GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Chromosome mapping of 18S rDNA and 5S rDNA by dual-color fluorescence *in situ* hybridization in the half-smooth tongue sole (*Cynoglossus semilaevis*)

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Genet. Mol. Res. 13 (4): 10761-10768 (2014)
Received November 21, 2013
Accepted May 27, 2014
Published December 18, 2014
DOI http://dx.doi.org/10.4238/2014.December.18.17

ABSTRACT. Half-smooth tongue sole (*Cynoglossus semilaevis*) is an important aquaculture flatfish in China. Cytogenetic analysis has revealed that its sex determination system is female heterogametic (ZZ/ZW). The W chromosome is morphologically larger and has been considered evolutionarily younger than any other chromosome in the set. However, the genetic origin and evolution process of this neo-chromosome remains unclear. In this study, 2 tandem arrays of rRNA genes were chosen to address this question. Both the major rDNA (18S rDNA) and the minor rDNA (5S rDNA) were located on the *C. semilaevis* chromosomes by fluorescence *in situ* hybridization (FISH). Six 18S rDNA signals were observed on the centromeric regions of 3 pairs of autosomes in both males and females. In females, there was an additional 18S rDNA signal mapping to the telomeric region of the W chromosome long arm. With respect to the 5S rDNA, 12 signals were mapped to the centromeric regions of six pairs of autosomes. Two-color FISH further confirmed that the two pairs

of the 5S rDNA signals were correspondingly located at the same positions of the same autosomes as those of the 18S rDNA signals. These results allowed us to speculate about the evolution process of the W chromosome. Chromosome fusions and repetitive sequence accumulations might have occurred in *C. semilaevis*. The synteny and non-synteny of *C. semilaevis* 18S rDNA and 5S rDNA might imply the original and evolutionary characteristics of this species. These findings will facilitate studies on karyotype evolution of the order Pleuronectiformes.

Key words: Fluorescence *in situ* hybridization; Major rDNA; Minor rDNA; Pleuronectiformes; Sex chromosome; *Cynoglossus semilaevis*

INTRODUCTION

Half-smooth tongue sole (*Cynoglossus semilaevis* Günther, 1873), a member of Pleuronectiformes, Soleoidei, Cynoglossidae, *Cynoglossus*, is one of the most commercially important aquaculture fish species in China. As a hotspot of molecular research, it has been studied from many aspects, such as the development of molecular DNA markers (Zhong et al., 2009), construction of fosmid libraries (Wang et al., 2009a), and creation of genetic linkage map (Liao et al., 2009). However, because of the existence of many small and similar chromosomes (Pardo et al., 2001), cytogenetic investigations of this species are impeded by the difficulties of karyotype analysis.

Chromosomal observations revealed that the karyotype of *C. semilaevis* was 2n = 42, NF = 42 (Zhou et al., 2005). Cytogenetic and genetic analyses revealed that its sex determination system is female heterogametic (ZZ/ZW) (Zhuang et al., 2006), with females possessing a W chromosome that is obviously larger than all the other chromosomes. Although some of the other genetically and/or cytogenetically studied Pleuronectiformes species possess obvious XX/XY or ZZ/ZW genetic sex determination systems, it was noteworthy that no distinct dimorphic sex chromosome has been identified (Kikuno et al., 1986; Bouza et al., 1994; Yamamoto, 1999; Carvalho et al., 2005). Therefore, the distinct heteromorphic W chromosome in *C. semilaevis* is evolutionarily a neo-sex chromosome, which might evolve during or after speciation. Although several sex-specific probes have been developed from the W chromosome (Wang et al., 2009b; Zhai et al., 2011) of *C. semilaevis*, the origin and evolution of this neo-chromosome remains unknown. To uncover the genetic basis of this chromosome, as well as the evolutionary status and taxonomy position of this species, further cytogenetic studies must be conducted.

Tandem arrays of rRNA genes are organized into 2 distinct multi-gene families composed of hundreds to thousands of copies. One family is represented by the major rDNA, 45S rDNA, which encodes the 18S, 5.8S, and 28S rRNAs. The second family is represented by the minor rDNA, 5S rDNA, which encodes the 5S rRNA (Martins and Galetti, 2001). There are usually one or more pairs of rDNA sites in the genome, which can be easily located by fluorescence *in situ* hybridization (FISH) with labeled DNA probes. The 5S rDNA repeats comprise conserved coding sequences of 120 base pairs (bp), which are separated from each other by non-transcribed spacer (NTS) sequences (Inafuku et al., 2000). It has been demonstrated that these conserved sequences can also be located by the NTS (Suzuki et al., 1996). The rRNA genes are worth studying because the sequences (particularly in the intergenic spacer region and NTS

region), the copy number of the repeats at each site, and the number of sites can evolve rapidly. Detecting the variation will help to explain some karyotype rearrangement processes that may be difficult to identify with other genetic marker methods (Galasso et al., 1995).

This study aimed to locate the 18S and 5S rDNAs on the chromosomes of *C. semilaevis* and to speculate about the chromosome evolution of this species.

MATERIAL AND METHODS

Sampling and chromosome preparation

Ten wild individuals (5 females and 5 males) of *C. semilaevis* were collected from the Yellow Sea adjacent to Yantai City, China. Their weights ranged from 350 to 600 g, and their body length ranged from 40 to 60 cm. Chromosome preparations were performed following the method of Wang et al. (2009c).

Probe labeling

The 18S rDNA and NTS primers (18S-F: 5'-GAGAAACGGCTACCACATC-3', 18S-R: 5'-ACAAATCGCTCCACCAAC-3', NTS-F: 5'-GGAGACCGCCTGGGAATAC-3', and NTS-R: 5'-ATCGGGCGTGTTCAGGGTG-3') were designed according to the homologous sequences of Japanese flounder (Accession No. EF126037 and AB154836). The 20 µL polymerase chain reaction (PCR) mixture contained 40 ng genomic DNA, 0.2 mM of each primer, 200 µM dNTPs, 2.5 mM Mg^{2+}, 1X PCR Buffer, and 1 U rTaq (TaKaRa, Dalian, China). The PCR conditions were as follows: 1 cycle of denaturation at 94°C for 5 min; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, with a final extension step at 72°C for 15 min. Products of about 900 and 800 bp could be separately amplified from the *C. semilaevis* genome. Via cloning, sequencing, and basic local alignment search tool analysis in the National Center for Biotechnology Information database, the 2 PCR products were demonstrated to be 18S rDNA and NTS sequences, respectively. The verified products were purified with the Cycle-Pure Kit (Omega, GA, USA). Probes for 18S rDNA and NTS were prepared with purified PCR products by DIG-Nick Translation Mix or Biotin-Nick Translation Mix according to manufacturer protocols (Roche, Mannheim, Germany). The labeled probes were precipitated with isopropyl alcohol and ammonium acetate at -20°C for 12 h and dissolved with 50% dextran sulfate and 20X saline sodium citrate (SSC) (v/v) at 4°C.

Fluorescence *in situ* hybridization (FISH)

FISH and probe detection were conducted according to Wang et al. (2009c) and Hu et al. (2011) with minor modifications. Aged slides were treated with 0.005% pepsin in 10 mM HCl at 37°C for 10 min. Then, they were washed 2 times with phosphate-buffered saline, rinsed in 70 and 100% ethanol each for 5 min, and air-dried. RNase-treated slides were denatured in 2X SSC containing 70% formamide at 75°C for 2 to 3 min, dehydrated with a precooled ethanol series of concentration gradients (70, 90, and 100%) for 5 min each, and air-dried. The 20 µL probe hybridization mix (containing about 10 ng/µL probes, 10% dextran sulfate, and 50% deionized formamide in 2X SSC) was denatured at 95°C for 5 min and chilled on ice immediately for at least 10 min. The denatured probe was applied to the slide under a cover slip. DNA-DNA *in situ* hybridization was performed in a humidity chamber at

37°C for 12 to 16 h. After hybridization, the slides were washed in 2X SSC with 50% formamide at 37°C for 10 min, 1X SSC at 37°C 3 times for 5 min each, and 2X SSC at room temperature for 1 to 2 min. The hybridized probes that were labeled with biotin or digoxigenin were detected with fluorescein isothiocyanate (Roche) or antidigoxigenin rhodamine (Roche) in a humidity chamber at 37°C for 1 h. Then, the slides were washed in washing buffer (0.1% Tween 20 in 4X SSC) at 37°C 3 times for 5 min each and 2X SSC for 1 to 2 min at room temperature. Chromosomes were counterstained with propidium iodide (only biotin) or 4',6-diamidino-2-phenylindole (both biotin and digoxigenin) for 10 min at room temperature in a cassette. Slides were observed using a Nikon E-600 epifluorescence microscope equipped with a CCD camera (COHU). The signals were collected using appropriate filter sets and the LUCIA software. At least 20 metaphases were examined in each individual.

RESULTS

The karyotype of *C. semilaevis* was observed to be 2n = 42 acrocentric chromosomes, which was in accordance with previous results (Zhou et al., 2005; Wang et al., 2009b). Compared to males, females had a unique and larger W chromosome with no homologous chromosome in the genome (Figure 1A). FISH with the 18S rDNA probe showed signals in the centromeric or subcentromeric regions of 3 pairs of autosomes in males (Figure 1B). However, in females, 7 signals were found in each metaphase spread, including 6 in the centromeric regions of 3 autosomal pairs and another signal in the subtelomeric region of the W chromosome long arm (Figure 1A). The 5S rDNA loci were detected on more chromosomes than the 18S rDNA loci. In total, 12 signals were identified in both the male and female genome, all of which were located in the centromeric region of autosomes (Figure 1C). No hybridization signal was found on the W chromosome. The signal intensities or "sizes" varied greatly among different chromosomes of the same metaphase spread, but the FISH results were consistent both among metaphases and among the 10 specimens. No individual-specific hybridization signal was observed.

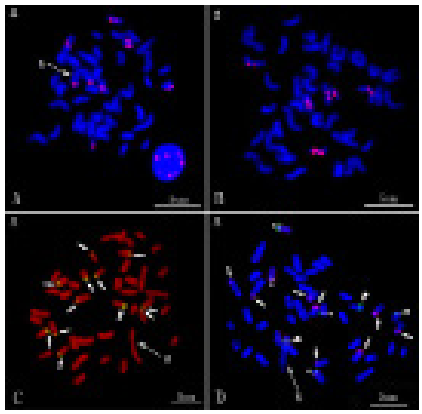

Figure 1. Distribution of 18S rDNA and 5S rDNA by fluorescence *in situ* hybridization (FISH) in mitotic chromosomes of *Cynoglossus semilaevis*. (**A**) 18S rDNA signals on the female metaphase spread, (**B**) 18S rDNA singnals on the male metaphase spread, (**C**) 5S rDNA signals on the female metaphase spread, and (**D**) 18S rDNA and 5S rDNA dual-color FISH signals on a female metaphase spread. The long arrows indicate W chromosomes, short arrows indicate 5S rDNA signals, arrow heads indicate 18S rDNA signals, and asterisks indicate 18S rDNA and 5S rDNA sympatric signals located at the same position. Bar = 5 μm.

Two-color FISH with 18S rDNA and 5S rDNA probes revealed that only the 18S rDNA signal could be identified on the W chromosome in the female genome. Syntenic signals of the 2 probes could be found on 2 pairs of autosomes. Besides, signals of 18S rDNA were also located on another pair of autosomes, and those of 5S rDNA were observed on another 4 pairs of autosomes (Figure 1D).

DISCUSSION

According to the existing cytogenetic studies in the order Pleuronectiformes, the 2n = 48 telocentric karyotype has been proposed to be ancestral (Pardo et al., 2001). The diploid number is relatively conserved in families Pleuronectidae and Paralichthyidae, ranging from 2n = 44 to 2n = 48. However, the diploid number is rather variable in Cynoglossidae, Scophthalmidae, Soleidae, and Bothidae, ranging from 2n = 28 to 2n = 48 (Pardo et al., 2001). In *Scophthalmus maximus* (2n = 44), 2 centric fusions have been proposed to explain the evolution of the turbot karyotype (Bouza et al., 1994). In Achiridae (2n = 34-40) and Soleidae (2n = 42), the reduction in the diploid number is possibly due to chromosome fusions in the ancestor of these families (Pardo et al., 2001; Carvalho et al., 2005). Therefore, it is rational to speculate that chromosome fusions may also occur in *C. semilaevis*. It has also been reported that sex chromosomes are labile and that their formation in fish is often associated with the accumulation of repetitive sequences or long interspersed repeat elements (Carvalho et al., 2005; Ellegren, 2011; Takehana et al., 2012) and the silencing of genes (Zhang et al., 2001). This study of the *C. semilaevis* W chromosome showed that a number of repetitive sequences accumulated in heterochromatic regions (data not shown), which was also found in mammals, birds, and other fishes (Mank et al., 2006; Ellegren, 2011).

In aquatic animals, especially in fishes, the numbers and locations of rDNA loci on chromosomes vary significantly. In Paralichthyidae (Wang et al., 2009c), Soleidae, and Scophthalmidae (Pardo et al., 2001), the 18S rDNA is generally located on 1 pair of autosomes. In *Apareiodon ibitiensis*, populations from different areas have 1 or 2 pairs of 18S rDNA FISH signals (Bellafronte et al., 2009). This may be caused by chromosome rearrangements and consequent dispersion throughout the genome (Moreira-Filho et al., 1984). As reported in many species, chromosome evolution from geographical isolation or reproductive isolation generates multiple 18S rDNA sites (Sola et al., 2000; Vicari et al., 2005). Three pairs of 18S rDNA signals and 6 pairs of 5S rDNA signals found in this study implied that the *C. semilaevis* genome had experienced a series of chromosome rearrangements and consequent dispersion. This phenomenon might result from chromosome translocation, insertion, or some undiscovered events during evolution among homologous or non-homologous chromosomes. It was interesting that the telomeric regions of the W chromosome long arm showed an explicit signal in all analyzed metaphases of females. The same phenomenon has been reported in *Oryzias hubbsi* and *O. javanicus* (Takehana et al., 2012), with 3 18S rDNA sites located on a pair of autosomes and the W chromosome in females. In *Triportheus auritus*, 5 18S rDNA fluorescence hybridization signals are present on 2 autosomal pairs and the W chromosome (Cioffi et al., 2012). A sex chromosome bearing rDNA loci has also been found in other fish species, such as *Fundulus diaphanous* (Howell and Black, 1979) and *Hoplias malabaricus* (Born and Bertollo, 2000).

The existence of 18S rDNA on the "evolutionarily young" W chromosome may result from chromosome translocation between non-homologous chromosomes after a novel W chro-

mosome is formed. Then, the 18S rDNA amplifies on the neo-W chromosome. The author of an existing study inclines to the hypothesis that nucleolus-organizing regions associated with terminal heterochromatin were more prone to chromosome rearrangements (Moreira-Filho et al., 1984). Besides, the accumulations of repetitive sequences on independently evolved sex chromosomes have been proved (Cioffi et al., 2012). The chromosome fusions and repetitive sequences accumulations may explain, to some extent, why the W chromosome was larger than the Z chromosome and autosomes.

The 5S rDNA locus has been considered to be located on 1 pair of chromosomes in most organisms including fishes. Martins and Galetti (1999) regard this as an ancient phenomenon in fishes. In the family Curimatidae, *Steindachnerina insculpta* has 1 pair of 5S rDNA sites and *Cyphocharax modesta* has 2 pairs (Santos et al., 2006). In the order Tetraodontiformes, *Sphoeroides greeleyi* and *S. spinosus* possess 1 pair of 5S rDNA sites, while *Cyclichthys spinosus* possesses 2 pairs (Noleto et al., 2007). In the order Pleuronectiformes, *Paralichthys olivaceus* (Paralichthyidae) and *Solea solea* (Soleidae) have 1 pair of 5S rDNA sites (Libertini et al., 2002; Fujiwara et al., 2007). Presumably, the 6 pairs of 5S rDNA signals in *C. semilaevis* might also have been generated from chromosome rearrangement, like the 18S rDNA. These 5S rDNA signals were all located in interstitial autosome segments, as observed in other fishes (Fujiwara et al., 1998; Martins and Galetti, 2001). Such a pattern may protect 5S rDNA clusters from evolutionary events (Noleto et al., 2007). The signal strength of hybridization that was observed in this study was variable in different positions, which might result from different copy numbers of the sequence. A similar situation exists in salmonid fish, where a lower copy number of rDNA clusters may lead to weaker signals (Fujiwara et al., 1998).

It was intriguing to find that 2 pairs of 18S rDNA and 2 pairs of 5S rDNA sites were correspondingly located at the same position of the *C. semilaevis* chromosomes, while the other signals were detected in different positions. In *Salmo salar* (Pendas et al., 1994) and *Paralichthys olivaceus* (Fujiwara et al., 2007), both the major and minor rDNA have 1 pair of signals located on the same chromosomes. In *Oncorhynchus mykiss* (Fujiwara et al., 1998), there are 2 pairs of 5S rDNA signals, 1 of which is syntenic with 18S rDNA and the other is on X chromosomes. In contrast, in *Hucho perryi* (Fujiwara et al., 1998), *Epinephelus marginatus* (Sola et al., 2000), *Cyclichthys spinosus* (Noleto et al., 2007), and many other teleosts, the major and minor rDNA signals are on different chromosome pairs. According to these previous studies, the location patterns between the major and minor rDNA sites are usually diverse among different families and conserved within the same family. In *Parodon* species, however, the localizations of both rDNAs are species-specific and can serve as genetic markers (Bellafronte et al., 2005). The synteny of 18S and 5S rDNA has been considered as an ancestral status in the fish genome (Fontana et al., 2003), but their non-synteny can prevent undesirable translocations of 5S sequences within 45S rDNA during evolution (Martins and Galetti, 1999). As observed in this study, *C. semilaevis* had both the original and the evolutionary characteristics. These 5S rDNA sites might acquire distinct structures and functions over a long evolutionary process.

Considering the cytogenetic studies on the Pleuronectiformes, the karyotype of *C. semilaevis*, with multiple major and minor rDNA sites and terminal 18S rDNA loci on the long arm of the W chromosome, might involve manifold rearrangements including translocation, inversion, and fusion. The existing studies on the order Pleuronectiformes were mainly focused on the families Bathidae, Paralichthydae, Soleidae, Achiridae, and Pleuronectidae (Berendzen et al., 2002). There was a lack of rDNA information about the family Cynoglos-

sidae. Therefore, it remains unclear whether these findings of 18S and 5S rDNA location in *C. semilaevis* represent the general characteristics of the family Cynoglossidae. Much information is needed to elucidate the evolutionary relationship between *C. semilaevis* and the other flatfish. Intensive studies are necessary to verify how 18S rDNA and the copy number of repetitive rDNAs impact the differentiation of the W chromosome.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Zhai Jieming of Laizhou Mingbo Aquatic Co. LTD. for generously providing the specimens that were used. Research supported by the National Natural Science Foundation of China (#30901098 and #31272646) and the '863' Hi-Tech Research and Development Program of China (#2012AA10A402).

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