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Molecular characterization and evolution of an interspersed repetitive DNA family of oysters

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Abstract When genomic DNA from the European flat oyster *Ostrea edulis* L. was digested by *Bcl*I enzyme, a band of about 150 bp was observed in agarose gel. After cloning and sequencing this band and analysing their molecular characteristics and genomic organization by means of Southern blot, in situ hybridisation, and polymerase chain reaction (PCR) protocols, we concluded that this band is an interspersed highly repeated DNA element, which is related in sequence to the flanking regions of (CT)-microsatellite loci of the species *O. edulis* and *Crassostrea gigas*. Furthermore, we determined that this element forms part of a longer repetitive unit of 268 bp in length that, at least in some loci, is present in more than one copy. By Southern blot hybridisation and PCR amplifications—using primers designed for conserved regions of the 150-bp *Bcl*I clones of *O. edulis*—we determined that this repetitive DNA family is conserved in five other oyster species (*O. stentina, C. angulata*, *C. gigas, C. ariakensis*, and *C. sikamea*) while it is apparently absent in *C. gasar*. Finally, based on the analysis of the repetitive units in these oyster species, we discuss the slow degree of concerted evolution in this interspersed repetitive DNA family and its use for phylogenetic analysis.

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Introduction

A large fraction of the eukaryotic genome consists of highly repeated DNA sequences. Typically, two main types of repetitive DNA families can be distinguished, tandem and interspersed repeats. Tandem repeats such as satellite DNA are normally subjected to a concerted mode of evolution or non-independent evolution, resulting in a sequence similarity of repeating units that is greater within than among species (Dover 1982). Due to this pattern of evolution, satellite-DNA sequences have important implications for taxonomic and phylogenetic analyses (see Elder and Turner 1995). However, the evolution and the utility of highly repeated interspersed DNA families have been less analysed, except in the case of transposable elements (Gaffney et al. 2003).

In mollusc genomes, several satellite-DNA sequences have been analysed and some of them even used as molecular markers for taxonomic (Canapa et al. 2000; Lapègue et al. 2002; Martínez-Lage et al. 2002; Klinbunga et al. 2003), phylogenetic (Littlewood 1994; Jozefowicz and Ò Foighil 1998; Ò Foighil and Taylor 2000) as well as phylogenetic and taxonomic analyses (Muchmore et al. 1998; López-Flores et al. 2004). With respect to other types of repetitive DNA in oysters, some recent publications report the use of microsatellite loci for analysing genetic diversity within and between populations (Zhang et al. 2005; Carlsson et al. 2006; Yu and Li 2007).

In this paper, we identify an interspersed repetitive DNA sequence, the *Bcl*I family, found initially in the genome of the European flat oyster, *Ostrea edulis*. After determining

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its molecular characteristics and its genomic organization in this species, we comparatively analyse its presence and sequence in six other species belonging to the mollusc genera *Ostrea* and *Crassostrea* in order to determine its evolution pattern.

Materials and methods

Sampling

We have analysed two species of oysters belonging to the genus *Ostrea* (*O. edulis* and *O. stentina*) and five from the genus *Crassostrea* (*C. angulata*, *C. gigas*, *C. gasar*, *C. ariakensis* and *C. sikamea*) as summarized in Table 1. The geographical origin and species identification have been described in detail elsewhere (López-Flores et al. 2004).

Identification, cloning, and sequencing of *Bcl*I repetitive DNA in *O. edulis*

Genomic DNA was extracted from abductor muscle of oysters as described by Winnepenninckx et al. (1993). Ten micrograms of *O. edulis* genomic DNA were digested overnight at 37°C with 15 units of *Bcl*I endonuclease (Roche) and fragments separated by electrophoresis on 3% agarose gel. A single prominent band of about 150 bp was excised from the gel, purified using GFX™ polymerase chain reaction (PCR) DNA and Gel Band Purification Kit (Amersham Biosciences) and ligated to the pUC19 plasmid (Roche). Ligation mix was used to transform DH5α-competent cells according to the recommendations of the supplier (Gibco BRL). Mini-preparations were made using Perfectprep® Plasmid Mini (Eppendorf) and recombinant plasmids (eight) were sequenced in both strands using Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems).

The complete unit of B*cl*I sequence was isolated by PCR using the primers invBcl-F (5′-GTCRACAGGGGATGCTT ACTCCTCCTAGGCAC-3′) and invBcl-R (5′-ATAGA-TAGTTGGGCAAACACRGACCCCTGGAC-3′) designed on the initial 150 bp *Bcl*I sequence in the opposite sense. Amplification was performed from 50-ng genomic DNA in 30 µl volume containing 100 µM of each primer, 10 mM dNTPs and 1.5 units of *Taq* polymerase (Amersham Biosciences). The PCR conditions consisted of one initial denaturing cycle of 5 min at 94°C, followed by 35 amplification cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C and a final extension cycle at 72°C for 10 min.

Southern-blot and dot-blot analysis of oyster species

For Southern-blot hybridisation, the genomic DNA of oyster species was digested with 20 restriction endonucleases: *Hind*III, *Eco*RI, *Bam*HI, *Sac*I, *Dra*I, *Cfo*I, *Pst*I, *Bcl*I, *Xba*I, *Msp*I, *Rsa*I, *Bgl*II, *Hinf*I, *Pvu*II, *Hae*III, *Alu*I, *Hin*dII, *Hpa*II, *Ssp*I, and *Nde*II. Digestions were carried out by applying three micrograms of DNA and 15 units of enzyme, in an overnight incubation at 37°C. Digested DNA fragments were electrophoretically separated and processed for transfer onto a nylon membrane by standard vacuum-transfer procedure (Sambrook and Russell 2001) using VacuGene™ XL Vacuum Blotting System (Amersham Biosciences).

For dot-blot hybridisation, genomic DNA from different oyster species were denatured in boiling water and diluted to different concentrations with 20xSSC. The DNA was fixed to the filter with 0.4 M NaOH for 2 min. Probes for Southern-blot and dot-blot hybridisations (a portion of *Bcl*I fragment eluted from agarose gel) were peroxidase-labelled by ECL™ Direct Nucleic Acid Labelling and Detection Systems (Amersham Biosciences), and hybridisation signals were detected according to the supplier's manual.

Isolation by PCR and characterization of *Bcl*I sequences in oyster species

The eight *Bcl*I cloned sequences of *O. edulis* were aligned by Megalign program of DNAstar package (LASERGENE) and the alignment was used to design the primers OsedBdir (5′-ARGATATAGGGCTCA-3′) and OsedBrev (5′-GATCAATCACATAAC-3′). Amplification by PCR of

Table 1 Information concerning oyster specimens used in this study

Species	Common name	Abbreviation	Clones	Accession number
O. edulis	European flat oyster	Oed	OedB5, OedB6, OedB19, OedB22, OedB23, OedB31, OedB46, OedB53,	AJ864926–AJ864934
O. stentina	Provence oyster	Ost	OstB4, OstB28, OstB29, OstB35, OstB37, OstB41	AJ864935–AJ864940
C. angulata	Portuguese oyster	Ca	CaB16, CaB21, CaB25, CaB27, CaB30, CaB31	AJ864902–AJ864907
C. gigas	Pacific oyster	Cg	CgB10, CgB13, CgB14, CgB20, CgB28, CgB40	AJ864908–AJ864913
C. gasar	Mangrove oyster	Cga	–	–
C. ariakensis	Suminoe oyster	Car	CarB6, CarB9, CarB22, CarB24	AJ864914–AJ864917
C. sikamea	Kumamoto oyster	Cs	CsB5, CsB31, CsB32, CsB33, CsB40	AJ864918–AJ864922

*Bcl*I sequence in the remaining oyster species was performed from 50 ng genomic DNA from two to three individuals as a template in 50 µl volume containing 175 µM of each primer, 10 mM dNTPs and 1.5 units of *Taq* polymerase (Amersham Biosciences). The PCR amplification profile consisted of one initial denaturing cycle of 5 min at 94°C, followed by 40 amplification cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension cycle at 72°C for 10 min. The annealing temperature was dropped to 45°C to facilitate amplification from *O. stentina* and *C. sikamea* species. The fragments obtained by PCR were separated electrophoretically, purified from agarose gels, and cloned using the pGEM-T Easy Vector Systems (Promega). Recombinant plasmids were sequenced as described above.

In all the 35 *Bcl*I sequences obtained from six oyster species (this sequence is apparently absent in *C. gasar*), multiple alignments were also performed using Megalign program of DNAstar package (LASERGENE). We computed basic sequence statistics using the program Genepro v. 6.1 (Riverside Scientific Ent., 1993). Polymorphism value (*P*) and nucleotide diversities (π) (Nei 1987) were determined with the program DnaSP v.3 (Rozas and Rozas 1999). The shared polymorphic sites and the genetic distances were analysed by satDNA Analyzer software (Navajas-Pérez et al. 2007), using Kimura's two parameters (Kimura 1980) and Jukes and Cantor distances (Nei 1987). The phylogenetic tree, based on the neighbour-joining method, was drawn using PhyloDraw software ver. 0.8 (Graphics Application Lab.).

Cytogenetic analyses

For FISH analyses, the chromosomes of *C. angulata* were obtained following the method of Cross et al. (2003), consisting mainly of feeding the oysters with phytoplankton for several days, followed for colchicine treatment, hypotonic shock of gills and application of Carnoy's fixative. A cell suspension from gills was splashed onto a previously heated slide. Fluorescent in situ hybridisation was performed according to the protocol described by Cross et al. (2005) with minor modifications. Briefly, the *Bcl*I probe (clone CaB16) was labelled with biotin-16-dUTP (Roche Biochemical Diagnostics) and chromosomes were hybridised overnight at 37°C. Post-hybridisation washes and immunocytochemical incubations were carried out as described by Cross et al. (2003, 2005).

Results and discussion

Molecular and cytogenetic characterization of the repetitive *Bcl*I family in *O. edulis*

The Southern-blot analysis revealed a single hybridisation signal in the case of the *Bcl*I repeated family instead of the typical ladder pattern from satellite DNA found for example in the previously characterized *Hind*III family in this species (Fig. 1).

This hybridisation pattern would initially indicate a highly homogeneous satellite-DNA family in which all of

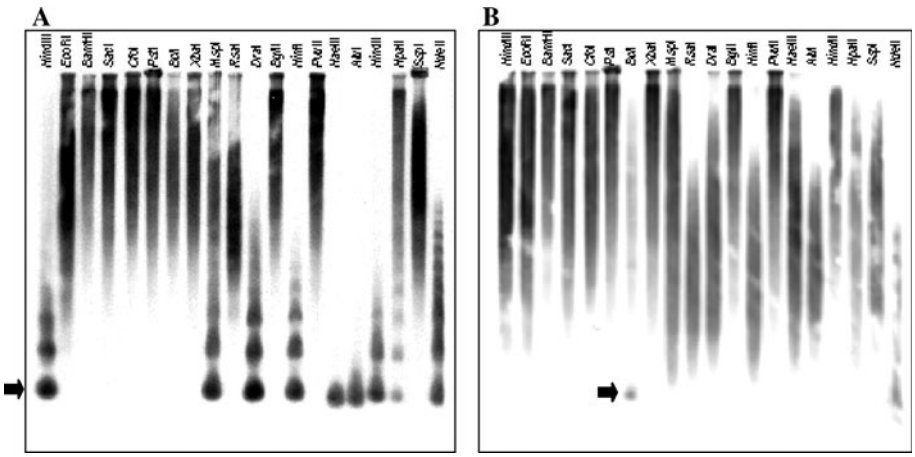

Fig. 1 Southern-blot of *O. edulis* DNA probed with *Hind*III satellite DNA (**a**) and *Bcl*I repetitive DNA (**b**). At the top of the panels, the name of the endonuclease corresponds to digestion in each line. *Arrowhead* shows every monomeric hybridisation signal. *Hind*III monomeric unit revealed cross-hybridisation with *Msp*I, *Dra*I, *Hinf*I, *Hae*III, *Alu*I, *Hind*II, *Hpa*II, and *Nde*II (**a**). Recognition sites for these eight enzymes are present in the sequence of *Hind*III satellite DNA, so all of the tandem patterns correspond to the same satellite-DNA family. *Bcl*I DNA fragments revealed that only the *Bcl*I band reacted positively with the probe (**b**)

the monomers were digested, giving the same size band and thus not showing a canonical satellite-DNA pattern. A similar satellite-DNA family has been described for example in a gastropod species (Muchmore et al. 1998), in which a tandem arrangement of one *Sal*I repetitive sequence was demonstrated in five species of Eastern Pacific abalone. For analysing this possibility, the total genomic DNA from *O. edulis* was partially digested with *Bcl*I, blotted onto a membrane and hybridised with the *Bcl*I repetition unit. The time course of *Bcl*I digestion again yielded only a single band of approximately 150 bp for which the hybridisation signal increased with extended digestion (data not shown) indicating that, in this case, the lack the multimeric fragments characteristic of satellite DNA did not appear due to insufficient digestion time. Thus, additional data were collected in order to clarify the genomic organization of this repetitive DNA.

After cloning and sequencing the *Bcl*I fragment of *O. edulis,* we obtained the sequence of eight repetitive units of 142–147 bp in length (see Fig. 2). The sequence analyses of different clones showed high variability. Within the eight *Bcl*I sequences the percentage of A + T was 50% on average without any internal direct or reverse repeating elements. Transitions were much more common than transversions ($R = 3.700$). The variability of the cloned sequences was estimated by the polymorphism value ($P = 0.284$) (42 polymorphic sites) and nucleotide diversity ($\pi = 0.095$) (see Table 2). These data reveal the absence of a high identity between monomers, again contradicting the idea that this repetitive DNA is a highly homogeneous satellite-DNA family.

On the other hand, and after a search of the EMBL/GenBank databases, the sequences showed identity to the flanking region of a (CT)n microsatellite locus from *O. edulis* (77% on average) (accession number AF297862; Sobolewska et al. 2001) and *C. gigas* (73% on average) (accession number AB091657). The association between microsatellites and repetitive elements such as transposable elements is well known. This association has been explained by the action of different mechanisms in which the transposable elements play a role in the origin and/or amplification of the microsatellite loci. In fact, the microsatellites within species can be grouped into families as a function of percentage sequence identity between their flanking regions, indicating their common origin (Meglécz et al. 2007). Other repetitive sequences, such as some satellite DNA, are often originated from transposable elements, as it is the case of the superfamily BIV160 conserved in the Class Bivalvia (Plohl et al. 2010) and which is part the *Hind*III satellite DNA characterized from oysters (López-Flores et al. 2004). Additionally, the role of transposition events in the mobilization mechanisms and evolution of satellite DNAs has also been proposed (Palomeque et al. 2006).

Fig. 2 **a** Multiple-sequence alignments of *Bcl*I sequenced clones from the seven oyster species studied. The complete sequence of clone five from *O. edulis* is shown. Primer sequences (OsedBdir, 1–15 and OsedBrev, 133–148) are underlined. *Dots* indicate identity with the first sequence and *dashes* indicate deletions. **b** Sequence alignment of *Bcl*I consensus sequence (BclIOed c.s.) obtained by restriction enzyme from *O. edulis* with the sequence amplified (C-BclI) using the primers invBcl-F and invBcl-R (*underlined*). *Dots* indicate identity with the first sequence and *dashes* indicate deletions. The A-box and B-box characteristics of SINE elements are highlighted. The complete unit of repetition are boxed with a *continue line* and the second partial unit boxed with *discontinue line* ▶

In our case, this repetitive DNA, is present in the genome of other oyster species, including *Crassostrea* species. Thus, the in situ hybridisation analysis in *C. angulata* species using the repetitive *Bcl*I sequence (clone CaB16) as a probe, showed many signals along all chromosomes without evidence of accumulated hybridisation signals in any chromosome region (Fig. 3). This result demonstrates the interspersed location of this *Bcl*I repetitive family. This location indicates the existence of two different types of repetitive sequences in the genome of oysters, exemplified by the interspersed *Bcl*I family and by the *Hind*III satellite-DNA family with a tandem centromeric location (López-Flores et al. 2004).

The southern-blot and in situ hybridisations would indicate that *Bcl*I is not a tandemly arranged DNA family. However, it is possible that we have not characterized the complete repetition, but rather have isolated only a fragment between two restriction *Bcl*I targets (separated about 150 bp) present in the repetitive unit. To check this possibility and clarify the genomic organization of this repetitive sequence, we designed a pair of primers (invBcl-F and invBcl-R) in 150-bp *Bcl*I sequences but in the opposite sense. The PCR amplified a band of about 400 bp which was sequenced and named complete-BclI (C-BclI). The analysis of this sequence enabled us to ascertain the complete size of the unit of this repetitive family. In this way, the alignment of the C-BclI sequence revealed a repetition unit of 268 bp and not of 148 bp isolated previously by *Bcl*I restriction enzyme (Fig. 2b). In this alignment, we found that the amplified C-BclI sequence includes approximately one and a half of the repetitive unit. Thus, we can deduce that, although the family is an interspersed repetitive DNA, at each locus, or at least at some loci, there is more than one repetition unit (at least two) because the PCR result implies the existence of contiguous units.

Then, *Bcl*I sequence of *O. edulis* presents length and interspersed distribution patterns similar to those of SINE elements. In this sense, degenerated sequences to A-box and B-box characteristics of SINE elements have been found in *Bcl*I unit (Fig. 2b). However, *Bcl*I sequences occur as tandem arrays of two or more copies, this being

A

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OedB5       AGAATATAGG GCTCACGGCG GGTGTGACTG GTCAACAGGG GATGCTTACT CCTCCTAGGC ACCTGATCCC ACCTCTGGTG  [ 80]
OedB6       ..G....G.. .......... ........C. .......... .......... .......A.. .......... ..........
OedB19      ..G....... .......... ........C. .......... .......... .---...... .......... ..........
OedB22      ..G....G.. ........AA ........C. .......... .......... .......... .......... ..........
OedB23      .AG.C...A. .......... ........C. ...GT..... ...A...... ...A.---.. .......... ...C.C....
OedB31      ..G....... ........T. ........C. ...G...... .......... .......... .T........ G........A
OedB46      .AG.C....T ......A.T. AA......C. .....G.... .......... ....T..... .......T.. .T.......A
OedB53      .A........ .....A...A ........C. ...G...... .......... .......... ........T. ..........
OstB4       ..G....... .......... ........C. .......... .......... .......... .......... ..........
Ost28       ..G....... .......... ..G.....C. ...G...... A..C...... .......... .......... ..........
OstB29      ..G....... .......... ..G.....C. ...G...... A..C...... .......... .......... ..........
OstB35      ..G....... .......... ..G.....C. ...G...... A..C...... .......... .......... ..........
OstB37      ..G....... .......... ..G.....C. ...G...... A..C...... .......... .......... ..........
OstB41      ..G....... .......... ........C. .......... .......... .......... .........A .......C..
CaB16       ..G....... .......... ........C. .......... ....A..... .......... .......... ..........
CaB21       ..G....... .......... .......G.. .......... .......... T......... .......... ....A.....
CaB25       ..G....... .......... .......G.. .......... .......... T......... .......... ....A.....
CaB27       ..G....... .....T.... T....T.... .......... .......... .......... .......... ..........
CaB30       ..G....... .......... .......G.. .......... .......... T......... .......... ....A.....
CaB31       ..G....... .......... .......G.. .......... .......... T......... .......... ....A.....
CgB10       ..G....... .......... ........C. .......... .......... .......... .......... ..........
CgB13       ..G....... .........A ........C. ...G...... .G.A...T.. .......... ....A..... ..G....T..
CgB14       ..G....... .......... ........C. ...G...... .......... A......... .......... ..........
CgB20       ..G....... .......... ........C. .......... .......... .......... .......... ..........
CgB28       ..G....... .......... ........C. .......... .......... .......... ........T. ..........
CgB40       ..G....... .........A ........C. ...G...... .G.A...T.. .......... ....A..... ..G....T..
CarB6       ..G....... .......... ........C. .......C.. .......G.. .......... .......... ..........
CarB9       .AG....... ........T. ........C. ...G...... .......... ..C....... .......... .A.......A
CarB22      ..G....... .......... .T......C. ...G...... .......... .......... .......--- ..G------.
CarB24      ..G....... ........T. ........C. ...G...... .......... ..C....... .......... .A.......A
CsB5        ..G....... .......... ........C. .......... .......... .......... .........A .......C..
CsB31       ..G....... .......... ........C. ...G...... ........G. .........A .......T. ..........
CsB32       ..G....... .........A .-.....AC. .......... .......... .......... .......... ..........
CsB33       ..G....... .......... ........C. .......... .......... .......... .........A .......C..
CsB40       ..G....... .......... ........C. .......... .......... .......... .........A .......C..

OedB5       TGTCCAAGGG TCCGTGTTTG CCCAACTATC TATTTT-GTA TTGTTTATAG G-AGTTATGA GATTGATC              [148]
OedB6       ......G... .......... .......... ......-... ...C...... .-........ ........
OedB19      ......G... ..-....... .......... ......-... ...C..G... .-G...G..G ........
OedB22      ......G... .......... .......... ......-... ...C..G... .-........ ........
OedB23      ......G... .......... .T........ ......TT.. ...C...... .-........ ........
OedB31      ......G... ........C. .......T.. ......-... ...C..G... .-....T... ........
OedB46      ....T.G... .......... .......C.T ......-... ..CC...... .G........ ........
OedB53      .A....G... .........T ........C.. ..A...-... ...C...... .-........ ........
OstB4       ......G... .......... T......... ......-... ...C..G... .-.......T ........
Ost28       ......G... ..T....... .......... ......-... ...C.....A .-.......T ........
OstB29      ......G... ..T....... .......... ......-... ...C.....A .-.......T ........
OstB35      ......G... ..T....... .......... ......-... ...C.....A .-.......T ........
OstB37      ......G... ..T....... .......... ......-... ...C.....A .-.......T ........
OstB41      ......TT.. .......... .......... ......-... ...C...... .-.......T ........
CaB16       ......G... .......... ....C..CC. ......-... ...C..G... A-.......T ........
CaB21       ......G... .......... .......... ......-... ...C..G... .-.......T ........
CaB25       ......G... .......... .......... ......-... ...C..G... .-.......T ........
CaB27       ......G... .......... .......... ......-... ...C.CG... .-.......T ........
CaB30       ......G... .......... .......... ......-... ...C..G... .-.......T ........
CaB31       ......G... .......... .......... ......-... ...C..G... .-.......T ........
CgB10       .A....G... .......... .....G.... ......-... ...C...... .-.......T ........
CgB13       ...------- -......... .G........ ......-... ..AC...... .-.......T ........
CgB14       ......G... .......... T......... ......-... ...C...... .-.......T ........
CgB20       ......G... .......... .......... ......-... ...C..G... .-.......T ........
CgB28       ......G... .......... .......... ......-... ...C..G... .-.......T ........
CgB40       ...------- -......... .G........ ......-... ..AC...... .-.......T ........
CarB6       .A.....A.. .......... .......... .......-C. ...C...... .-.......T ........
CarB9       .A....GC.. ..T....... .......C.. .G....TA.. ...C....G. A-.......T ........
CarB22      .AC..CT... ..T....... T......... ......-... ...C.....T .-.......T ........
CarB24      .A....GC.. ..T....... .......C.. .G....TA.. ...C....G. A-.......T ........
CsB5        ......TT.. .......... .......... ......-... ...C...... .-.......T ........
CsB31       .A....G... .........A .......C.A ......-... ...C...... .-.......T ........
CsB32       ......G... .........T .......... ......-... ..CC...... .-.......T ........
CsB33       ......TT.. .......... .......... ......-... ...C...... .-.......T ........
CsB40       ......TT.. .......... .......... ......-... ...C...... .-.......T ........
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B

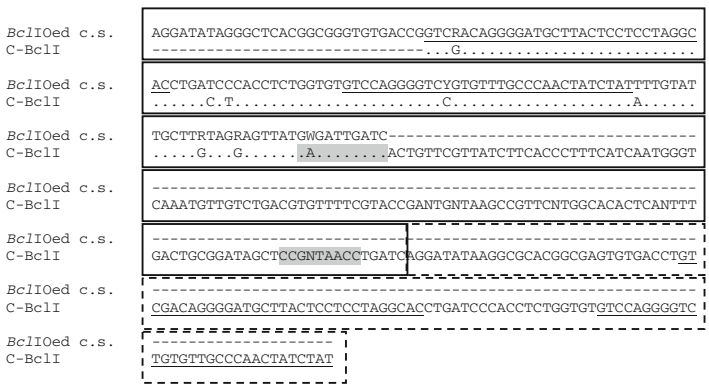

Table 2 Variability between cloned *Bcl*I sequences

	Polymorphism value (P)	Nucleotide diversity (π)
O. edulis	0.284	0.095
O. stentina	0.082	0.036
C. angulata	0.089	0.034
C. gigas	0.110	0.055
C. ariakensis	0.156	0.100
C. sikamea	0.102	0.049

Polymorphism value corresponds to the variable number of nucleotide sites of all the nucleotides from the cloned sequences of a taxon while nucleotide diversity corresponds to nucleotide differences averaged per site between two sequences

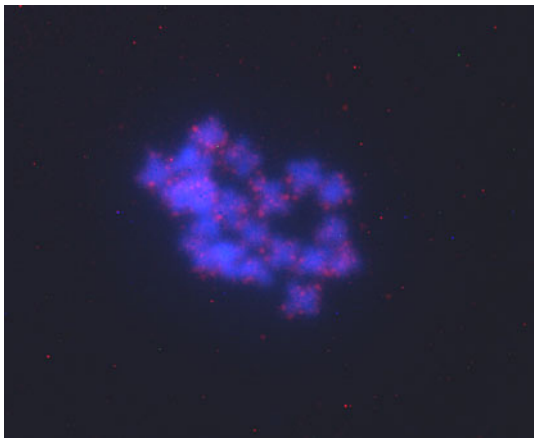

Fig. 3 Representative fluorescence in situ hybridisation of *Bcl*I family on *C. angulata* chromosomes

Table 3 Pairwise divergence values between species according to Kimura's two-parameter model (Kimura 1980) for the *Bcl*I fragment

	O. edulis	*O. stentina*	*C. angulata*	*C. gigas*	*C. ariakensis*
O. edulis					
O. stentina	0.094				
C. angulata	0.094	0.072			
C. gigas	0.092	0.063	0.069		
C. ariakensis	0.130	0.099	0.119	0.104	
C. sikamea	0.092	0.063	0.072	0.062	0.100

unusual in these short interspersed sequences, as almost all *Alu* elements occur as single copies in euchromatic regions (El-Sawy and Deininger 2005). This rare organization has been described for an interspersed repeat sequence found in *Drosophila ananassae,* and it has been related to processes and mechanisms that increase the number of the repeat sequences in the genome (Nozawa et al. 2006).

Conservation and evolution of the *Bcl*I repetitive DNA family among different oyster species

The *Hin*dIII satellite-DNA family (related in origin to a transposable oyster element) is conserved in all oyster species (López-Flores et al. 2004). To check the conservation degree of *Bcl*I repetitive DNA within the genome of oysters, we analysed this family by means of Southern-blot or dot-blot hybridisation methods, for the oyster species *O. stentina*, *C. angulata*, *C. gigas, C. gasar*, *C. ariakensis*, and *C. sikamea*. We used *Bcl*I clones from *O. edulis* as probes. Both analyses showed only slight hybridisation signals over the high-molecular-weight genome DNA in the case of *O. stentina*, *C. angulata* and *C. gigas* species, although post-hybridisation washes were made at low stringency. These faint hybridisation signals could be due to a lower representation of the repetitive *Bcl*I sequence in the genomes of *O. stentina*, *C. angulata*, and *C. gigas* with respect the genome of *O. edulis*.

We also tried to detect the presence of *Bcl*I repetitive family in several oyster species using the PCR technique. We designed a new pair of specific primers, OsedBdir and OsedBrev, faced and based in the *Bcl*I 150 bp sequence isolated in *O. edulis* by restriction enzymes (see Fig. 2a). Using this method, we initially amplified a single band between 100 and 200 bp in length from the genomes of *C. angulata*, *C. gigas, C. ariakensis*, and *C. sikamea,* and from *O. stentina* when PCR conditions were relaxed. No amplification was found from the *C. gasar* genome even under low-astringency conditions.

Thus, for six oyster species, we have comparatively analysed 27 sequences obtained by PCR as well as the eight sequences from *O. edulis* by the *Bcl*I restriction enzyme. The alignment of all these 35 sequences is shown in Fig. 2a. Intraspecific variability is low in general, varying between the maximum of 10% in *C. ariakensis* and 3.4% in *C. angulata* (Table 2). Also, the *Bcl*I sequences are highly similar between species, showing interspecific divergence similar to intraspecific variation (Table 3).

Usually, the repetitive sequences in eukaryotic genomes, present lower divergence within species than between species as consequence of molecular drive, leading to a pattern called concerted evolution (Dover 1986). Few investigations have reported non-concerted evolution in tandem repetitive sequences (Robles et al. 2004; Luchetti et al. 2006; Plohl et al. 2010). These authors found similar values of divergence within and between taxa. But, while the *Bcl*I family, with a relatively high degree of preservation (present in six species of the seven analysed from two different genera) shows low sequence change, in other cases such as the very old satellite-DNA BIV160, a high sequence divergence is observed (Plohl et al. 2010).

Thus, different factors affect the rate of concerted evolution, such as number, structure, distribution and diversity within the genome of the repeats, functional requirements and population factors (see for example the aforementioned Robles et al. 2004; Luchetti et al. 2006; Plohl et al. 2010). In our case, the slow concerted evolution observed in this repetitive family could be due to its peculiar organization. The *Bcl*I sequences are dispersed throughout chromosomes. This type of arrangement is the least favourable for concerted evolution because it reduces the chance of unequal crossing over and gene conversion (reviewed in Graur and Li 1999). On the other hand, the existence of few repetitions in each locus results in a high homogenisation rate by intrachromosomal gene conversion. However, this event is random for each locus, allowing different nucleotide change for the different loci of each chromosome. In fact, in a family of similar interspersed tandem arrays found in *D. ananassae*, the sequences within the same locus were more similar than those between separated loci, showing concerted evolution within the locus (Nozawa et al. 2006). Thus, in our case, the amplification by PCR of sequences from different loci would explain the absence of differences between intra- and interspecific distances and therefore, the apparent slow rate concerted evolution observed for the *Bcl*I sequences isolated from oyster species.

Phylogenetic considerations in oysters using *Bcl*I family

Although this family shows a slow rate of change, we tried to use the *Bcl*I sequence as a phylogenetic marker to clarify the relationships between oyster species, comparing the results with those based on the other repetitive sequences found in oysters, such as the *Hind*III satellite-DNA family (López-Flores et al. 2004) and with mitochondrial data (Ò Foighil et al. 1998).

In oysters, due to their phenotypic plasticity and wide distribution, are known to have numerous taxonomic misclassifications, and some of their phylogenetic relationships are controversial (Korringa 1952; Stenzel 1971; Boudry et al. 2003).

Initially, we detected similar intraspecific and interspecific divergence values for *Bcl*I sequences of oyster species (Table 3). However, when we analysed the sequence alignment, we detected polymorphic sites that were shared between species two by two. These sites can be considered ancestral variation, as the polymorphisms are present in both species, and appeared before their separation (Navajas-Pérez et al. 2005). These polymorphisms might be an overestimation of the intraspecific variability, but it would not be true divergence.

Thus, we have recalculated these data taking into account these shared polymorphic sites and they were excluded in the estimation of the rates of sequence change (Table 4). We have used these distances clues regarding the phylogenetic relationships between the oysters species analysed. In the resulting distance tree (Fig. 4), the species of *Ostrea* genera (*O. stentina* and *O. edulis*) were separated from species of *Crassotrea* genera (*C. angulata, C. gigas, C. ariakensis* and *C. sikamea*), confirming the existence of the two genera as independent taxa. On the other hand, the species *C. angulata*, *C. gigas*, and *C. ariakensis* (as well as *C. sikamea*) are grouped, being closely related. This finding is similar to that reported by Ò Foighil et al. (1998) (using mitochondrial DNA) and Lopez-Flores et al. (2004) (using the *Hind*III satellite-DNA family), confirming the Asian origin of these species.

In summary, after characterising the new *Bcl*I repetitive DNA family in the genome of seven commercial oyster species, we conclude, that: (1) *Bcl*I repetitive sequences are interspersed but it is possible to find more than one unit per locus, this being unusual for this type of short interspersed element; (2) the *Bcl*I sequences are related to a flaking region of a microsatellite locus, and represent the second repeated-DNA family isolated from the oyster genome, after *Hin*dIII satellite-DNA family, which in turn is derived from a transposable element; (3) the *Bcl*I family is poorly

Table 4 Average number of nucleotide substitutions per site between oyster species using Jukes and Cantor distance and excluding shared polymorphic sites between species

	O. edulis	*O. stentina*	*C. angulata*	*C. gigas*	*C. ariakensis*
O. edulis					
O. stentina	0.070				
C. angulata	0.099	0.114			
C. gigas	0.111	0.106	0.149		
C. ariakensis	0.050	0.070	0.099	0.104	
C. sikamea	0.073	0.083	0.103	0.135	0.073

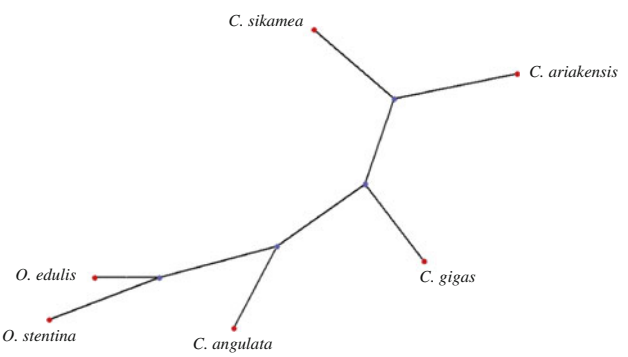

Fig. 4 Phylogenetic tree of relationships between different oyster species, based on the neighbour-joining method, and obtained using the genetic distances from Table 4

represented in the genome of all the species analysed with the exception of *O. edulis,* and it is apparently absent from the *C. gasar* genome; (4) the low rate of concerted evolution found in this repetitive sequence could be due to its peculiar genome organization; (5) phylogenetic analyses using *Bcl*I repetitive family separated *Crassotrea* species (*C. sikamea*, *C. gigas*, *C. ariakensias*, and *C. angulata*) from Ostrea species (*O. stentina* and *O. edulis*).

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