

The 5S rDNA of the bivalve *Cerastoderma edule*: nucleotide sequence of the repeat unit and chromosomal location relative to 18S-28S rDNA

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Abstract – The whole 5S rDNA repeated unit of the bivalve *Cerastoderma edule* was amplified by PCR and several clones were sequenced. In addition, the PCR product from several individuals was digested with restriction enzymes. The results obtained indicate that 5S rDNA is organized in tandem repeats of 544–546 bp, 120 of which could represent the coding region and 424–426 the spacer region. Minimal intra- and inter-individual variation was detected, always within the spacer region. In comparison to the published 5S rRNA sequences of three other bivalves, *C. edule* displays a maximum of four different nucleotide positions. A specific probe of *C. edule* 5S rDNA was generated by PCR and used for FISH. Five chromosome pairs were identified that carried a cluster of 5S rDNA at the telomere of the long arm. After performing FISH with a heterologous 18S-28S rDNA probe and C-banding, absence of linkage between 5S and 18S-28S rDNA was demonstrated. © Inra/Elsevier, Paris

5S rRNA gene / non-transcribed spacer / *Cerastoderma edule* / FISH

Résumé – L'ADNr 5S chez le bivalve *Cerastoderma edule* : séquence nucléotidique de l'unité de répétition et localisation chromosomique par rapport à l'ADNr 18S-28S. L'unité de répétition complète de l'ADNr 5S a été amplifiée par PCR chez le bivalve *Cerastoderma edule* et plusieurs clones ont été séquencés. En outre, le produit de PCR de plusieurs individus a été digéré par des enzymes de restriction. Les résultats obtenus indiquent que l'ADNr 5S est organisé sous forme de répétitions en tandem dont l'unité mesure 544–546 pb, parmi lesquelles 120 pourraient représenter la région

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codante et 424–426 la région de l'espaceur non codante. Des variations intra- et interindividuelles minimales ont été détectées, toujours dans la région de l'espaceur. Par rapport aux trois autres séquences d'ARNr 5S publiées chez les bivalves, *C. edule* présente un maximum de quatre positions nucléotidiques différentes. D'autre part, une sonde spécifique pour l'ADNr 5S de *C. edule* a été générée par PCR et utilisée dans des essais de FISH. Cinq paires chromosomiques portent un groupement d'ADNr 5S sur le télomère du bras long. Après avoir réalisé la FISH avec une sonde d'ADNr 18S-28S hétérologue et le marquage des bandes C, l'absence de liaison entre l'ADNr 5S et 18S-28S a été démontrée. © Inra/Elsevier, Paris

gènes d'ARNr 5S / espaceur non transcrit / *Cerastoderma edule* / FISH

1. INTRODUCTION

The 5S ribosomal DNAs (5S rDNA) of many eukaryotes has been cloned and characterized. In most cases, it is organized as clusters of tandem repeats of several hundred base pairs (bp), consisting of a coding region and a non-transcribed spacer region [14]. Accumulated data demonstrate that, while the coding region is highly conserved among taxa, both with respect to length and nucleotide sequence, the spacer region evolves more rapidly and can show variation both within and between species (e.g. [7, 19]).

In addition to the gene encoding the 5S rRNA, many species contain gene variants and pseudogenes, differing from the gene by a variable number of substitutions and deletions [5, 18, 24]. Moreover, it has been well documented that *Xenopus laevis* has three types of 5S rDNA sequences with developmentally regulated expression [32]. More than one type of 5S rDNA sequence with differential expression was also seen in the chicken [13] and some fish [12]. As is true for several other families of tandemly repeated genes, the 5S rDNA repeats evolve concertedly [3], i.e. in intra-specific comparisons a high degree of sequence similarity is usually observed between independent repeats. Thus, 5S rDNA sequences are regarded as potentially useful in revealing phylogenetic relationships.

In contrast to genes encoding 18S, 5.8S and 28S rRNA (18S-28S rDNA), where chromosomal location can be determined by selective staining of nucleolus organizer regions and in situ hybridization, 5S rDNA can only be detected by in situ hybridization. This could explain the fact that, in general, there is less information available on the chromosomal location of 5S rDNA. In bivalve molluscs, very little attention has been paid to 5S rDNA. To date, only the 5S rRNA of three species belonging to different subclasses has been sequenced: *Solemya velum* (Protobranchia), *Calyptogena magnifica* (Heterodonta) [25] and *Mytilus edulis* (Pteriomorphia) [6]. The chromosomal location of 5S rDNA was determined in a pectinid species, *Aequipecten opercularis* [10].

This work provides for the first time the nucleotide sequence of the whole 5S rDNA repeated unit of a bivalve species, the cockle *Cerastoderma edule* (Heterodonta, Cardiidae), and an analysis of the intra- and inter-individual variation. In addition, it reports the chromosomal location of 5S rDNA and its physical relation to 18S-28S rDNA.

2. MATERIALS AND METHODS

Specimens of *C. edule* were collected from several locations (Pontevedra, Vilanova de Arousa, Ponteceso, Ría do Burgo and Cedeira) along the Galician coast (NW Spain). Genomic DNA was extracted from muscle tissue according to Winnepenninckx et al. [31].

2.1. PCR amplification, cloning and sequencing

The amplification mixture used for PCR (50 μ L) contained 500 ng of genomic template DNA, 1 μ M each primer, 250 μ M dNTPs, 1.25 U of Taq polymerase (Boehringer Mannheim) and the buffer recommended by polymerase suppliers. The primers were 5'-CAACGTGATATGGTCGTAGAC-3' (A) and 5'-AACACCGGTTCTCGTCCGATC-3' (B), obtained from the 5S rRNA sequence of the mussel *M. edulis* [6], and 5'-CAAGCACAGAGGCAGGAG-3' (C) and 5'-CGATCCGCGGTTTACCTG-3' (D) obtained from the *C. edule* 5S rDNA spacer region. Thirty standard PCR amplification cycles were performed at an annealing temperature of 64 °C with primers A and B and 56 °C with primers C and D. The PCR product generated with both sets of primers was purified using GeneClean (BIO 101, INC), ligated into the plasmid pGEM-T Easy, using pGEM-T Easy Vector System II (Promega), and subsequently transformed into *E. coli JM109* cells. Recombinant clones were selected as white colonies on ampicillin plates containing X-gal and IPTG. Plasmid DNA purification of four clones (two with insert obtained with primers A and B, and the other two with insert obtained with primers C and D) was carried out as described by Sambrook et al. [23]. Both strands of each clone were sequenced by the dideoxy-sequencing method with the AutoRead kit (Pharmacia). Automatic sequencing was performed on an A.L.F. express sequencer (Pharmacia). Sequences were aligned using CLUSTAL V with both fixed and floating gap penalties of 10 [9]. The nucleotide sequences have been deposited in the EMBL DNA data base under the accession numbers AJ132196–132199.

2.2. Chromosome preparation and FISH

Metaphases were obtained from gill cells following the procedure described by Thiriot-Quévèreux and Ayraud [28]. A specific probe of *C. edule* 5S rDNA was produced by PCR using the primers A and B. Labelling was obtained using the PCR procedure described above, but with a different dNTP concentration (100 μ M dATP, 100 μ M dCTP, 100 μ M dGTP, 160 μ M dTTP and 35 μ M digoxigenin-11-dUTP). A recombinant plasmid containing 18S, 5.8S and 28S genes plus intergenic spacers of *Drosophila melanogaster* was used as probe to localize 18S–28S rDNA. After extraction by alkaline lysis [23], the whole plasmid was labelled with digoxigenin-11-dUTP employing the Boehringer Mannheim nick translation kit. FISH was carried out as in Insua et al. [10], but the post-hybridization washing was carried out with a 65 % formamide solution in the case of 5S rDNA. C-banding was performed according to the method of Sumner [26] but slides were stained with acridine orange following Martínez-Lage et al. [15]. The examination of chromosome spreads was performed with a Nikon fluorescence photomicroscope equipped with appropriate filters and photographs were taken with Kodak Ektachrome film.

3. RESULTS

The 5S rDNA repeat unit of *C. edule* was amplified by PCR using primers A and B, designed from the 5S rRNA sequence of *M. edulis* [6]. PCR amplification produced a single band of approximately 550 bp. This amplification product was cloned and then two clones were sequenced (Ce1 and Ce2). Two additional primers, C and D, derived from the spacer region of the just sequenced *C. edule* 5S rDNA, were also used to produce a new PCR amplification of the 5S rDNA repeat unit. A single band of 550 bp was also obtained, and after cloning two clones were sequenced (Ce3 and Ce4).

The complete repeat unit consists of 544–546 bp and the alignment of full-length sequences of the four clones consists of 548 bp (*figure 1*). Comparison with available bivalve sequences [6, 25] allows us to infer that the coding region starts 5' with GTC and ends with CTT to give a 5S rRNA size of 120 nucleotides (*figure 2*). However, the 5S rRNA from mussel *M. edulis* ends with ACA and has a size of 119 nucleotides [6]. Therefore, the assignment of the 3' end and the 5S rRNA size must be considered tentative. The inferred coding region of the *C. edule* 5S rDNA is invariable between clones, ignoring the sequence corresponding to primer A in clones Ce1 and Ce2.

Several sequences of the eukaryotic 5S rDNA involved in the transcription can be identified in the sequences obtained from *C. edule*: internal control region [22]; sequence elements related to upstream regulatory regions of the coding region as TATATA [17]; and terminator sequences composed of four thymidine residues [1] located downstream of the coding region (*figure 1*). The G/C content, determined in the consensus sequence of the four clones, is higher in the coding region (54.2 %) than in the spacer region (45.8 %).

The spacer region showed some variation, ranging from 424 to 426 bp in length. Eight variable sites were detected in the alignment, four of which correspond to gaps and four to nucleotide substitutions (*figure 1*). Nevertheless, three of the clones (Ce1, Ce2 and Ce3) are almost identical, only two gaps associated with a run of thymidine residues at the 5' end being observed. To further examine the extent of the variation, the 5S rDNA was amplified with primers A and B from nine additional individuals collected along the Galician coast. The product obtained was digested with the enzymes *Alu* I, *Hae* III, *Rsa* I and *Taq* I. No variation was found in the restriction pattern generated by these enzymes, except in one individual which showed intra-individual variation concerning the *Rsa* I restriction pattern. This had three bands as is to be expected from the sequences determined here, but also an additional band, resulting from the absence of one enzyme target in the spacer region.

Comparison of the 5S rDNA coding sequence of *C. edule* with previously published sequences of other bivalve species (*figure 2*) reveals no differences with *Calyptogena magnifica* (Heterodonta, Vesicomidae), and four nucleotide differences with *Solemya velum* (Protobranchia, Solemyidae) and *Mytilus edulis* (Pteriormorphia, Mytilidae).

The chromosomal location of the 5S rDNA was determined by FISH, using a specific probe obtained by PCR. Forty-one metaphases, belonging to four individuals, were analysed. The pattern most frequently observed displays a total of nine hybridization sites distributed on the telomere of the long arms of five chromosome pairs (*figure 3a*). Since most chromosomes in the karyotype

	<i>A</i>	<i>B</i>	
Ce1	5' <u>GTCTACGACCATATCACGTTG</u> ?? <u>AACACCGGTTCTCGTCCGATCACCGA</u> agttaagcaac		60
Ce2		
Ce3G.....AA.....		
Ce4G.....AA.....		
Ce1	gtcgAGcccggT <u>TAGTACTtggatgggtgaccgctg</u> GGAATACCGGTGCTGTAGACTT		120
Ce2		
Ce3		
Ce4		
Ce1	CACATCTCtttt-AATTTTTTTTTCTTTCTTTTTTTTTTGTCAAACGTTTTTCATGAG		180
Ce2--.....		
Ce3-.....		
Ce4C.A.....--.....		
Ce1	AGCAGCATCGCCGTCATCATCACCATCACCCAGTACTCTTGCTTTTTT-GCGATATCAAC		240
Ce2		
Ce3		
Ce4	.A.....G.....T.....		
		<i>C</i> <i>D</i>	
Ce1	TCATTTTATTCATTA AA ACTGGTAGGGCCCTG <u>CCTCGCCTCTGTGCTTGCCGATCCGG</u>		300
Ce2		
Ce3?.....		
Ce4?.....		
Ce1	<u>GTTTACCTGTCCAAAAATCGTCTGATGAGTTACGCACCTGCCTGGCTATAAATAGCACCA</u>		360
Ce2		
Ce3		
Ce4		
Ce1	GCAACGCGTTGGTTGTAGGTTTTGTGGGCTTTCTGGGCAATGCTCGGCTCTCTGGAAGGC		420
Ce2		
Ce3		
Ce4		
Ce1	CTATCTGGTCCCGGAAAAGGCAAACCTACTCTGACCCGTCCAGACCATCTGTGAAGCTAG		480
Ce2		
Ce3		
Ce4C.....		
Ce1	ACGACAGACGACTTtatataAAGGATCGGCATGCAAAGTATAAATAGCCCGAGAAATGGG		540
Ce2		
Ce3		
Ce4		
Ce1	TGTCCCAT 3'		548
Ce2		
Ce3		
Ce4		

Figure 1. Aligned nucleotide sequences of four cloned 5S rDNA repeats from *C. edule*. Dots represent residues identical to those of the reference sequence; dashes indicate alignment gaps; question marks denote missing information; boldface type corresponds to coding sequence; lower-case indicates putative regulatory and terminator sequences; underlined represents primers. Clones Ce1-2 were obtained with primers A and B, and clones Ce3-4 with primers C and D.

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Ce 5' GTCTACGGCCATATCACGTTGAAAACACCGGTTCTCGTCCGATCACCGAAGTTAAGCAAC 60
Cm .....
Sv .....C.....G.....
Me .....A.....

Ce GTCGAGCCCGGTTAGTACTTGGATGGGTGACCGCCTGGGAATACCGGGTCTGTAGACTT 3' 120
Cm .....
Sv .....G.....A.....
Me .....T.....A-
    
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Figure 2. Alignment of the coding region of the *C. edule* (Ce) 5S rDNA with those of *C. magnifica* (Cm), *S. velum* (Sv) and *M. edulis* (Me). Dots represent residues identical to those of the reference sequence; dashes indicate alignment gaps.

of *C. edule* are submetacentric (12 pairs), and the remaining chromosomes are subtelocentric (4 pairs) or telocentric (3 pairs), with small differences in size [11], identification of chromosome pairs carrying 5S rDNA cannot be accurately determined.

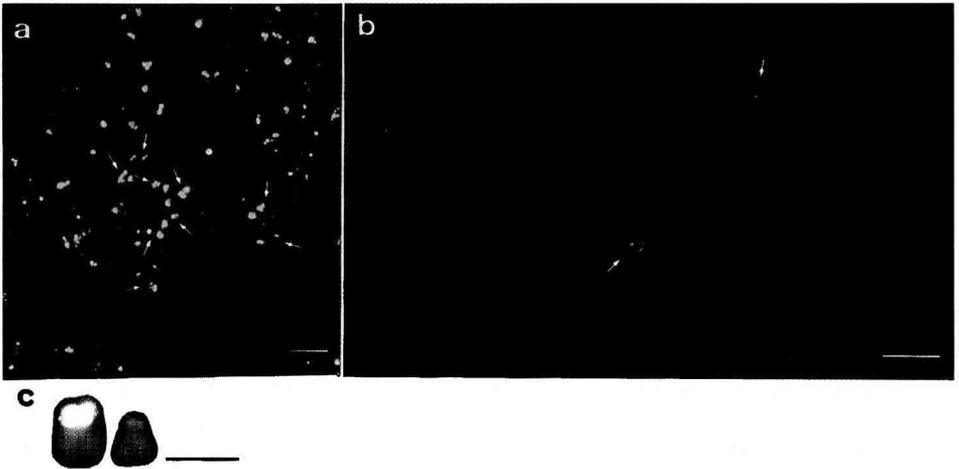


Figure 3. Propidium iodide counterstained metaphases after FISH with 5S (a) and 18S-28S rDNA probes (b); chromosome pair with constitutive heterochromatin regions of different size between homologous chromosomes as revealed by C-banding (c). Arrows indicate hybridization signals. Asterisk indicates the chromosome with the largest heterochromatin region showing less intensity of propidium iodide staining at this region (short arms). Scale bar = 5 μm.

To establish the relative position of 5S rDNA and 18S-28S rDNA, FISH was carried out with a heterologous 18S-28S rDNA probe. Forty-seven metaphases from four individuals were examined. In all cases, hybridization signals were found spread along the short arms of one pair of chromosomes characterized by having short arms of different size between homologous chromosomes (*figure 3b*). After performing C-banding, this pair showed constitutive heterochromatin regions of unequal size between homologous chromosomes (*figure 3c*). Propidium iodide staining was less intense at these heterochromatin regions, especially at the largest one. This made it possible to recognize them after FISH (*figure 3a*). Hybridization signals with 5S rDNA probe were not observed

in these heterochromatin regions (*figure 3a*) indicating that 5S and 18S-28S rDNA are not linked.

4. DISCUSSION

This is the first report on the characterization of the whole 5S rDNA repeat unit in a bivalve species. The results obtained suggest that *C. edule* 5S rDNA exhibits the conventional tandem arrangement. Evidence is provided by the fact that the PCR amplification of the 5S rDNA unit was obtained using contiguous primers located in opposite orientation. The length of the inferred 5S gene is 120 bp and the spacer region ranges between 424 and 426 bp. Totalling 544–546 bp, this size is intermediate between other 5S rDNA repeat units observed in invertebrates such as the 300–450 bp of some Diptera [8] and the 589 bp of a crustacean species [20].

The four sequenced clones of *C. edule* display an identical coding region and potential regulatory elements are identifiable in all of them. Therefore, the occurrence of gene variants or pseudogenes among the repeat units analysed here can be considered improbable.

In the spacer region of 5S rDNA, three of the clones analysed are almost identical as only two variable sites were found and these appear to result from reduction/expansion of the thymidine-rich region at the 5' end. Excluding the variable sites associated with these thymidine regions, the fourth clone displays differences at five nucleotide positions. As the clones analysed were obtained by PCR, it cannot be excluded that some of the nucleotide variations were due to Taq polymerase misincorporation. The occurrence of this minimal intra-individual variation and the identification of the same restriction patterns after enzyme digestion in the individuals examined suggest that 5S rDNA would be an appropriate region for assessing the relationships between *C. edule* and other bivalves.

Comparison of the 5S rDNA coding sequence of *C. edule* and the three available sequences of bivalves shows little interspecific variation. *C. edule* and *Calyptogena magnifica*, two species whose superfamilies are considered to have a common ancestor in Late Paleozoic [16], share the same sequence. On the other hand, *C. edule* differs from *S. velum* and from *M. edulis* at only four nucleotide positions, although *C. edule* and *S. velum* share a common ancestor in Precambrian and *C. edule* and *M. edulis* in Early Paleozoic [16]. This might indicate that phylogenetic information provided by the coding sequence may be limited, but a sufficiently large species sample and comparison of the supposedly fast-evolving spacer sequences could establish a utility of 5S rDNA for phylogeny estimations.

FISH revealed a total of nine hybridization sites. Thus, one of the pairs seems to bear 5S rDNA in heterozygosity; that is, it is absent in one chromosome or is present in such a low copy number that it is not sufficient for detection by FISH. Since chromosome pairs cannot be distinguished unambiguously after propidium iodide counterstaining it is not possible to determine if it is always the same pair that shows 5S rDNA in heterozygosity. Differences between homologous chromosomes concerning the size of the 5S rDNA hybridization signals were observed in amphibians [30] and this type of variation is not unusual in the case of repetitive sequences located at telomeres. Also, 18S-28S rDNA and

heterochromatin regions identified here in *C. edule* display different sizes. Sister chromatid exchanges and unequal meiotic crossing-over events could cause the size of rDNA clusters to fluctuate randomly so that heterozygosity of the rDNA clusters is the rule, and homozygosity the exception.

The distribution of 5S rDNA in *C. edule* is very different from that found in the bivalve analysed so far, *A. opercularis*. In this pectinid species, 5S rDNA was identified at two sites of one arm of a metacentric pair [10]. The differences between these two species contrast, for example, with the tendency of 5S rDNA in mammals to be localized in the terminal region of a pair of chromosomes [27]. However, they are similar to those found in other groups such as anuran amphibians where both number as well as position can be very different between species [30].

Unlike the 5S rDNA, the location of 18S-28S rDNA in *C. edule* is restricted to short arms of one chromosome pair, as was already observed in individuals of *C. glaucum* populations using silver staining [29]. Both types of rDNA have been found to be linked in several animals and plants [2, 4, 21], nevertheless, the most common situation in higher eukaryotes is the absence of linkage. The results obtained in this work show that this also occurs in *C. edule*.

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