

CHROMOSOMAL MAPPING OF THE MAJOR RIBOSOMAL RNA GENES IN THE DWARF SURFCLAM (*MULINIA LATERALIS* SAY)

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ABSTRACT Chromosomal location of the major ribosomal RNA genes (rRNA) were studied in the dwarf surfclam (*Mulinia lateralis*, Say) using fluorescence *in situ* hybridization (FISH). FISH probes for the rRNA genes were made by polymerase chain reaction (PCR), labeled with digoxigenin-11-dUTP and detected with fluorescein-labeled antidigoxigenin antibodies. *Mulinia lateralis* had a diploid number of 38 chromosomes and all chromosomes were telocentric. FISH with the rRNA probe produced positive and consistent signals on two pairs of chromosomes: Chromosome 15 with a relative length of 4.6% and Chromosome 19, the shortest chromosome. Both loci were telomeric. The rRNA location provides the first physical landmark of the *M. lateralis* genome.

KEY WORDS: FISH, chromosomes, major rRNA genes, genome duplication, *Mulinia lateralis*

INTRODUCTION

The dwarf surfclam *Mulinia lateralis* (Say 1822) is a marine bivalve that is naturally distributed along the Atlantic coast of North America. It is a small species with an adult size of about 15–20 mm. It has a short generation time and can reach sexual maturation in 1–2 mo. *Mulinia lateralis* is dioecious with an XX-female and XY-male system of sex determination (Guo & Allen 1994). It can be strip-spawned and easily cultured in captivity. Triploidy and gynogenesis can be artificially induced in this species (Guo & Allen 1994, Yang & Guo 2006). There have been some interests in using *M. lateralis* as a model bivalve species for genetic research (Calabrese 1969, Guo & Allen 1994). One limiting factor is that we know little about the genome of this species. We only know that it has a haploid number of 19 chromosomes (Wada et al. 1990) with one cross-over per chromosome (Guo & Allen 1996), and it shares the vertebrate telomeric sequence, (TTAGGG)_n (Wang & Guo 2001). No DNA-based genetic markers have been developed, and no genes have been mapped in this species. In an effort to learn more about the genome of this species, we studied chromosomal location of the major ribosomal RNA (rRNA) genes using fluorescence *in situ* hybridization (FISH). Chromosomal mapping of genes and DNA fragments are important for the identification of chromosomes, which has been difficult in marine bivalves. Traditional banding techniques have not provided the level of resolution and consistence that are needed for reliable chromosome identification (Guo et al. 2007). By direct hybridization to DNA targets on chromosomes, FISH can provide unambiguous labeling and identification of chromosomes, which is essential for studies on genome organization and chromosomal evolution.

The major rRNA genes, 18S, 5.8S and 28S, code for the RNA components of ribosomes that are essential for protein synthesis. They are highly conserved genes clustered in tandem repeats. Each repeating unit contains all three genes in the order of 18S-5.8S-28S, with two internal transcribed spacers (ITS) in-

between. Each locus may contain hundreds of repeating units. Because they present in tandem repeats, rRNA genes provide a large target and can be easily detected with FISH. FISH involves the labeling of a DNA fragment with a reporter molecule, which is hybridized to its target sequence on chromosomes and detected under fluorescence microscope (Guo et al. 2007). FISH has been used for gene mapping and chromosome characterization in some clam species. The vertebrate telomeric repeat (TTAGGG)_n has been assigned to the telomeres of *Donax trunculus* (González-Tizón et al. 1998, Plohl et al. 2002), *Mercenaria mercenaria*, *Mulinia lateralis* (Wang & Guo 2001), and *Dosinia exoleta* (Hurtado & Pasantes 2005). The major ribosomal RNA genes have been mapped to the chromosomes of seven species: *Donax trunculus* (González-Tizón et al. 1998, Martínez et al. 2002), *Macoma nasuta*, *Nuttallia nuttallii* (González-Tizón et al. 2000), *Cerastoderma edule* (Insua et al. 1999), *Solen marginatus* (Fernández-Tajes et al. 2003), *Dosinia exoleta* (Hurtado & Pasantes 2005), and *Mercenaria mercenaria* (Wang & Guo 2007). Here we report the chromosomal mapping of the major rRNA genes in the dwarf surfclam, *M. lateralis*.

MATERIALS AND METHODS

Chromosome Preparation

The dwarf surfclams used in this study were from a wild population from Rhode Island. Adult clams were collected by Dr. Timothy Scott and immediately shipped to our laboratory. Metaphases were prepared using early embryos according to published protocols (Guo et al. 2007). Six females and five males of *M. lateralis* were used for embryo production. The sex of ripe individuals was determined by the gonad color visible through the transparent shells: pink for females and milky white for males. Eggs and sperm were obtained by dissecting. Eggs were washed by passing through a 60- μ m nytex screen to remove large tissue debris and rinsed on a 25- μ m screen. Eggs were resuspended in seawater and fertilized by adding sperm. Excessive sperm were removed at 15 min postfertilization by rinsing

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fertilized eggs on a 25- μ m screen. Embryos were resuspended and incubated at 23°C. After 6–8 h of incubation, embryos were harvested into a 15-mL tube. To obtain elongated chromosomes, embryos were exposed to a colchicine treatment (0.005%) for 20 min. The colchicine solution is then removed, and 9 parts of a hypotonic treatment (0.075 M KCl) was added to every part of embryo suspension. After 15 min, the hypotonic treatment was replaced with 3:1 (v:v) methanol and acetic acid. The fixative was changed twice. Fixed samples were stored at 4°C. Metaphase spread was made using an air-dry method (Guo et al. 2007). Slides were stored at –20°C until use.

Probe Construction

Clam genomic DNA was extracted from adductor muscle according to Doyle and Doyle (1987). Internal transcribed spacers between the 18S and 5.8S (ITS1), and between 5.8S and 28S (ITS2) were amplified by PCR, and used as FISH probes. PCR primer sequences were 5'-GGTTCTGTAGGTGAACCTGC and 5'-CTGCGTTCTTCATCGACCC for ITS1, and 5'-GGGTCGATGAAGAACGCAG and 5'-GCTCTCCCGCTTCACTCG for ITS2. Probes were labeled with digoxigenin-11-dUTP (alkali-stable) by PCR incorporation. Digoxigenin-11-dUTP and all other PCR reagents were purchased from Roche Diagnostics (Indianapolis, IN). PCR was performed in a 25 μ L reaction mixture containing 1X PCR buffer with 1.5 mM of MgCl₂, 0.4 mg/mL of BSA, 0.2 mM each of dATP, dCTP, and dGTP; 0.13 mM of dTTP, 0.07 mM of Digoxigenin-11-dUTP, 0.5 U of Taq DNA polymerase, 1 μ M of each primer; and 1 μ g of clam genomic DNA. The thermal cycling parameters were 35 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C, with a final extension of 5 min at 72°C. Amplified products were verified on 2% agarose gels. DIG-labeled PCR products were purified with G-50 columns and used as FISH probes.

Fluorescence in situ Hybridization

FISH was performed as previously described with minor modifications (Guo & Allen 1997, Guo et al. 2007). Briefly, metaphase slides were pretreated with 2 \times SSC (0.3M sodium chloride, 0.03 M sodium citrate, pH 7.0) at 37°C for 30 min. Slides were then dehydrated for 5 min each in 70%, 80%, and 95% ethanol and air-dried. Metaphase spreads were then denatured in 2 \times SSC containing 70% deionized formamide at 72°C for 2 min, dehydrated for 5 min in cold ethanol, and air-dried. To prepare the probe for hybridization, 1.0 μ L of the purified PCR product was added to 25 μ L of Hybrisol VI (Oncor) and incubated at 72°C for 5 min. The denatured probe (26 μ L) was applied to the slide and covered with a 22 \times 40 mm glass cover-slip. The slides were sealed with rubber cement and incubated overnight in a humidified chamber at 37°C. After hybridization, the slides were washed in appropriate SSC solutions at 72°C for 5 min and twice in 1X phosphate buffer detergent (PBD, 0.1 M NaH₂PO₄, 0.4% BSA, 0.1% Tween-20, pH 7.4) at room temperature for 2 min each. For detection, fluorescein-labeled antidigoxigenin antibody (25 μ L) was added to each slide and covered with a plastic coverslip and incubated at 37°C for 15 min. The slides were then washed 3 times for 2 min each in 1 \times PBD at room temperature in the dark. Twenty microliters propidium iodide/antifade (0.5 μ g/mL) was added to each slide and covered with a glass coverslip. Hybridization

signals were analyzed and documented using a Nikon microscope equipped with a 3CCD camera and the Image-Pro Plus image analysis system.

RESULTS

PCR Amplification

PCR amplification with ITS1 and ITS2 primer pairs generated a single fragment each. In the absence of digoxigenin-11-dUTP, ITS1 primers generated an approximately 950 bp fragment, and ITS2 primers generated a 550 bp fragment (Fig. 1, A lane 2 and 4). The incorporation of digoxigenin-11-dUTP significantly decreased the mobility of both fragments, with the ITS1 fragment shifted to about 1,200 bp and ITS2 fragment to about 700 bp (Fig. 1, A: lane 3 and 5).

Karyotype

The use of propidium iodide as a counterstain allowed the visualization and identification of chromosome pairs (Fig. 2, A). Most metaphases (167 of 192) screened in this study had 38 chromosomes. In a few metaphases, the loss of one or two chromosomes was observed, probably because of excessive disruption during chromosome spreading. Fourteen good metaphases that showed no chromosome overlapping were selected for karyotype analysis. All chromosomes were telocentric and chromosomes were paired by length alone. Because the difference in length is small (Table 1), the pairing and chromosomal assignment of some chromosomes are arbitrary and uncertain.

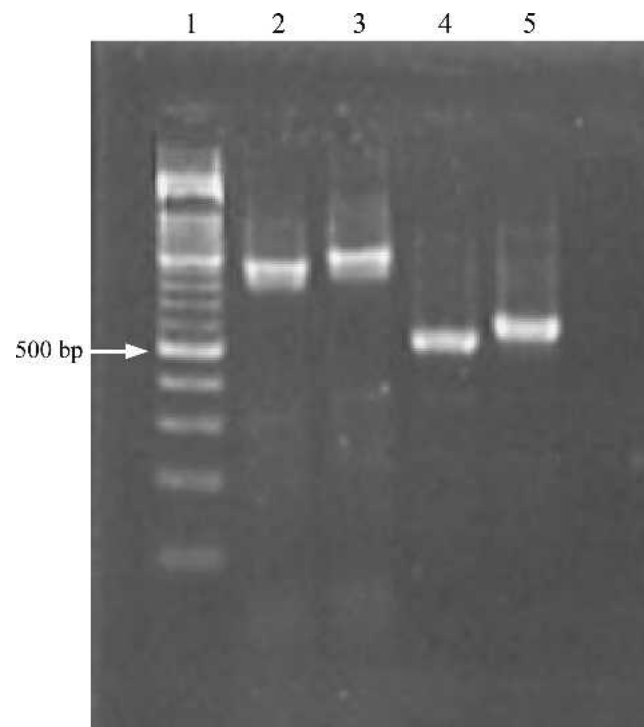


Figure 1. PCR amplification of internal transcribed spacers between 18S and 5.8S (ITS1) and 5.8S and 28S RNA genes (ITS2) in *Mulinia lateralis*. Lane 1, 100-bp ladder; Lane 2, ITS1 fragment; Lane 3, Digoxigenin-labeled ITS1; Lane 4, ITS2 fragment; Lane 5, Digoxigenin-labeled ITS2. Arrows indicate the location of 500-bp fragment.

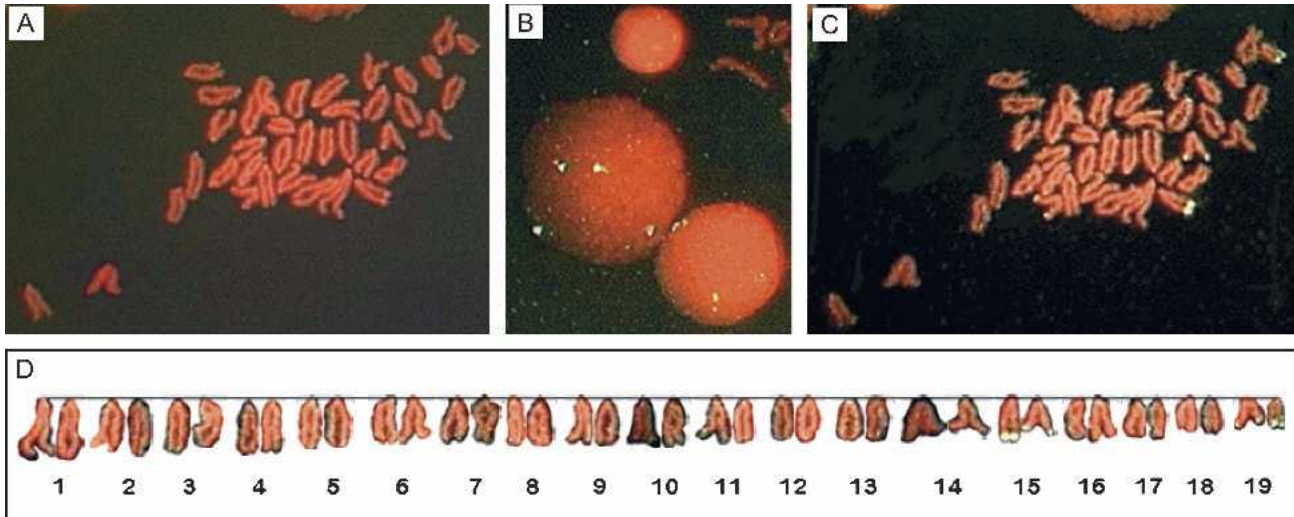


Figure 2. Fluorescence *in situ* hybridization (FISH) with the major rRNA gene probes on interphase nuclei and metaphase chromosomes in *Mulinia lateralis*: (A) metaphase stained with the counterstain propidium iodide alone; (B) four signals on interphase nuclei after FISH; (C) metaphase after FISH showing rRNA genes on telomeric regions of two pairs of chromosomes (arrows); and (D) karyotype showing rRNA genes on Chromosome 15 and 19.

FISH

After FISH with either probe, interphase nuclei consistently showed 4 clusters of signals per nucleus (Fig. 2, B). Similarly, positive signals were observed on metaphase chromosomes. At a wash stringency of $2 \times$ SSC, the probe produced positive FISH signals mostly on two pairs of chromosomes with considerable background signals. To reduce nonspecific signals, FISH was conducted at $1 \times$ SSC and rRNA loci were clear with little/no background signals.

After the increase in stringency, specific FISH signals for ITS1 were consistently observed on telomeric regions of two

pairs of chromosomes (Fig. 2, C). Analysis of FISH signals from 14 metaphases revealed that one pair of signals was on Chromosome 15, which had a relative length of 4.64, and the other on Chromosome 19, the smallest chromosome. The fluorescence signals on Chromosome 15 were slightly more intense than those on Chromosome 19. FISH with ITS2 produced the same results as ITS1 (data not shown). The karyotype of *M. lateralis* with two rRNA loci is presented in Figure 2, D. The identity of Chromosome 19 is certain because it is clearly the shortest one. We named the other rRNA-bearing chromosome as Chromosome 15, although it has similar length with Chromosome 14 and 16 (Fig. 2, D).

TABLE 1.

Karyotype analysis of the dwarf surfclam (*Mulinia lateralis*) chromosomes.

Chromosome Number	Relative Length (Mean \pm SD)
1	7.11 \pm 0.36
2	6.57 \pm 0.17
3	6.26 \pm 0.15
4	6.02 \pm 0.16
5	5.88 \pm 0.11
6	5.75 \pm 0.09
7	5.59 \pm 0.09
8	5.50 \pm 0.07
9	5.35 \pm 0.08
10	5.26 \pm 0.51
11	5.15 \pm 0.08
12	5.03 \pm 0.11
13	4.90 \pm 0.12
14	4.81 \pm 0.13
15	4.64 \pm 0.24
16	4.57 \pm 0.16
17	4.29 \pm 0.09
18	3.83 \pm 0.26
19	3.48 \pm 0.28

DISCUSSION

This study confirms that *M. lateralis* has a haploid number of 19 chromosomes, as previously reported (Wada et al. 1990, Wang & Guo 2001). All chromosomes of *M. lateralis* are telocentric, which is rare among bivalves studied so far. Most clam and scallop species studied so far have a haploid number of 19 mostly metacentric or submetacentric chromosomes (Thiriou-Quévieux 1994, Thiriou-Quévieux 2002). Most species of Ostreidae have a haploid of 10 metacentric chromosomes. It has been suggested that a whole genome duplication event has occurred during the evolution of Bivalvia; clams and scallops with 19 chromosomes represent the tetraploid lineage; and oysters with 10 chromosomes represent the diploid lineage (Wang & Guo 2004).

The genome duplication hypothesis is partly based on the observation that some scallop species can tolerate considerable chromosome loss. The ability to tolerate significant chromosome loss is typical of duplicated genomes. Most clams studied so far have 19 pairs of metacentric chromosomes (76 chromosome arms), whereas some have significantly reduced chromosome numbers or arms. Karyotypes with metacentric chromosomes are generally considered as more pleisomorphic than those with telocentric and subtelocentric chromosomes (Thiriou-Quévieux 1994). The fact that *M. lateralis* karyotype has

19 telocentric chromosomes or 38 chromosome arms, suggests that *M. lateralis* may have experienced significant chromosome loss (50% of chromosome arms) during its evolution. If so, the ability of *M. lateralis* tolerating significant chromosome loss would provide another piece of evidence supporting the genome duplication hypothesis.

This study provides unambiguous chromosomal mapping of the major rRNA genes in *M. lateralis*. As far as we can determine, this is the first time that specific genes are assigned to chromosomes of *M. lateralis*. Results of this study clearly demonstrate that the major rRNA genes have two loci in *M. lateralis*: one on Chromosome 15 and the other on Chromosome 19. Both locations were telomeric. Before this study, chromosomal location of ribosomal RNA genes had been determined in seven clam species: two loci in the razor clam, *S. marginatus* (Fernández-Tajes et al. 2003), and hard clam *M. mercenaria* (Wang & Guo 2007), whereas only one locus in *Donax trunculus* (González-Tizón et al. 1998, Martínez et al. 2002), *Macoma nasuta*, *Nuttallia nuttallii* (González-Tizón et al. 2000), cockle *Cerastoderma edule* (Insua et al. 1999), and *Dosinia exoleta* (Hurtado & Pasantes 2005). Although all oyster species have one major rRNA locus, whereas some clams including *M. lateralis* have two, the number of rRNA loci may not be indicative of whole-genome duplication, as considerable variation in the number of the major RNA loci exists among clams and scallop species. It seems that the number and location of the major rRNA loci can go through rapid evolutionary change. Species with two major rRNA loci provide a unique opportunity for studying mechanism of concerted evolution of repetitive genes. For example, it would be interesting to study if the presence of an extra locus would speed up or slow up sequence divergence in the rRNA genes and their internal transcribed spacers. The dwarf surf-

clam, because of its short generation time, should be a good model for the study of gene conversion in artificially designed crosses.

In this study, FISH signals on one chromosome pair were considerably stronger than those on the other pair (Fig. 2). Although *in situ* hybridization is not strictly quantitative, differences in signal strength should reflect variation in copy number of the target loci (Ito et al. 2000). Because the strength of the major rRNA gene signals differed between Chromosome 15 and 19, it is possible and even likely that the two loci vary in copy number. Similar results have been observed in the hard clam (Wang & Guo 2007). It would be interesting to confirm if the difference in signal strength is indeed caused by difference in copy number, and how copy number varies between the two loci and among different species.

This study provides positive identification of two chromosomes of *Mulinia lateralis*. Further efforts are needed to develop FISH probes and identify all 19 chromosomes. The identification of the two chromosomes carrying the major rRNA loci may eventually help us to understand how the duplication occurred during evolution—whether it involved whole chromosome duplication or terminal translocation.

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