromosome 17, accompanied by at least a deletion, is hypothesized for the formation of the neo-Y chromosome (Fig. 5). The association between the repetitive DNA including two kinds of rDNA and the sex chromosome were also present. These results will expand the knowledge of the genome structure in Sciaenid, and provide a clue for identifying the sex chromosome in other Sciaenid species.

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