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Nucleolar organizing regions in *Crassostrea angulata*: chromosomal location and polymorphism

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Abstract

Specimens of *Crassostrea angulata* collected from several natural populations, with or without metal contamination, located along the Southwestern coast of Spain were cytogenetically analyzed. The diploid number was 20 and all 10 chromosome pairs were metacentric. The nucleolar organizing regions (NORs) were identified on the telomeric region of the chromosome pair 10 by several methods, including silver nitrate staining ($AgNO_3$), chromomycin A_3 staining (CMA_3) and fluorescence *in situ* hybridization (FISH). One or two primary Ag-NORs in the metaphase cells were most frequently found. Very infrequently, one or two secondary NORs were seen. High Ag-NOR and rDNA polymorphisms in both the size and/or the number occurred intra-individually, inter-individually and inter-populationally. However some population-specific Ag-NOR polymorphisms were found to reach or be approaching to a level of significant difference. A comparison of number and frequencies between FISH-rDNA signals and Ag-NORs showed that some NORs were not transcriptionally active. Moreover, presence of pollutants on the medium could be favoring the presence of two NORs per cell more than acting modifying expression on the NORs.

Introduction

The nucleolar organizing region (NOR) on the chromosome contains a multiple-copy cluster of 5.8, 18 and 28 S ribosomal RNA genes (rDNA). The NOR in the transcriptionally active state contains DNA, RNA and protein. NORs can be visualized by several techniques. The silver nitrate staining (Ag-NOR staining) only stains those NORs which have expressed themselves during the last interphase, because silver binds to a complex of acidic protein associated with the nucleolus and nascent pre-RNA (Jordan, 1987). The rDNA, corresponding to NORs, can be located on chromosomes using *in situ* hybridization (ISH) with an rDNA probe, in particular fluorescence *in situ* hybridization (FISH). A fluorescence staining with chromomycin A_3 (CMA_3) dyes detects chromosomal DNA sequences rich in GC. CMA_3-staining is believed to show all the structural NORs on chromosomes in cold-blooded vertebrates, especially in fish (Ameniya & Gold, 1990). FISH in combination with CMA_3-staining can allow us to precisely identify the NOR-bearing chromosomes.

The Ag-NOR has been a useful chromosome marker, polymorphisms of which, including number, location and size, are often species specific. With regards to oysters, Ag-NORs have been found in two chromosome pairs in the species *Ostrea edulis* and *O. densellamelosa* (Thiriot-Quiévreux & Insua, 1992) and in the species *Crassostrea sikamea*, *C. virginica* and *C. ariakensis* (Leitao et al., 1999). However, only one pair of homologous chromosomes carrying Ag-NORs have been described in *C. gasar*, *C. angulata* (Leitao et al., 1999) and *C. gigas* (Thiriot-Quiévreux & Insua, 1992). In addition, polymorphisms in both size and number of active Ag-NORs

per cell and individual are well-known characteristics (Howell, 1982). It has been suggested that Ag-NORs indicate the metabolic activity of the cell, which in turn, can tell the physiological stages because NOR activity was higher in larvae than in juvenile mussels (Martínez-Expósito, Pasantes & Méndez, 1994). However, the silver staining method does not allow us to know whether the observed variations in number of Ag-NORs are caused by differences in transcriptional activity of rDNA or by structural changes on the bearing chromosomes such as deletions, duplications or translocations of the regions containing the rDNA genes.

C. angulata is an oyster species naturally inhabiting the Southwestern Coast of the Iberian Peninsula, mainly at river mouths and heads of estuaries. Organisms, living at intertidal areas, have to respond to a variety of environmental conditions produced by tides, seasonal changes, differences in salinity etc. Thus, they need systems of gene regulation which provide them with metabolic plasticity. The genetic control of such mechanisms is likely to involve the NOR activity, as it has been suggested in mussels (Insua & Méndez, 1998). Indeed, we have found a correlation between isozyme genotypes and the presence of metals in the environment (Cross, Vega & Rebordinos, 2002).

In the present study, we examined the karyotype and the NOR locations or the rDNA sites in oyster populations of *C. angulata* from several sites along the Southwestern Coast of Spain, differing in the degree of metal contamination. The methods of Ag-NOR staining, CMA_3 staining, and FISH were used to identify the NOR locations or the rDNA sites. We attempted to analyze if there were correlations between Ag-NOR, rDNA, CMA_3-stained NOR and different oyster populations.

Materials and methods

Material

Samples of *C. angulata* were collected from natural populations at two estuaries and three unpolluted and nearshore marine sites along the Southwestern Coast of Spain. The estuarine sites were located at the mouth of the Guadalquivir river (La Jara) and a nearby area (Corrales). These sites have been found to be highly contaminated by metals, mainly Cd, Cu, Fe, Pb and Zn (Blasco, Arias & Saénz, 1999). The marine locations include El Chato, Sancti Petri and Roche which have not been polluted by metals. A map indicating these five sampling locations is shown in Figure 1.

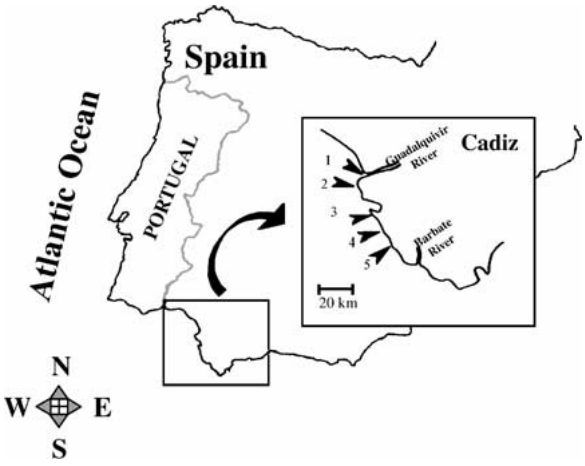

Figure 1. Collection sites of the natural populations of *Crassostrea angulata* studied along the Southwestern Coast of Spain. 1: La Jara, 2: Corrales, 3: El Chato, 4: Sancti Petri, 5: Roche. 1 and 2 are metal-contaminated sites.

Karyotype, Ag-NOR and CMA_3 staining

Collected oysters, ranging from one to two cm in size, were kept in well-aerated tanks in the laboratory and fed *ad libitum* for two days with a mix of *Tetraselmis chuii*, *Isochrysis* ssp., and *Chaetoceros* ssp., in order to activate their somatic growth. After being incubated in seawater containing 0.005% colchicine for 8 h, the gills were dissected and treated for 1 h in 0.4% KCl. Then they were fixed in Carnoy's solution. Several lamellas from the gills were homogenized in 45% acetic acid and a drop of the cell suspension was splashed onto a slide heated to 43°C (Thiriot-Quiévreux, 1984). Slide preparations were stained with Giemsa following the procedure of Thiriot-Quiévreux and Ayraud (1982).

Well-spread metaphases were photographed. After karyotyping, chromosomes of 10 metaphases were measured using the Adobe Photoshop 5.5 program and data analysis were processed using the Microsoft Excel program. The following indexes were calculated: relative length (RL = chromosome length/total haploid length $\times$ 100), centromeric index (CI = short arm length/entire chromosome length) and arm ratio (AR = short arm length/long arm length). The classification of chromosomes based on centromere position followed that of Levan, Fredga and Sandberg (1964): metacentric if $32.5 < Ci < 50$ or $0.588 < AR < 1.000$; submetacentric if $25.0 < Ci < 32.5$ or $0.333 <$

Table 1. Measurements and classification of *C. angulata* chromosomes

Chromosome pair no.	RL		CI		AR		Chromosome type
	Mean	SD	Mean	SD	Mean	SD	
1	12.61	0.82	42.20	3.69	0.74	0.11	m
2	11.69	0.70	43.01	3.66	0.76	0.11	m
3	11.18	0.45	43.68	4.49	0.78	0.14	m
4	10.60	0.51	41.83	2.96	0.71	0.10	m
5	10.18	0.26	41.83	4.30	0.72	0.13	m
6	9.85	0.26	42.24	4.74	0.74	0.14	m
7	9.39	0.43	41.43	4.21	0.69	0.10	m
8	8.91	0.64	43.81	3.17	0.78	0.10	m
9	8.34	0.69	42.97	4.06	0.76	0.11	m
10	6.94	0.58	42.08	3.46	0.73	0.10	m

RL = relative length.
CI = centromeric index.
AR = arm ratio.
SD = standard deviation.
m = metacentric.

AR < 0.88; and subtelocentric if $12.5 <$ Ci < 25.0 or $0.143 <$ AR < 0.333.

The nucleolus organizer regions (Ag-NORs) in metaphases and nucleoli in interphases were detected by silver nitrate staining (Howell & Black, 1980).

CMA_3 staining was carried out according to Schweizer (1976) with minor modifications. Slides were treated with 0.5 mg/ml CMA_3 and counterstained with 0.1 mg/ml distamycin A (DA) for 20 min. Slide preparations were mounted with a solution containing glycerol and McIlvaine buffer pH 7.0 (1:1). All the steps were carried out in darkness and slides were kept at 37°C for 48 h before the microscopic examination. Photographs were made using a fluorescence microscope equipped with a standard blue excitation filter.

FISH

A probe pDm 238 (Roiha et al., 1981), containing 5.8-18-28S rDNA genes and spacers of *Drosophila melanogaster*, cloned into the plasmid pBR322 was used to detect the rDNA clusters on chromosomes. Whole plasmid was labeled with digoxigenin-11-dUTP according to the manufacturer's instructions (Boehringer Mannheim). Slides were pretreated with RNAse and pepsin according to Weigant et al. (1991), and then chromosomes were denatured in 70% formamide/2 × SSC for 2.5 min at 83°C. Each slide was hybridized with 20 µl of the hybridization mixture containing 4 ng/µl of labeled and denaturated probe dissolved in hybridization solution (50% formamide, 10% dextran sulphate, 2 × SSC, 50 mM sodium phosphate). Hybridizations were proceeded at 37°C in a moist chamber overnight. Hybridization signals were detected by three incubations of 30 min each at 37°C in the order of the following immunological reagents: mouse anti-digoxigenin, rabbit anti-mouse-FITC (fluorescein isothiocyanate) and goat anti-rabbit-FITC. The chromosomes were counterstained with 20 µl of 50 ng/ml propidium iodide in Vectashield antifade (Vector). Photomicrographs were obtained using a fluorescence microscope (Zeiss Axioskop 2 plus) equipped with a digital camera.

Results and discussion

Examinations of metaphase chromosome spreads revealed the karyotype of *C. angulata*. In general, all five populations under study showed the same chromosome constitution. The diploid number ($2n$) was 20, as it is in the *Ostreidae* family (Lubet, 1976). Chromosomal classifications based on the centromeric position were averaged from 10 metaphases and adopted the nomenclature of Levan, Fredga and Sandberg (1964) (Table 1). It appeared that all 10 homologous pairs are metacentric, as described by Thiriot-Quievreux (1984). However, Leitao et al. (1999) considered the 8th chromosome pair as a submetacentric, which differed from that of Thiriot-Quievreux (1984)

Table 2. Counts and frequencies of metaphase spreads with 1–4 Ag-NORs from several individuals representing each sampling population of *C. angulata*

Pop./individual	Metaphases with different numbers of Ag-NOR				Total metaphases
	1	2	3	4	
La Jara 1	12	7	1		20
La Jara 2	10	9			19
La Jara 3	53	72	1		126
La Jara 4	21	34	1		56
La Jara 5	25	23	1		49
La Jara 6	20	8			28
La Jara 7	11	9	1		21
%	47.65	50.78	1.57		
Corrales 1	17	14			31
Corrales 2	14	12	1		27
Corrales 3	19	9	1		29
Corrales 4	15	24	2		41
Corrales 5	21	13			34
Corrales 6	12	21			33
Corrales 7	11	9	1		21
%	50.46	47.22	2.31		
E1 Chato 1	7	10			17
E1 Chato 2	13	8			21
E1 Chato 3	34	13			47
%	63.53	36.47			
Sancti Petri 1	10	15			25
Sancti Petri 2	8	9			17
Sancti Petri 3	11	9			20
Sancti Petri 4	11	9			20
Sancti Petri 5	20	8	1	1	30
Sancti Petri 6	17	14	1		32
%	53.47	44.44	1.39	0.69	
Roche 1	14	10	1		25
Roche 2	7	9			16
Roche 3	28	29			57
Roche 4	7	13	1		21
Roche 5	9	9	2		20
Roche 6	33	17	1		51
Roche 7	28	26	1		55
Roche 8	7	9			16
%	50.96	46.74	2.93		
TOTAL	525	481	18	1	1025
%	51.51	46.92	1.75	0.09	

as a result of the origin of the oysters. In fact, values in Leitao's study (AR = 0.59; CI = 36.84) indicated that the chromosome pair number 8 would be metacentric according to Levan's nomenclature.

Two primary Ag-NORs were located on the telomeric regions of the chromosome pair 10, as reported by Leitao et al. (1999). On rare occasions, one or two secondary Ag-NORs (1.75 and 0.09%, respectively,

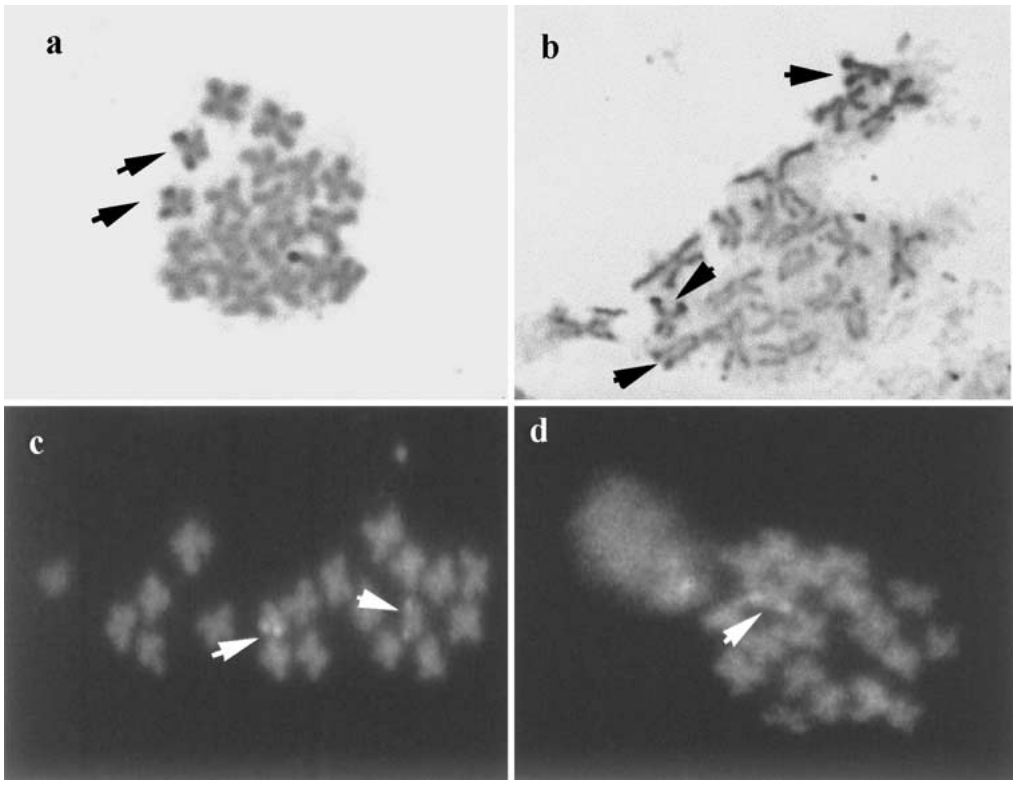

Figure 2. Silver-stained metaphase spreads ((a), (b)) showing two (a) and three (b) Ag-NOR-bearing chromosomes in *C. angulata*. CMA_3-stained metaphase spreads ((c), (d)) showing (c) NOR heteromorphism on a chromosome pair and (d) only one CMA_3 stained NOR chromosome.

Table 2) were observed (Figure 2(a), (b)). The secondary Ag-NORs were seen on different chromosomes. The primary Ag-NORs were present on one or both of the 10th chromosome pair. In this study, 51% of metaphases showed one Ag-NOR and 46.92% showed two (Table 2). Moreover, the sizes of Ag-NORs were highly variable intra- and inter-individually, as well as inter-populationally.

The number and locations of Ag-NORs has generally been found characteristic, although variable among oysters (Thiriot-Quievreux & Insua, 1992). *C. gigas* contains one chromosome pair bearing telomeric NORs, heteromorphic in size. The NORs of *O. edulis* and *O. denselamellosa* were also characterized by variable sizes. In *Mytilus*, wide variability in NOR sizes has also been described (Dixon, McFadzen & Sisley, 1986), as well as in other molluscs (Vituri & Catalano, 1990), fish (Foresti, Almeida-Toledo & Toledo, 1981), amphibians (Schmid, 1982) and other vertebrate groups (Bickman & Rogers, 1985).

It has been suggested that chromosomal NORs can play a role in inferring phylogenetic relationships, mainly based on a primitive pair of NOR-bearing chromosomes in most vertebrates (Ameniya & Gold, 1990). Our results reinforce the theory, with the oysters with a pair of NORs, as *C. gigas*, being more primitive than those with two pairs, as *O. edulis and O. denselamellosa* (Thiriot-Quievreux & Insua, 1992).

The variability of the Ag-NOR was analyzed by examining 1025 metaphases from 31 individuals representing five natural populations. Two of these populations (La Jara and Corrales) inhabit seriously metal-contaminated waters. The other three populations (El Chato, Sancti Petri and Roche) live in unpolluted areas (Figure 1). Populations, differing with the degree of metal pollution, were studied to determine the effect of metal on Ag-NOR expression. Differences in the number and frequencies of Ag-NOR between populations were compared by contingency χ^2 test (Table 3). Most probability values were higher than 0.05. However, a significant difference was obtained when data of El Chato versus La Jara were compared. The comparisons of El Chato versus Roche

Table 3. *P* values obtained from the comparison of Ag-NOR frequencies between populations through χ^2 contingency analysis

	La Jara	Corrales	Chato	Sancti Petri	Roche
La Jara	–	0.629	0.023*	0.291	0.548
Corrales		–	0.064	0.536	0.994
Chato			–	0.316	0.068
Sancti Petri				–	0.489
Roche					–

* $P \leq 0.05$.

and El Chato versus Corrales data resulted in *p* values approaching a level of significant difference, but these values could be as a result of the statistics performed on a small number of metaphases.

The controlling mechanism of the NOR expression has yet to be determined in molluscs. Genetic control of the NOR activity is not known and the number of NORs varies during the development. The variability could not be related to a definitive factor, such as age, etc. Thus, in avian species, variation in Ag-NOR number has not been observed from the embryonic stage to adult birds (Bloom & Bacon, 1985). However, in mammals, an increase in the number of Ag-NORs during the development occurs in mice (Dyban et al., 1990), whereas the opposite tendency has been described in humans (Das et al., 1986). Mussels also displayed a sharp decrease during the transformation from larvae to juvenile (Martínez-Expósito, Pasantes & Méndez, 1994). It is considered that the control of rDNA transcriptional activity causing Ag-NOR expression could be species specific. On the other hand, the NOR activity could be highly influenced by environmental conditions. For example, an inverse correlation between number of Ag-NOR and the altitude has been described in the fish *Astyanax scabripinnis* (Marco-Ferro et al., 2001). Notwithstanding, the association has been complicated by the connection between NORs and B-chromosomes. In addition, influences of B-chromosomes in the expression of rDNA have been described in grasshopper (Camacho, Sharbel & Beuekeboom, 2000) and rye (Delgado et al., 1995).

Number and frequencies of Ag-nucleoli between populations were also examined and comparable to the results of Ag-NORs. Nucleoli in interphase cells, like NORs in metaphase cells, can be visualized by silver staining. A maximum number of four Ag-nucleoli (Table 4) and frequencies of 1–4 Ag-nucleoli similar to those of Ag-NORs (Table 2) were observed. All populations showed the occurrence of four nucleoli except the Sancti Petri population (Figure 3). The Chato had proportionally lower Ag-NOR number than the other populations (Table 3), but similar frequencies of different nucleolus number. Comparison between frequencies of Ag-NOR (Table 2) and nucleoli (Table 4) showed similar values for each population with each one of the techniques indicating that (i) every Ag-NOR sequence would organize one nucleolus and (ii) no differences could be established between polluted and unpolluted populations.

It has been suggested that the presence in the genome of multiple repeated rDNA sequences as well as multiple NORs, each containing highly repeated rDNA gene sequences, must be under strong positive selection, since the number of NORs generally appears to exceed the number of nucleoli (Flavell & Martini, 1982). The cooperation of several NORs in forming a common nucleolus has been observed at prophase for a variety of different organisms, when several nucleolar chromosomes are attached to the same nucleolus by their NORs (Stahl, 1982). NOR number could be integrated into cell metabolism via the nucleolus due to different physiological states between cells or between individuals, so that, some enzymes like RNA polymerase is coordinated with the concentration of amino acids, intracellular energy levels and rates of protein synthesis (Dixon, McFadzen & Sisley, 1986).

In order to locate rDNA genes and to examine the variability in both the number and size with relation to the copy number of rDNA genes, CMA_3-staining and FISH analysis was carried out. CMA_3 staining stains GC-rich DNA sequences which have been associated with the NOR regions in some organisms (Schmid, 1982; Pardo et al., 2001). CMA_3 staining confirmed the locations of Ag-NORs on the telomeric regions of the chromosome pair 10 and did not stain any other chromosome regions. However, differences in structural NOR were seen between a pair of homologues (Figure 2(c)), and some metaphases showed only one CMA_3-positively stained chromosome (Figure 2(d)). FISH analysis showed that the site of 18-28 S rDNA was telomeric on the long arm of the chromosome pair 10, the same as the Ag-NOR and CMA_3-positive locations (Figure 4). The analysis of the silver staining showed the proportion of individuals with one or two Ag-NORs to be about half and half, whereas the FISH analysis revealed two rDNA sites in most individuals. This indicated that a certain proportion of rDNA sites were transcriptionally silent (Table 5) and did not form

Table 4. Number of nucleoli in several populations of *C. angulata*

Population	Number of individuals	Nuclei with different number of nucleoli				Total nucleoli
		1	2	3	4	
La Jara	11	681	736	20	3	1441
%		47.26	51.07	1.39	0.21	
Corrales	7	439	371	11	1	822
%		53.41	45.13	1.34	0.12	
E1 Chato	7	469	470	7	3	949
%		49.42	49.42	0.74	0.32	
Sancti Petri	9	551	502	6	0	1059
%		52.03	47.40	0.57	0	
Roche	17	1014	1022	23	8	2066
%		49.08	49.47	1.11	0.39	
Total	51	3154	3101	72	15	6342
%		49.7	48.8	1.13	0.23	

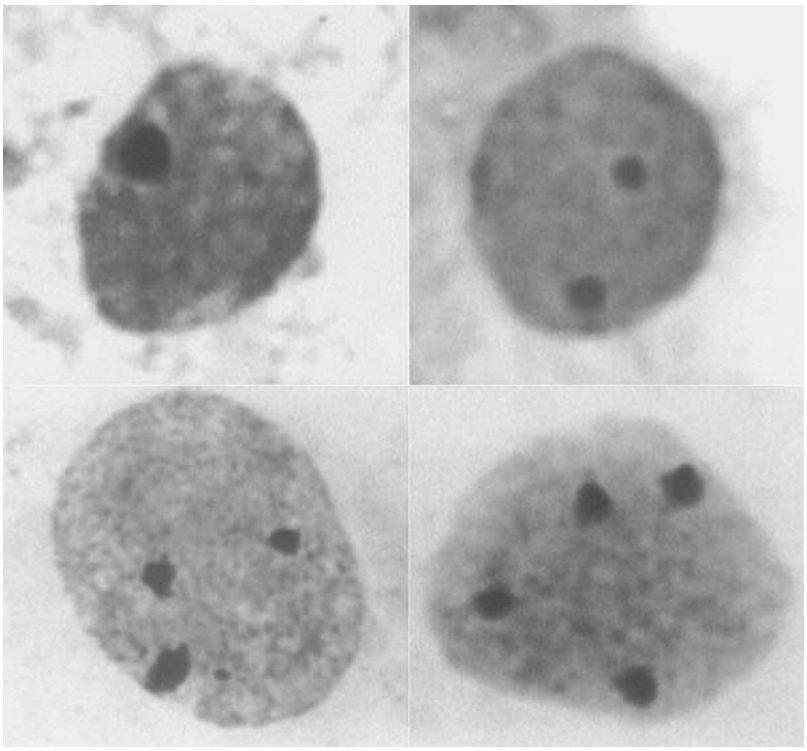

Figure 3. Silver stained interphase cells of *C. angulata* showing 1, 2, 3 and 4 nucleoli, respectively.

Ag-NORs. Moreover, comparison of the frequencies of two rDNA sites between metal-polluted and metal-unpolluted populations showed that a higher proportion of two rDNA signals were present in metal-polluted populations (94 v.s. 87%, respectively). It may be due to a high selection pressure exerted by the polluted environment favoring the presence of two rDNA sites, while in a less restrictive environment the

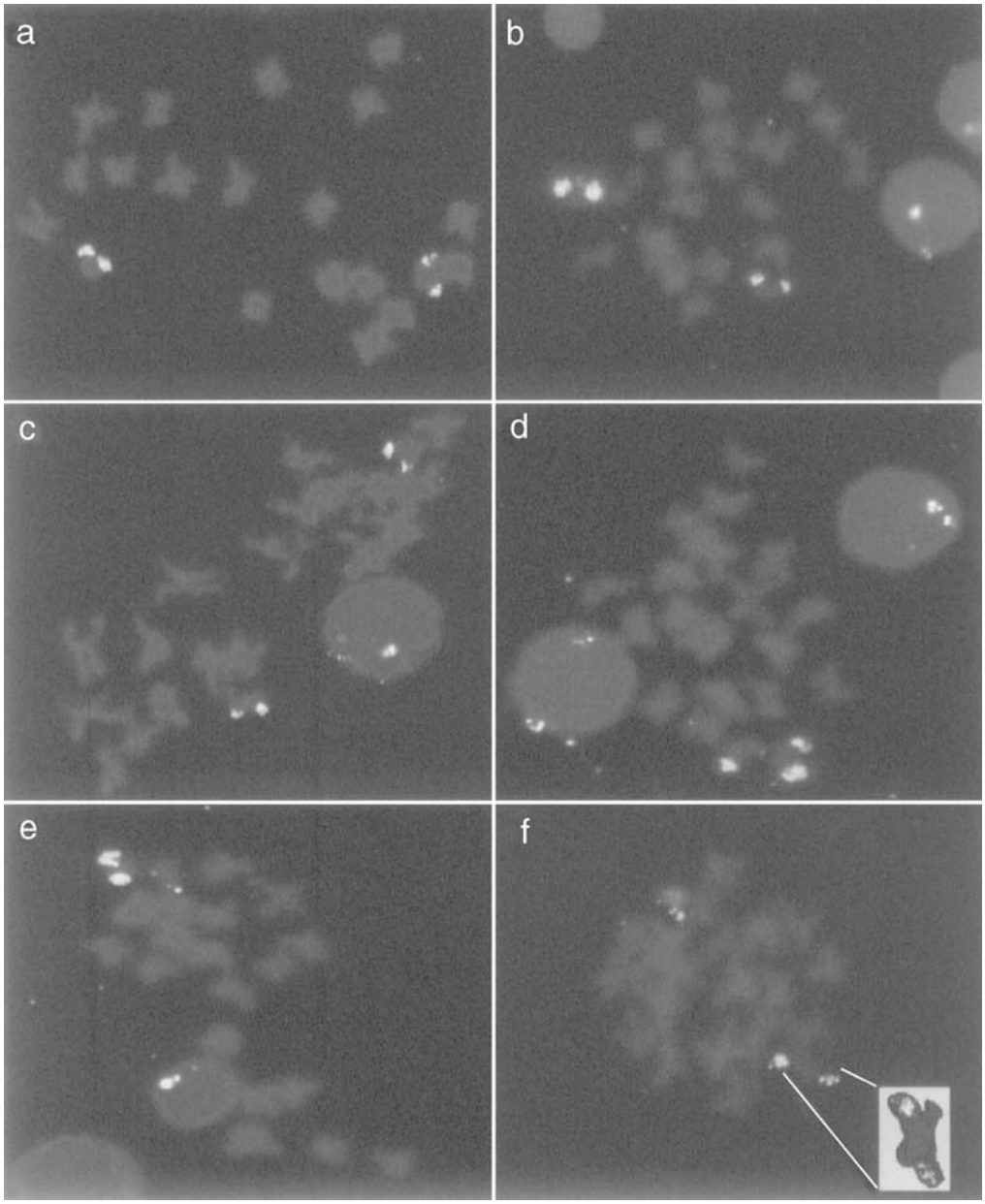

Figure 4. C. angulata metaphases showing the 5.8-18-28S rDNA chromosomal locations. ((a) and (b)) NOR size heteromorphism between a homologue chromosome pair. (c) NOR size heteromorphism between sister chromatids. (d) Absence of rDNA on one sister chromatid. (e) Absence of rDNA on an homologue chromosome. (f) A cytogenetic abnormality due either to a translocation of rDNA site on one of sister chromatids or to an arm crossing.

presence of either one or two could be tolerated. On this sense, isozyme data obtained from individuals of these populations have shown a genotypic selection by certain metallic ions (Cross, Vega & Rebordinos, 2002).

Size heteromorphisms of the FISH-rDNA signal between both homologues and between sister chromatids occurred intra- and inter-individually, as did those of Ag-NORs (Figure 4(a), (b)). This indicates that the copy number of rDNA is variable. This unusual occurrence between sister chromatids was likely caused by mistakes of either deleting or duplicating the rDNA clusters during the DNA replication (Figure 4(c)). Severe deletions could give

Table 5. Counts of metaphase spreads with one or two FISH-rDNA hybridization signals in individuals representing two populations of *C. angulata*

Individual	Signal on one choromosome	Signal on two chromosomes
Sancti Petri 1	6	17
Sancti Petri 2	2	17
Sancti Petri 3	2	16
Sancti Petri 4	2	23
Sancti Petri 5	1	27
Sancti Petri 6	4	10
Sancti Petri 7	1	11
Corrales 1	0	6
Corrales 2	0	8
Corrales 3	1	2

rise to the missing of rDNA on one sister chromatid (Figure 4(d)), which would become a cell line with only one NOR-bearing chromosome (Figure 4(e)). Moreover, this FISH technique revealed a possible cytogenetic abnormality, either due to a translocation on a chromatid of a NOR-chromosome or to an incorrect arrangement of the chromosome disposition in the metaphase, as a consequence of an arm crossing (Figure 4(f)).

The high cytogenetic variability of these regions is in agreement with the high genetic variability found with other markers (Cross, Vega & Rebordinos, 2002) and could be related to the plasticity of *C. angulata* to survive in a wide range of environmental conditions (salinity, contamination, etc.).

Zurita et al. (1998) showed that the level of transcriptional activity of NORs is directly related to the copy number of ribosomal genes. Variation in the copy number of rDNA genes has been described in several organisms in both directions, additional loci (de Lucchini et al., 1993) and deleting loci (Garrido Ramos et al., 1995). Some studies have indicated that variation in rDNA copy number is operated by selection. For instance, a 50% reduction of the rDNA copy number caused deaths in *Xenopus* (Miller & Knowland, 1972). In another case, an optimum rDNA copy number has been favored for growth in *Neurospora crassa* (Russell & Roland, 1986) and in *Salmo trutta* the average NOR copy number has been predominant, indicating the selection against extremes (Martínez et al., 1993).

Our data showed that presence of pollutants on the environment could act at level of DNA positively selecting presence of two NOR sequences more than to the level of NOR expression. Additional studies are being carried out in order to confirm this proposition.

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References

Ameniya, C.T. & J.R. Gold, 1990. Cytogenetic studies in North American minnows (*Cyprinidae*). XVII. Chromosomal NOR phenotypes of 12 species, with comments on cytosystematic relationships among 50 species. Hereditas 112: 231–247.

Bickham, K.W. & D.S. Rogers, 1985. Structure and variation of the nucleolus organizer regions in turtles. Genetica 67: 171–184.

Blasco, J., A. Arias & V. Sáenz, 1999. Heavy metals in organisms of the River Guadalquivir estuary: possible incidence of the Aznalcóllar disaster. Sci. Total Environ. 242: 249–259.

Bloom, S.E. & L.D. Bacon, 1985. Linkage of the major histocompatibility (*B*) complex and the nucleolar organizer in the chicken. J. Hered. 76: 146–154.

Camacho, J.P., T.F. Sharbel & L.W. Beuekeboon, 2000. B-chromosome evolution. Philos. T. Roy. Soc. B. 355: 163–178.

Cross, I. & L. Rebordinos, 2002. Effect of marine pollution on genetic structure in populations of bivalve *Crassostrea angulata*. Mar. Sci. (in press).

Das, B.C., R. Rani, A.B. Mitra & U.K. Lekhra, 1986. The number of silver staining NORs (rDNA) in lymphocytes and newborns and its relationship to human development. Mech. Ageing Dev. 36: 117–123.

de Lucchini, S., I. Nardi, G. Barsacchi & R. Batistoni, 1993. Molecular cytogenetics of the ribosomal (18S+28S and 5S) DNA loci in primitive and advanced urodele amphibians. Genome 36: 762–773.

Delgado, M., L. Cecilio-Morais, N. Neves, R.N. Jones & W. Viegas, 1995. The influence of B chromosomes on rDNA organization in rye interphase nuclei. Chromosome Res. 3: 487–491.

Dixon, D.R., I.R.B. McFadzen & K. Sisley, 1986. Heterochromatic marker regions (nucleolar organiser) in the chromosomes of the common mussel, *Mytilus edulis* (Mollusca: Pelecypoda). J. Exp. Mar. Biol. Ecol. 97: 205–212.

Dyban, A.P., E.L. Severova, O.V. Zatsepina & Y.S. Chentsov, 1990. The silver-stained NOR and argentophilic proteins in early mouse embryogenesis: a cytological study. Cell Differ. Dev. 29: 165–179.

Flavell, R.B. & G. Martini, 1982. The genetic control of nucleolus formation with special reference to the common breadwheat, pp. 113–128 in The Nucleolus, edited by E.G. Jordan & C.A. Cullis. Cambridge University Press, Cambridge.

Foresti, F.L., L.F. Almeida-Toledo & S.A. Toledo, 1981. Polymorphic nature of nucleolus organizer regions in fishes. Cytogenet. Cell Genet. 31: 137–144.

Garrido-Ramos, M.A., M. Jamilena, R. Lozano, S. Cárdenas, C. Ruiz-Rejón & M. Ruiz-Rejón, 1995. Cytogenetic analysis of gilthead seabream *Sparus aurata* (Pisces, Perciformes), a deletion affecting the NOR in a hatchery stock. Cytogenet. Cell Genet. 68: 3–7.

Howell, W.M. & D.A. Black, 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: 1-step method. Experientia 36: 1014–1015.

Howell, W.M., 1982. Selective staining of nucleolus organizer regions (NORs). Cell Nucleus 11: 90–142.

Insua, A. & J. Méndez, 1998. Physical mapping and activity of ribosomal RNA genes in mussel *Mitylus galloprovincialis*. Hereditas 128: 189–194.

Jordan, G., 1987. At the heart of the nucleolus. Nature 329: 489–499.

Leitao, A., P. Boudri, J.P. Labat & C. Thiriot-Quievreux, 1999. Comparative karyological study of cupped oyster species. Malacologia 41: 175–186.

Levan, A., K. Fredga & A.A. Sandberg, 1964. Nomenclature for centromere position in chromosomes. Hereditas 52: 201–220.

Lubet, P., 1976. L'espèce chez les Lamellibranches marins. Mém. Soc. Zool. Fr. 38: 341–368.

Marco-Ferro, D.A., D.M. Néo, O. Moreira-Filho & L.A.C. Bertollo, 2001. Nucleolar organizing regions 18 S and 5 S rDNA in *Astyanax scabripinnis* (Pisces, Characidae): populations distribution and functional diversity. Genetica 110: 55–62.

Martínez, P., A. Viñas, C. Bouza, J. Castro & L. Sánchez, 1993. Quantitative analysis of the variability of nucleolar organizer regions in *Salmo trutta*. Genome 36: 1119–1123.

Martínez-Expósito, M.J., J.J. Pasantes & J. Méndez, 1994. NOR activity in larval and juvenile mussel (*Mytilus galloprovincialis* Lmk.). J. Exp. Mar. Biol. Ecol. 175: 155–165.

Miller, L. & J. Knowland, 1972. The number and activity of ribosomal RNA genes in *Xenopus laevis* embryos carrying partial deletions in both nucleolar organizers. Biochem. Genet. 6: 65–73.

Pardo, B.G., C. Bouza, J. Castro, P. Martínez & L. Sánchez, 2001. Localization of ribosomal genes in Pleuronectiformes using Ag-, CMA_3-banding and *in situ* hybridization. Heredity 86: 531–536.

Roiha, H., J.R. Miller, L.C. Woods & D.M. Glover, 1981. Arrangements and rearrangements of sequences flanking the two types of rDNA insertion in Drosophyla melanogaster. Nature 290: 749–753.

Russell, P.J. & K.D. Roland, 1986. Magnification of rDNA gene number in a *Neurospora crassa* strain with a partial deletion of the nucleolus organizer. Chromosoma 93: 333–340.

Schmid, M., 1982. Chromosome banding in Amphibia. VII. Analysis of the structure and variability of NORs in Anura. Chromosoma 87: 327–344.

Schweizer, D., 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58: 307–324.

Stahl, A., 1982. The nucleolus and nucleolus chromosomes, pp. 1–24 in The Nucleolus, edited by E.G. Jordan & C.A. Cullis. Cambridge University Press, Cambridge.

Thiriot-Quiévreux, C. & N. Ayraud, 1982. Les caryotypes de quelques especes de Bivalves et de Gastéropodes marins. Mar. Biol. 70: 165–175.

Thiriot-Quiévreux, C., 1984. Analyse comparée des caryotypes d'ostreidae (Bivalvia). Cah. Biol. Mar. XXV: 407–418.

Thiriot-Quiévreux, C. & A. Insua, 1992. Nucleolar organiser region variation in the chromosomes of three oyster species. J. Exp. Mar. Biol. Ecol. 157: 33–40.

Vituri, R. & E. Catalano, 1990. Spermatocyte chromosome banding studies in *Buccinulum corneum* (Prosobranchia: Neogastropoda): variation in silver-NOR banding pattern. Mar. Biol. 104: 259–263.

Weigant, J., T. Ried, P.M. Nederlof, M. van der Ploeg, H.J. Tanke & A.K. Raap, 1991. *In situ* hybridization with fluoresceinated DNA. Nucleic Acids Res. 19: 3237–3241.

Zurita, F., A. Sánchez, M. Burgos, R. Jiménez & R. Díaz de la Guardia, 1998. Interchromosomal, intercellular and interindividual variability of NORs studied with silver staining and *in situ* hybridization. Heredity 78: 229–234.