EIDO Escola Internacional de Doutoramento

TESE DE DOUTORAMENTO

Caracterización citogenética de bivalvos veneroideos y algunos de sus parásitos

Cytogenetic characterization of veneroid bivalves and some of their parasites

Daniel García Souto

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Mención internacional

Universidade Vigo

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Cytogenetic characterization of veneroid bivalves and some of their parasites

Dirixida polo doutor Juan José Pasantes Ludeña

Año: 2017

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Aquéllos que me busquen, sabrán que he existido.

Los demás no tienen necesidad de saberlo.

Gustav Mahler

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Estimado lector anónimo, sea Ud. consciente de que, más allá de la loable función de calzar algún que otro mueble cojo, esta tesis es la consecución, el pináculo quizás, de una etapa de considerable esfuerzo en mi vida. Pero ante todo, sepa que se encuentra ante el trabajo de muchas personas.

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SUMMARY

Bivalves are a group of exclusively aquatic mollusks characterized by presenting a laterally compressed body whose soft parts are covered by a shell (Gosling 2015). More than 8500 species are currently accepted (World Register of Marine Species, WoRMS, http://www.marinespecies.org/) and they are distributed worldwide inhabiting marine, brackish and, to a lesser extent, freshwater ecosystems. The class Bivalvia includes a considerable number of species of great economic interest that have been traditionally subjected to extensive exploitation on commercial purposes. Their ecological role is of high relevance as they constitute both the basis of trophic networks and the physical support for the development of numerous benthonic animal species, both vertebrates and invertebrates. Bivalve mollusks also act as intermediary hosts in the life cycle of many digenean trematodes, parasitic metazoans that may have a huge impact on bivalve populations, both natural and cultured.

Although the classification of bivalves is a rather controversial subject and recent phylogenetic analyses (Bieler et al. 2014) propose six monophyletic lineages within Bivalvia, WoRMS still utilizes four subclasses, Protobranchia, Pteriomorphia, Paleoheterodonta and Heterodonta. The subclass Heterodonta includes more than two thirds of the extant bivalves, extremely diverse in form, size, anatomy and lifestyle. As molecular phylogenies (Bieler et al., 2014) have verified that some of the orders, superfamilies and families in this group are polyphyletic, bivalve taxonomy is under continuous revision. A good example is the traditional order Veneroida, which, despite including some of the largest families (Veneridae, 680 species; Mactridae 180; Donacidae, 100; Tellinidae, 500), is neither morphologically homogeneous nor monophyletic being, therefore, currently taxonomically questioned (Huber 2010, 2015).

Most chromosomal studies in bivalves just described chromosome numbers and, in some cases, karyotypes according to size and morphology (Nakamura 1985; Thiriot-Quiévreux 1994). This kind of analysis led to propose that chromosome numbers were relatively stable within families, $2n = 20$ in Ostreidae, $2n = 28$ in Mytilidae and Pteridae and $2n = 38$ in most of the remaining, including Veneridae (Thiriot-Quiévreux 1994). However, further studies showed that some genera and families included species with different diploid numbers.

Nevertheless, this does not appear to be the case in Heterodonta since the vast majority of the species are $2n = 38$. In addition, karyotypes are usually characterized by presenting similar chromosomes in terms of size and morphology (Nakamura 1985; Thiriot-Quiévreux 1994, 2002). This homogeneity precludes the unequivocal identification of chromosome pairs and, consequently, interspecific chromosomal homologies deducted from comparing homogenously stained metaphases are quite presumptive. Therefore correct comparisons of veneroid karyotypes require methods that allow accurate chromosome identification; among those, chromosome banding and fluorescence *in situ* hybridization (FISH).

FISH mapping in bivalves has been mostly restricted to 45S rRNA genes (50 species), 5S rRNA genes (25), core histone genes (15), linker histone genes (6) and telomeric sequences (25) (Thiriot-Quiévreux 2002; Leitão and Chaves 2008; Petrović et al. 2009; Pérez-García et al. 2010a, 2010b, 2011, 2014a, 2014b; Bouilly et al. 2010; Carrilho et al. 2011; Hurtado et al. 2011; González-Tizón et al. 2013; Li et al. 2016; García-Souto et al. 2017). FISH mapping, mainly applied in Mytilidae, Ostreidae and Pectinidae, have made possible establishing more reliable karyotypes and identifying some of the chromosomal changes accompanying bivalve evolution (Pérez-García et al. 2014b) but also contributing to a better understanding of their phylogenies. In addition, the application of these techniques has shown that, contrasting the presumed karyotypic conservation provided by classical techniques, important variations can appear. For instance, Atlantic species of oysters differ from their Pacific congeners by the chromosomal location of the 45S rDNA, therefore demonstrating that even apparently highly conserved karyotypes diverge (Wang et al., 2004). On the other hand, some satellite DNAs has been also mapped by FISH in these families (Clabby et al. 1996; Wang et al. 2001; Martínez-Lage et al. 2002; Cross et al. 2005; Odierna et al. 2006; Biscotti et al. 2007; Bouilly et al. 2008; López-Flores et al. 2010; Hu et al. 2011; Petraccioli et al. 2015).

As for Heterodonta, chromosomal mapping is much scarcer. To date, 45S rDNAs have been located by FISH in 18 species, 5S rDNAs in 9, core histone genes in 2 and telomeric sequences in 12 (Insua et al. 1999; González-Tizón et al. 2000; Wang and Guo 2001, 2007, 2008; Martínez et al. 2002; Plohl et al. 2002; Fernández-Tajes et al. 2003, 2008; Hurtado and Pasantes 2005; Petrović et al. 2009; Bouilly et al. 2010; Carrilho et al. 2011; Hurtado et al. 2011; González-Tizón et al. 2013; Pérez-García et al. 2014a). Almost all species are $2n = 38$ and present rather similar karyotypes but considerable

differences in the chromosomal distribution of those sequences, thus demonstrating their potential as cytogenetic markers for deducing their evolutionary relationships. Regarding satellite DNAs, they have been only mapped in the manila clam, *Ruditapes philippinarum* (Passamonti et al. 1998) and the abrupt wedge shell, *Donax trunculus* (Petrović et al. 2009).

Cytogenetic studies in digenean trematodes are even scarcer than in bivalves. Despite being the largest group of metazoan endoparasites comprising more than 18000 extant species, chromosomal numbers and karyotypes have been described in only 300 and FISH mapping was limited to 45S rDNAs and/or telomeric sequences in nine (Hirai et al. 1989, 2000; Baršienė 1993; Hirai y Lo Verde 1996; Bell et al. 1998; Petkevičiūtė et al. 2003; Špakulová and Casanova 2004; Reblánová et al. 2011; Zadesenets et al. 2012a, 2012b; Petkevičiūtė et al. 2012, 2014, 2015; Hirai 2014; Sofi et al. 2015).

Therefore, in order to improve our cytogenetic knowledge in both species of the order Veneroida and some of the digenean trematodes that parasite them, we analyzed the chromosomes of 20 bivalve species belonging to the families Veneridae (*Ruditapes philippinarum*, *R. decussatus*, *Venerupis corrugata*, *Clausinella fasciata*, *Chamelea gallina*, *C. striatula*, *Venus verrucosa*, *Venus casina*, *Dosinia exoleta*, *Dosinia lupinus* and *Petricola litophaga*), Mactridae (*Spisula subtruncata*, *S. solida* and *Mactra stultorum*), Donacidae (*Donax trunculus* and *D. vittatus*) and Tellinidae (*Bosemprella incarnata*, *Macomangulus tenuis*, *Moerella donacina* and *Serratina serrata*) and 10 taxa of digenean parasites *Bucephalus minimus*, *B. australis*, *Prosorhynchoides carvajali* (Bucephaloidea), *Monascus filiformis* (Gymnophalloidea), *Parorchis acanthus* (Echinostomatoidea), *Cryptocotyle lingua* (Opisthorchioidea), Cercaria longicaudata, *Monorchis parvus* (Monorchioidea), *Diphterostomum brusinae*, and *Bacciger bacciger* (Microphalloidea) isolated from 12 molluskan intermediate hosts. We mostly applied diverse combinations of base-specific (4'-6-diamidino-2-phenylindole DAPI, AT-rich; chromomycine A_3, CMA, GC-rich) and unespecific (propidium iodide, PI) fluorochromes, C-banding and 5-methyl-cytosine immunodetection and mapped repetitive DNA probes (45S and 5S rDNAs, core histone genes, telomeric sequences and satellite DNAs) to their chromosomes.

Chromosome preparations were obtained following previously published methods (Méndez et al. 1990; Pasantes et al. 1990). After exposing the bivalves overnight to colchicine, gills and gonads were removed, treated with 50% and 25% sea water and fixed in ethanol/acetic acid. Isolated digenean paralarvae were also immersed in 50% and 25% seawater and fixed in ethanol/acetic acid. Fixed samples were disaggregated in 60% acetic acid and the resulting cellular suspensions were dropped onto slides preheated to 50 °C.

Fluorochrome staining was performed as described by Pérez-García et al. (2010b). After controlling the quality of the chromosome preparations by phase contrast microscopy (Nikon), selected slides were stained for 2 h with CMA (0.25 mg/mL), counterstained with DAPI (0.14 μg/mL) for 8 min, washed in tap water, air-dried and mounted with antifade (Vectashield, Vector). Slide visualization and photography were performed using a Nikon Eclipse-800 microscope equipped with an epifluorescence system. Separated images for each fluorochrome were obtained with a DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon). Merging of the images was performed with Adobe Photoshop. Following visualization and photography, chromosome preparations were re-stained with a combination of DAPI and PI (0.07 μg /mL), washed in tap water, air-dried, mounted in antifade, and photographed again.

DNA was extracted either using standard chloroform/isoamyl alcohol or the EZNA Mollusc DNA Kit (OMEGA). When necessary, fragments of the mitochondrial COI gene were amplified by PCR employing the standard barcoding primers LCO1490 and HCO2198 (Folmer et al. 1994). The fragments of the mitochondrial 16S rRNA gene were amplified by means of primers 16L29 (Schubart et al. 2001) and 16SBr (Palumbi 1996). The complete ITS2 of the major rDNA was amplified using primers ITS3 and ITS4 (White et al. 1990). On FISH mapping purposes, universal primers LR10R and LR12 retrieved from Vilgalys lab website (http://www.biology.duke.edu/fungi/mycolab/primers.htm) were used to amplify a fragment of the 28S rDNA. Amplifications of the entire 5S rDNA repeat and the H3 histone gene used primers described by Pérez-García et al. (2010a, 2010b) and Giribet and Distel (2003), respectively.

DNA sequences were amplified in a GeneAmp PCR system 9700 (Applied Biosystems) in 50 μL solutions containing 125 ng of genomic DNA, 50 μM each dNTP, 50 μM each primer, 1xPCR buffer, 15 μM MgCl2 and 5 U of JumpStart™ Taq DNA Polymerase (Sigma). Amplifications included an initial denaturation step at 95 ºC (2 min), 35 amplification cycles and a final extension at 72 °C (5 min). PCR products were examined by electrophoresis on 2% agarose gels

The amplified sequences were purified (FavorPrepTM GEL/PCR Purification Kit, Favorgen) and sequenced (CACTI, University of Vigo) in both directions in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were edited with BioEdit v. 7.1.11 (Hall 1999) and aligned with Muscle set to default parameters using MEGA7 (Kumar et al. 2016). Sequence similarity searches were performed using the Basic Local Alignment Search Tool algorithm (BLAST), available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The MegaBLAST algorithm set to default parameters was employed against both NCBI nucleotide collection and Barcode of Life Data System (BOLD) databases.

Metaphase chromosome spreads were single, double and sequentially hybridized using 5S and 45S rDNA and H3 histone gene probes (Pérez-García et al. 2011). 45S rDNA probes were labeled with biotin-16-dUTP (Roche Applied Science) and/or digoxigenin-11-dUTP (10x DIG Labeling Mix, Roche Applied Science) using a nick translation kit (Roche Applied Science). Histone H3 gene and 5S rDNA probes were directly labeled by PCR either with biotin-16-dUTP (20 μM) or digoxigenin-11-dUTP (5 μM).

Chromosome preparations were digested with RNase and pepsin before denaturation (70 ºC, 2 min) and hybridized overnight at 37 ºC. Biotin was detected with fluorescein isothiocyanate (FITC) conjugated avidin and biotinylated anti-avidin (Vector) whereas digoxigenin was detected with anti-digoxigenin antibodies conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Sigma). Chromosome preparations were counterstained with DAPI, mounted with antifade and examined by fluorescence microscopy. Separated images for each fluorochrome were recorded, pseudo-colored and merged as indicated above. In addition, we also performed FISH with a telomeric $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) following the protocol indicated by the supplier.

Chromosome counting and karyotype analysis were performed in at least 10 specimens per species (5 males, 5 females). For each species, at least 10 complete metaphase plates showing FISH signals were used to construct karyotypes. Chromosome and arm lengths were carefully measured and relative lengths and centromeric indices calculated.

In concordance with previous data, all Veneroida species studied herein were $2n = 38$ and their karyotypes mostly presented chromosomes showing an almost continuous gradation of decreasing lengths. In 16 of the 20 species GC-rich regions were limited to NORs but in the remaining four, the Donacidae *Donax trunculus* and *D. vittatus* and the Mactridae *Mactra stultorum* and *Spisula subtruncata*, additional, mostly intercalary, GC-rich heterochromatic bands were present. Moreover, a novel satellite DNA was identified as the major component of this GC-rich heterochromatin in *S. subtruncata*. As foreseen by the satellite DNA library hypothesis, this satellite DNA was also present in *M. stultorum* and *S. solida*, showing a highly conserved nucleotide sequence but a dramatic diminution in the number of repeats. The methylation status of this satellite was explored by both bisulfite genomic sequencing and immunodetection and the results were compared with those of the whole genome, as detected by methylation-sensitive amplification polymorphism (MSAP) and Enzyme-Linked ImmunoSorbent Assays (ELISA), showing a methylation level that was high for bivalve standards and triplicated the mean methylation of *S. subtruncata* genome.

In regards to FISH mapping data, the distribution patterns of H3 histone gene, 5S rDNA and 45S rDNA clusters in Veneroida showed some peculiarities. In the family Veneridae a total of 18 loci for histone H3 gene clusters were identified in the 11 species analyzed. Six of the species displayed a single core histone gene cluster whilst *Chamelea striatula* showed four clusters and the remaining four species presented two. Fifteen of these histone gene clusters were subterminal, two were intercalary, and the remaining one subcentromeric. A single major rDNA cluster was present in all venus clams but *Chamelea striatula* that showed two. In contrast, single 5S rDNA clusters were detected in five of the species, two in other five and three in the remaining one, *Petricola litophaga*. The signals were on different chromosome pairs but in *Clausinella fasciata* and *Chamelea striatula* that showed a chromosome bearing both histone gene and rDNA clusters, and *Ruditapes decussatus* that presented overlapping 5S rDNA and 45S rDNA signals.

The number and location of these clusters, together with the analysis of the nucleotide sequences of fragments of the mitochondrial cytochrome c oxidase subunit I (COI) and 16S rDNA and the whole nuclear internal transcribed spacer 2 (ITS2), allowed us to elucidate the taxonomic status of the striped venus clams *Chamelea gallina* and *C. striatula*. The comparison of four geographically distant Atlantic

and Mediterranean populations demonstrated clear differences in the number and distribution of rDNA and histone gene clusters. The consistency in the 5S rDNA signal pattern and the mapping differences for both 45S rDNA and H3 histone gene signals found between *C. gallina* and *C. striatula* were of a similar magnitude to those for *Venus casina* and *V. verrucosa* and *Dosinia exoleta* and *D. lupinus*. Conversely, even though some mapping differences among *C. gallina* populations and among *C. striatula* specimens were also found, they were comparatively narrower than those between taxa and similar to those found in other bivalve species; thusly constituting the standard intraspecific variation. The sequence data obtained also indicated that these two taxa are separated species.

In the family Mactridae, FISH experiments employing 45S rDNA probes demonstrated that major rDNA clusters were located at subterminal regions in the three species studied. Both *Spisula solida* and *Mactra stultorum* showed two 45S rDNA signals, subterminally located on the short arms of two chromosome pairs 17 and 19 and on the long arms of chromosome pairs 3 and 4, respectively. In contrast, a single 45S rDNA cluster was detected on the long arms of chromosome pair 18 in *Spisula subtruncata*. All three trough shells showed a single 5S rDNA cluster subterminal on the short arms of chromosome pair 5 in *Spisula solida* and on the long arms of chromosome pair 3 in *Spisula subtruncata*, and intercalary on the short arms of chromosome pair 15 in *Mactra stultorum*. Although H3 histone gene clusters also mapped to a single locus in the three analyzed species, their locations differed and were intercalary to the long arms of subtelocentric chromosome pairs 8 in *Spisula solida* and 12 in *Mactra stultorum* but subcentromeric to the long arms of metacentric chromosome pair 14 in *Spisula subtruncata*.

Donacidae wedge shell species displayed H3 histone gene clusters intercalary to the long arms of chromosome pair 17 but this chromosome was subtelocentric in *D. trunculus* and telocentric in *D. vittatus*. 45S rDNAs mapped to a single locus intercalary to the short arms of subtelocentric chromosome pair 6 in *D. trunculus*. Most *D. vittatus* specimens also displayed major rDNAs in the short arms of chromosome pair 6, telocentric. In contrast, 45S rDNA is subcentromeric to the long arms of chromosome pair 6, metacentric, in all *D. vittatus* specimens collected in Samil. The number of 5S rDNA clusters displayed by *D. trunculus* and *D. vittatus* was different. Whilst subterminal signals on the long arms of a single subtelocentric chromosome pair were present in the two wedge shells, *D. trunculus* presented an additional signal intercalary to the short arms of metacentric chromosome pair 2. The intra- and interspecific karyotype differences seem to be the results of multiple pericentric inversions.

As for the other veneroid families, tellin clams also showed interspecific divergences in the number and location of 45S rDNA, 5S rDNA and H3 histone gene clusters. *Bosemprella incarnata*, *Macomangulus tenuis* and *Moerella donacina* displayed a single 45S rDNA cluster, whilst *Serratina serrata* presented two. One (*B. incarnata* and *S. serrata*) or two (*M. tenuis* and *M. donacina*) 5S rDNA clusters were also present in these species. In contrast, all four tellin clams showed single H3 histone gene clusters, although at different locations.

In the digenean trematode species studied, diploid numbers varied from a minimum of $2n = 12$ for *B. bacciger* to a maximum of $2n = 22$ for *P. acanthus*. They also showed striking differences in chromosome composition. Whereas *C. lingua* and *P. acanthus* karyotypes were exclusively composed of exclusively metacentric and subtelocentric chromosomes, respectively, the remaining taxa presented diverse combinations of chromosome types. Single- and double-color FISH experiments demonstrated the presence of both 45S and 5S rDNA clusters at a single locus in all taxa, but displaying differences in chromosomal location. In *B. minimus* and *P. acanthus* 45S and 5S rDNA clusters were located on different chromosome pairs, whilst the remaining species showed overlapping signals at a single location on one chromosome pair. FISH mapping also demonstrated the presence of highly uncondensed, DAPI negative unstained regions coincident with major rDNA signals. 45S rDNA signals appeared at the condensed chromatin at both sides of the uncondensed regions but without any sign of telomeric signals. These regions were subcentromeric in *B. australis*, *P. carvajali*, *P. acanthus*, *C. lingua*, *M. parvus*, and *B. bacciger*, thus yielding apparently higher chromosome numbers than its species respective modal number after DAPI staining. In the remaining taxa, *B. minimus*, *M. filiformis*, C. longicaudata and *D. brusinae*, many of the metaphase plates showed clear DAPI-negative secondary constrictions that separated relatively small pieces of chromatin from the chromosome. This approach also allowed for the detection of B chromosomes on *B. bacciger* isolated from *Ruditapes decussatus* from a minimum of 1 to a maximum of 19 per metaphase plate. These supernumerary metacentric chromosomes presented telomeric signals at both ends and were characterized by showing long arms strongly stained with DAPI also evident on the interphase nuclei.

RESUMEN

Los bivalvos son un grupo de moluscos exclusivamente acuícolas caracterizados por presentar un cuerpo comprimido lateralmente cuyas partes blandas están recubiertas por una concha (Gosling 2015). Las más de 8500 especies aceptadas en la actualidad según el World Register of Marine Species (WoRMS, http://www.marinespecies.org/) se distribuyen por todo el planeta ocupando ecosistemas tanto marinos como salobres y, en menor medida, de agua dulce. Los bivalvos incluyen un buen número de especies de gran interés económico y juegan un papel ecológico fundamental en ambientes bentónicos. Los bivalvos son, además, hospedadores intermediarios de digeneos parásitos, que pueden llegar a tener un enorme impacto en poblaciones, tanto naturales como de cultivo, de bivalvos.

Si bien la clasificación de los bivalvos es un tema bastante controvertido y análisis filogenéticos recientes (Bieler et al. 2014) proponen la existencia de seis linajes monofiléticos en la clase Bivalvia, WoRMS todavía usa cuatro subclases, Protobranchia, Pteriomorphia, Paleoheterodonta, y Heterodonta. La subclase Heterodonta incluye más de dos tercios de los bivalvos actuales y es extremadamente diversa en cuanto a forma, tamaño, anatomía y hábitos de vida y las filogenias moleculares más recientes han constatado que algunos de los órdenes, superfamilias y familias incluidos en ella son grupos para o polifiléticos (Bieler et al. 2014). Debido a ello, su taxonomía está en continua revisión. Un buen ejemplo de esta situación es el tradicional orden Veneroida que en la actualidad ya no se considera ni morfológicamente homogéneo ni monofilético y, por tanto, es cuestionado a pesar de su enorme importancia pues engloba algunas de las familias con mayor número de especies descritas (Veneridae, 680 especies; Mactridae, 180; Donacidae, 100; Tellinidae, 500) (Huber 2010, 2015).

La mayoría de los estudios cromosómicos en bivalvos se limitan a la descripción del número cromosómico y el cariotipo organizado en función de la morfología y el tamaño de los pares cromosómicos (Nakamura 1985; Thiriot-Quiévreux 1994). Los análisis de este tipo realizados a lo largo del pasado siglo llevaron a proponer que los números cromosómicos eran relativamente estables dentro de cada familia de bivalvos y que se agrupaban en torno a los $2n = 20$ habituales en Ostreidae, los $2n = 28$ de Mytilidae y Pteridae y los $2n = 38$ de la mayoría de las restantes familias, incluida Veneridae (Thiriot-Quiévreux 1994). No obstante, el incremento en el número de especies analizadas ha demostrado que algunas familias y géneros de bivalvos presentan especies con distintos números cromosómicos.

Esto no parece ser el caso en la subclase Heterodonta puesto que la inmensa mayoría de las especies analizadas son $2n = 38$. Además, los cariotipos de estas especies suelen presentar cromosomas en los que morfología y tamaño muestran escasas diferencias (Nakamura 1985; Thiriot-Quiévreux 1994, 2002). Esta homogeneidad dificulta la identificación de pares cromosómicos concretos y hace que las homologías cromosómicas interespecíficas descritas sean una suposición. Así pues, a fin de comparar los cariotipos de bivalvos en general y de veneroideos en particular, es necesario explorar otras metodologías, entre ellas las técnicas de bandeo cromosómico o la hibridación *in situ* fluorescente (FISH).

En bivalvos se han mapeado mediante FISH genes rRNA 45S en unas 50 especies, genes rRNA 5S en unas 25, genes de las histonas del núcleo en 15, genes de las histonas de unión en seis y secuencias teloméricas en unas 25 (Thiriot-Quiévreux 2002; Leitão y Chaves 2008; Petrović et al. 2009; Pérez-García et al. 2010a, 2010b, 2011, 2014a, 2014b; Bouilly et al. 2010; Carrilho et al. 2011; Hurtado et al. 2011; González-Tizón et al. 2013; Li et al. 2016; García-Souto et al. 2017). Estas técnicas han posibilitado el establecimiento de cariotipos más fiables y una mejor comprensión de los cambios cromosómicos que han acompañado su evolución (Pérez-García et al. 2014b) pero también han contribuido a entender mejor sus filogenias. Además, la aplicación de estas técnicas en especies de estas familias ha permitido comprobar que frente a la presunta conservación cariotípica que se deducía tras aplicar técnicas citogenéticas clásicas, la situación cromosómica de estas secuencias puede presentar variaciones considerables. Por ejemplo, las especies atlánticas de ostras se diferencia de sus congéneres pacíficas por la posición de los genes rRNA 45S, demostrando que incluso cariotipos aparentemente muy conservados muestran divergencias (Wang et al. 2004). A estos mismos grupos se refiere la mayoría de las localizaciones cromosómicas de DNA satélites en bivalvos (Clabby et al. 1996; Wang et al. 2001; Martínez-Lage et al. 2002; Cross et al. 2005; Odierna et al. 2006; Biscotti et al. 2007; Bouilly et al. 2008; López-Flores et al. 2010; Hu et al. 2011; Petraccioli et al. 2015).

En Heterodonta la aplicación de técnicas de mapeo cromosómico es todavía más escasa. Hasta la fecha se han localizado mediante FISH los genes rRNA 45S en 18 especies, los genes rRNA 5S en 9, los genes de las histonas del núcleo en 2 y secuencias teloméricas en 12 (Insua et al 1999; González-Tizón et al. 2000; Wang y Guo 2001, 2007, 2008; Martínez et al. 2002; Plohl et al. 2002; Fernández-Tajes et al. 2003, 2008; Hurtado y Pasantes 2005; Petrović et al. 2009; Bouilly et al. 2010; Carrilho et al. 2011;

Hurtado et al. 2011; González-Tizón et al. 2013; Pérez-García et al. 2014a). Casi todas estas especies son 2*n* = 38 y sus cariotipos relativamente similares pero presentan considerables diferencias en la distribución cromosómica de estas secuencias, demostrando, por tanto, su interés como marcadores para deducir relaciones de parentesco evolutivo. Por lo que se refiere a ADN satélites, las únicas especies de la subclase Heterodonta en las que se han localizado este tipo de secuencias son la almeja fina, *Ruditapes philippinarum* (Passamonti et al. 1998) y la coquina, *Donax trunculus* (Petrović et al. 2009).

Si los estudios citogenéticos en bivalvos son escasos, todavía lo son mucho más en trematodos digeneos. Pese a ser el grupo mayoritario de metazoos endoparásitos con más de 18000 especies, sólo se han descrito números cromosómicos y cariotipos en unas 300 y se han localizado mediante FISH secuencias teloméricas y/o genes de los rRNA 45S en 9 (Hirai et al. 1989, 2000; Baršiené 1993; Hirai y Lo Verde 1996; Bell et al. 1998; Petkevičiūtė et al. 2003; Špakulová y Casanova 2004; Reblánová et al. 2011; Zadesenets et al. 2012a, 2012b; Petkevičiūtė et al. 2012, 2014, 2015; Hirai 2014; Sofi et al. 2015).

Por ello, para incrementar el conocimiento citogenética en especies de Veneroida y de algunos de los trematodos digeneos que las parasitan, analizamos los cromosomas de 20 especies de Veneridae (*Ruditapes philippinarum*, *R. decussatus*, *Venerupis corrugata*, *Clausinella fasciata*, *Chamelea gallina*, *C. striatula*, *Venus verrucosa*, *Venus casina*, *Dosinia exoleta*, *Dosinia lupinus* and *Petricola litophaga*), Mactridae (*Spisula subtruncata*, *S. solida* and *Mactra stultorum*), Donacidae (*Donax trunculus* and *D. vittatus*) y Tellinidae (*Bosemprella incarnata*, *Macomangulus tenuis*, *Moerella donacina* and *Serratina serrata*) y de 10 taxones de parásitos digeneos, *Bucephalus minimus*, *B. australis*, *Prosorhynchoides carvajali* (Bucephaloidea), *Monascus filiformis* (Gymnophalloidea), *Parorchis acanthus* (Echinostomatoidea), *Cryptocotyle lingua* (Opisthorchioidea), Cercaria longicaudata, *Monorchis parvus* (Monorchioidea), *Diphterostomum brusinae* y *Bacciger bacciger* (Microphalloidea) aislados a partir de 12 moluscos que actúan como huéspedes intermedios. La aproximación general consistió en aplicar diversas combinaciones de fluorocromos base-específicos (4'-6-diamidino-2-phenylindol DAPI, DNA rico en AT; cromomicina A_3, CMA, DNA rico en GC) e inespecíficos (ioduro de propidio, PI), bandas C e inmunodetección de 5-methyl-cytosine además de mapear sondas de DNA repetido (rDNA 45S y 5S, genes de histonas, secuencias telomericas y DNA satélites) sobre sus cromosomas.

Se obtuvieron preparaciones cromosómicas utilizando métodos previamente publicados (Méndez et al. 1990; Pasantes et al. 1990). Después de tratar los bivalvos con colchicina, se diseccionaron branquias y gónadas y se sumergieron en agua de mar al 50% y al 25% antes de fijarlas en una mezcla de etanol y ácido acético. Las paralarvas de digeneos fueron tratadas del mismo modo. En ambos casos las muestras ya fijadas fueron desagregadas en una disolución de ácido acético al 60% hasta obtener suspensiones celulares que se dejaron caer gota a gota sobre portaobjetos precalentados a 50 °C.

La tinción con fluorocromos se llevó a cabo tal y como describen Pérez-García et al. (2010b). Después de controlar su calidad mediante microscopía de contraste de fases (Nikon), las preparaciones fueron teñidas con CMA (0.25 mg/mL), contrateñidas con DAPI (0.14 μg/mL), lavadas en agua corriente, se dejaron secar al aire y se montaron (Vectashield, Vector). Las preparaciones se estudiaron y fotografiaron mediante microscopía de fluorescencia (Nikon Eclipse-800). Se tomaron imágenes separadas para cada fluorocromo con una cámara CCD DS-Qi1Mc (Nikon) controlada con el programa NIS-Elements (Nikon). La superposición de las imágenes se realizó con Adobe Photoshop. Tras ser fotografiadas, las preparaciones se retiñeron con una combinación de DAPI y PI (0.07 μg /mL), se lavaron en agua corriente, se dejaron secar al aire, se montaron y se volvieron a fotografiar.

La extracción de DNA se realizó mediante cloroformo/isoamilalcohol o con el EZNA Mollusc DNA Kit (OMEGA). En los casos necesarios se amplificaron por PCR fragmentos del gen COI mitocondrial usando los cebdores LCO1490 y HCO2198 (Folmer et al. 1994). Para amplificar un fragmento del gen rRNA 16S mitocondrial se usaron los cebadores 16L29 (Schubart et al. 2001) y 16SBr (Palumbi 1996). El segmento ITS2 del rDNA 45S se amplificó con los cebadores ITS3 e ITS4 (White et al. 1990). Para su uso como sondas FISH, se utilizaron los cebadores universales LR10R y LR12 (Vilgalys lab website, http://www.biology.duke.edu/fungi/mycolab/primers.htm) para amplificar un fragmento del 28S rDNA. Para amplificar el 5S rDNA y el gen de la histona H3 se usaron los cebadores descritos por Pérez-García et al. (2010a, 2010b) y por Giribet y Distel (2003), respectivamente.

Las secuencias de DNA se amplificaron (GeneAmp PCR system 9700, Applied Biosystems) en volúmenes de 50 μL que contenían 125 ng de DNA genómico, 50 μM de cada dNTP, 50 μM de cada cebador, 1x tampón de PCR, 15 μM de MgCl2 y 5 U de JumpStart™ Taq DNA Polymerase (Sigma). Las

amplificaciones incluyeron una desnaturalización inicial a 95 ºC (2 min), 35 ciclos de amplificación y una extensión final a 72 °C (5 min).

Las secuencias amplificadas fueron purificadas (FavorPrepTM GEL/PCR Purification Kit, Favorgen) y secuenciadas (CACTI, Universidad de Vigo) en ambos sentidos en un ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) usando un BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Las secuencias se editaron con BioEdit v. 7.1.11 (Hall 1999) y se alinearon con Muscle usando los parámetros por defecto en MEGA7 (Kumar et al. 2016). Se realizaron búsquedas de secuencias con el algoritmo Basic Local Alignment Search Tool (BLAST), disponible en el National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Se empleó el algoritmo MegaBLAST, con parámetros por defecto, para comparar las secuencias con las existentes en las bases de datos del NCBI y del Barcode of Life Data System (BOLD).

Los cromosomas se hibridaron con sondas de rDNA 5S y 28S y de genes de la histona H3 (Pérez-García et al. 2011). La sondas 28S rDNA se marcaron con biotina-16-dUTP (Roche Applied Science) o digoxigenina-11-dUTP (10x DIG Labeling Mix, Roche Applied Science) usando un kit de *nick trans*lation (Roche Applied Science). Las sondas para el gen de la histona H3 y para el rDNA se marcaron directamente por PCR con biotina-16-dUTP (20 μM) o digoxigenina-11-dUTP (5 μM).

Las preparaciones recibieron tratamientos con RNasa y pepsina, se desnaturalizaron (70 ºC, 2 min) y se hibridaron a 37 ºC. La biotina fue detectada con avidina conjugada con isotiaocianato de fluoresceína (FITC) y anti-avidina biotinilada (Vector). La digoxigenina se detectó con anticuerpos anti-digoxigenina conjugados con isotiocianato de tetrametil rodamina (TRITC) (Sigma). Una vez contrateñidas con DAPI, se montaron y se examinaron al microscopio. Se tomaron imágenes para cada fluorocromo por separado, se seudocolorearon y se superpusieron de la manera indicada más arriba. También se realizaron experimentos FISH utilizando una sonda telomérica $(C_3TA_2)_3$ péptido nucleica (PNA) (Applied Biosystems) siguiendo el protocolo indicado por el suministrador.

Se realizaron análisis cromosómicos y cariotipicos sobre un mínimo de 10 ejemplares por especie (5 machos, 5 hembras). Para cada especie, se construyeron cariotipos a partir de un mínimo de 10 metafases completas con señales de FISH y se midieron en ellas las longitudes de los brazos cromosómicos. A partir de estos datos se calcularon longitudes relativas e índices centroméricos.

En concordancia con datos previos, todas las especies aquí estudiadas poseen $2n = 38$ y sus cariotipos suelen estar formados por cromosomas que muestran una distribución de longitudes cuasi continua. En 16 de las 20 especies las regiones ricas en GC coincidieron con los NORs pero las cuatro restantes, *Donax trunculus*, *D. vittatus*, *Mactra stultorum* y *Spisula subtruncata*, presentaron bandas intercalares adicionales. Además, se identificó un nuevo DNA satélite como el componente principal de la heterocromatina rica en GC de *S. subtruncata*. Como predice la *library hypothesis*, este DNA satélite también fue detectado en *M. stultorum* y *S. solida*, mostrando en ellas una gran conservación de la secuencia pero una drástica reducción en el número de copias. Se determinó también el grado de metilación de este satélite mediante secuenciación genómica con bisulfito e inmunodetección de 5-metil-citosina y los resultados se compararon con los detectados mediante polimorfismos de amplificación sensible a la metilación (MSAP) y ensayos por inmunoabsorción ligados a enzimas (ELISA). La metilación del satélite fue alta para lo habitual en bivalvos y triplicó la media de metilación del genoma de *S. subtruncata*.

En relación al mapeo mediante FISH, los patrones de distribución de las agrupaciones de genes de la histona H3, del rDNA 5S y del rDNA 45S en especies de Veneroida presentaron algunas peculiaridades. En la familia Veneridae se identificaron un total de 18 *loci* para las agrupaciones de genes de histona H3 en las 11 especies analizadas. En seis de las especies apareció una única agrupación mientras que *Chamelea striatula* presentó cuatro y las cuatro especies restantes dos. Quince de estos *loci* son subterminales, dos intercalares y el restante subcentromérico. Excepto *Chamelea striatula* todas estas especies presentaron una única agrupación 45S rDNA. Por el contrario, para el 5S rDNA sólo apareció una única localización en cinco especies, dos en otras cinco especies y tres en la restante, *Petricola litophaga*. Las señales aparecieron en cromosomas distintos salvo en *Clausinella fasciata* y *Chamelea striatula* en las que un único par era portador de agrupaciones de genes de histonas y de rDNA, y *Ruditapes decussatus* con señales 5S rDNA y 45S rDNA solapantes.

El número y la localización de estas agrupaciones, unido al análisis de las secuencias nucleotídicas de fragmentos del gen de la subunidad I de la citocromo oxidasa (COI) y del rDNA 16S

mitocondriales y el espaciador interno transcrito 2 (ITS2) del rDNA 45S nuclear, permitió determinar el estatus taxonómico de las chirlas *Chamelea gallina* y *C. striatula*. La comparación de ejemplares procedentes de cuatro poblaciones atlánticas y mediterráneas mostró claras diferencias entre ellas. La constancia en la distribución de las agrupaciones rDNA 5S y la variabilidad en las del rDNA 45S y de los genes de histonas en *C. gallina* y *C. striatula* fueron de una magnitud similar a las de *Venus casina* y *V. verrucosa* y las de *Dosinia exoleta* y *D. lupinus*. Al contrario, aunque se detectaron pequeñas diferencias intraespecíficas tanto en *C. gallina* como en *C. striatula* éstas fueron mucho menores que entre taxones y similares a las mostradas por otras especies de bivalvos. La comparación de las secuencias nucleotídicas confirmó que estos taxones son especies diferentes.

En Mactridae las agrupaciones 45S rDNA son subterminales en las tres especies. Tanto *Spisula solida* como *Mactra stultorum* presentaron dos agrupaciones para el 45S rDNA en los brazos cortos de los pares 17 and 19 y en los brazos largos de los pares 3 y 4, respectivamente. La única agrupación encontrada en *Spisula subtruncata* se encuentra en el brazo largo del par 18. Las tres mactras presentan una única agrupación 5S rDNA, subterminal en el brazo corto del par 5 en *Spisula solida* y en el brazo largo del par 3 en *Spisula subtruncata*, e intercalar en el brazo corto del par 15 en *Mactra stultorum*. A pesar de que también hay una única agrupación de genes de histona H3 en las tres especies, sus localizaciones difieren, intercalar en el brazo largo del par subtelocéntrico 8 en *Spisula solida* y del 12 en *Mactra stultorum* pero subcentromerico en el brazo largo del par metacentrico 14 en *Spisula subtruncata*.

Las coquinas presentaron agrupaciones de genes de la histona H3 intercalares en el brazo largo del par 17, subtelocentrico en *D. trunculus* y telocentrico en *D. vittatus*. El rDNA 45S es intercalar en el brazo corto del par 6, subtelocéntrico en *D. trunculus*. La mayoría de los ejemplares de *D. vittatus* presentan el rDNAs 45S en el brzo corto del par 6 que en este caso es telocéntrico. Por el contrario, el rDNA 45S es subcentromerico en el brazo largo del par 6, metacéntrico, en todos los especímenes de *D. vittatus* recolectados en Samil. El número de agrupaciones rDNA 5S es diferente en *D. trunculus* y *D. vittatus*. En ambas especies hay una agrupación subterminal en el brazo largo del par subtelocéntrico 10 pero *D. trunculus* presenta también una segunda agrupacion en el brazo corto del par metacéntrico 2. Las diferencias cariotípicas pudieran ser fundamentalmente debidas a inversiones pericéntricas.

Como en las otras familias, las telinas también presentaron diferencias en número y localización de las agrupaciones rDNA 45S, rDNA 5S y de genes de la histona H3. *Macomangulus tenuis*, *Moerella donacina* y *Bosemprella incarnata* portan un único rDNA 45S mientras que *Serratina serrata* presenta dos. Con respecto al rDNA 5S, también aparecen especies con una (*B. incarnata* and *S. serrata*) o dos (*M. tenuis* and *M. donacina*) agrupaciones. Por el contrario, las cuatro especies muestran una única agrupación de genes de la histona H3, aunque situados en lugares diferentes.

En las especies de tremátodos digeneos estudiadas, los números diploides variaron desde un mínimo de 2*n* = 12 en *B. bacciger* a un máximo de 2*n* = 22 en *P. acanthus*. Estos parásitos también mostraron grandes diferencias en la morfología de sus cromosomas. Mientras que los cariotipos de *C. lingua* y *P. acanthus* se componen de cromosomas exclusivamente metacéntricos y subtelocéntricos, respectivamente, el resto de los taxones presentó diversas combinaciones de tipos cromosómicos. Todas las especies presentaron una única agrupación del rDNAs 45S y otra del 5S aunque la localización fue diferente. En *B. minimus* and *P. acanthus* ambos rDNAs aparecen en pares cromosómicos distintos mientras que en las restantes especies las señales solapan en un único par. Los experimentos mediante FISH también permitieron probar la existencia de regiones DAPI negativas, muy descondensadas, coincidentes con el rDNA 45S. Las señales para el 45S rDNA aparecen en la cromatina condensada que flanquea esas regiones y que no son verdaderos telómeros pues carecen de señales teloméricas. La presencia de regiones subcentroméricas de ese tipo en *B. australis*, *P. carvajali*, *P. acanthus*, *C. lingua*, *M. parvus*, y *B. bacciger*, provoca que una tinción sencilla con DAPI diese números cromosómicos aparentemente superiores a la dotación diploide. En los taxones restantes, *B. minimus*, *M. filiformis*, C. longicaudata y *D. brusinae*, muchas de las metafases mitóticas mostraron constricciones secundarias claramente DAPI negativas que separaban un pequeño fragmento de cromatina del resto del cromosoma. En este estudio también se detectaron de 1 a 19 cromosomas B en los ejemplares de *B. bacciger* aislados de *Ruditapes decussatus*. Estos cromosomas metacéntricos supernumerarios presentan señales teloméricas en ambos extremos y se caracterizan por mostrar brazos largos fuertemente teñidos con DAPI, evidentes también en los núcleos interfásicos.

INTRODUCTION

Bivalves are a group of exclusively aquatic mollusks characterized by presenting a laterally compressed body whose soft parts are covered by a shell (Gosling 2015). More than 8500 species are currently accepted (Huber 2010, 2015; World Register of Marine Species, WoRMS, http://www.marinespecies.org/) and they are distributed worldwide inhabiting marine, brackish and, to a lesser extent, freshwater ecosystems. Taxa within this group are usually benthic and mostly diggers (clams) but also sessile (mussels) or showing some swimming abilities (scallops).

Although some bivalves are detritivores and other carnivores, the evolutionary success and radiation of these organisms has been attributed to their filtration-feeding through a pair of modified gills (Plazzi et al. 2011). Most bivalves are dioecious and present an annual reproductive cycle along which gonads progressively mature. Spawning is seasonal and, with a few exceptions, the fertilization external. Typically, after a few pelagic larval stages (trocophora, veliger), mature larvae (pediveliger) settle in suitable substrates before undergoing metamorphosis and beginning the juvenile phase (Gosling 2015).

The class Bivalvia includes a considerable number of species of great economic interest that have been traditionally subjected to extensive exploitation on commercial purposes. Their ecological role is of high relevance as they constitute both the basis of trophic networks and the physical support for the development of numerous benthonic animal species, both vertebrates and invertebrates. Bivalve mollusks also act as intermediary hosts in the life cycle of many digenean trematodes, parasitic metazoans that may have a huge impact on bivalve populations, both natural and cultured.

The most peculiar characteristic of bivalves, on which their classification is based, is the presence of a calcareous shell formed by two **valves**, articulated on a toothed **hinge** and joined by a flexible **ligament** in its dorsal part. A pair of adductor muscles connects the inner sides of both valves, keeping them together and controlling their opening and closing. The mantle, a thin tissue membrane upholstering the soft organs of the animal inside the pallial cavity, internally covers the valves. Coinciding with the margin of the mantle, it appears a curved line on the inner side of the shell which usually connects with two marks at the insertion point of the adductor muscles, the **pallial line** and the **scars** of the adductor muscles, respectively.

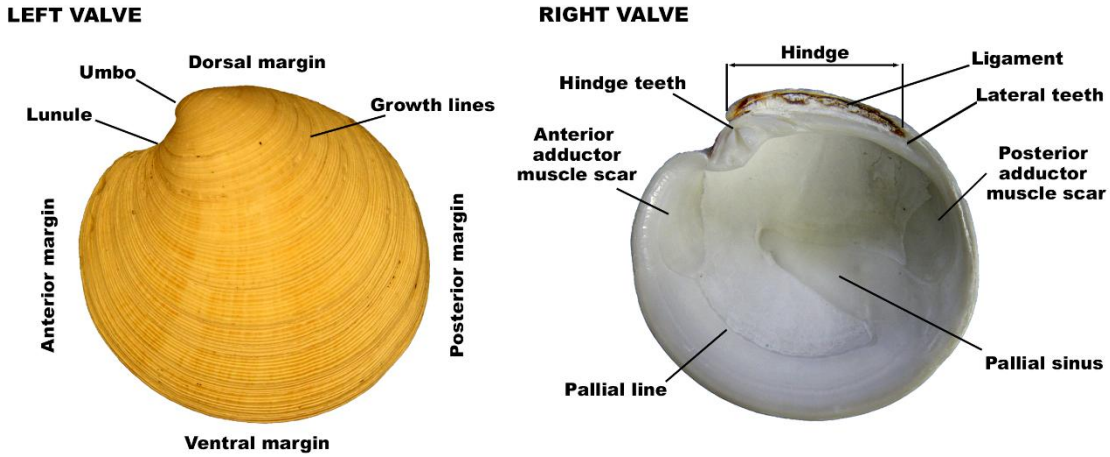


Figure 1: Internal and external shell features of a bivalve, the rayed artemis *Dosina exoleta*

Some species of bivalves have frontal siphons that channel water into the pallial cavity. As siphons are retracted into the closed shell, an anterior disruption in the pallial line, the pallial sinus, is usually detected. The visceral mass of these organisms includes a muscular foot with digging and locomotive functions, a pair of gills responsible for the gaseous exchange and, in some cases, the food filtering, a digestive system closely associated with an open circulatory system, composed of a set of sinuses and a heart with two atria and a single ventricle, and a nervous system including three pairs of ganglia and two neural cords (Huber 2010).

Although the classification of bivalves is a rather controversial subject and recent phylogenetic analyses (Bieler et al. 2014) propose six monophyletic lineages within Bivalvia, WoRMs still utilizes four subclasses, Protobranchia, Pteriomorphia, Paleoheterodonta and Heterodonta. The subclass Heterodonta includes more than two thirds of the extant bivalves, extremely diverse in shape, size, anatomy and lifestyle. As molecular phylogenies (Bieler et al. 2014) have verified that some of the orders, superfamilies and families in this group are polyphyletic, bivalve taxonomy is under continuous revision. A good example is the traditional order Veneroida, which, despite including some of the largest families (Veneridae, 680 species; Mactridae 180; Donacidae, 100; Tellinidae, 500), is neither morphologically homogeneous nor monophyletic being, therefore, currently taxonomically questioned (Huber 2010, 2015).

Beneath family level the chaos is even higher. The lack of diagnostic and morphological criteria makes that easily identifiable, economically important species were continuously reassigned to different genera on the basis of rather limited DNA sequence analyses. For instance, *Paphia aurea*, the golden carpet shell, was *Tapes aureus* and *Venerupis aurea* prior being *Polititapes aureus*, all changes resulting from a mitochondrial 16S rDNA phylogeny constructed with samples of only 14 out of a total of 680 species of Veneridae (Canapa et al. 2003; Huber 2010).

The scarcity of systematic studies in these groups turns species assignment to genus into an extremely difficult task and, in fact, this assignment is usually recognized only as temporary, while awaiting deeper phylogenetic reviews. On the other hand, the ever growing amount of introduction of non indigenous bivalve species outside their native ranges of distribution throughout the last decades, either accidentally or deliberately, further complicates this task. Many of these alien species are unknown in the new environment and, as a consequence, mistakenly identified as variants of native ones. In addition, the analyses of DNA sequences, mainly mitochondrial cytochrome oxidase I (COI) genes, in different bivalve specimens have exponentially increased the description of presumptive cryptic species in these organisms. Although in some cases this may be true, in many others this could be due to misidentifications of alien specimens (Garcia-Souto et al. 2017).

Most chromosomal studies in bivalves just described chromosome numbers and, in some cases, karyotypes according size and morphology (Nakamura 1985; Thiriot-Quiévreux 1994). This kind of analysis led to propose that chromosome numbers were relatively stable within families, $2n = 20$ in Ostreidae, $2n = 28$ in Mytilidae and Pteridae and $2n = 38$ in most of the remaining, including Veneridae (Thiriot-Quiévreux 1994). However, further studies showed that some genera and families included species with different diploid numbers. For instance, although 14 of the 41 species of Mytilidae are $2n = 28$, the range in the remaining goes from $2n = 22$ to $2n = 32$. Moreover, in some genera all species have the same chromosome number (i.e. *Mytilus*, 2n = 28) while in others there are variation (*Perna*: $2n = 28$ and $2n = 30$; *Brachidontes*: $2n = 28$, $2n = 30$ and $2n = 32$). This indicates that speciation in this family has been accompanied, in some cases, by changes in chromosome number (Garcia-Souto et al. 2017).

Nevertheless, this does not appear to be the case in Heterodonta since the vast majority of the species are 2*n* = 38. In addition, karyotypes are usually characterized by presenting chromosomes similar in size and morphology (Nakamura 1985; Thiriot-Quiévreux 1994, 2002). This homogeneity precludes the unequivocal identification of chromosome pairs and, consequently, interspecific chromosomal homologies deducted from comparing homogenously stained metaphases are quite presumptive. Therefore correct comparisons of veneroid karyotypes require methods that allow accurate chromosome identification; among those, chromosome banding and fluorescence *in situ* hybridization (FISH).

The description, and subsequent widespread application, of chromosome banding techniques in mammals along the 1970s was revolutionary for cytogenetics. The discovery that quinacrine induced differential staining patterns on chromosomes (Q-bands, Caspersson et al. 1968, 1969, 1970) prompted researches to develop diverse banding techniques to accurately identify human and mammal chromosomes (Ferguson-Smith 2015). These techniques usually enable the selective detection of chromosomal regions on the basis of chromatin characteristics such as base-pair composition (Q and G bands, restriction bands), condensation level (C bands and restriction bands), or activity (Ag-NOR and N bands). However, despite descriptions of multiple structural bands in oysters (Rodríguez-Romero et al. 1979) and mussels (Moore et al. 1986; Méndez et al. 1990; Martínez-Lage et al. 1994), the results obtained in bivalves are hardly reproducible and show much lower quality than those in birds and mammals. In contrast, the banding techniques detecting heterochromatin (C bands) and nucleolar organizing regions (NORs; Ag-NOR and N bands) also gave consistent and repeatable results in bivalve chromosomes (Thiriot-Quiévreux 2002; Leitão and Chaves 2008). In spite of that, as most bivalves are poor in heterochromatin and show NORs at similar positions, these techniques are not good instruments for chromosome identification in bivalves (Thiriot -Quiévreux 2002; Leitão and Chaves 2008).

Separate mention requires chromosome fluorochrome staining. The combination of fluorochromes showing different DNA base pair binding affinities allows detecting GC-rich and GC-poor chromosome regions. Those fluorochromes can be classified into three categories depending on whether they bind to AT (quinacrine, Hoechst 33258,

DAPI, distamycin A) or GC (chromomycin A3, mithramycin) or show unspecific binding (propidium iodide). The use of these fluorochromes in mammals and birds allowed detecting patterns of GC-rich and GC-poor regions along their chromosomes. In contrast, GC-rich regions are infrequent in other vertebrates and usually coincident with the NORs (Schmid and Guttenbach 1988). The same applies to most species of bivalves and, therefore, these techniques are usually not very informative (Thiriot-Quiévreux 2002; Leitão and Chaves 2008; Pérez-García et al 2010a, 2010b, 2011, 2014). However, there are exceptions to this rule and some bivalves do present many GC-rich bands that help in identifying particular chromosomes. This is the case of the abrupt wedge shell *Donax trunculus* (Martínez et al. 2002; Petrović et al. 2009) and the zebra mussel *Dreissena polymorpha* (Woznicki and Boron 2003; Boron et al. 2004), both presenting multiple intercalary GC-rich heterochromatic bands.

The absence of clear banding patterns in bivalves also prompted the use of restriction endonucleases to digest bivalve chromosomes as an interesting alternative. This methodology has been successfully applied to mussels (Martínez-Lage et al. 1994), pectinids (Gajardo et al. 2002), oysters (Leitão et al. 2004), clams (Leitão et al. 2006; Teixeira de Sousa et al. 2012) and cockles (Leitão et al. 2006), giving results that, despite their consistency and reproducibility, have the disadvantage of being greatly dependent on the level of condensation of the chromosomes. Given the absence of successful cell culture techniques in bivalves, obtaining adequate numbers of not too condensed metaphase plates is a hard task. The application of replication banding techniques presents similar difficulties as it depends on the incorporation of base analogs, such as 5-bromo-2'-deoxyuridine (BrdU), to DNA during the S phase of the cell cycle. Although these techniques allowed studying cell cycle kinetics, replication banding patterns and sister chromatids exchanges in mussels (Martínez-Expósito et al. 1994; Pasantes et al. 1996; Torrreiro et al. 1999), the impossibility of synchronizing cell populations hampers a correct control of BrdU incorporation at the initial or the final part of the S phase and, therefore, makes difficult the recreation and interpretation of the resulting replication bands.

Taking into account all of the above, the chromosomal mapping of DNA sequences by FISH is the most direct method for identifying particular chromosomes in

bivalves and, consequently, to construct more reliable karyotypes. This technology has been widely employed since 1980s for chromosome identification, DNA sequence localization and chromosomal rearrangement analyses in many organisms (Levsky and Singer 2003). In addition, starting from cytogenetic data of closely related species and providing that some chromosome markers show variability among them, a sequence of chromosomal changes by which they had diverged from a hypothetical ancestral karyotype could be deduced. Although still somewhat exceptional in bivalves, FISH using repetitive DNA probes has been systematically applied to many species of plants and animals. In contrast, detection of single- or low-copy sequences by FISH is still mostly restricted to pachytene and polytene chromosomes or extended chromatin fibers.

FISH mapping in bivalves have been mostly restricted to 45S rRNA genes (50 species), 5S rRNA genes (25), core histone genes (15), linker histone genes (6) and telomeric sequences (25) (Thiriot-Quiévreux 2002; Leitão and Chaves 2008; Petrović et al. 2009; Pérez-García et al. 2010a, 2010b, 2011, 2014a, 2014b; Bouilly et al. 2010; Carrilho et al. 2011; Hurtado et al. 2011; González-Tizón et al. 2013; Li et al. 2016; García-Souto et al. 2017). FISH mapping, mainly applied to Mytilidae, Ostreidae and Pectinidae, makes possible establishing more reliable karyotypes and identifying some of the chromosomal changes accompanying bivalve evolution (Pérez-García et al. 2014b) but also contributed to better understand their phylogenies. In addition, the application of these techniques has shown that, contrasting the presumed karyotypic conservation provided by classical techniques, important variations appear. For instance, Atlantic species of oysters differ from their Pacific congeners by the chromosomal location of the 45S rDNA, therefore demonstrating that even apparently highly conserved karyotypes diverge (Wang et al. 2004). On the other hand, some satellite DNAs has been also mapped by FISH in these families (Clabby et al. 1996; Wang et al. 2001; Martínez-Lage et al. 2002; Cross et al. 2005; Odierna et al. 2006; Biscotti et al. 2007; Bouilly et al. 2008; López-Flores et al. 2010; Hu et al. 2011; Petraccioli et al. 2015).

As for Heterodonta, chromosomal mapping is much scarcer. To date, 45S rDNAs have been located by FISH in 18 species, 5S rDNAs in 9, core histone genes in 2 and telomeric sequences in 12 (Insua et al. 1999; González-Tizón et al. 2000; Wang

and Guo 2001, 2007, 2008; Martínez et al. 2002; Plohl et al. 2002; Fernández-Tajes et al. 2003, 2008; Hurtado and Pasantes 2005; Petrović et al. 2009; Bouilly et al. 2010; Carrilho et al. 2011; Hurtado et al. 2011; González-Tizón et al. 2013; Pérez-García et al. 2014a). Almost all species are 2*n* = 38 and present rather similar karyotypes but considerable differences in the chromosomal distribution of those sequences, thus demonstrating their potential as cytogenetic markers for deducing their evolutionary relationships. Regarding satellite DNAs, they have been only mapped in the manila clam, *Ruditapes philippinarum* (Passamonti et al. 1998) and the abrupt wedge shell, *Donax trunculus* (Petrović et al. 2009).

Cytogenetic studies in digenean trematodes are even scarcer than in bivalves. Despite being the largest group of metazoan endoparasites comprising more than 18000 extant species, chromosomal numbers and karyotypes have been described in only 300 and FISH mapping was limited to 45S rDNAs and/or telomeric sequences in nine (Hirai et al. 1989, 2000; Baršienė 1993; Hirai y Lo Verde 1996; Bell et al. 1998; Petkevičiūtė et al. 2003; Špakulová and Casanova 2004; Reblánová et al. 2011; Zadesenets et al. 2012a, 2012b; Petkevičiūtė et al. 2012, 2014, 2015; Hirai 2014; Sofi et al. 2015).

OBJECTIVES

As outlined in the introduction, although the order Veneroida includes some of the largest families of bivalves, the knowledge of their chromosomes lags behind that of many other invertebrate groups, including other bivalves. The same applies to the many digenean trematodes that use them as intermediate hosts. Because of that, as the main aim of this work was to broaden cytogenetic data on these organisms, we selected species belonging to four of the largest veneroid families (Veneridae, Mactridae, Donacidae and Tellinidae), and digenean parasites infecting them, in order to accomplish the following objectives:

1. Establish the karyotypes of the veneroid species *Clausinella fasciata*, *Chamelea striatula*, *Venus casina*, *Dosinia lupinus*, *Petricola litohpaga* (Veneridae), *Spisula subtruncata* (Mactridae), *Donax vittatus* (Donacidae), *Bosemprella incarnata*, *Moerella donacina* and *Serratina serrata* (Tellinidae) and confirm those of *Ruditapes decussatus*, *Ruditapes philippinarum*, *Venerupis corrugata*, *Chamelea gallina*, *Venus verrucosa*, *Dosinia exoleta* (Veneridae), *Mactra stultorum*, *Spisula solida* (Mactridae), *Donax trunculus* (Donacidae) and *Macomangulus tenuis* (Tellinidae).

2. Establish the karyotypes of the digenan taxa *Bucephalus minimus Bucephalus australis*, *Prosorhynchoides carvajali*, *Monascus filiformis* (Bucephalata), Cercaria longicaudata, *Monorchis parvus* (Monorchiata) *Diphterostomum brusinae* and *Bacciger bacciger* (Xiphidiata) and confirm those of *Parorchis acanthus* (Echinostomata) and *Cryptocotyle lingua* (Opisthorchiata).

3. Determine the presence of GC-rich and/or GC-poor regions on them.

4. Localize the position occupied by 45S rDNAs, 5S rDNAs, H3 histone genes and/or telomeric sequences in the chromosomes of those species.

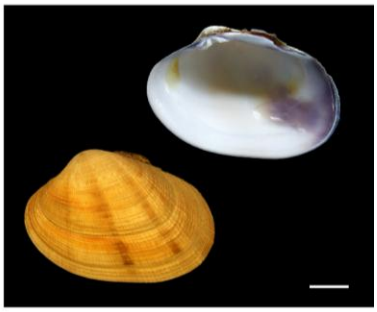
5. Analyze the utility of those sequences as chromosomal markers.

6. Assess the possible utility of the ameliorated karyotypes in taxonomic, phylogenetic and other problems.

VENERIDAE

Ruditapes philippinarum
(Adams y Reeve, 1850)
Almeja japonesa

Ruditapes decussatus
(Linnaeus, 1758)
Almeja fina



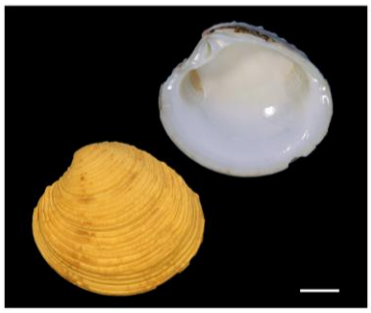
Venerupis corrugata
(Gmelin, 1791)
Almeja babosa

Clausinella fasciata
(da Costa, 1778)

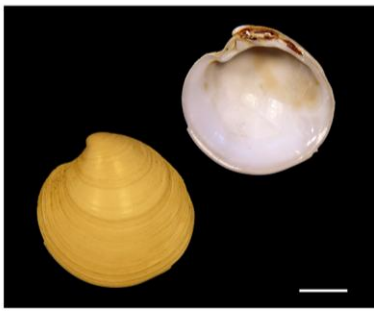
Chamelea gallina
(Linnaeus, 1758)
Chirla

Chamelea striatula
(da Costa, 1778)
Chirla

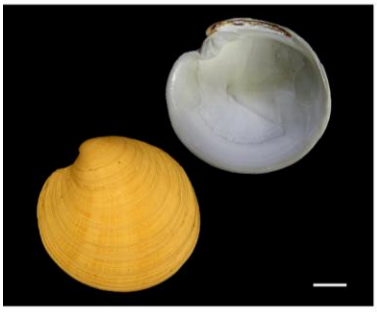
Venus verrucosa
(Linnaeus, 1758)
Escupiña grabada



Venus casina
(Linnaeus, 1758)



Dosinia lupinus
(Linnaeus, 1758)



Dosinia exoleta
(Scopoli, 1777)
Reloj

Petricola lithophaga
(Retzius, 1788)

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MOLECULAR CYTOGENETICS

RESEARCH Open Access

Divergent evolutionary behavior of H3 histone gene and rDNA clusters in venerid clams

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Abstract

Background: Histone H3 gene clusters have been described as highly conserved chromosomal markers in invertebrates. Surprisingly, in bivalves remarkable interspecific differences were found among the eight mussels and between the two clams in which histone H3 gene clusters have already been located. Although the family Veneridae comprises 10 % of the species of marine bivalves, their chromosomes are poorly studied. The clams belonging to this family present 2n = 38 chromosomes and similar karyotypes showing chromosome pairs gradually decreasing in length. In order to assess the evolutionary behavior of histone and rRNA multigene families in bivalves, we mapped histone H3 and ribosomal RNA probes to chromosomes of ten species of venerid clams.

Results: In contrast with the reported conservation of histone H3 gene clusters and their intercalary location in invertebrates, these loci varied in number and were mostly subterminal in venerid clams. On the other hand, while a single 45S rDNA cluster, highly variable in location, was found in these organisms, 5S rDNA clusters showed interspecific differences in both number and location. The distribution patterns of these sequences were species-specific and mapped to different chromosomal positions in all clams but *Ruditapes decussatus*, in which one of the minor rDNA clusters and the major rDNA cluster co-located.

Conclusion: The diversity in the distribution patterns of histone H3 gene, 5S rDNA and 28S rDNA clusters found in venerid clams, together with their different evolutionary behaviors in other invertebrate taxa, strongly suggest that the control of the spreading of these multigene families in a group of organisms relies upon a combination of evolutionary forces that operate differently depending not only on the specific multigene family but also on the particular taxa. Our data also showed that H3 histone gene and rDNA clusters are useful landmarks to integrate nex-generation sequencing (NGS) and evolutionary genomic data in non-model species.

Keywords: Venerid clams, Chromosome, Fluorescent *in situ* hybridization, Histone genes, Ribosomal RNA genes

Background

The analysis of the chromosome changes encompassing the evolution of a group of organisms relies on the accurate identification of their chromosomes. When chromosome-specific painting probes are not available, as frequently happens in invertebrates, and karyotypes are composed by chromosomes gradually decreasing in length, chromosomal identification turns into a very difficult task. In those cases, the hybridization of highly conserved repetitive sequences, among which ribosomal RNA (rRNA) and histone genes are paramount, usually constitutes a first step in finding chromosome-specific probes.

Eukaryotic genomes present multiple copies of genes encoding histones, the basic proteins responsible of packaging DNA into chromatin. The histone multigene family includes five main types of genes, those encoding the histones of the nucleosome core particle (H2A, H2B, H3 and H4) and those for the linker histones (H1) [1]. rRNA genes are also organized in multigene families, one expressing for the 18S, 5.8S and 28S rRNAs (45S rDNA) and the other for the 5S rRNA [2]. Histone and rRNA genes in invertebrates are usually organized in tandem arrays clustered in one or more chromosomal

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positions, although other organizations have also been described [3, 4]. The evolutionary dynamics of both histone gene and rDNA clusters has been analyzed in only a few groups of these organisms, i.e. grasshoppers [5, 6], beetles [7, 8], aphids [9] and moths [10]. Whereas histone gene clusters were extremely conserved in number and location in all these groups, 45S and 5S rDNAs showed high degrees of variation.

In bivalves, the genomic organization of the histone genes has been studied using molecular methodologies in species belonging to the families Mytilidae [11–14], Pectinidae [15] and Veneridae [16], showing, in all of them, a tandemly arranged organization. Usually, the clusters comprise only core histone genes but repeated clusters including linker histone and/or other genes have also been reported. In these organisms histone H3 genes have been mapped to chromosomes in only eight mussels [13, 14, 17–20], four scallops [21], one oyster [22] and two clams [23]. In comparison with 45S and 5S rDNA clusters, surprisingly remarkable differences in number and location of the histone H3 gene clusters were found, more outstandingly among mussels and clams.

The venerid clams of the family Veneridae (Rafinesque 1815) represent almost 10 % of the species of marine bivalves [24]. Phylogenetic relationships among the species of this family were the subject of many recent investigations using DNA sequences whose results, in some cases, challenged the traditional, morphologically based classification [25–29]. In contrast, the chromosomal characterization of the venerid clams lags far beyond the knowledge achieved for other bivalve families. Classical venerid cytogenetics was limited to determine chromosome numbers and karyotypes in a few species [30–32]. More recently, a restriction endonuclease banding pattern was described in *Ruditapes decussatus* [33] and some repetitive DNA sequences were mapped by fluorescent *in situ* hybridization (FISH). The location of telomeric sequences and/or major and minor rDNA was reported for *Mercenaria mercenaria* [34, 35], *Dosinia exoleta* [36, 37], *Ruditapes decussatus* and *Ruditapes philippinarum* [37, 38], *Polititapes aureus* and *Polititapes rhomboides* [23] and *Venerupis corrugata* and *Venus verrucosa* [37]. On the other hand, histone gene clusters, as indicated above, were only mapped to chromosomes of *Polititapes aureus* and *Polititapes rhomboides* [23].

In order to get a better understanding of the evolutionary behavior of these multigene families in these organisms, we have hybridized H3 gene, 5S rDNA and 28S rDNA probes to mitotic and meiotic chromosomes of ten species of clams of the family Veneridae, *Ruditapes philippinarum*, *Ruditapes decussatus*, *Venerupis corrugata*, *Clausinella fasciata, Chamelea gallina*, *Venus verrucosa*, *Venus casina*, *Dosinia exoleta*, *Dosinia lupinus* and *Petricola litophaga*.

Results

FISH experiments identified a total of 14 *loci* for the histone H3 gene in the 10 species analyzed (Figs. 1 and 2). We detected a single core histone gene cluster in six of the species and two clusters in the remaining four. Regarding their chromosomal location, 11 of the histone clusters were close to the telomeres, two were intercalary, and the remaining one close to the centromere. The subterminal location of the clusters was confirmed by FISH on synaptonemal complex spreads (Fig. 3). A summary of the data obtained in this work, together with the other currently available FISH mapping data for the family Veneridae, is presented in Table 1. The species were arranged following the molecular phylogenetic tree suggested by [29] (Chen et al.) and assigned both to their proposed clade groups (A1, A2, A3, A4, B1) and the subfamilies of the traditional classification (Tapetinae, Chioninae, Venerinae, Dosininae and Petricolinae). Histone H3 gene clusters mapped to a single locus in two of the three analyzed species included in clade A1 (Tapetinae: *Ruditapes philippinarum* and *Ruditapes decussatus*), to two of the four species in clade A2 (Venerinae: *Venus verrucosa* and *Venus casina*), and in the two species in clade A4 (Dosininae: *Dosinia exoleta* and *Dosinia lupinus*). In the remaining four species, one in clade A1 (Tapetinae: *Venerupis corrugata*), two in clade A2 (Chioninae: *Clausinella fasciata* and *Chamelea gallina*), and one in clade B1 (Petricolinae: *Petricola litophaga*), core histone gene clusters mapped to two loci situated in different chromosome pairs.

In order to investigate the location of the core histone gene clusters in relation to rDNAs, we performed double and sequential FISH experiments using core histone gene, major rDNA and 5S rDNA probes in the five species of clams in which the location of rDNA sequences was already known (Fig. 1) and in the other five in which not previous data were available (Fig. 2). Whereas all species presented a single major rDNA cluster per haploid genome, the number of 5S rDNA clusters was one in five of the species (*Venerupis corrugata*, *Clausinella fasciata, Venus verrucosa*, *Venus casina* and *Dosinia lupinus*), two in four (*Ruditapes philippinarum*, *Ruditapes decussatus*, *Chamelea gallina* and *Dosinia exoleta*) and three in the remaining one, *Petricola litophaga* (Table 1). Taking into account that two of the three 5S rDNA clusters detected in *Petricola litophaga* were close together, sometimes giving double FISH signals and other times a single signal, we performed FISH on prophase I meiotic bivalents to clarify the true nature of the signals. As shown in Fig. 4, three clearly different signals were detected, two of them on the same bivalent.

Clausinella fasciata was the only species in which a single chromosome harbors both histone gene and rDNA cluster; in this species, a subterminal histone gene cluster

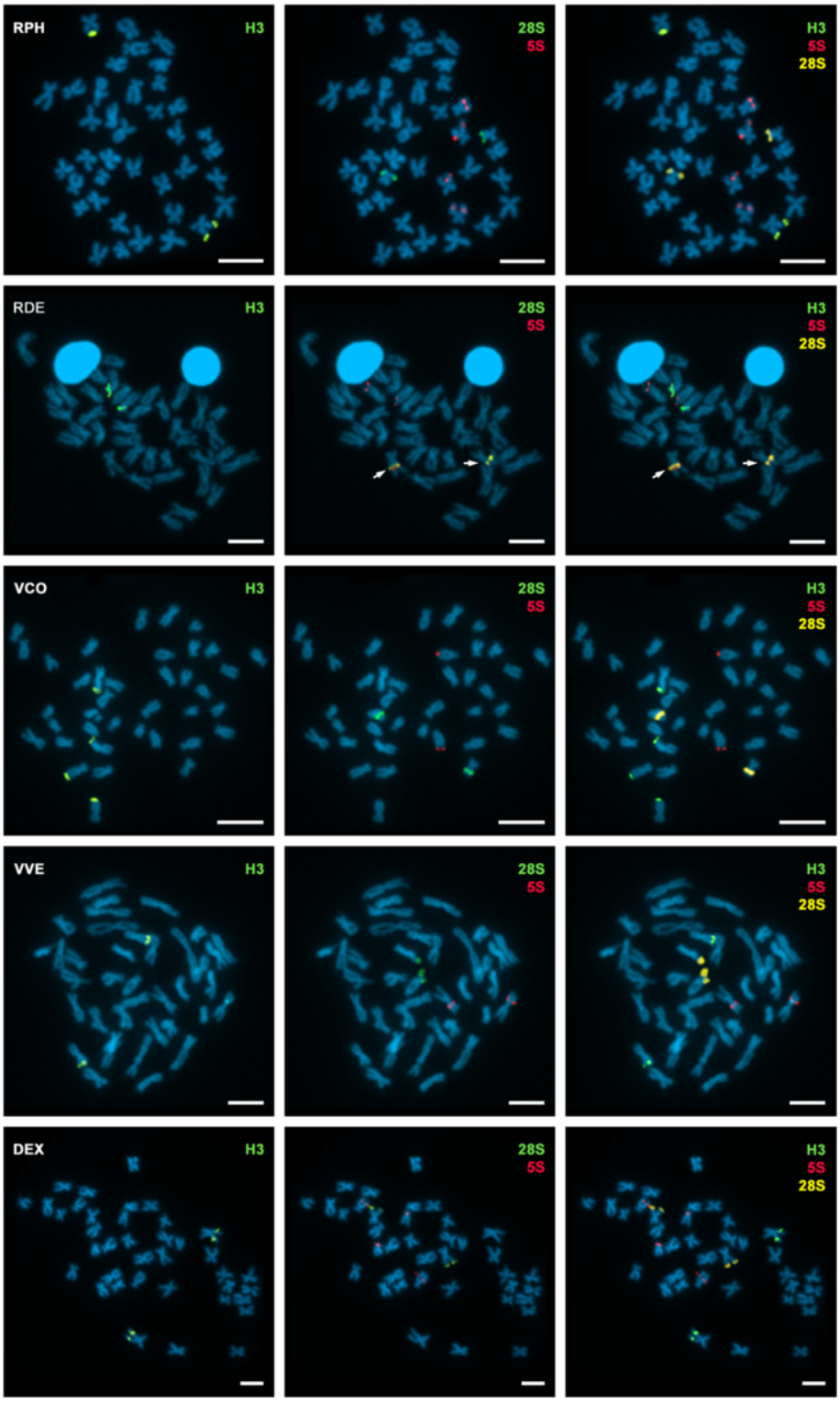

Fig. 1 Chromosomal location of H3 histone genes in venerid clams. H3 histone gene (H3) probes mapped by FISH to mitotic chomosomes of *Ruditapes philippinarum* (RPH), *Ruditapes decussatus* (RDE), *Venerupis corrugata* (VCO), *Venus verrucosa* (VVE) and *Dosinia exoleta* (DEX). To ascertain the chromosomal position of core histone gene clusters in relation to rDNA clusters, the same metaphases were rehybridized using 5S rDNA (5S) and major rDNA (28S) probes. Excluding 5S and major rDNA in RDE (arrows), all signals are on different chromosome pairs. Scale bars, 5 μm

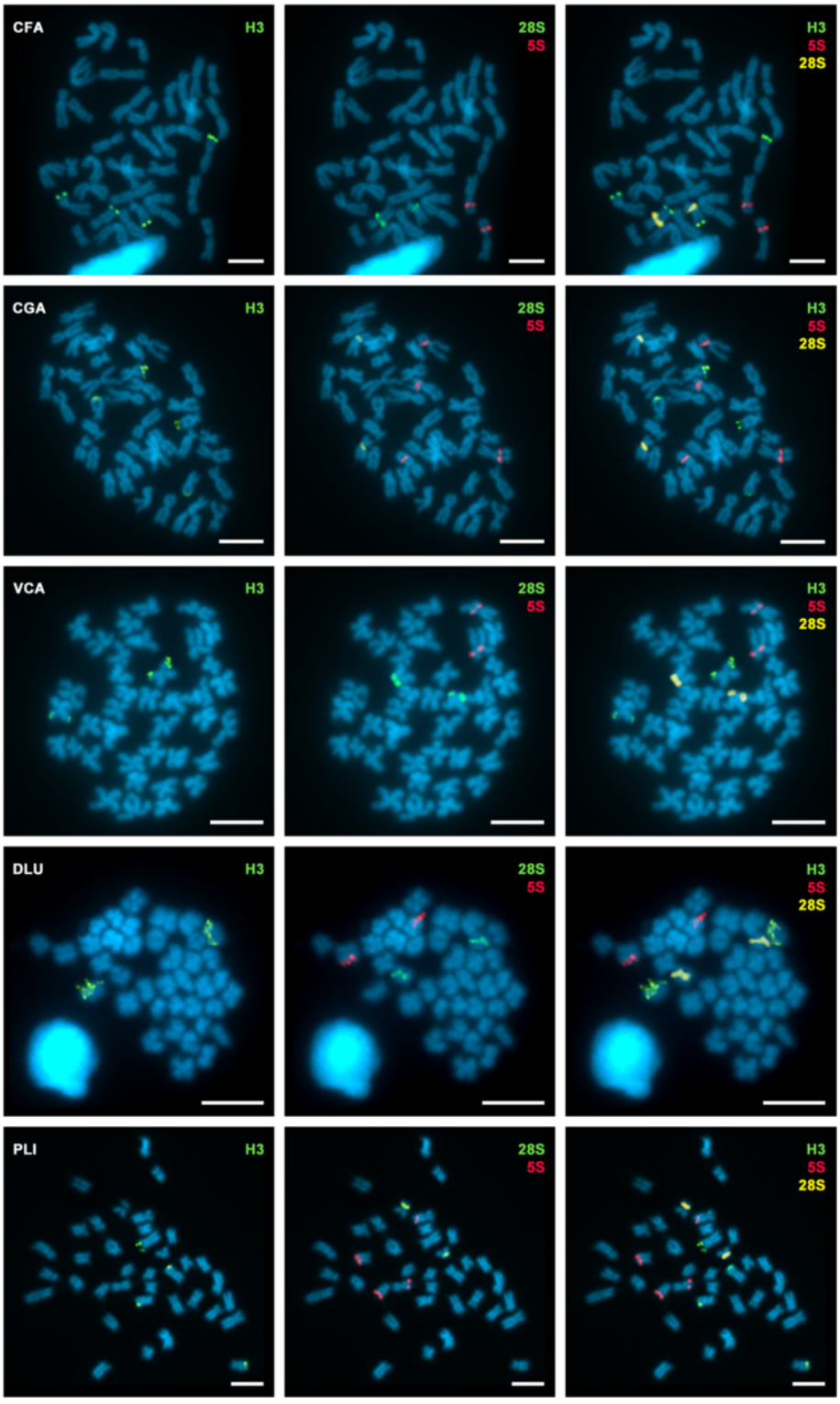

Fig. 2 Chromosomal location of H3 histone genes (H3), 5S rDNA (5S) and major rDNA (28S) in venerid clams. Single FISH using H3 histone gene probes mapped to chromosomes of *Clausinella fasciata* (CFA), *Chamelea gallina* (CGA), *Venus casina* (VCA), *Dosinia lupinus* (DLU), and *Petricola litophaga* (PLI), followed by double-FISH using 5S rDNA (5S) and major rDNA (28S) probes on the same metaphase plates. All signals for the different probes appear at different chromosome pairs with the exception of H3 histone gene and major rDNA in *Clausinella fasciata* (CFA, first row). Scale bars, 5 μm

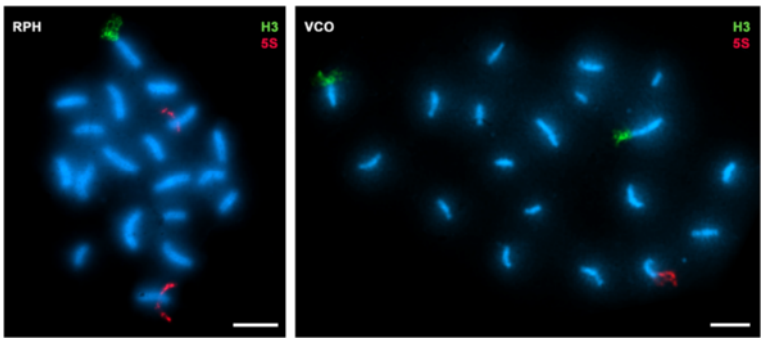

Fig. 3 Subterminal H3 histone gene clusters in venerid clams. Examples of FISH to surface spread synaptonemal complexes of *Ruditapes philippinarum* (RPH) and *Venerupis corrugata* (VCO) clearly denote the subterminal location of the H3 histone gene clusters (H3, green). 5S rDNA clusters (5S, red) are also subterminal in VCO but intercalary in RPH. Scale bars, 5 μm

Table 1 Chromosomal location of core histone gene and rDNA clusters in venerid clams

Clade	Subfamily	Species	Histone genes	Major rDNA	5S rDNA	References
A1	Tapetinae	*Ruditapes philippinarum*	4q ter (m)	8p ter (m)	5q ic (st)	[29, 30] this study
					6q ic (st)	
		Ruditapes decussatus	4p cen (st)	3q ic (sm)	3q ic (sm)	[29, 30] this study
					8q ter (st)	
		Polititapes aureus	2p ter (m)	5p ic (m)	17q ter (st)	[15]
			2q ter (m)			
			3q ter (m)			
			8q ter (m)			
		Venerupis corrugata	2q ter (m)	10q ic (m)	9q ter (st)	[29] this study
			4q ter (sm)			
		Polititapes rhomboides	5q ic (m)	17q ter (st)	9p ter (m)	[15]
			12q cen (st)			
A2	Chioninae	*Clausinella fasciata*	10q ter (m)	10q cen (sm)	5p ic (sm)	this study
			12p ter (sm)			
		Chamelea gallina	15q ter (m)	13p cen (sm)	5p ic (sm)	this study
			18q ter (st)		9q ic (m)	
	Venerinae	*Venus verrucosa*	13q ter (m)	12p ter (sm)	9q ic (m)	[29] this study
		Venus casina	9q ter (m)	16q ter (st)	6p ic (sm)	this study
A3	Chioninae	*Mercenaria mercenaria*	unknown	10q ic (sm)	unknown	[27]
				12p ter (st)		
A4	Dosininae	*Dosinia exoleta*	2q ic (m)	3q ter (m)	13q ic (sm)	[28, 29] this study
					15p ter (sm)	
		Dosinia lupinus	9q ic (m)	12q ic (m)	14q ter (sm)	this study
B1	Petricolinae	*Petricola litophaga*	3p ter (m)	19p cen (m)	5q ic (m)	this study
			14q ter (m)		12q ic (st)	
					12q ic (st)	

p short arm, *q* long arm
cen subcentromeric, *ic* intercalar, *ter* subterminal
(m) metacentric, *(sm)* submetacentric, *(st)* subtelocentric

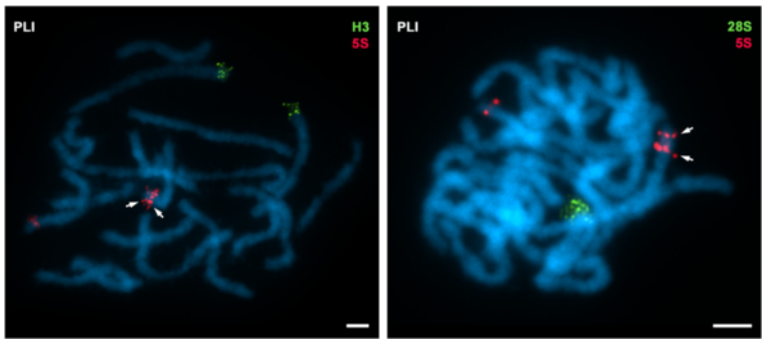

Fig. 4 5S rDNA clusters on *Petricola litophaga*. FISH of H3 histone gene (H3), 5S (5S) and 28S (28S) rDNA probes to prophase I meiotic bivalents of *Petricola litophaga* (PLI) clearly show the presence of two distinct 5S rDNA signals (arrows) on a single bivalent. Scale bars, 5 μm

and a subcentromeric major rDNA cluster appeared in the long arm of chromosome 10 (Fig. 2). Confirming previously published results, in *Ruditapes decussatus* the signals for one of the 5S rDNA clusters and the major rDNA cluster overlap on chromosome 3 (Fig. 1).

Figure 5 presents an ideogrammatic representation of the karyotypes of the 13 species of Veneridae for which histone gene and/or rDNA mapping results are currently available.

FISH experiments using a vertebrate telomeric $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe gave terminal signals at the ends of the sister chromatids of every mitotic chromosome in all clam species. No intercalary signals were detected.

Discussion

Multigene families are useful cytogenetic markers not only for studying chromosomal evolution but also for the correct interpretation of the data obtained via NGS. Aligning and assembling NGS data is a hard task in many non-model organisms mainly due to the obstacles posed by the abundance of repetitive DNA sequences. The physical location of repetitive gene families will help in this task.

In this work we have demonstrated the presence of remarkable interspecific differences in the physical location of H3 histone gene clusters in venerid clams. These clusters have been described as highly conserved chromosomal markers in other invertebrate groups. Our data, together with previously published results [23], showed variation for the number of core histone gene sites in venerid clams, with six species carrying a single cluster, five presenting two clusters and one showing four clusters (Table 1). The observed variation in the number of core histone gene clusters did not present any obvious relationship with the currently taxonomic classification of the family Veneridae or its most represented clades. In clade A1 (Tapetinae) there were species showing one (*Ruditapes philippinarum* and *Ruditapes decussatus*), two (*Venerupis corrugata* and *Polititapes rhomboides*) and four (*Polititapes aureus*) histone gene clusters. A similar divergence also applied to clade A2, including species of the subfamilies Venerinae and Chioninae, in which two species (*Venus verrucosa* and *Venus casina*) showed a single cluster and the other two species (*Clausinella fasciata* and *Chamelea gallina*) presented two clusters.

These differences in the number of core histone gene clusters in related species are coincident with results reported for other bivalve families. While three species of scallops and one mussel showed a single core histone gene cluster [17, 21], one scallop and six mussels presented two [17, 18, 20, 21] and one mussel had four [19]. This behavior differs with those described for other invertebrate groups in which histone gene clusters have been reported to be a highly conservative cytogenetic marker [5–10].

In contrast with the variability in number, 75 % of the H3 histone gene clusters detected in venerid clams (15 of a total of 20) were located at subterminal chromosome positions. This is unusual for bivalves and also for other invertebrates; in fact, barely a 30 % of the core histone gene clusters detected in mussels of the family Mytilidae (5 of 17) [17–20] and only a 40 % of those reported in the scallops of the family Pectinidae (2 of 5) [21] were subterminal. For other invertebrates, the subterminal position of the histone gene signal has only been described in three grasshoppers [5].

Taking into account the above mentioned data, the presumably ancestral situation in venerids is a single subterminal core histone gene cluster. Although the mechanisms that allowed increasing the number of clusters remain to be determined, the presence of most of these sequences in close proximity to the telomeres might facilitate their spreading to non-homologous chromosomes. Subtelomeric chromosomal regions are characterized in many eukaryotes by accumulating repeat sequences and harbor many breakpoints [39, 40]. These features, together with the telomere clustering in meiotic cells, probably favors their implication in sequence exchanges between

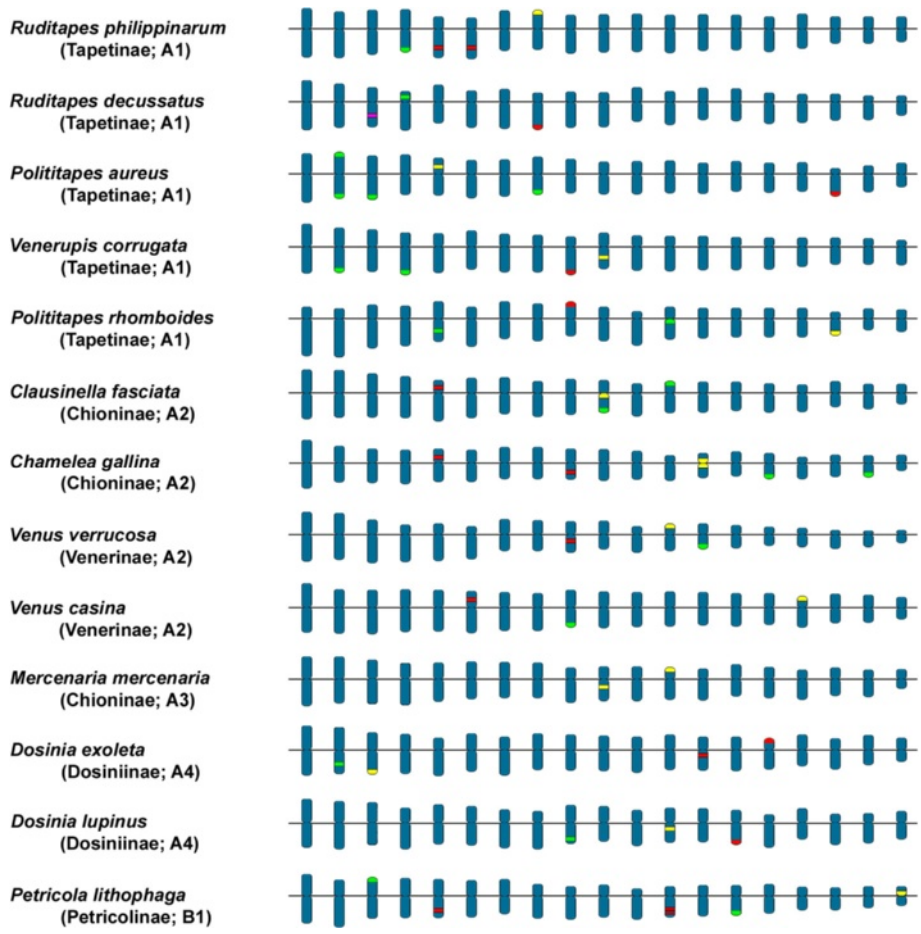

Fig. 5 Ideograms showing the chromosomal location of H3 histone genes, 5S rDNA and major rDNA in thirteen species of Veneridae. The green areas represent the H3 histone gene clusters, the red areas the 5S rDNA clusters and the yellow areas the major rDNA clusters. The magenta area in *Ruditapes decussatus* indicates overlapping of major and 5S rDNA signals

non-homologous chromosomes and contribute to their highly dynamic behavior [41].

Regarding rDNA, the clams of the family Veneridae showed variation in both number and chromosomal location of the 5S rDNA clusters but only in location of the 45S rDNA clusters. Whereas all species had a single 45S rDNA cluster, the number of 5S rDNA clusters varied; seven species showed a single cluster, six species two and *Petricola litophaga* three. The conservation in the number of major rDNA clusters in Veneridae was not paired by their chromosomal location; subterminal, intercalary and subcentromeric locations were found. The 5S rDNA clusters were either subterminal or intercalary. As happened with H3 histone gene clusters, the variant 45S and 5S rDNA arrangements did not concord with the taxonomic distribution of the species of the family Veneridae; species belonging to the same clade or subfamily showed 45S and 5S rDNA clusters differing in number and chromosome location (Table 1).

These results partially differ with those found in other bivalve families. While mytilid mussels showed one to four major rDNA and two to five 5S rDNA clusters [17–20, 42], both Pectinidae and Ostreidae species presented one or two major and 5S rDNA clusters [31, 43–47].

The evolutionary dynamics of the major rDNA clusters in venerid clams is similar to the one reported for tortricid moths [10] but the opposite to the described in other invertebrate groups in which both the number and the position of these sequences has been reported as highly variable [5–8]. In contrast, the behavior of the 5S rDNA is common to some other invertebrate taxa in which it has been described as a highly variable chromosomal marker [5–8] whose movement has been attributed to transposition and/or unequal crossover [48].

In conclusion, the diversity in the distribution patterns of histone H3 gene, 5S rDNA and 28S rDNA clusters found in venerid clams, together with their different evolutionary behaviors in other invertebrate taxa, strongly

suggest that the control of the spreading of these multigene families in a group of organisms relies upon a combination of evolutionary forces that operate differently depending not only on the specific multigene family but also on the particular taxa. On the other hand, our data clearly showed that the number and position of the H3 histone gene and rDNA clusters are species-specific in venerid clams and that the complexity of their evolutionary patterns make them useful landmarks that can contribute to integrate NGS and evolutionary genomic data in non model species.

Methods

Venus clam specimens

Samples of the Japanese carpet shell *Ruditapes philippinarum* (Adams and Reeve 1850), the grooved carpet shell *Ruditapes decussatus* (Linnaeus 1758), the pullet carpet shell *Venerupis corrugata* (*pullastra*) (Gmelin 1791), the banded venus *Clausinella fasciata* (da Costa 1778), the warty venus *Venus verrucosa* (Linnaeus 1758), the pale venus *Venus casina* (Linnaeus 1758), the rayed artemis *Dosinia exoleta* (Linnaeus 1758), the smooth artemis *Dosinia lupinus* (Linnaeus 1758), and the boring petricola *Petricola litophaga* (Retzius1788) were collected from natural and cultured populations in Ría de Pontevedra and Ría de Vigo (NW Spain). Samples of the striped venus *Chamelea gallina* (Linnaeus 1758) were collected from natural populations in the Gulf of Valencia (E Spain). The nomenclature used for these taxa follows the World Register of Marine Species database [49].

Chromosome preparation

Mitotic metaphase and meiotic prophase I spreads were prepared as previously described [50]. In brief, after exposing the clams to colchicine (0.005 %, 12 h), gills and gonads were removed. The tissues were treated with diluted sea water (50 %, 25 %, 1 h) and fixed in ethanol/acetic acid (3:1, 1 h). The cell suspension obtained after dissociating the tissue (60 % acetic acid) was dropped onto heated slides.

Synaptonemal complexes were spread as indicated by Hurtado and Pasantes [36]. Suspensions of male gonadic cells were spread on slides using 0.1 M sucrose and 0.5 % Triton X-100, fixed with paraformaldehyde (4 %), rinsed in distilled water and air-dried.

DNA extraction, PCR amplification and probe labeling

DNA was extracted using the method published by Winnepenninckx et al. [51]. The tissue was homogenized in hexadecyltrimethylammoniumbromide (CTAB) buffer and digested with pronase (1.5 mg/mL, 60 °C). The DNA was extracted with chloroform/isoamyl alcohol (24/1).

FISH probes were amplified by polymerase chain reaction (PCR). Reactions used 50 ng DNA, 1x PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 1 μM each primer and 1 U BIOTAQ DNA polymerase (Bioline) in a volume of 20 μl. A fragment of the 28S rRNA gene of the major rDNA repeat was amplified using universal primers [52]. Primers designed from the sequence of the 5S rRNA of *M. edulis* [53] were used to amplify the whole repeat of the 5S rDNA. The amplification of the histone H3 genes used primers described by Giribet and Distel [54].

After an initial denaturation at 95 °C, 30 cycles (95 °C, 20 s; 48 °C, 20 s; 72 °C, 30 s) of amplification and a final extension step of 7 min at 72 °C were applied in a GeneAmp PCR system 9700 (Applied Biosystems). Electrophoresis on 2 % agarose gels demonstrated that single PCR products were obtained. 28S rDNA probes were labeled by nick translation (Roche Applied Science) with biotin-16-dUTP (Roche Applied Science) and/or digoxigenin-11-dUTP (10x DIG Labeling Mix, Roche Applied Science). Histone H3 gene and 5S rDNA probes were labeled by PCR either with biotin-16-dUTP (20 μM) or digoxigenin-11-dUTP (5 μM). The labeled PCR products were precipitated before FISH.

Fluorescent *in situ* hybridization (FISH)

Single and double FISH experiments using biotin and digoxigenin labeled histone H3 gene and 28S and 5S rDNA probes were performed following previously published methods [19, 37]. Slides were pre-treated with RNase and pepsin before denaturating them for 2 min at 70 °C (mitotic chromosomes) or 80 °C (meiotic chromosomes). Hybridizations were performed overnight at 37 °C. Signal detection was carried out with fluorescein avidin and biotinylated anti-avidin for the biotinylated probes and with mouse anti-digoxigenin and anti-mouse TRITC for the probes labeled with digoxigenin. Slides were counterstained for 8 min with 4′-6-diamidin-2-fenilindol (DAPI: 0.14 μg mL^{-1} in 2xSSC) and mounted in antifade (Vectashield, Vector). In addition we also performed FISH with a vertebrate telomeric $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) following the protocol indicated by the supplier.

A Nikon Eclipse-800 microscope equipped with an epifluorescence system was used to record a minimum of 20 metaphase plates per probe or combination of probes in 10 specimens (5 male, 5 female) per species. Separated images for each fluorochrome were obtained with a DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon). Merging of the images was performed with Adobe Photoshop.

For each species, karyotypes were constructed from 10 complete metaphase plates showing FISH signals.

Relative lengths and centromeric indices were determined. Chromosomes nomenclature follows Levan et al. [55].

Abbreviations

CFA: *Clausinella fasciata*; CGA: *Chamelea gallina*; DAPI: 4′,6-diamidino-2-phenylindole; DEX: *Dosinia exoleta*; DLU: *Dosinia lupinus*; FISH: Fluorescence *in situ* hybridization; NGS: Next-generation sequencing; PCR: Polymerase chain reaction; PLI: *Petricola litophaga*; PNA probe: Peptide nucleic acid probe; RDE: *Ruditapes decussatus*; rDNA: Ribosomal DNA; RPH: *Ruditapes philippinarum*; rRNA: Ribosomal RNA; TRITC: Tetramethyl rhodamine isothiocyanate; VCA: *Venus casina*; VCO: *Venerupis corrugata*; VVE: *Venus verrucosa*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DGS did most part of the cytogenetic procedures and collaborated on the molecular work, the bibliographic review and the writing of this paper. CPG helped with the cytogenetic procedures and the writing of the manuscript. PM participated in developing the molecular techniques and helped in the writing. JJP coordinated the study, helped in developing the laboratory techniques and cytogenetic analyses and coordinated the writing of the manuscript. All authors read and approved the final manuscript.

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Resolving the taxonomic status of *Chamelea gallina* and *C. striatula* (Veneridae, Bivalvia): A combined molecular cytogenetic and phylogenetic approach

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The authors declare that there is no conflict of interests regarding the publication of this article

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Abstract

The striped venus clams *Chamelea gallina* and *C. striatula* are commercially important bivalves inhabiting European and North African coastal waters. The taxonomic status of these taxa has been the subject of debate for decades. In order to elucidate this issue, we generated 5S and 28S ribosomal RNA and H3 histone gene probes and mapped them by fluorescent *in situ* hybridization to the chromosomes of morphologically identified striped venus clams, collected from four geographically distant Atlantic and Mediterranean populations. The nucleotide variation at the three DNA markers, i.e., the nuclear internal transcribed spacer 2 (ITS2), the mitochondrial cytochrome c oxidase subunit I (COI) and the large ribosomal subunit rRNA (16S) fragments, was also studied and resultant phylogenetic trees evaluated. Striking differences in both the chromosome distribution of these genes and in the clustering of the samples on the phylogenetic trees observed provide clear evidence that *C. gallina* and *C. striatula* are separated species.

1. Introduction

The striped venus clams *Chamelea gallina* (Linnaeus, 1758) and *C. striatula* (da Costa, 1778) (Bivalvia: Veneridae), the only extant species in the genus *Chamelea* Mörch, 1853, are filter feeding, sand burrowing bivalves inhabiting European and North African coastal waters. Even though these taxa are commercially exploited and economically valuable, for decades their taxonomic status has been a matter of debate [1] that is still not completely settled. The confused status of these taxa is partially due to difficulties associate in using of shell and siphon morphology, alongside geographical origin to identify them. *C. striatula* is distinguished from *C. gallina* by possessing a more pointed and ridged shell and longer siphons [2] and by residing in the Atlantic [3]. On the basis of shell and siphon morphologies, *C. striatula* and *C. gallina* have been considered members of a single polymorphic species, two geographically isolated subspecies or two different species. The lack of modern genetic studies applied to differentiate *C. gallina* and *C. striatula* also contribute to their uncertain status. The genetic distances estimated after studying seven polymorphic enzymes in samples obtained from striped venus clam mixed beds in the

south of Portugal [2], supporting their status as separated species, is, as of yet, the only genetic study applied to these taxa.

Although shell shape analyses [4] and geometric morphometric methods [3] have helped in accurately predicting the origin of some *C. gallina* samples and differentiating *C. gallina* from *C. striatula*; establishing taxonomic boundaries within bivalves has always been hindered by a lack of diagnostic morphological features [5]. This is due to an extensive parallelism of interspecific variability as a result of convergent evolution in response to the same environment alongside a degree of phenotypic divergence among populations of a single taxon developed on different substrates. Therefore, it is inaccurate to rely on morphology alone to delimit species boundaries and further criteria have to be analyzed [5].

In comparison with many other groups of organisms, cytogenetic analyses in clams of the family Veneridae are scarce. All species studied to date show a diploid chromosome number of $2n = 38$ [6, 7] and their karyotypes are mostly composed of chromosome pairs showing small differences in size and morphology, therefore making almost impossible their accurate identification [7]. As a resolution to this problem, along the last two decades highly conserved repetitive DNA sequences have been used as probes in fluorescent *in situ* hybridization (FISH) experiments to identify chromosome pairs in bivalve species [8-9] some of which were venus clams [7,10-14]. Most commonly used DNA sequences for these types of studies are ribosomal RNA (rRNA) and histone genes as they are usually organized in tandems and clustered at one or more chromosomal positions. The employment of this approach in mussels of the *Mytilus edulis* species complex has allowed for differentiating *M. edulis* and *M. galloprovincialis* from *M. trossulus* by the chromosomal location of one of the H3 histone gene clusters and the number and location of the major rRNA gene (rDNA) clusters [15].

Chromosomal studies on striped venus clams are rarer than in other venerids. To the best of our knowledge, there is no published karyological information for *C. striatula*. For *C. gallina*, information is mostly limited to two reports describing chromosome number and karyotype composition and the location of major rDNA, 5S rDNA and H3 histone gene clusters [7, 16].

In regards to molecular phylogenetic analysis, many studies have utilized the mitochondrial cytochrome c oxidase subunit I (COI) gene as the standard most accurate

marker for delimitating and clarifying the taxonomic status of animal species, including venerid clams [17]. The mitochondrial 16S rRNA gen has also been successfully employed for species identification in clams [17, 18]. These mitochondrial sequences are known to evolve faster than nuclear genes, making them ideal tools for detecting differences among closely related species and among populations within a species. In addition, the sequences of the internal transcribed spacer 2 (ITS2) of the 18S-5.8S-28S nuclear rDNA, and their corresponding secondary structures of the ITS2 rRNAs, have also probed suitable for assessing phylogenetic and phylogeographic relationships among many animal taxa, some of them venerid clams [19].

In order to contribute to a better understanding of the evolutionary history and diversification within the genus *Chamelea*, we used an integrated approach based, on the one hand, on the comparison of the karyotypes constructed after mapping by FISH three tandemly repeated gene families (5S rDNA, 28S rDNA and H3 histone genes) and, on the other, in the molecular phylogenies obtained from one nuclear (ITS2) and two mitochondrial (COI and 16S rRNA genes) sequences in striped venus clams collected from two Atlantic populations (Ría de Pontevedra, NW Spain; Gulf of Cádiz, S Spain) and two Mediterranean ones (Gulf of Valencia, E Spain; Adriatic Sea, E Italy). The genetic evidence obtained in this work confirms the consideration of *C. gallina* and *C. striatula* as separated species.

2. Materials and methods

2.1. Sampling and identification. Specimens of striped venus clams were collected (Figure 1) from different localities in Ría de Pontevedra (Atlantic coast, NW Spain) and identified as *C. striatula*, through their morphological attributes. Other striped venus clam samples, identified as *C. gallina*, were collected from natural beds in Valencia (Gulf of Valencia, Mediterranean coast, E Spain), and from local market places in coastal towns of the Gulf of Cádiz (Atlantic coast, S Spain) and the Adriatic Sea (Mediterranean coast, E Italy). The nomenclature utilized for these taxa follows the World Register of Marine Species (WoRMS) database (http://www.marinespecies.org/). In all cases, striped venus clams were transported alive to the lab, maintained in tanks of 5 L filtered seawater at 18 ± 1°C and fed on microalgae to promote somatic growth.

2.2. Chromosome preparation. Chromosome spreads were obtained as previously published [20, 21]. Following an *in vivo* colchicine (0.005%, 10 h) treatment, striped venus clams were dissected and gills and gonads immersed in diluted sea water (50%, 20 min; 25%, 20 min). After fixation in ethanol /acetic acid (3:1; 3 times 20 min), pieces of tissue were disaggregated in 60% acetic acid and the cell suspensions spread onto warm microscope slides [22, 23].

2.3. DNA extraction and PCR amplification. DNA was extracted from adductor muscles using a phenol-chloroform-isoamyl alcohol method [24]. A fragment of the mitochondrial COI gene was amplified by PCR employing the standard barcoding primers *LCO1490* and *HCO2198* [25]. A fragment of the mitochondrial 16S rRNA gene was amplified by means of primers *16L29* [26] and *16SBr* [27]. The complete ITS2 of the major rDNA was amplified using primers *ITS3* and *ITS4* [28]. On FISH mapping purposes, universal primers *LR10R* and *LR12* retrieved from Vilgalys lab website [29] were used to amplify a fragment of the 28S rDNA. Amplifications of the entire 5S rDNA repeat and the H3 histone genes used primers described by Pérez-García et al. [22, 23] and Giribet and Distel [30], respectively.

DNA sequences were amplified in a GeneAmp PCR system 9700 (Applied Biosystems) in 50 μL solutions containing 125 ng of genomic DNA, 50 μM each dNTP, 50 μM each primer, 1xPCR buffer, 15 μM $MgCl_2$ and 5 U of JumpStart™ Taq DNA Polymerase (Sigma). Amplifications included an initial denaturation step at 95 ºC (2 min), 35 amplification cycles (Supplementary Table 1) and a final extension at 72 °C (5 min). PCR products were examined by electrophoresis on a 2% agarose gels.

2.4. DNA sequencing and phylogenetic analysis. Amplified mitochondrial 16S rRNA and COI genes and nuclear ITS2 rDNA sequences were purified (FavorPrep™ GEL/PCR Purification Kit, Favorgen) and sequenced (CACTI, University of Vigo) in both directions in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were edited with BioEdit v. 7.1.11 [31] and aligned with Muscle set to default parameters using MEGA7 [32]. Sequence similarity searches were performed using the Basic Local

Alignment Search Tool algorithm (BLAST), available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The MegaBLAST algorithm set to default parameters was employed against both NCBI nucleotide collection and NCBI nucleotide collection and Barcode of Life Data System (BOLD) databases. After removing primers, maximum likelihood (ML) phylogenetic analyses were performed. The best-fit nucleotide substitution models were selected (COI gene: JC+G; 16S rRNA gene: HKY+G; ITS2: T92) by the AIC criterion employing JModelTest 2 [33, 34]. ML reliability was assessed with 500 bootstrap replicates. All phylogenetic analyses were performed on MEGA7 [32]. Nucleotide diversity (pi) and uncorrected pairwise *p*-distances were estimated using DnaSP v5 [35] and MEGA7 [32], respectively.

2.5. Fluorescence in situ hybridization (FISH). Single, double and sequential FISH experiments using 5S and 28S rDNA and H3 histone gene probes were performed on metaphase chromosome spreads obtained from striped venus clams collected in all four regions. Biotin and digoxigenin labeled probes were generated either directly by PCR or by nick translation [36]. Chromosome slides were digested with RNase and pepsin before denaturation (70 ºC, 2 min) and hybridized overnight at 37 ºC. Biotin was detected with fluorescein isothiocyanate (FITC) conjugated avidin and biotinylated anti-avidin (Vector) whereas digoxigenin was detected with anti-digoxigenin antibodies conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Sigma). Chromosome slides were counterstained with 4′-6-diamidin-2-fenilindol (DAPI, 0.14 μg/mL in 2xSSC) and mounted with antifade (Vectashield, Vector).

Chromosome preparations were examined with a Nikon Eclipse-800 microscope equipped with an epifluorescence system [36]. Separated images for each fluorochrome were recorded and pseudo-colored using a DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon). Merging of the images was performed with Adobe Photoshop.

3. Results

3.1. Karyotypes and chromosomal mapping of rDNA and H3 histone gene clusters

All striped venus clams analyzed showed mitotic metaphase plates presenting 38 chromosomes (Figure 2). The karyotypes constructed for the four striped venus clam populations (Figure 2) were roughly similar for 18 of the chromosome pairs (7 metacentric, 6 meta/submetacentric, 2 submetacentric and 3 subtelocentric) but the remaining one (#19 for comparative purposes) showed morphological differences. This chromosome pair was subtelocentric in both *C. striatula* and the Italian *C. gallina* and metacentric in the two Spanish *C. gallina* populations.

FISH mapping of 5S rDNA probes showed intercalary signals located in two metacentric chromosome pairs (5p and 9q) in all striped venus clams regardless of origin and whether they were morphologically identified as *C. gallina* or *C. striatula* (Figure 2). In contrast, the number and the distribution of the signals corresponding to both 28S rDNA and H3 histone gene probes evinced some differences. All striped venus clam specimens presented at minimum a single 28S rDNA signal situated in the neighborhood of the centromere on chromosome pair 19; whereas this is the only signal in *C. gallina*, an additional signal subterminal to 8q appeared in *C. striatula* (Figure 2). Likewise, although H3 histone gene probes were subterminal to 15q and 17q in all striped venus clams, further signals were present in both the Italian *C. gallina* population (6q) and in *C. striatula* (6q and 5q).

Double-color FISH mapping using 5S rDNA and H3 histone gene probes also confirmed the presence of signals for both probes on *C. striatula* chromosome pair 5. This chromosome pair only bears 5S rDNA clusters in *C. gallina*.

In all four populations, variation in signal patterning was low, and aberrance was reduced to a pericentric inversion or an additional 28S rDNA signal in four of the 55 *C. striatula* specimens and one additional 28S rDNA signal in two of the 28 specimens of the Italian *C. gallina*.

3.2. DNA sequence variation and genetic divergence

A fragment of 629 bp, excluding primers, of the mitochondrial COI gene was sequenced for 20 striped venus clams, five per population (GenBank acc. nos. KY547747 to KY547766). The sequences showed 89 polymorphic sites and 78 differentially fixed mutations. The nucleotide diversity was 0.0556 (Table 1) and the genetic distance between

taxa 13.20% (Table 2). The comparison of these sequences with those stored in GenBank showed that our *C. striatula* sequences coincided in a 99% with those from North Sea *C. gallina* samples [37]. The *C. gallina* COI sequences displayed a high level of homology (98%) with samples from the Adriatic Sea (GenBank acc. nos. DQ458474 and KR078004) and a market place in the Canary Islands (GenBank acc. no. DQ184835). Pairwise genetic distances between populations inferred from the mitochondrial COI gene sequences (Table 2) showed that *C. striatula* is clearly distinct from the three *C. gallina* populations. Likewise, the ML tree recovered by MEGA on these sequences (using *Dosinia exoleta* as outgroup) revealed two well supported clades, one formed by the specimens morphologically identified as *C. striatula* and the other by the specimens identified as *C. gallina* with bootstrap values of 100% and 86%, respectively (Figure 3).

The sequenced 16S rRNA gene fragments were 468 bp long in the specimens collected from Pontevedra (*C. striatula*) and 466 bp long in all *C. gallina* specimens (GenBank acc. nos. KY547767 to KY547786). The difference in length was due to six between-taxa gaps; two single nucleotide and one dinucleotide insertions in *C. striatula* and two single nucleotide insertions in *C. gallina*. There were 51 polymorphic sites, 48 of them taxa specific, providing a genetic distance between taxa of 11.44%. The nucleotide diversity was 0.0443 (Table 1). These 16S rRNA gene sequences were also compared with the six sequences stored in GenBank. The *C. striatula* sequences were identical to sequences from England (GenBank acc. nos. DQ280041 and KX713203) whereas those from *C. gallina* matched the sequences of specimens from Turkey (GenBank acc. no. AM085110), Adriatic Sea (GenBank acc. no. AJ548762), NW Spain (GenBank acc. no. JF808193) and a market in the Canary Islands (GenBank acc. no. DQ184735). Again, both the pairwise distances (Table 2) and the ML tree recovered (not shown) differentiated specimens belonging to the two taxa (bootstrap values of 100% and 72%).

The amplified ITS2 fragments (GenBank acc. nos. KY508254 to KY508283) were the most inconsistent in length. Whilst all specimens of the Mediterranean populations of *C. gallina* displayed 498 bp long fragments, in the Atlantic specimens the ITS2 was 496 bp long; these differences in length were resultant of a dinuleotide insertion (or deletion) and six nucleotide substitutions. In contrast, five specimens of the *C. striatula* population displayed 498 bp long ITS2 fragments whereas the remaining 10 exhibited 495 bp long fragments, due to a trinucleotide insertion and two point mutations. Sequence analysis of

the ITS2 for all striped venus clams revealed 34 polymorphic sites and, excluding gaps, 13 differentially fixed mutations between the two taxa. The nucleotide diversity was 0.0202 (Table 1) and the genetic distance between taxa 3.52% (Table 2). While the Atlantic *C. gallina* sequences obtained in this work were identical to those from Tyrrhenian Sea specimens (GenBank acc. nos. HE965773 and HE965774), the two Mediterranean populations ITS2 sequences coincided with those from Adriatic Sea samples (GenBank acc. nos. HE965771 and HE965772). No *C. striatula* ITS2 sequences were found in GenBank. The pairwise genetic distances between populations inferred from nuclear ITS2 sequences (Table 2) also depicted that *C. striatula* is clearly separated from the three *C. gallina* populations and the ML tree also recovered two clades with bootstrap values of 100% and 84% (Figure 3).

In addition, the predicted folding shapes of the *C. gallina* ITS2 rRNAs (Supplementary Figure 1) were identical to those previously published for the same species [19]; those for *C. striatula* were concordant with the structure proposed for Veneridae [19]. Most point mutations detected (17 of a total of 20), including those differentiating taxa and populations, were clustered on the DIV-DVI stems, the less conserved area in terms of primary sequence [19].

4. Discussion and conclusion

The taxonomic status of the striped venus clams *C. gallina* and *C. striatula* has been a matter of debate for decades [1]. Although the World Register of Marine Species have recognized them as separated species since 2004 [38, 39], the distribution ranges of these taxa displayed in WoRMs, from the North Atlantic to the Eastern Mediterranean, still overlap in almost their entirety. Moreover, even as recently as last year COI gene sequences obtained from North Sea samples, therefore *C. striatula*, were stored in GenBank under the specific name *C. gallina* [37]. In order to resolve this issue we applied a molecular cytogenetic approach, from the perspective of chromosomal distribution of three gene families [7, 12-14], alongside comparing the sequences of mitochondrial and nuclear DNA markers, that are increasingly being utilized in phylogenetic studies [5, 17-19, 40, 41], to further insight and strengthen evidence.

The diploid chromosome number of $2n = 38$ obtained in this work is in accordance with those previously described for *C. gallina* [7, 16] and all other Veneridae studied to date [6-8, 12-14]. The karyotype compositions obtained were also fundamentally coincidental with that proposed for *C. gallina* [16].

Discordant with their conserved chromosome numbers, the species of Veneridae present clear differences in the number and distribution of rDNA and histone gene clusters on their chromosomes [7, 12-14]. These differences are also present in closely related, congeneric species and are usually accompanied of an almost complete absence of intraspecific variability [7, 12-14].

Our results demonstrated that this is also the case for the two striped venus clam taxa studied. The consistency in the 5S rDNA signal pattern and the mapping differences for both 28S rDNA and H3 histone gene signals found between *C. gallina* and *C. striatula* are of a similar magnitude to those reported for *Venus casina* / *V. verrucosa* and *Dosinia exoleta* / *D. lupinus* [7]. Conversely, although we found some mapping differences among *C. gallina* populations and among *C. striatula* specimens, these differences were comparatively narrower than those between taxa and similar to those found in other bivalve species [23, 36]; thusly constituting the standard intraespecific variation.

The sequence data obtained also indicated that these two taxa are separated species. All individual ML trees recovered by MEGA on the COI gene, 16S rRNA gene and ITS2 sequences (using *Dosinia exoleta* as outgroup) revealed two clearly separated, well supported clades, one formed by the specimens morphologically identified as *C. striatula* and the other by the specimens identified as *C. gallina*. Furthermore, the genetic distances between the *C. striatula* population and any of the *C. gallina* populations were indubitably higher than those between any two *C. gallina* populations for both mitochondrial and nuclear sequences. The magnitudes of these genetic distances were fully concordant with those previously reported for congeneric species of Veneridae, both for the mitochondrial COI gene [5] and the nuclear ITS2 [19] sequences analyzed.

In conclusion, the results obtained in this study after employing two mitochondrial and one nuclear DNA markers together with three chromosomal markers in four geographically distant populations of striped venus clams clearly demonstrate that *C. gallina* and *C. striatula* are well differentiated species.

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Figure legends

FIGURE 1: Collection localities and representative shells of the striped venus analyzed.

FIGURE 2: FISH mapping of 5S rRNA (5S, red), 28S rRNA (28S, magenta) and H3 histone gene (H3, green) probes to mitotic metaphase chromosomes of striped venus clams *Chamelea striatula* from Pontevedra (CST Pon) and *Chamelea gallina* from Cadiz (CGA Cad), Valencia (CGA Val) and Italy (CGA Ita) counterstained with DAPI. The corresponding karyotypes and schematic representations of the signal bearing chromosomes are also included. Note that all chromosome pairs present a single signal with the exception of CST Pon chromosome pair 5 that bears both 5S rDNA and H3 histone gene clusters. Scale bars, 5 μm.

FIGURE 3: Maximum likelihood trees based on mitochondrial COI gene and nuclear ITS2 sequences of striped venus clams using *Dosinia exoleta* as outgroup together with an

schematic representation of the main chromosomal differences. Numbers in internal nodes indicate maximum-likelihood bootstrap support values (500 replicates).

Supplementary Figure legend

SUPPLEMENTARY FIGURE 1: Secondary structures predicted for the *Chamelea striatula* (CST Pon 495 bp; CST Pon 498 bp) and *Chamelea gallina* (CGA Cad 496 bp; CGA Ita 498 bp) ITS2 rRNA. Nucleotide changes between taxa are marked in yellow and those inside taxon in red.

Table 1: Nucleotide diversity (pi) of COI gene, 16S rDNA and ITS2 sequences in *Chamelea gallina* and *Chamelea striatula*

	COI gene		16S rDNA		ITS2	
	n	pi	n	pi	n	pi
Chamelea striatula						
CST Pon	5	0.0022	5	0.0000	15	0.0021
Chamelea gallina						
CST Cad	5	0.0000	5	0.0000	5	0.0000
CST Val	5	0.0000	5	0.0000	5	0.0000
CST Ita	5	0.0057	5	0.0035	5	0.0000
All CST	15	0.0060	15	0.0030	15	0.0063
All *Chamelea*	20	0.0556	20	0.0443	30	0.0202

Table 2. Pairwise *p* distances between striped venus clam populations using mitochondrial COI and 16S rRNA genes and nuclear ITS2 sequences. Interspecific distances in bold

Populations		COI gene	16S rDNA	ITS2
CST Pon	CGA Cad	**0.1304**	**0.1121**	**0.0396**
CST Pon	CGA Val	**0.1320**	**0.1164**	**0.0330**
CST Pon	CGA Ita	**0.1335**	**0.1147**	**0.0330**
CGA Cad	CGA Val	0.0095	0.0043	0.0132
CGA Cad	CGA Ita	0.0086	0.0047	0.0132
CGA Val	CGA Ita	0.0048	0.0022	0.0000
CST	CGA	**0.1320**	**0.1144**	**0.0352**

FIGURE 1

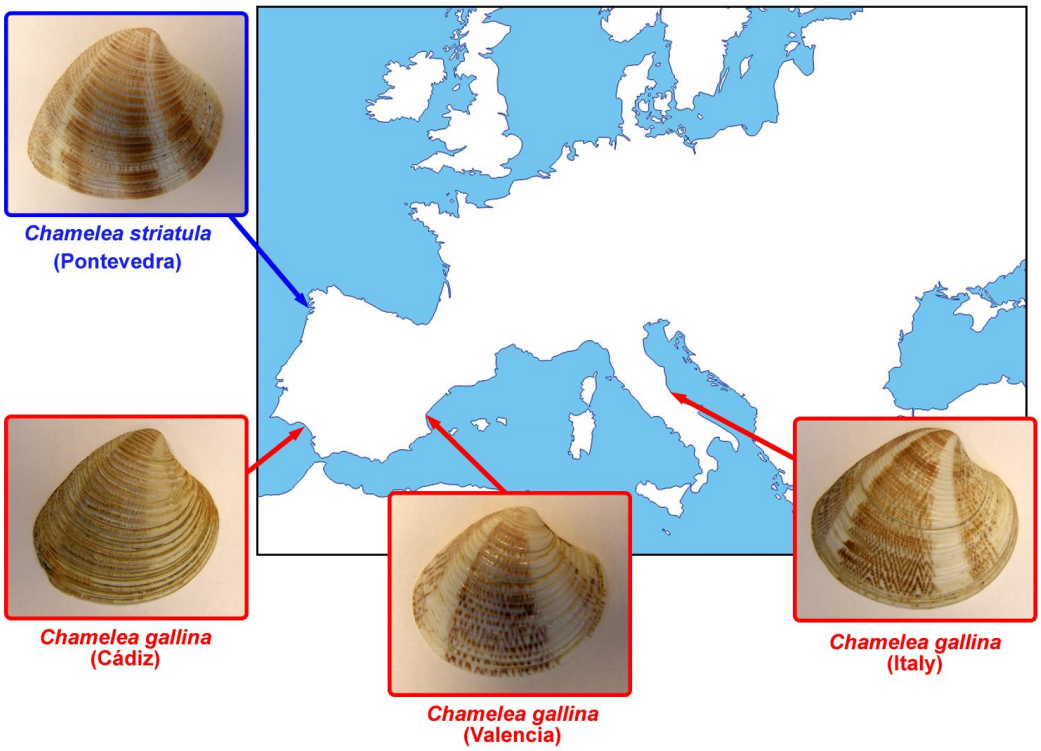

Chamelea striatula
(Pontevedra)
Chamelea gallina
(Cádiz)
Chamelea gallina
(Valencia)
Chamelea gallina
(Italy)

FIGURE 2

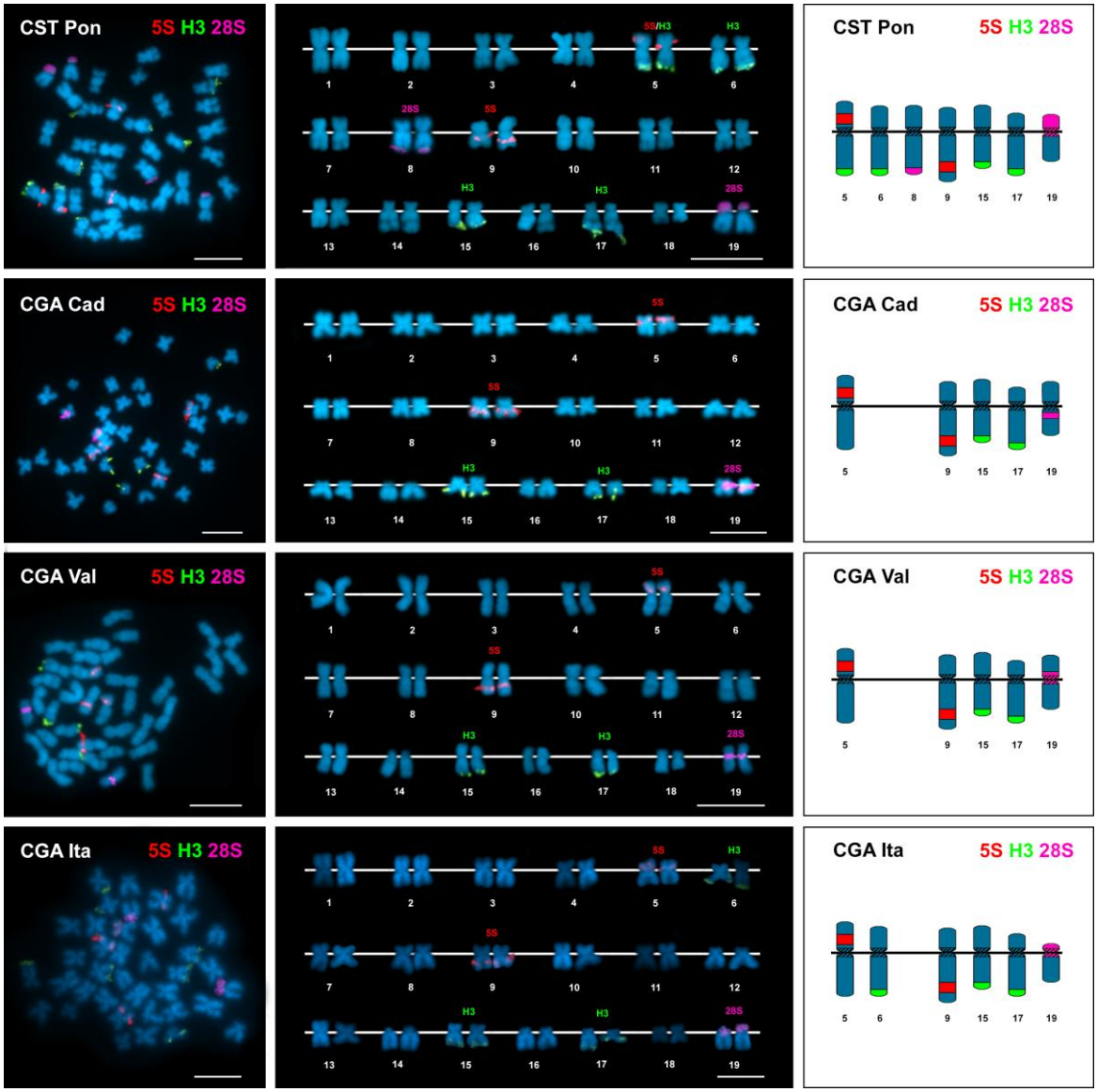
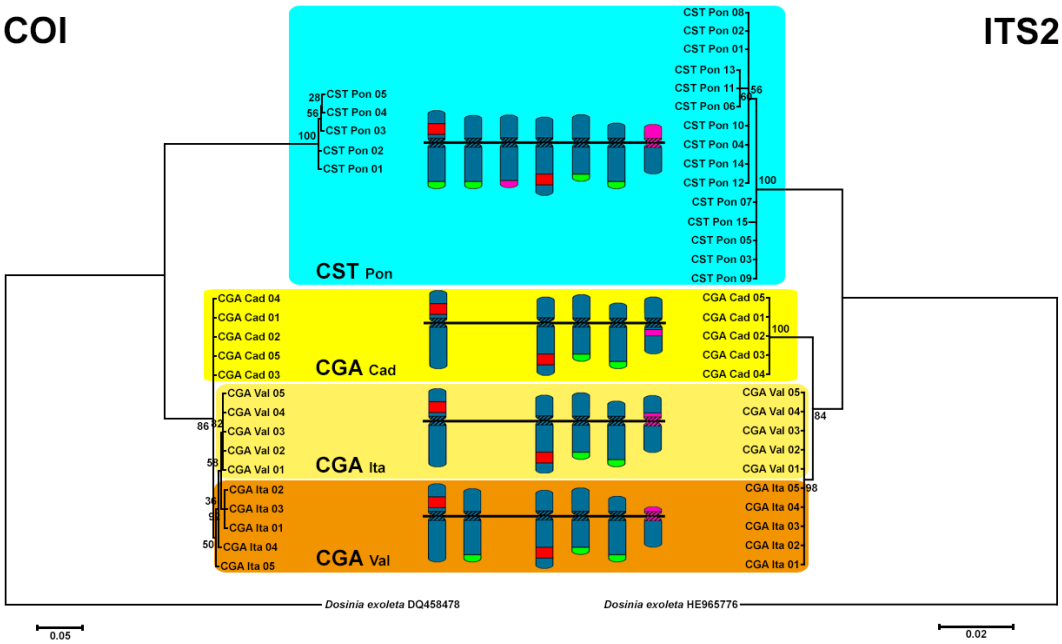

CST Pon
5S H3 28S
5S/H3
H3
28S
5S
H3
H3
28S
CST Pon
5S H3 28S
CGA Cad
5S H3 28S
5S
5S
H3
H3
28S
CGA Cad
5S H3 28S
CGA Val
5S H3 28S
5S
5S
H3
H3
28S
CGA Val
5S H3 28S
CGA Ita
5S H3 28S
5S
H3
5S
H3
H3
28S
CGA Ita
5S H3 28S

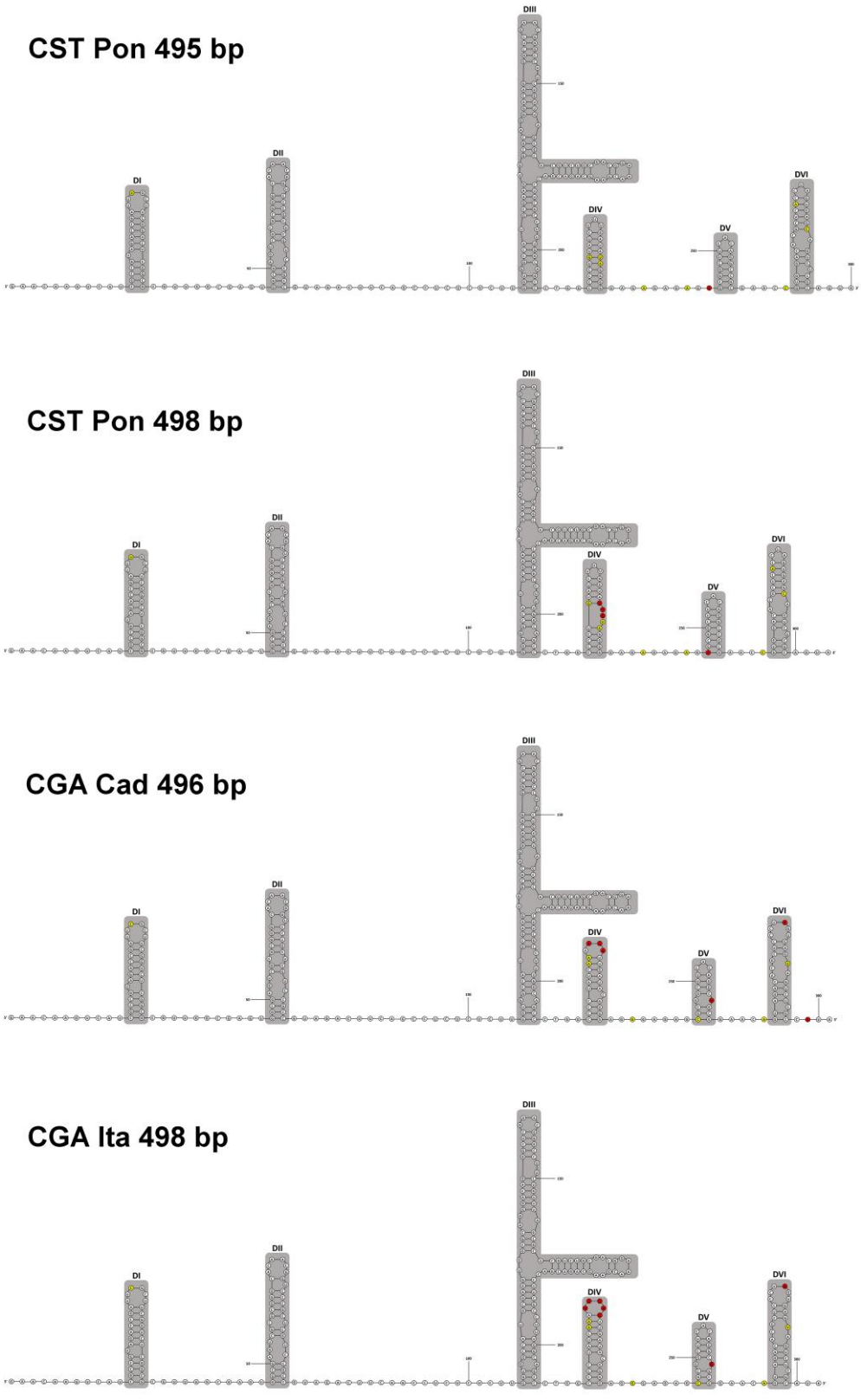
FIGURE 3


COI
ITS2
CST Pon 08
CST Pon 02
CST Pon 01
CST Pon 13
CST Pon 11
CST Pon 06
CST Pon 10
CST Pon 04
CST Pon 14
CST Pon 12
CST Pon 07
CST Pon 15
CST Pon 05
CST Pon 03
CST Pon 09
28
56
100
CST Pon 05
CST Pon 04
CST Pon 03
CST Pon 02
CST Pon 01
56
60
100
CST Pon
CGA Cad 04
CGA Cad 01
CGA Cad 02
CGA Cad 05
CGA Cad 03
CGA Cad 05
CGA Cad 01
CGA Cad 02
CGA Cad 03
CGA Cad 04
100
CGA Cad
CGA Val 05
CGA Val 04
CGA Val 03
CGA Val 02
CGA Val 01
CGA Val 05
CGA Val 04
CGA Val 03
CGA Val 02
CGA Val 01
84
86
92
58
CGA Ita 02
CGA Ita 03
CGA Ita 01
CGA Ita 04
CGA Ita 05
CGA Ita 05
CGA Ita 04
CGA Ita 03
CGA Ita 02
CGA Ita 01
98
34
9
50
CGA Ita
CGA Val
Dosinia exoleta DQ458478
Dosinia exoleta HE965776
0.05
0.02

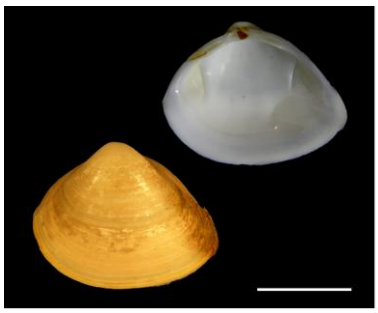
Supplementary Table 1. Parameters used in the PCR

Sequence	Denaturation	Annealing	Elongation
COI	95 ºC, 30 s	55 ºC, 20 s	72 ºC, 20 s
16S rDNA	95 ºC, 30 s	48 ºC, 30 s	72 ºC, 30 s
ITS2	95 ºC, 30 s	50 ºC, 30 s	72 ºC, 30 s
28S rDNA	95 ºC, 20 s	48 ºC, 20 s	72 ºC, 15 s
5S rDNA	95 ºC, 30 s	48 ºC, 30 s	72 ºC, 30 s
h3	95 ºC, 15 s	48 ºC, 15 s	72 ºC, 15 s

SUPPLEMENTARY FIGURE 1



MACTRIDAE



Spisula subtruncata
(Gray, 1837)
Clica

Spisula solida
(Linnaeus, 1758)
Almeja blanca

Mactra stultorum
(Linnaeus, 1758)
Huevo

genes

MDPI

Article

Molecular Cytogenetics in Trough Shells (Mactridae, Bivalvia): Divergent GC-Rich Heterochromatin Content

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Abstract: The family Mactridae is composed of a diverse group of marine organisms, commonly known as trough shells or surf clams, which illustrate a global distribution. Although this family includes some of the most fished and cultured bivalve species, their chromosomes are poorly studied. In this work, we analyzed the chromosomes of *Spisula solida*, *Spisula subtruncata* and *Mactra stultorum* by means of fluorochrome staining, C-banding and fluorescent in situ hybridization using 28S ribosomal DNA (rDNA), 5S rDNA, H3 histone gene and telomeric probes. All three trough shells presented 2n = 38 chromosomes but different karyotype compositions. As happens in most bivalves, GC-rich regions were limited to the nucleolus organizing regions in *Spisula solida*. In contrast, many GC-rich heterochromatic bands were detected in both *Spisula subtruncata* and *Mactra stultorum*. Although the three trough shells presented single 5S rDNA and H3 histone gene clusters, their chromosomal locations differed. Regarding major rDNA clusters, while *Spisula subtruncata* presented a single cluster, both *Spisula solida* and *Mactra stultorum* showed two. No evidence of intercalary telomeric signals was detected in these species. The molecular cytogenetic characterization of these taxa will contribute to understanding the role played by chromosome changes in the evolution of trough shells.

Keywords: trough shells; chromosome; heterochromatin; fluorescent in situ hybridization; histone genes; ribosomal RNA genes

1. Introduction

The family Mactridae (Lamarck 1809) is composed of a diverse group of marine organisms, commonly known as trough shells, duck clams or surf clams, showing a global distribution [1] and including some of the most fished and cultured bivalve species. Although trough shells are among the better known bivalves, some questions about their biology are not clear. In contrast with most other bivalve families in which many genera are available, in the family Mactridae there are only a few recognized genera and some groups do not match to any of them. Phylogenetic relationships among species of this family, and among specimens of single putative species, were the subject of some recent investigations using DNA sequences [2–5], giving increasing evidence of cryptic speciation in the group [2,4].

The chromosomal characterization of trough shells is far beyond the knowledge achieved for other bivalve families [6,7]. Classical studies were limited to determining chromosome numbers in a few species [8–12]. More recently, the karyotypes of the dwarf surf clam *Mulinia lateralis* [13], the fat gaper (horse clam) *Tresus capax* (*Lutraria maxima*) [14,15], and the sunray surf clam *Mactra chinensis* [16] were described. Regarding molecular cytogenetic techniques, fluorescent in situ hybridization (FISH)

was only applied to map telomeric sequences [17] and major ribosomal DNAs (rDNAs) [18] to the chromosomes of *Mulinia lateralis*.

In order to cytogenetically characterize the trough shells *Spisula solida*, *Spisula subtruncata* and *Mactra stultorum*, we studied their chromosomes by means of chromomycin A3 (CMA)/4′,6-diamidino-2-phenylindole (DAPI) and DAPI/propidium iodide (PI) fluorescence staining, C-banding and FISH using major rDNA, 5S rDNA, H3 histone genes and telomeric probes. Our results highlighted chromosomal similarities and differences among these taxa that can help to understand the role played by chromosome changes in the evolution of trough shells.

2. Experimental Section

2.1. Trough Shell Specimens

Samples of the thick trough shell *Spisula solida* (Linnaeus 1758), the cut trough shell *Spisula subtruncata* (da Costa 1778) and the rayed trough shell *Mactra stultorum* (Linnaeus 1758) were collected from natural populations in Ría de Pontevedra and Ría de Vigo (NW Spain).

2.2. Chromosome Preparation, Fluorochrome Staining and C-Banding

Chromosome preparations were obtained from gill and gonadic tissues following previously published methods [19,20]. After exposing the trough shells overnight to colchicine (0.005%), gills and gonads were removed, treated with 50% (20 min) and 25% (20 min) sea water and fixed in ethanol/acetic acid (3:1) three times for 20 min each time. Small pieces of the fixed tissues were dissociated in 60% acetic acid and the cell suspensions were dropped onto slides heated to 50 °C.

Fluorochrome staining was performed as previously described [21,22]. Some of the chromosome preparations were stained with CMA (0.25 mg/mL, 2 h), counterstained with DAPI (0.14 μg/mL, 8 min) and mounted with antifade (Vectashield, Vector, Burlingame, USA). After visualization and photography, the same chromosome preparations were re-stained with a combination of DAPI and PI (0.07 μg/mL, 8 min), mounted in antifade and photographed again. Chromosome counts were performed in at least 200 metaphase plates obtained from 10 specimens (5 males, 5 females) of each species. To detect heterochromatic regions on these species, some preparations were also C-banded using barium hydroxide [23] and stained with acridine orange (0.1 mg/mL).

2.3. DNA Extraction, PCR Amplification and Probe Labeling

Total genomic DNA was extracted from ethanol-preserved adductor muscles following a classical method [24] with slight modifications [25]. Small pieces of tissue were homogenized in hexadecyltrimethylammonium bromide (CTAB) buffer and digested with pronase (1.5 mg/mL, 60 °C, overnight) (Sigma Aldrich, St. Louis, MO, USA) and RNase A from bovine pancreas (1 mg/mL, 37 °C, 1 h) (Sigma Aldrich, St. Louis, MO, USA). The DNA was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and stored at 4 °C until further use.

FISH probes were generated by polymerase chain reaction (PCR) in 20 μL reactions containing 50 ng DNA, 1× PCR buffer, 0.5 mM of each dNTP (Thermo Fisher Scientific. Waltham, MA, USA), 2.5 mM $MgCl_2$, 1 μM of each primer and 1 U BIOTAQ DNA polymerase (Bioline, London, UK). As shown in Table 1, universal primers [26] were used to amplify a fragment of the 28S rRNA gene of the major rDNA repeat. The whole repeat of the 5S rDNA was amplified using primers described in [27]. H3 histone genes were amplified using the primers proposed by [28]. Following a denaturation step at 95 °C for 5 min, 30 cycles of amplification (Table 1) and a final extension of 7 min at 72 °C were performed in a GeneAmp PCR system 9700 (Applied Biosystems. Foster City, CA, USA). PCR products were checked by electrophoresis in 2% agarose gels. 28S rDNA probes were labeled with biotin-16-dUTP (Roche Applied Science, Penzberg, Germany) and/or digoxigenin-11-dUTP (10× DIG Labeling Mix, Roche Applied Science) by nick translation (Roche Applied Science. Penzberg, Germany). H3 histone gene and 5S rDNA probes were labeled with either biotin-16-dUTP (20 μM) or digoxigenin-11-dUTP (5 μM) by PCR. The labeled PCR products were precipitated before FISH.

Table 1. Primers and parameters used in the PCR.

Probe	Sequences of the Primers	Denaturation	Annealing	Elongation
28S rDNA	LR10R: 5′GACCCTGTTGAGCTTGA3′ LR12: 5′GACTTAGAGGCGTTCAG3′	95 °C, 20 s	48 °C, 20 s	72 °C, 30 s
5S rDNA	F: 5′CAACGTGATATGGTCGTAGAC3′ R: 5′AACACCGGTTCTCGTCCGATC3′	95 °C, 20 s	44 °C, 20 s	72 °C, 20 s
H3 histone gene	F: 5′ATGGCTCGTACCAAGCAGACVGC3′ R: 5′ATATCCTTRGGCATRATRGTGAC3′	95 °C, 15 s	48 °C, 15 s	72 °C, 15 s

2.4. Fluorescent in Situ Hybridization (FISH)

Single- and double-color FISH and re-hybridization experiments were performed as previously published [29]. Preparations were digested with RNase A (Sigma Aldrich, St. Louis, MO, USA) and pepsin (Sigma Aldrich, St. Louis, MO, USA) before denaturation (70 °C, 2 min). After overnight hybridization at 37 °C, biotin-labeled probes were detected with fluorescein avidin and biotinylated mouse anti-avidin antibodies (Vector, Burlingame, CA, USA) and digoxigenin-labeled ones were detected with mouse anti-digoxigenin and anti-mouse rhodamine antibodies (Sigma Aldrich, St. Louis, MO, USA). Slides were counterstained with DAPI (0.14 µg/mL in 2× saline sodium citrate) and mounted in antifade (Vectashield, Vector, Burlingame, CA, USA). FISH with the vertebrate telomeric $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) was performed following the protocol indicated by the supplier.

A minimum of 20 metaphase plates per probe or combination of probes in 10 specimens (5 male, 5 female) per species were recorded using a Nikon Eclipse-800 microscope (Tokio, Japan) equipped with an epifluorescence system. Separated images for each fluorochrome were obtained with a DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon). Merging of the images was performed with Adobe Photoshop CS2 (San Jose, CA, USA).

Ten of the best metaphase plates showing FISH signals were used to construct karyotypes for each species. Chromosome and arm lengths were carefully measured and relative lengths and centromeric indices were determined.

3. Results

The diploid chromosome numbers determined for *Spisula solida*, *Spisula subtruncata* and *Mactra stultorum* were 2n = 38 (Figures 1–3). *Spisula solida* showed a karyotype composed of five metacentric, four meta/submetacentric, three submetacentric, three submeta/subtelocentric and four subtelocentric chromosome pairs (Figure 1e). The karyotype of *Spisula subtruncata* presented eight metacentric, one meta/submetacentric, two submetacentric, three submeta/subtelocentric, four subtelocentric and one telocentric chromosome pairs (Figure 2e). In *Mactra stultorum*, the karyotype was composed of five metacentric, three meta/submetacentric, four submetacentric, four submeta/subtelocentric, and three subtelocentric chromosome pairs (Figure 3e).

The presence of AT- and/or GC-rich chromosomal regions was examined using a combination of AT-specific (DAPI), GC-specific (CMA) or unspecific (PI) fluorochromes. DAPI staining revealed two DAPI negative regions, subterminal to the short arms of two chromosome pairs in *Spisula solida* (Figure 1a); these regions were clearly stained with both CMA and PI (Figure 1b,c). In contrast, *Spisula subtruncata* showed eight chromosome pairs displaying DAPI−/CMA+ regions, seven of them at intercalary locations and one subterminal (Figure 2a–c). *Mactra stultorum* showed six pairs of intercalary and two pairs of subterminal DAPI−/CMA+ bands (Figure 3a–c). As detected by C-banding (Figure S1), these DAPI−/CMA+ regions were heterochromatic.

FISH experiments using 28S rDNA probes demonstrated that major rDNA clusters were located at subterminal DAPI−/CMA+ regions in these taxa. Both *Spisula solida* and *Mactra stultorum* showed two major rDNA signals, subterminally located on the short arms of chromosome pairs 17 and 19 (Figure 1d,e) and on the long arms of chromosome pairs 3 and 4 (Figure 3d,e), respectively. In contrast,

a single major rDNA cluster was detected on the long arms of chromosome pair 18 in *Spisula subtruncata* (Figure 2d,e).

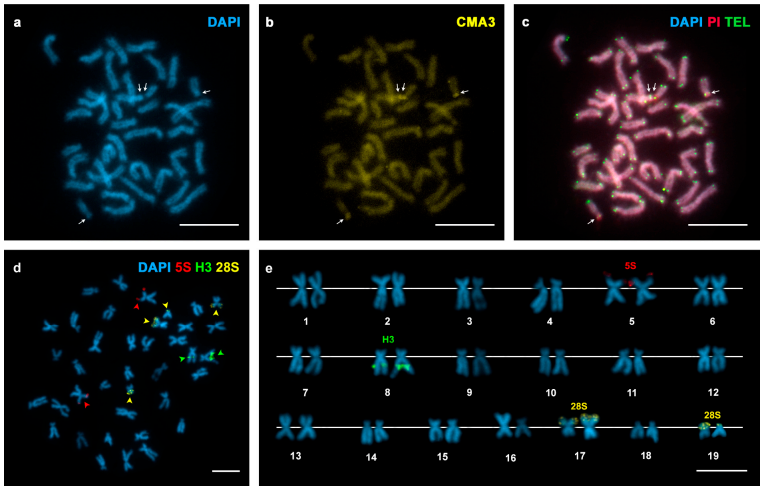

Figure 1. Fluorochrome staining and fluorescent in situ hybridization (FISH) mapping of telomeric (TEL), ribosomal DNA (rDNA) and histone gene probes to mitotic chromosomes of *Spisula solida*. (**a**) 4′,6-diamidino-2-phenylindole (DAPI)-stained metaphase plate showing DAPI dull regions on the short arms of two chromosome pairs (arrows); (**b**) Chromomycin A3 (CMA) staining of the same metaphase plate showed that DAPI dull regions were CMA bright (arrows); (**c**) FISH mapping of telomeric sequences to the same metaphase plate counterstained with DAPI/propidium iodide (PI) shows signals on the telomeres. Note that the four DAPI negative regions are stained with PI (arrows); (**d**) FISH mapping of 28S rDNA (yellow arrowheads), 5S rDNA (red arrowheads) and H3 histone gene (green arrowheads) probes on a metaphase counterstained with DAPI and the corresponding karyotype (**e**). Scale bars = 5 μm.

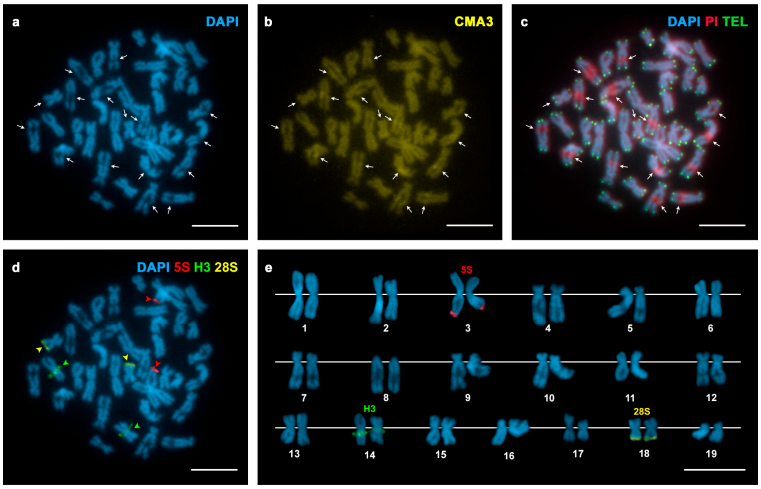

Figure 2. Fluorochrome staining and FISH mapping of telomeric, rDNA and histone gene probes to mitotic chromosomes of *Spisula subtruncata*. (**a**) DAPI-stained metaphase plate showing DAPI dull regions (arrows) subterminal (1) and intercalary (7) to the long arms of eight chromosome pairs; (**b**) CMA staining of the same metaphase plate showed that the DAPI dull regions were stained with CMA (arrows); (**c**) FISH mapping of telomeric sequences to the same metaphase plate counterstained with DAPI/PI shows green signals at the ends of all chromatids and DAPI−/PI+ regions stained in red (arrows); (**d**) FISH mapping of 28S rDNA (yellow arrowheads), 5S rDNA (red arrowheads) and H3 histone gene (green arrowheads) probes on the same metaphase counterstained with DAPI and the corresponding karyotype (**e**). Scale bars = 5 μm.

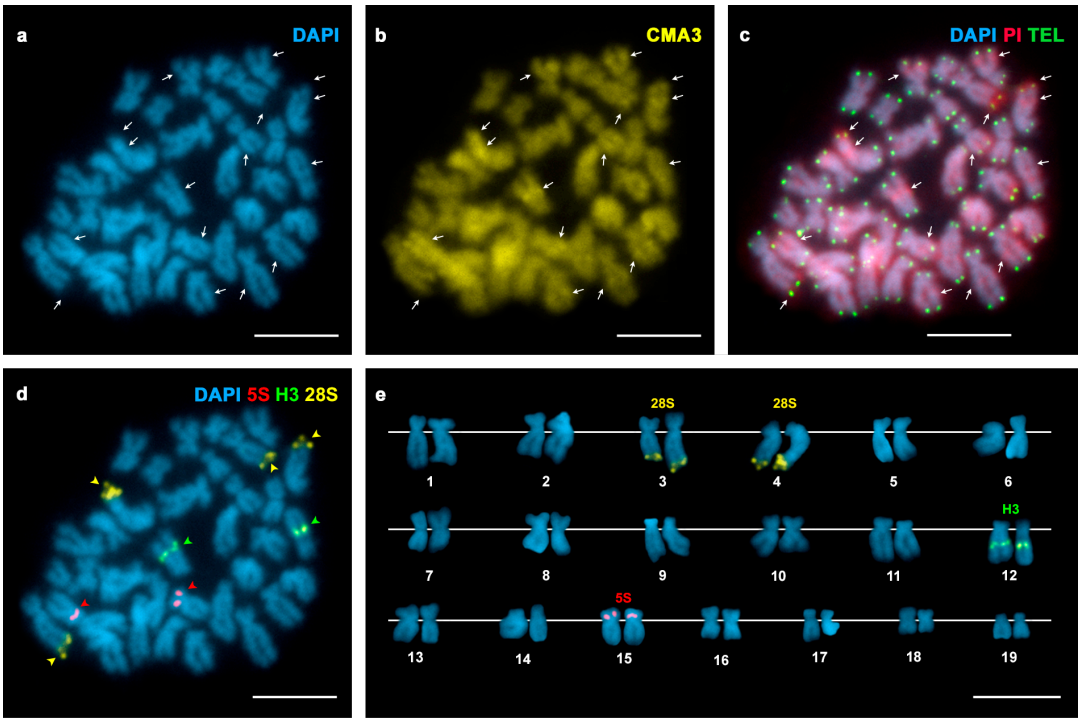

Figure 3. Fluorochrome staining and FISH mapping of telomeric, rDNA and histone gene probes to mitotic chromosomes of *Mactra stultorum*. (**a**) DAPI-stained metaphase plate showing DAPI dull regions (arrows) subterminal (2) and intercalary (6) to the long arms of seven chromosome pairs; (**b**) CMA staining of the same metaphase plate showed that the DAPI dull regions were stained with CMA (arrows); (**c**) FISH mapping of telomeric sequences to the same metaphase plate counterstained with DAPI/PI shows green signals at the ends of all chromatids and DAPI−/PI+ red-stained regions (arrows); (**d**) FISH mapping of 28S rDNA (yellow arrowheads), 5S rDNA (red arrowheads) and H3 histone gene (green arrowheads) probes on the same metaphase plate counterstained with DAPI and the corresponding karyotype (**e**). Scale bars = 5 µm.

In order to investigate the location of the major rDNA clusters in relation to the 5S rDNA and the core histone gene clusters, we performed double and sequential FISH experiments using 28S rDNA, 5S rDNA and H3 core histone gene probes. All three trough shells showed a single 5S rDNA cluster subterminal on the short arms of chromosome pair 5 in *Spisula solida* (Figure 1d,e) and on the long arms of chromosome pair 3 in *Spisula subtruncata* (Figure 2d,e), and intercalary on the short arms of chromosome pair 15 in *Mactra stultorum* (Figure 3d,e). Although H3 histone gene clusters also mapped to a single locus in the three analyzed species, their locations differed and were intercalary to the long arms of subtelocentric chromosome pairs 8 in *Spisula solida* (Figure 1d,e) and 12 in *Mactra stultorum* (Figure 3d,e) but subcentromeric to the long arms of metacentric chromosome pair 14 in *Spisula subtruncata* (Figure 2d,e). Apart from the histone gene cluster in *Mactra stultorum* chromosome pair 12, neither histone gene nor 5S rDNA clusters were located on the chromosome pairs that bear DAPI−/CMA+ heterochromatic bands.

In order to detect telomeric sequences in these species, we also performed FISH experiments using a vertebrate telomeric $(C_3TA_2)_3$ PNA as probe. As shown in Figures 1c, 2c and 3c, telomeric signals were detected exclusively at the ends of the sister chromatids of every mitotic chromosome without any indication of intercalary signals.

A summary of the results obtained in this study, together with previously published cytogenetic data in species of the family Mactridae, can be observed in Table 2.

Table 2. Chromosome numbers, karyotypes and fluorescent in situ hybridization (FISH) mapping data in trough shells.

Species	2n	Karyotype	28S rDNA	5S rDNA	Histone Genes	Telomeric Sequences	References
Mactra chinensis	38	10 sm, 9 st					[11]
	38	10 m, 8 sm, 1 st/t					[16]
Mactra stultorum	38						[10]
	38	5 m, 3 m/sm, 4 sm, 4 sm/st, 3 st	3q ter (st), 4q ter (st)	15p cen (sm)	12q ic (st)	terminal	This study
Mactra sp.	36						[8]
Mulinia lateralis	36						[8]
	38	19 t					[13]
	38	19 t				terminal	[17]
	38	19 t	15q ter (t), 19q ter (t)				[18]
Raeta (Labiosa) plicatella	36						[8]
Spisula solida	38	5 m, 4 m/sm, 3 sm, 3 sm/st, 4 st	17p ter (sm), 19p ter (st)	5p ter (sm)	8q ic (st)	terminal	This study
Spisula solidissima	38	4 m, 5 sm, 10 st/t					[12]
Spisula subtruncata	38	8 m, 1 m/sm, 2 sm, 3 sm/st, 4 st, 1 t	18q ter (m)	3q ter (m)	14q cen (m)	terminal	This study
Tresus capax	34	10 m, 7 sm	no signals				[14]
(*Lutraria maxima*)	34	10 m, 6 sm, 1 st/t					[15]

m: metacentric; sm: submetacentric; st: subtelocentric; t telocentric; p: short arm; q: long arm; cen: subcentromeric; ic: intercalar; ter: subterminal.

Ideogrammatic representations of the trough shell karyotypes showing the location of the GC-rich heterochromatic regions together with 28S rDNA, 5S rDNA and H3 histone gene clusters are displayed in Figure 4.

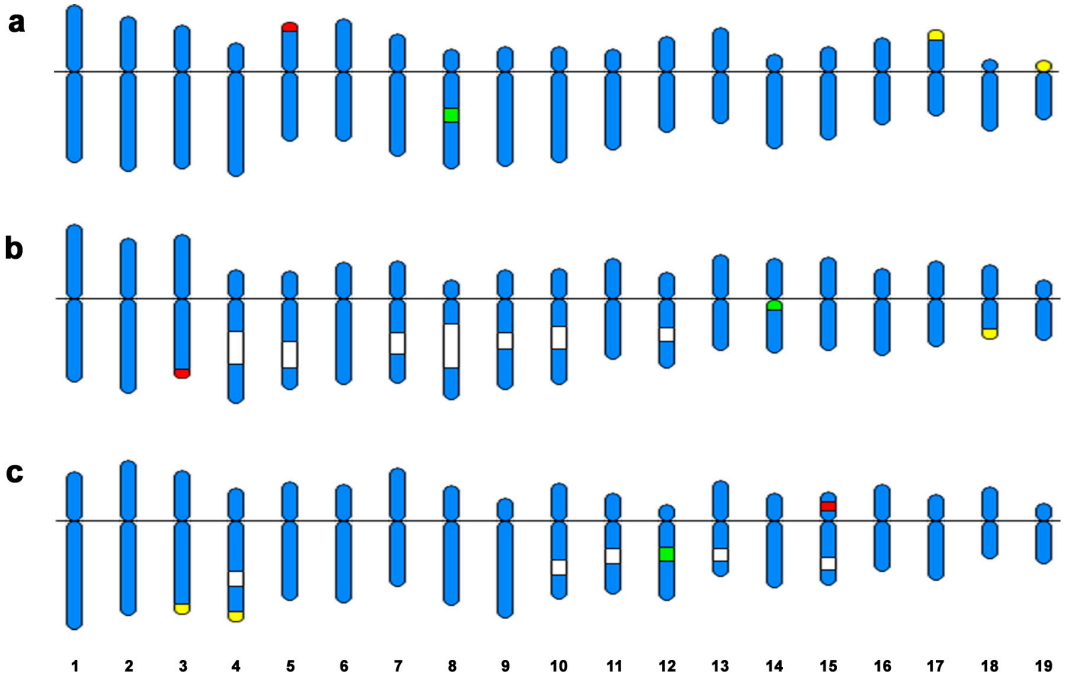

Figure 4. Ideogrammatic karyotypes of the trough shells: (**a**) *Spisula solida*; (**b**) *Spisula subtruncata*; and (**c**) *Mactra stultorum*. DAPI dull (CMA bright/C positive) bands are depicted in white. 28S rDNA signals, drawn in yellow, are coincident with the subterminal DAPI−/CMA+/C+ bands. 5S rDNA clusters are portrayed in red. H3 histone gene clusters, green, are coincident with DAPI−/CMA+/C+ bands in *Mactra stultorum*.

4. Discussion

Chromosome numbers have been described in only a few species of the family Mactridae (Table 2) [8–18]. The diploid chromosome numbers of 2n = 38 recorded in this work for *Mactra stultorum*, *Spisula solida* and *Spisula subtruncata* are in accordance with previous studies on seven mactrid taxa; however, they do differ from the 2n = 36 reported for *Raeta* (*Labiosa*) *plicatella* [8] and the 2n = 34 described in *Tressus capax* (*Lutraria maxima*) [14,15].

Regarding karyotype composition, striking differences were detected among karyotypes of the species belonging to the family Mactridae. While all chromosome pairs were telocentric in *Mulinia lateralis* [13,17,18], the other three species in which karyotypes were previously described, *Mactra chinensis* [11,16], *Spisula solidissima* [12] and *Tresus capax* (*Lutraria maxima*) [14,15], mostly showed metacentric and submetacentric chromosome pairs. The karyotypes of *Mactra stultorum*, *Spisula solida* and *Spisula subtruncata* here described also presented a high proportion of metacentric and submetacentric chromosome pairs.

The application of diverse combinations of base-specific fluorochromes to the staining of bivalve chromosomes has mostly detected GC-rich regions coincident with the nucleolus organizing regions (NORs) [7,21,22,27–31]. Our results showed that this was also the case for *Spisula solida* in which the two GC-rich regions detected were coincident with the two major rRNA gene clusters. In contrast, the presence of seven intercalary GC-rich regions, in addition to the single GC-rich NOR, in *Spisula subtruncata* and the six intercalary GC-rich regions located outside the two NORs in *Mactra stultorum* are a rare phenomenon in bivalves. In fact, these kind of GC-rich bands were previously reported only for the wedge shell *Donax trunculus* [32,33] and the zebra mussel

Dreissena polymorpha [34,35]. As also happened in *Donax trunculus* [33], C-banding demonstrated that these GC-rich regions were heterochromatic in both *Spisula subtruncata* and *Mactra stultorum* thus pointing to a probable abundance of GC-rich satellite DNA in these species that is going to be analyzed in the near future. In the meantime, the occurrence of high amounts of intercalary GC-rich heterochromatin in *Spisula subtruncata* and *Mactra stultorum*, but not in *Spisula solida*, suggests that heterochromatin amplification mechanisms (unequal exchange, transposition...) are operating in some trough shell taxa. It is tempting to think that this situation also happens in cryptic species [2,4]; if this were the case, cytogenetic research could contribute to both solving identification issues and establishing phylogenetic relationships within these taxa.

Although multigene families are useful cytogenetic markers for studying chromosomal evolution, their use in bivalves is quite scarce. With respect to the order Veneroida, major rDNA clusters have been previously mapped in 23 species [7,25,33,36,37], 5S rDNA clusters in 14 [7,25], core histone gene clusters in 12 [7] and telomeric sequences in 18 [7,25,33,36–38], most of them in species of the family Veneridae. The only species of Mactridae in which the location of some of these sequences was known was *Mulinia lateralis* [17,18].

The presence of two 28S rDNA signals subterminal to two chromosome pairs in *Mulinia lateralis* [18] coincided with our results for *Mactra stultorum* and *Spisula solida* but differed with the single pair bearing these signals in *Spisula subtruncata*. With regards to the chromosomal location of 5S rDNA and histone gene clusters, no previous data were available for any species of Mactridae. The occurrence of single minor rDNA and histone gene clusters in the trough shells *Spisula solida*, *Spisula subtruncata* and *Mactra stultorum* (Table 2) is similar to the situation found in some species of venerid clams [7].

Concerning telomeric sequences, the hybridization signals obtained at chromosome ends in *Spisula solida*, *Spisula subtruncata* and *Mactra stultorum* after using the vertebrate telomeric repeat as probe is coincident with results found in most other bivalves, including the mactrid *Mulinia lateralis* [17], and further support the molecular data obtained in *Donax trunculus* showing that bivalve telomeres were constituted by tandem repeats of the hexanucleotide that also constitutes the vertebrate telomeric sequence [38].

In summary, the results obtained in this work highlighted chromosomal similarities and differences among these taxa that can contribute to a better understanding of the role played by chromosome changes in the evolution of trough shells.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/7/8/47/s1, Figure S1: C-banding in *Spisula solida*, *Spisula subtruncata* and *Mactra stultorum*.

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Methylation profile of a satellite DNA constituting the intercalary CG-rich heterochromatin of the cut trough shell *Spisula subtruncata* (Bivalvia, Mactridae)

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Abstract

Tandemly repeated DNAs usually constitute significant portions of eukaryotic genomes. In bivalves, however, repetitive DNAs are habitually not widespread. In our search for abundant repetitive DNAs in trough shells, we discovered a novel satellite DNA, SSUsat, which constitutes 1.3% of the genome of *Spisula subtruncata*. As foreseen by the satellite DNA library hypothesis, we confirmed that this satellite DNA is also present in two other Mactridae species, showing a highly conserved nucleotide sequence together with a dramatic diminution in the number of repeats. Predominantly located at the GC-rich intercalary heterochromatin of *S. subtruncata*, SSUsat displays several DNA methylation peculiarities. The level of methylation of SSUsat is high (3.38%) in comparison with bivalve standards and triplicates the mean of the *S. subtruncata* genome (1.13%). Methylation affects not only the cytosines in CG dinucleotides but also those in CHH and CHG trinucleotides, a feature common in plants but scarce and without any clear known relevance in animals. SSUsat segments enriched in methylated cytosines partly overlap those showing higher sequence conservation. The chromosome-specific differences in the distribution of over- and under-methylated SSUsat monomers additionally emphasize the complexity of the methylation processes that shape repetitive genome compartments.

Keywords

cytosine methylation, fluorochrome chromosome banding, FISH, trough shells, satellite DNA

Introduction

Along recent years, the application of molecular genetic techniques to the study of marine organisms has supplied a plethora of information on the structure and evolution of bivalve genomes[1,2,3]. In accordance with data from other eukaryotes in which repetitive sequences constitute an important fraction of the genome[4], it has been estimated that in some bivalves about one third of the genomes is represented by repetitive sequences[2,3]. In spite of that, our knowledge about repetitive sequences in bivalves, as well as in other organisms, is still fragmentary, mainly due to technical difficulties in their sequencing, assembling and annotation[2,3].

Satellite DNAs (satDNAs), constituted by arrays of tandem head-to-tail DNA repeats, are paramount among repetitive sequences and can be located in centromeric, intercalary and/or subtelomeric chromosomal regions[5,6]. Though several satellites differing in both length and sequence may coexist within a single genome, monomers from the same family usually display low sequence divergence within a species due to concerted evolution[5]. However, under reproductive isolation and in absence of selective pressure, mutations may be differentially homogenized and fixed among lineages, resulting in the outcome of new specific satDNA variants. In this way, closely related species sharing a set (a library) of satDNAs may show a differential spreading of some of these variants across their genomes due to divergent contraction or amplification events[7,8]. The library model can explain the observation that some satDNAs are overrepresented in one species but either absent or present in low copy numbers in closely related taxa[9,10,11,12,13,14,15,16,17].

Although it has been proposed that satDNAs can act as heterochromatin modulators, participating in gene expression control and play a role in the dynamics of centromere assembly[18,19,20], the real functions of satDNAs still remain unknown and the epigenetic mechanisms operating on them are far from understood. As happens in other invertebrates[21], the genomes of bivalves studied until now show highly methylated tracts, mostly corresponding to genes, interspersed with methylation depleted areas in intergenic regions [22,23]. Invertebrate taxa are also characterized by presenting a rather sparse methylated repetitive DNA fraction, ranging from low to almost negligible depending on the species[22,24,25], contrastingly to vertebrates and plants. In regards to bivalves, the oyster *Crassostrea gigas* presents unmethylated long terminal repeats (LTRs) and long interspersed elements (LINEs), whereas short interspersed elements (SINEs) show a certain degree of methylation that reaches the mean genomic level for satDNAs[26]. Additionally, as revealed by the use of restriction enzymes sensitive to methylation, the DTF2 satellite of the wedge shell *Donax trunculus* displayed methylation[27,28]. The differences in the methylation behaviour of different types of sequences in bivalves do not suggest a mere DNA silencing role for DNA methylation in these organisms, therefore reinforcing the possibility of alternative functions within invertebrate genomes[24,29].

In the past four decades, the use of base-specific fluorochromes as chromomycin A3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI) to stain metaphase chromosomes have presented a distinction between GC-rich and GC-poor chromosome bands in many organisms. In bivalves, these bands are relatively scarce and GC-rich regions are mostly coincidental with nucleolus organizing regions (NORs) [30,31,32,33,34,35]. The exceptions to this behavior are the wedge shell *Donax trunculus* [27,36], the zebra mussel *Dreisena polymorpha* [37,38] and the trough shells *Mactra stultorum* and *Spisula subtruncata*[39] in which many GC-rich bands non-

coincident with the NORs appear. Furthermore, C-banding demonstrated that these regions were heterochromatic in both wedge shells[27] (27 Petrović et al. 2009) and trough shells[39] (39 García-Souto et al. 2016). The molecular composition of this GC-rich heterochromatin is still unknown. In a previous work, we mapped the abundant (2%), GC-rich DTF2 satDNA that exhibits CpG methylation to subtelomeric regions of many *D. trunculus* chromosomes[27] (27 Petrović et al. 2009); in the majority of cases, far away from the predominantly intercalary, GC-rich heterochromatin.

In this study we have recorded a novel satDNA, conserved within the examined species of the bivalve family Mactridae, which is the main component of the GC-rich heterochromatin in the cut trough shell *Spisula subtruncata*. The methylation status of the *S. subtruncata* satellite was explored by both bisulfite genomic sequencing and immunocytological detection and the results were compared with those of the whole genome, as detected by methylation-sensitive amplification polymorphism (MSAP) and ELISA assays.

Results

Characterization of SSUsat

Partial digestions of *Spisula subtruncata* genomic DNA with *Pvu*II and *Hae*III REs yielded identical ladder-like multimer bands of a 315 bp monomer unit (Fig. 1a), indicative of the presence of a satellite DNA in the genome of *S. subtruncata*. Southern blotting, using the 315 bp fragment recovered from the *Hae*III digestion as a probe, gave signals on all bands of both *Hae*III and *Pvu*II ladders, with multimers distinctive up to several kb (Fig. 1b). This regularity

of the ladder signals is consistent with the presence of an abundant satellite DNA presumably organized into long arrays within the *S. subtruncata* genome. The same approach did not reveal any signal in either of the other two Mactridae species (*Spisula solida* and *Mactra stultorum*) nor in a species of the related family Donacidae (*Donax trunculus*).

Dot blot analysis performed at high stringency conditions (68 °C) showed that these repeats accounted for approximately 1.3% of the *S. subtruncata* genome (Supplementary Fig. 1a). Medium (65 °C) and low (60 °C) stringent dot blots indicated that related, less homologous sequences are also abundant (2.5 to 4%) in *S. subtruncata* (Supplementary Fig. 1b, c), suggesting that a large number of repeats related to SSUsat coexist within the cut trough shell genome. This approach did not detect homologous sequences in the genomes of *S. solida*, *M. stultorum* and *D. trunculus*.

DNA fragments recovered from the gel bands corresponding to putative *S. subtruncata* monomers, dimers, trimers and tetramers were cloned and sequenced. The SSUsat representative sequence was deposited in the NCBI GenBank database under the accession number KY657249. The SSUsat consensus sequence is displayed in Figure 2 (see Supplementary Fig. 2 for the alignment of the 39 monomeric sequences obtained from 10 monomers, 6 dimers, 3 trimers, and 2 tetramers). 38 out of 39 monomers were 315 bp long, and only one monomer showed a single nucleotide deletion. In addition to the monomer length conservation, 39 monomeric sequences also showed a low overall nucleotide diversity (Pi = 0.0294), having GC-content of 44.05%. Similarity analysis of SSUsat monomeric sequences derived from the cloned multimers did not reveal any form of higher order repeat organization.

The SSUsat consensus sequence was blasted against both NCBI GenBank and Repbase[40], a database that contains a collection of different types of known repetitive sequences and mobile elements. Neither of the searches yielded positive results, thus confirming that this is a novel satellite DNA sequence, not previously described within any other species. Besides, local blast searches against *Crassostrea gigas* genome and partial genomic libraries of other bivalve species (*Ruditapes decussatus, Ruditapes philippinarum*) also demonstrate that SSUsat is a novel satDNA.

To confirm that the 39 SSUsat monomer variants analyzed here were accurate representatives of those present in the genome of *S. subtruncata*, Southern blotting was performed on genomic DNA digested with restriction enzymes characterized by presenting single cleavage sites situated in either variable or conserved regions of the SSUsat. *Hae*III and *Taq*I, single cutters with conserved restriction sites on all sequenced monomers, gave restriction profiles showing a prominent 315 bp band accompanied with few multimers (Supplementary Fig. 3). In contrast, *Pvu*II, a single cutter showing targets in a variable region, ergo only in a reduced proportion of the monomers (Supplementary Fig. 2), showed a prominent high molecular weight smear corresponding to uncut, target-free multimers (Supplementary Fig. 3). Additionally, the methylation status of the sequence was inferred by using *Hpa*II and *Msp*I, isoschizomeres whose restriction targets (three on SSUsat) only differ on cytosine methylation. The presence of the same sub-monomeric size fragments in both digestions indicates that majority of the SSUsat monomers are not methylated at the target positions. However, the slightly more intensive hybridisation signal observed in the *Msp*I digestion reaction (Supplementary Fig. 3b) suggests that a certain portion of SSUsat monomers could be subject to methylation.

Sequence conservation of SSUsat

In order to examine the presence of SSUsat in related species by using PCR methods, specific PCR primers were designed based on the SSUsat DNA consensus sequence (Fig. 2). In contrast to Southern and dot-blot experiments, the more sensitive PCR was successful in both *S. solida* and *M. stultorum* (Fig. 3a). Southern blotting using as a probe labeled SSU monomeric variants from *S. subtruncata*, revealed that SSUsat homologous sequences were indeed present in both species (Fig. 3b). On the other hand, no specific PCR amplification was obtained in distantly related *D. trunculus*, suggesting that *D. trunculus* lacks target sequences similar enough to be amplified with the selected primers (Fig. 3). Full-size monomers (21 from *S. solida*, and 11 from *M. stultorum*) were obtained from ~500 bp PCR-fragments, and used in further analyses.

The 71 SSUsat sequences, coming from *S. subtruncata*, *S. solida*, and *M. stultorum*, were aligned and compared (Supplementary Fig. 2). Among them, 66 sequences were 315 bp long, one showing a single nucleotide insertion and five having a single nucleotide deletion. The overall interspecific nucleotide diversity was low (Pi = 0.0290) and comparable to the intraspecific diversities (*S. subtruncata*: 0.0294, *S. solida*: 0.0340, *M. stultorum*: 0.0290), thus denoting a high degree of sequence similarity within and among species. Phylogenetic analysis of the SSUsat sequences clearly revealed clustering of monomer variants from different taxa, indicating a lack of correlation between monomer variants and a given taxon (Fig. 4). Regarding nucleotide diversity, its distribution along the satellite sequence was not homogeneous; variable positions were mostly piled up at four 15 bp long regions alternating with three highly conserved regions (Fig. 5a).

Chromosomal localization of the SSUsat DNA

The chromosomal location of the SSUsat was determined by FISH on mitotic metaphase plates previously stained with DAPI/CMA and DAPI/PI to evidence GC-rich chromatin regions (Fig. 6a, b). As previously shown[39], the karyotype of *S. subtruncata* is composed of 2n = 38 chromosomes and presents CMA positive / DAPI negative, intercalary GC-rich bands on the long arms of chromosome pairs 4, 5, 7, 8, 9, 10, and 12, and a subterminal band, coincident with the NOR, on the long arm of pair 18 (Fig. 6g). As determined by barium hydroxide C-banding, these GC-rich bands were heterochromatic[39]. Although most of the FISH signals obtained with the SSUsat probes overlapped the heterochromatic regions situated in those chromosome pairs (Fig. 6c, g), additional SSUsat signals, apparently situated outside heterochromatic regions, were detected on the long arms of the chromosome pairs 3, 6, and 13 (asterisks in Fig. 6g). Whilst on the other eight chromosome pairs (1, 2, 11, 14, 15, 16, 17, and19) SSUsat signals were not detected.

FISH using the SSUsat probe on *S. solida* and *M. stultorum* chromosome spreads revealed inconclusive results, possibly resultant of a low number of copies of SSUsat monomers in these species.

***Cytosine methylation in the genome of* S. subtruncata**

The distribution of methylated regions on the chromosomes of *S. subtruncata* was analyzed by immunostaining employing an antibody against 5-MeC. The overall fluorescence was rather low and image acquisition required long exposure times. As shown in Figure 6d-f, the 5-MeC signals were homogeneously distributed along most regions of the *S. subtruncata*

chromosomes (Fig. 6d, g). In contrast, the most prominent GC-rich heterochromatic band, which presented the strongest SSUsat FISH signals, did not display any fluorescence (chromosome 8, arrows in Fig. 6). Although these results clearly show a low degree of methylation of some of the GC-rich heterochromatic regions, this methodology does not allow for a quantitative measurement of the global genome methylation. Therefore other approaches were employed to better assess the methylation degree of the cut trough shell genome.

The detection of possible differences in the degree of cytosine methylation among *S. subtruncata* adult specimens was determined through a MSAP experiment. Comparisons of banding patterns obtained after *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I digestions demonstrated that out of a total of 288 methylation-susceptible loci, 103 (36.79%) were polymorphic. Surprisingly, no significant intraindividual differences were found between *Hpa*II and *Msp*I restriction banding profiles (AMOVA; $\Phi_{PT} = 0.0000$, $p = 0.513$). Furthermore, the principal component analysis of the MSAP data showed that the *Hpa*II and *Msp*I profiles from a single specimen are quite close, without any other clustering trend (Supplementary Fig. 4). These results strongly indicate that the level of cytosine methylation in adult cut trough shell genome is very low and were further corroborated by data obtained by ELISA immunoassays which demonstrated that less than 1.3% of the genome (1.13 ± 0.22) was methylated in *S. subtruncata*.

In a further attempt to establish the degree of methylation of the SSUsat sequences, we treated genomic DNA with bisulfite and sequenced the bisulfite modified monomers. To avoid biases associated with spontaneous point mutations from cytosine to thymine in the native DNA, methylation data was normalized by dividing the proportion of cytosine after bisulfite

transformation by the proportion of thymine and cytosine in the initial monomer pool. The efficiency of the bisulfite conversion, checked by treating PCR amplified monomers in parallel with genomic DNA samples, was 98.7%. The results, outlined in Table 1, indicate that the 3.38% of the cytosine sites in the SSUsat are methylated, three times the genomic mean**,** without main differences between strands (3.75% and 3.00%) and that the proportion of methylated cytosine was higher at CG sites (5.65%) than at CHH (2.69%) and CHG (1.79%) ones. Regarding the methylation status of the three CCGG restriction targets for *Hpa*II/*Msp*I, most of them were unmethylated and two showed internal cytosine hemimethylation, therefore being cut by the two isoschizomers. On the other hand, our results also indicated that methylation is unevenly distributed along the satellite (Fig. 5b) and correlated with sequence conservation both for the direct (ρ = -0.1340, p = 0.0213) and the complementary (ρ = -0.2505, p = 0.0000) strands and also for both strands taken together (ρ = -0.2975, p = 0.0000). Furthermore, the highest methylated regions (nucleotide positions 60-89 and 209-221) coincide with two of the three most conserved domains (nucleotide positions 41-89 and 199-232) in the monomer sequence.

Discussion

SatDNA sequences have been found in many bivalve mollusc species. These organisms have been considered a good model for the study of satDNA dynamics, with satellite families ubiquitous at higher taxonomic levels[16,17], and fast evolving ones, restricted to a limited number of congeneric species[13,15]. The SSUsat described here is the first satellite DNA sequence found in trough shell genomes and, as BLAST searches did not reveal significant similarity with any other DNA sequence stored in GenBank, representing a new satellite family. The 1.3% SSUsat content in the genome of *S. subtruncata* was among the highest

detected until now for a homogeneous satDNA family in bivalves, being comparable with the abundance of the DTF2 satellite of the wedge shell *D. trunculus* [27,41] or with the BIV160 satDNA of *R. decussatus*, which represents 2% of its genome[16]. The different amplification and distribution of the SSUsat in the genomes of three trough shell species, abundant and presumably organized into long arrays in *S. subtruncata* and low copy in *S. solida* and *M. stultorum*, but absent in the more distant *D. trunculus*, is concordant with the library model of satellite DNA evolution[7,19].

Although initially thought to be junk DNA, an accumulating amount of evidence from the last few years has suggested that satellite DNAs are involved in heterochromatin dynamics, centromere function and epigenetic silencing[6,42,43]. The irregular distribution of nucleotide diversity along satellite monomers is considered an indication of the evolutionary constraints imposed on segments of monomer sequences, whilst more variable segments would correspond to regions of a lesser relevance[44,45]. The non-randomness of the nucleotide diversity in the SSUsat, showing three highly conserved domains interspersed with four variable regions, constitute an additional example of that paradigm.

As in many other invertebrates, GC contents of bivalve genomes are relatively low: 31.65% in the mussel *Mytilus galloprovincialis*[3], 33.69% in the pearl oyster *Pinctada imbricata fucata*[1], 35.30% in the freshwater clam *Corbicula fluminea* (https://www.ncbi.nlm.nih.gov/genome/15808) and 36.60% in the oyster *Crassostrea gigas*[2]. Heterochromatin content is also scarce in bivalves and only the wedge shell *D. trunculus* [27,36], the zebra mussel *Dreissena polymorpha*[37,38] and the trough shells *S. subtruncata* and *M.stultorum*[39] characteristically present relatively high amounts of C-banded heterochromatin. Differential staining with base-specific fluorochromes demonstrated that these

heterochromatic bands were GC-rich, i.e. showed higher GC content than the surrounding euchromatin[27,39]. As the SSUsat is the main component of the heterochromatic bands in *S. subtruncata* and its GC-richness is around 44%, it can be inferred that the overall GC-content of *S. subtruncata*, must be below that value as it is in other bivalves.

Although DNA methylation has been traditionally interpreted as an epigenetic mark in the eukaryotic genome, regulating both gene transcription and heterochromatin formation and inactivating transposition[21], most cytosine methylation data was obtained from a few vertebrate and plant genomes and even less from invertebrates[22,46]. DNA methylation patterns in invertebrates differs from those found in vertebrates, being generally characterized by low methylation levels[22,25]; this is also the case in bivalves[22,29,46]. In concordance, the level of cytosine methylation of the genome for *S. subtruncata* is very low (1.3%) but showing about thrice that value for the SSUsat. Increased methylation levels have also been reported for satellite DNAs in other bivalves such as the wedge shell *D. trunculus*[27] and the oyster *C. gigas*[26]. In the latter, the satDNA methylation is similar to the genome level but twice as high than intergenic regions with no annotations; however, it is not randomly distributed but preferentially affects the most conserved repetitive sequences, that is, the younger and most prone to be active. Interestingly, although SSUsat monomer sequence is highly conserved among Mactridae species, methylation along the SSUsat monomer is inversely correlated with nucleotide diversity, and the two highest methylated regions in SSUsat coincide with two of the three most conserved sequence segments.

SSUsat also presents two more interesting distinctive methylation characteristics. On the one hand, methylation affects not only to the cytosine on CG dinucleotides but also, though in a lesser extent, to CHH and CHG trinucleotides; this is an abundant phenomenon playing

functional roles in plants but scarce and without clear relevance in animals[26,47]. On the other hand, the clear absence of methylation signals on the heterochromatic regions of one chromosome pair could indicate that methylated and non-methylated SSUsat monomers are not randomly distributed along heterochromatin. Somewhat similar results were previously found for the satellite family pEV in the wild beet *Beta procumbens* in which, as demonstrated by immunostaining with an antibody against 5-MeC, the methylation of the heterochromatic regions bearing the pEV satellite showed a certain degree of variation[20]. Whether this is a consequence of particular life stage- or tissue-specific methylation/demethylation processes or any other cause needs to be evaluated.

Materials and Methods

Biological samples

Adult specimens of the cut trough shell *Spisula subtruncata* (da Costa, 1778), the thick trough shell *Spisula solida* (Linnaeus, 1758), the rayed trough shell *Mactra stultorum* (Linnaeus, 1758) and the wedge shell *Donax trunculus* (Linnaeus, 1758) were collected from natural populations in Galicia (NW Spain).

Cloning, sequencing, and sequence analysis

Genomic DNA was isolated from adductor muscles using the E.Z.N.A. ® Mollusc DNA Kit (OMEGA). *S. subtruncata* genomic DNA was subjected to partial digestion with *Hae*III (New England Biolabs), and the products were separated by agarose gel electrophoresis producing a

ladder-like profile. Fragments up to tetramer were recovered from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen).

DNA fragments were cloned into XL10-Gold Ultracompetent Cells (Agilent Technologies) using a *Sma*I-digested pUC19 plasmid vector. Positive clones were purified using the High Pure Plasmid Isolation Kit (Roche) and commercially sequenced (Macrogen Inc., Amsterdam, the Netherlands) using M13/pUC forward and reverse primers. The resulting sequences were aligned using ClusterW via MEGA V7[48] (48 Kumar et al. 2016) applying default parameters.

On the basis of the *S. subtruncata* aligned sequences (SSUsat) and using the Lasergene PrimerSelect software (Dnastar), we designed a set of specific primers (SSUsatPR1: 5'-AACCGCGGTGCAAGTTCG-3'; SSUsatPR2: 5'-TAACCTGTTTTCGCGATGTTTACG-3') in order to explore the conservation of the sequences in closely (*S. solida, M. stultorum*) and distantly (*D. trunculus*) related species. PCR conditions included an initial denaturation at 94 °C for 3 min and 35 cycles of 94 °C for 20 s, 63 °C for 20 s and 72 °C for 1 min. A final extension at 72 °C for 7 min was applied. All reactions were performed in an Applied Biosystems 2720 Thermal Cycler. PCR products (comprising a full-size monomer plus flanking regions) were cloned into XL10-Gold Ultracompetent Cells (Agilent Technologies) using the pGEM®-T Easy Vector System (Promega). Positive clones were colony-PCR amplified with the vector specific primers (M13F, M13R-40) and sequenced as described above. The sequences were aligned using ClusterW via MEGA V7[48] applying default parameters. Phylogenetic analysis was carried out with the Maximum Likelihood method based on the Kimura 2-parameter model plus Gamma implemented in MEGA V7[48]. The distribution of the nucleotide diversity (Pi) was estimated using DnaSP v5[49] as described in

Plohl et al. [16]. Sequence segments were considered conserved or variable only if Pi differed from the mean value in more than two times the standard deviation.

Southern and dot blot hybridisation

Genomic DNAs were digested with *Hae*III (Fermentas), *Hpa*II (Fermentas), *Msp*I (Promega), *Pvu*II (New England Biolabs) and *Taq*I (Roche). Restriction products were resolved by gel electrophoresis, transferred to positively charged nylon membranes (Roche), and hybridized overnight with the DIG-labelled SSUsat probe. To test homology in low, moderate and high stringency conditions, different experimental temperatures (60, 65 and 68 °C) were used. Signals were detected with alkaline phosphatase conjugated anti-digoxigenin antibodies (Roche) using CDP-Star (Roche) as substrate.

The proportions of the satellite present in the genomes of *S. subtruncata*, *S. solida*, *M. stultorum* and *D. trunculus* were estimated by dot blotting. Serial dilutions of genomic DNAs (10 - 250 ng) and cloned SSUsat (0.3 - 10 ng) were dot-blotted onto positively charged nylon membranes (Roche) and hybridized with SSUsat probes. Hybridisation signals were quantified using Image J (http://imagej.nih.gov/ij/).

Immunodetection and fluorescent in situ hybridisation

Mitotic chromosomes were obtained as indicated in García-Souto et al. [35,39]. Specimens were treated with colchicine (0.005%, 8 h) before dissection. Gills were hypotonised in diluted seawater and fixed with ethanol-acetic acid (3:1). Gill fragments were immersed in acetic acid (60%) and the resulting cell suspension dropped onto glass slides.

Immunodetection of 5-methylcytosine (5-MeC) was performed with mouse anti-5-MeC (Eurogentec) following supplier indications as modified by Covelo-Soto et al.[50]. After detection with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibodies (Sigma), chromosomes were counterstained with CMA (0.25 mg/mL) and DAPI (0.14 μg/mL in 2x SSC). Chromosome slides were mounted with antifade (Vectashield, Vector). After microscopy and image acquisition, chromosome preparations were re-stained with DAPI and propidium iodide (PI: 0.07 μg/mL in 2x SSC) and photographed again.

Fluorescent *in situ* hybridisation (FISH) with digoxigenin-labelled SSUsat probes was performed as described previously[35,39,51]. Chromosome spreads were treated with RNase and pepsin before denaturation in 70% formamide (70 °C, 2 min). After overnight hybridisation and stringency washes (50 % formamide and 1xSSC, 45 ºC), signal detection was accomplished with mouse anti-digoxigenin, goat anti-mouse tetramethylrhodamine isothiocyanate (TRITC) and rabbit anti-goat TRITC antibodies (Sigma). Slides were then counterstained with DAPI and mounted with antifade.

Chromosome analysis and imaging was performed with a Nikon Eclipse E800 microscope equipped with an epifluorescence system and a DS-Qi1Mc CCD camera controlled by NIS-Elements (Nikon). At least 20 complete metaphase plates from five individuals of each species were examined. Processing of the images was performed with Adobe Photoshop.

Bisulfite sequencing

Genomic DNA of *S. subtruncata* was converted with sodium bisulfite, using the EZ DNA Methylation™ Kit (ZYMO Research) as recommended by the manufacturer. After

transformation, entire SSUsat monomers were recovered from both strands using four sets of primers, two of them targeting the direct strand (SSUsat1MD F/R: 5'-AACCGCGGTGYAAGTTYGA-3', 5'-ATAACCTRTTTTCRCRATRTTTAC-3'; SSUsat2MD F/R: 5'-CCGGAAGTTACACATTTYGGA-3', 5' CAAAAAATGRCCTCCAACTTRCA-3') and the other two the complementary strand (SSUsat1MC F/R: 5'-CAAAAAATGGGYTYGAAYTTGYA-3', 5'-CCGGAARTTACACATTTCRRA-3'; SSUsat2MC F/R: 5'-ATAACYTGTTTTYGYGATGTTTAY-3', 5'AACCGCGRTRCAARTTCR A-3'). Following an initial denaturation at 95 °C (5 min), 35 amplification cycles of 95 °C (15 s), 52 °C (15 s) and 72 °C (15 s) and a final extension step at 72 °C (7 min) were applied. Amplicons were cloned using the NZY-A PCR cloning kit (NZYTECH) and sequenced using the kit vector primers. Bisulfite sequenced monomers were aligned with the untransformed SSUsat library using MEGA V7[48]. All conserved cytosine positions were screened for methylation events. Sequences corresponding to the primers were excluded from the analysis.

The relationship between methylation level and sequence conservation was assessed by means of the Spearman correlation coefficient.

ELISA and Methylation-Sensitive Amplified Polymorphism

The overall 5-MeC content in the genome of S. *subtruncata* was determined using a monoclonal antibody specific for 5-MeC (5-MeC ELISA Kit, ZYMO Research) following the manufacturer's instructions. The results were expressed as the proportion of 5-MeC in the DNA sample. The standard curve was generated using the controls included in the kit.

A methylation sensitive amplified polymorphism (MSAP) assay[52,53] was performed on the same samples. This assay is based on the differential cleavage reactivity of the isoschizomers *Hpa*II and *Msp*I to the cytosine methylation of their target and permits the assessment of differences in methylation patterns within and among specimens. After *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I digestion of genomic DNA from eight specimens of *S. subtruncata,* the restriction profiles obtained by capillary electrophoresis (ABI Prism 310 Genetic Analyzer, Applied Biosystems) using a 500 ROX size standard (GeneScan) were scored with GeneMapper v.3.7 (Applied Biosystems) and compared using the R package MSAP[54] and GeneAlEx 6.502[55].

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files)

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Contributions

D.G-S., B.M., P.M. and J.J.P. designed the research. D.G-S., B.M., E.S. and P.M. participated in the experimental work. D.G-S., B.M., M.P., P.M. and J.J.P. analysed and interpreted the data. B.M., P.M. and J.J.P. financed the research. D.G-S. and J.J.P. drafted the manuscript. All other authors assisted in revising the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors declare no competing financial interests.

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Table 1 Proportion (%) of methylated cytosines in SSUsat monomers according C site type and DNA strand

Site	Strand		
	Direct	Complementary	Both
CG	6.18	5.11	5.65
CHG	2.97	0.61	1.79
CHH	2.11	3.28	2.69
All C	3.75	3.00	3.38

Figure legends

Figure 1: Southern blot hybridisation analysis of SSUsat repeats in *Spisula subtruncata.*

Partial digestion with *Pvu*II and *Hae*III of *Spisula subtruncata* genomic DNA (**a**) followed by Southern blotting and hybridisation with the SSUsat monomer probe (**b**) yielded identical ladder-like multimer bands of a 315 bp monomer unit

Figure 2: Consensus sequence of the SSUsat monomers from *Spisula subtruncata*.

Restriction sites for *Msp*I/*Hpa*II, *Pvu*II and *Taq*I are underlined. The positions of the PCR primers are indicated by green and red arrows

Figure 3: PCR amplification and Southern blot hybridisation of SSUsat.

PCR amplification of SSUsat (a) in *Spisula subtruncata* (SSU), *Spisula solida* (SSO), *Mactra stultorum* (MST) and *Donax trunculus* (DTR). Corresponding Southern blot hybridisation using SSUsat probe (**b**). Blank represents PCR reaction without DNA template

Figure 4: SSUsat maximum likelihood tree.

Maximum likelihood tree based on SSUsat sequences isolated from *Spisula subtruncata*, *Spisula solida* and *Mactra stultorum*. The tree is based on the alignment shown in Suppl. Fig. 2. Numbers in internal nodes indicate bootstrap support values (500 replicates)

Figure 5: Nucleotide diversity and cytosine methylation in SSUsat.

Distribution of the nucleotide diversity (**a**), computed using a 10 bp overlapping sliding window, along the SSUsat sequence showing conserved (pinkish) and variable (yellowish) regions; the red lines indicate mean (± 2 SD) diversity (Pi). The proportion of methylated cytosines (**b**), computed using a 10 bp overlapping sliding window, for direct (green line) and complementary (blue line) strands of the SSUsat; red lines indicate mean (+ 2 SD) methylation values

Figure 6: Chromosomal location of GC-rich bands, SSUsat and methylated regions in *Spisula subtruncata*.

DAPI/CMA (**a**) followed by DAPI/PI staining (**b**) and FISH using the SSUsat probe (**c**) on the same metaphase plate and the corresponding karyotype (**g**) show that SSUsat signals mainly co-locate with the CMA positive, DAPI negative, GC-rich intercalary heterochromatic regions on chromosome pairs 4, 5, 7, 8, 9, 10 and 12 and with the subterminal GC-rich heterochromatin associated with the NOR on chromosome pair 18. Additional intercalary

SSUsat signals are also present in regions not showing conspicuous CMA+/DAPI- staining in the long arms of chromosome pairs 3, 6 and 13 (asterisks in **g**). Immunolocalization of 5-methylcytosine (5-MeC) in the same metaphase plate (**d-f**) and the corresponding karyotype (**g**) mainly shows a homogeneous distribution of the 5-MeC signals with two main exceptions; the absolute lack of methylation signals on the biggest, intercalary CMA+/DAPI-, GC-rich, heterochromatic, SSUsat-bearing band on the long arm of chromosome 8 (arrows in **a**-**g**) and the strong methylation showed by the subterminal NOR in one of the homologous chromosomes of pair 18. Scale bars, 5μm

Supplementary Figure legends

Supplementary Figure 1: Quantification of SSUsat by dot blot hybridisation.

High (**a**), medium (**b**) and low (**c**) stringency dot blot analysis of the SSUsat DNA from *Spisula subtruncata* (SSU), *Spisula solida* (SSO), *Mactra stultorum* (MST) and *Donax trunculus* (DTR). The amounts of dot blotted DNA (ng) are indicated

Supplementary Figure 2. Nucleotide alignment of SSUsat.

Nucleotide alignment of SSUsat nucleotide sequences isolated from *Spisula subtruncata* (**SSU**), *Spisula solida* (**SSUinSSO**) and *Mactra stultorum* (**SSUinMST**). Points and dashes indicate identical nucleotides and deletions, respectively

Supplementary Figure 3. Southern blotting of ER digested *Spisula subtruncata* genomic DNA.

Southern blotting of *Spisula subtruncata* genomic DNA digested either with REs that have single cleavage sites in conserved (*Hae*III and *Taq*I) or variable (*Pvu*II) regions of the

SSUsat, or with two isoschizomers (*Hpa*II and *Msp*I) with three restriction targets in the SSUsat monomer sequence

Supplementary Figure 4. MSAP principal component analysis.

Principal component analysis of the *Eco*RI+*Hpa*II (red) and *Eco*RI+*Msp*I (blue) restriction band profiles (MSAP) for eight *Spisula subtruncata* specimens (1 to 8) shows that the only clustering is between each pair of profiles from the same specimen, without any other clustering trend

Figure 1:

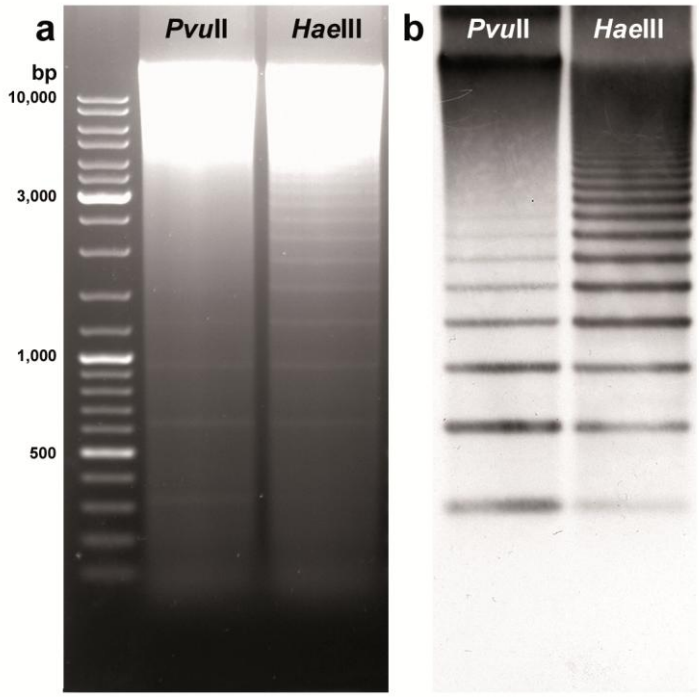


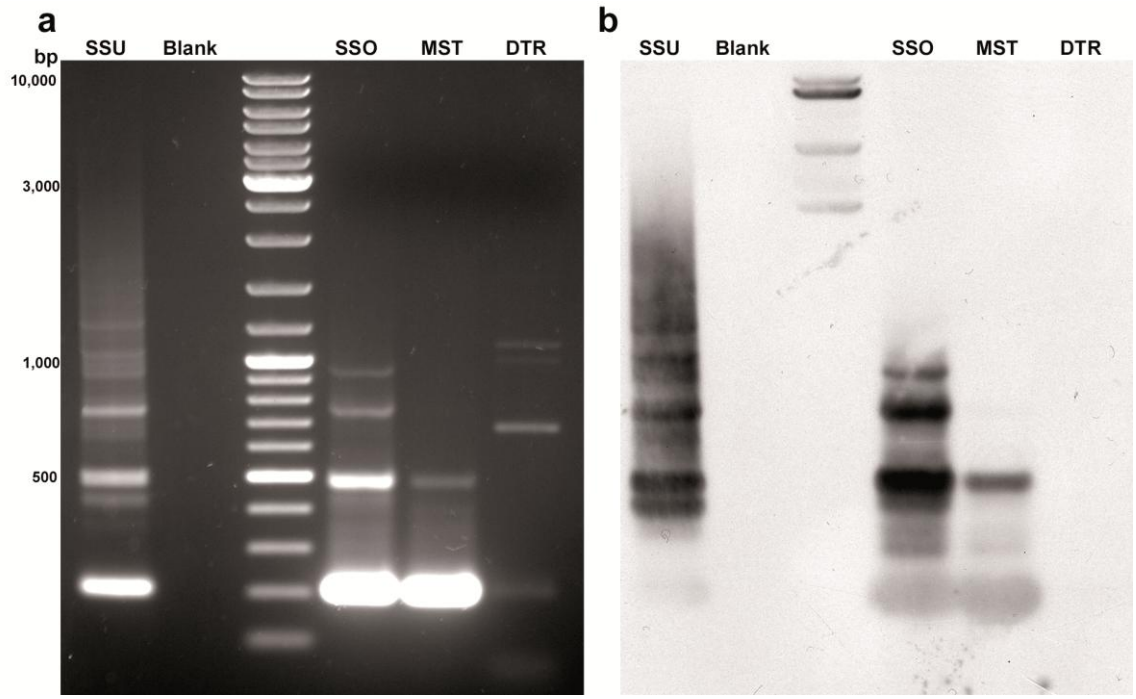
Figure 2

```
1   CCGAACCTAA AAAACCCAAA AAAAACCGGA TGTTCAGAGG TGAACCGGAA GTTACACATT TCGGATACGT
                              MspI/HpaII            MspI/HpaII
          SSUsatPR2
71  AAACATCGCG AAAACAGGTT ATTTTATGAA AACATCGGTT TTTTTGGTGC GAATGCACTG AAATTTGCAC

141 CATTTTCCTA ACTTTACTGC CCGAGCTTAA CTTGAAAATT TCAGCTCCCC CAAACGCATA GGAGCCGAAC
                                                PvuII
     SSUsatPR1
221 CGCGGTGCAA GTTCGAGCCC ATTTTTTGAC CGGTAAAAAG CCTGTAGAAC GCGTATTTAT GCACCGATTG
                  TaqI              MspI/HpaII

291 AGCTGAAATT TTGCATGAAT GCCTCCCTTG AGTGG
```

Figure 3:



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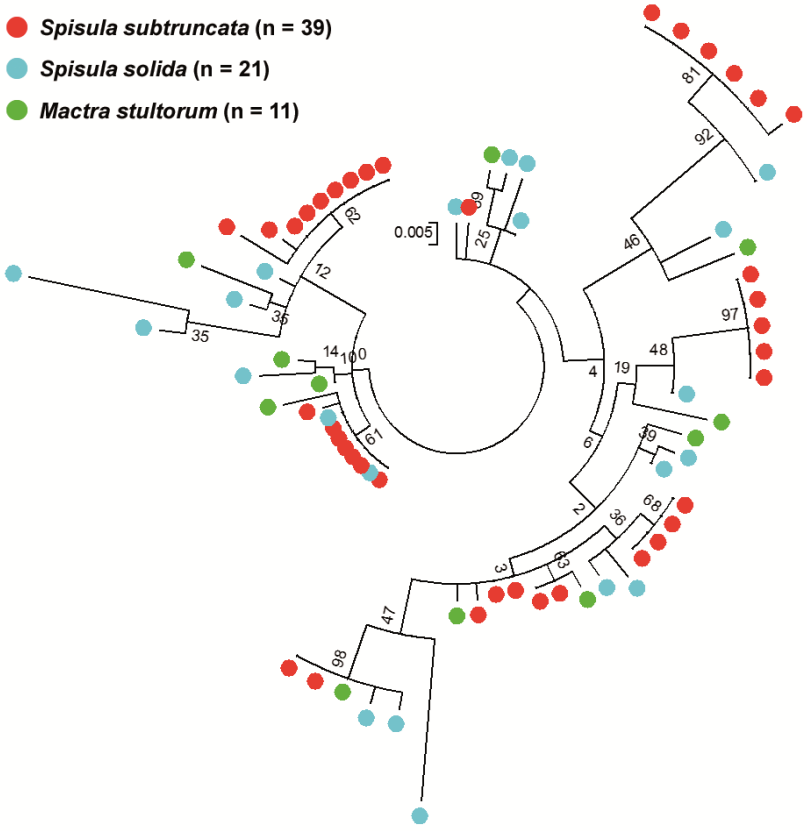

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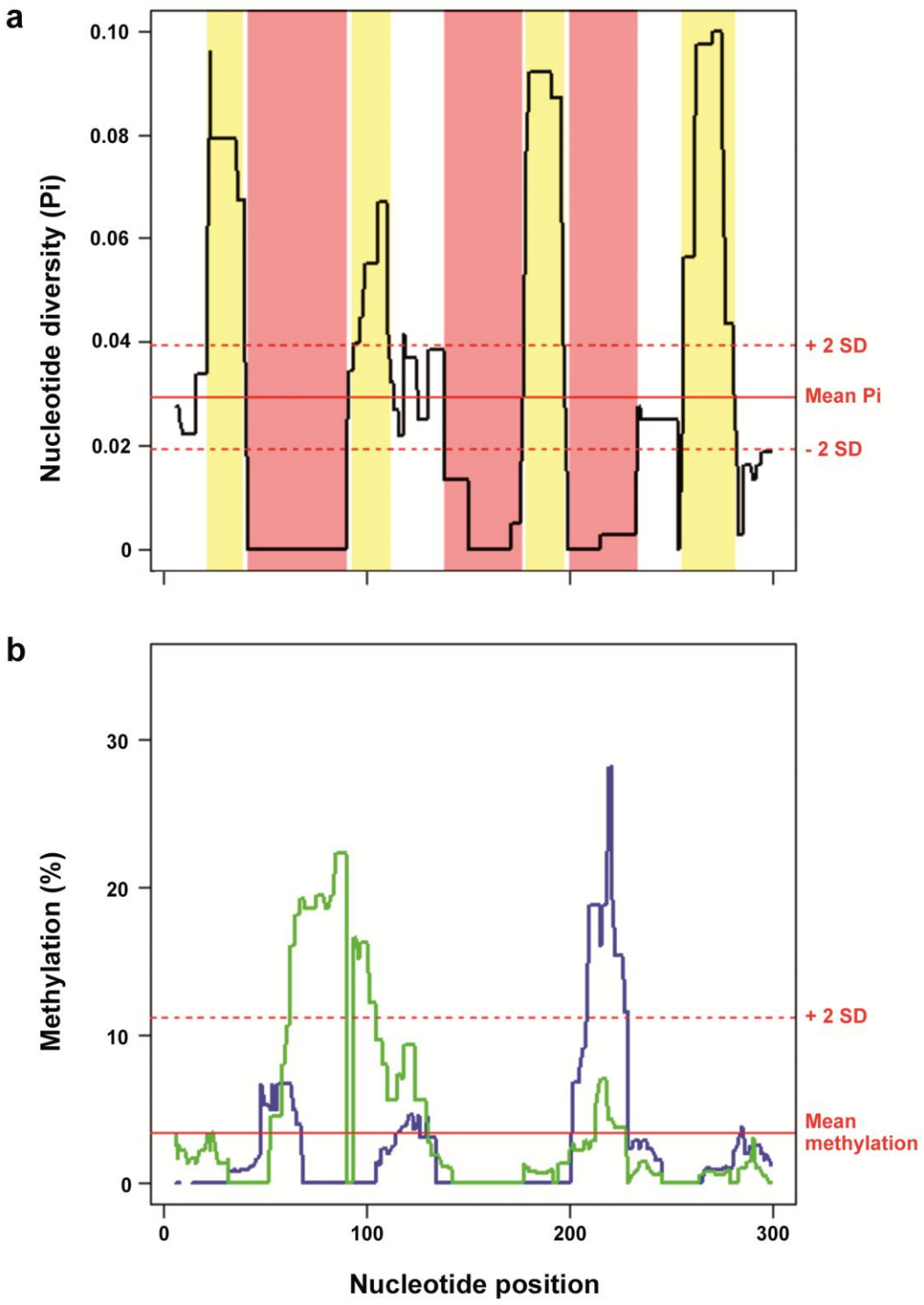
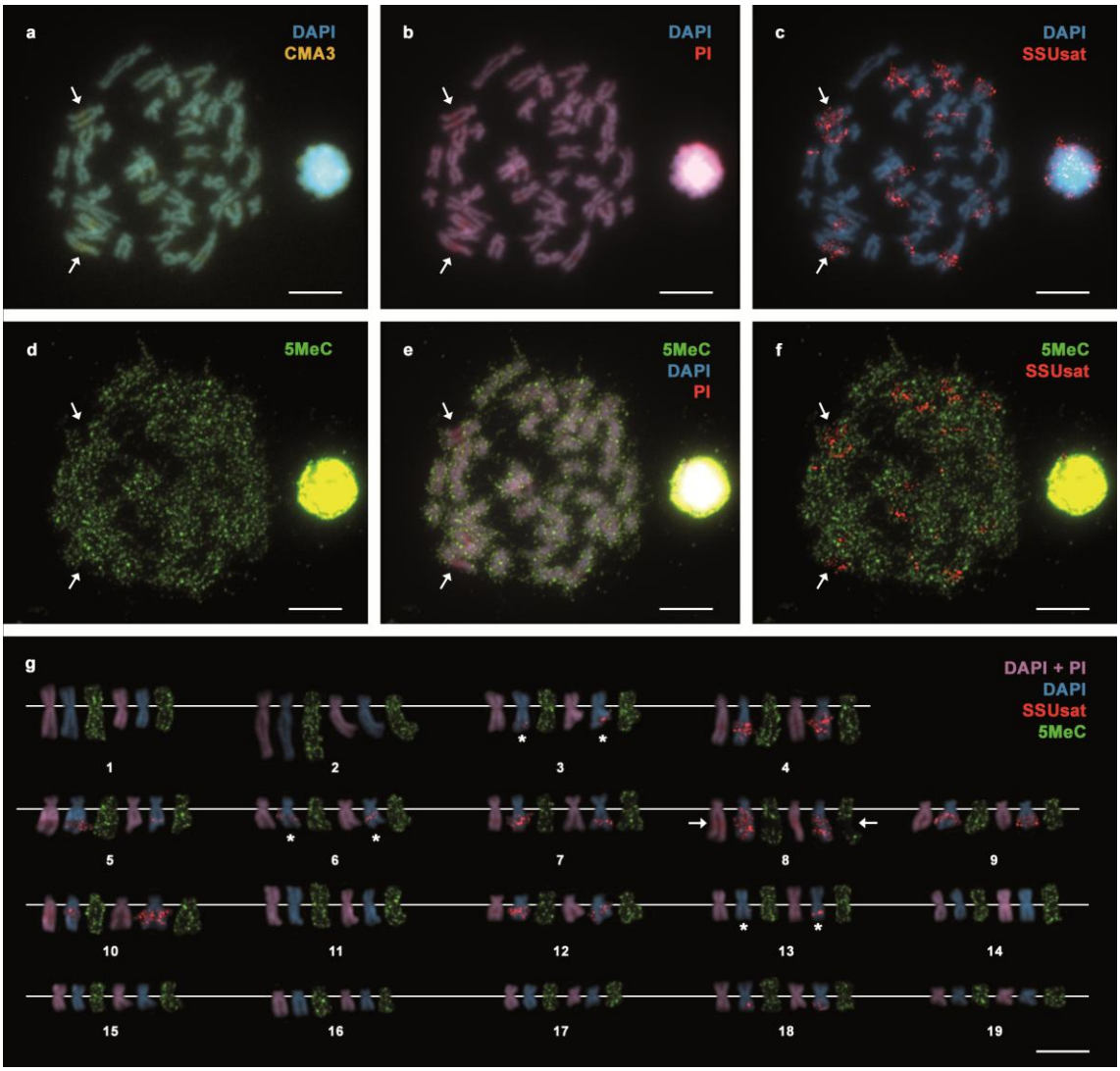
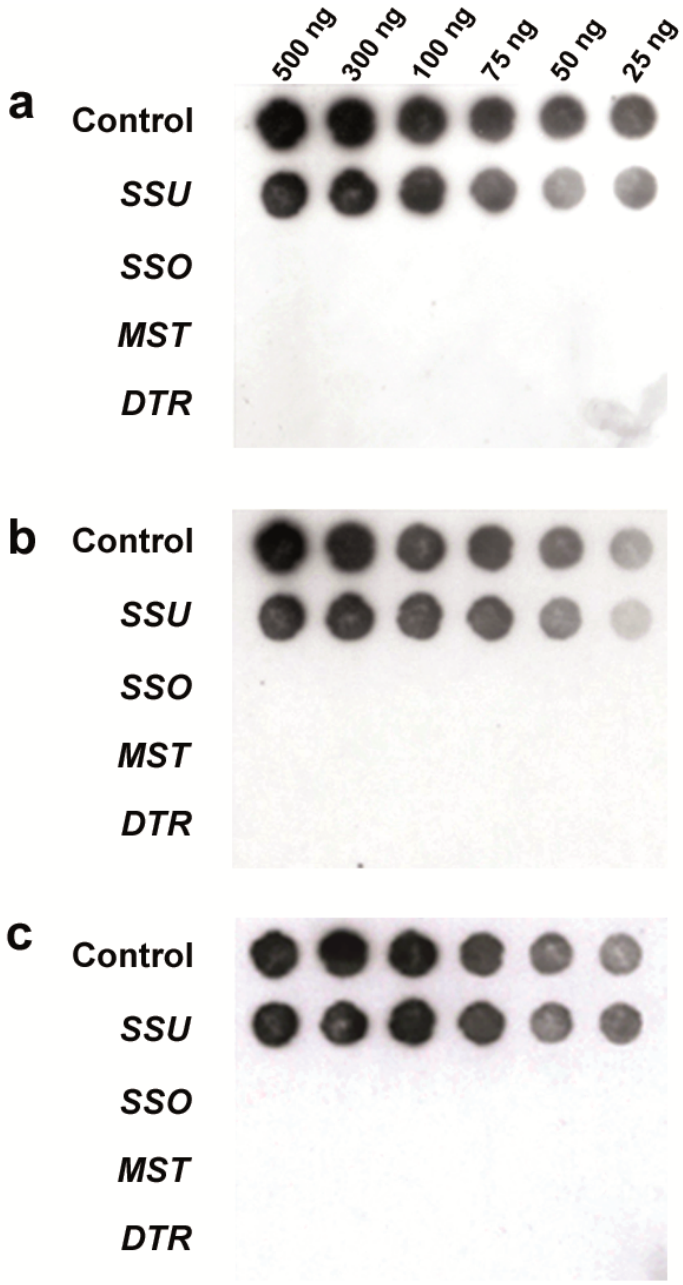

a
Nucleotide diversity (Pi)
0.10
0.08
0.06
0.04
0.02
0
+ 2 SD
Mean Pi
- 2 SD
b
Methylation (%)
30
20
10
0
+ 2 SD
Mean methylation
0
100
200
300
Nucleotide position

Figure 6


a
DAPI
CMA3
b
DAPI
PI
c
DAPI
SSUsat
d
5MeC
e
5MeC
DAPI
PI
f
5MeC
SSUsat
g
DAPI + PI
DAPI
SSUsat
5MeC
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Supplementary Figure 1:




10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
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SSU22a 159
SSU22c 159
SSU22b 159
SSU1 159
SSU5 159
SSU4 159
SSU14a 159
SSU14b 159
SSU19a 159
SSU19b 159
SSU7 159
SSU8 159
SSU18a 159
SSU18b 159
SSU17a 159
SSU17b 159
SSU2 159
SSU9 159
SSU21b 159
SSU21c 159
SSU21a 159
SSU20b 159
SSU20a 159
SSU10 158
SSU37a 159
SSU39b 159
SSU39d 159
SSU39c 159
SSU39a 159
SSU37b 159
SSU37c 159
SSU37d 159
SSU3 159
SSU6 159
SSU30c 159
SSU16a 159
SSU16b 159
SSU30a 159
SSU30b 159
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SSUinSSO2 159
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SSUinMST20 159
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SSUinMST25 159


170 180 190 200 210 220 230 240 250 260 270 280 290 300 310
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SSU17b 315
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SSU39d 315
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SSUinMST32 315
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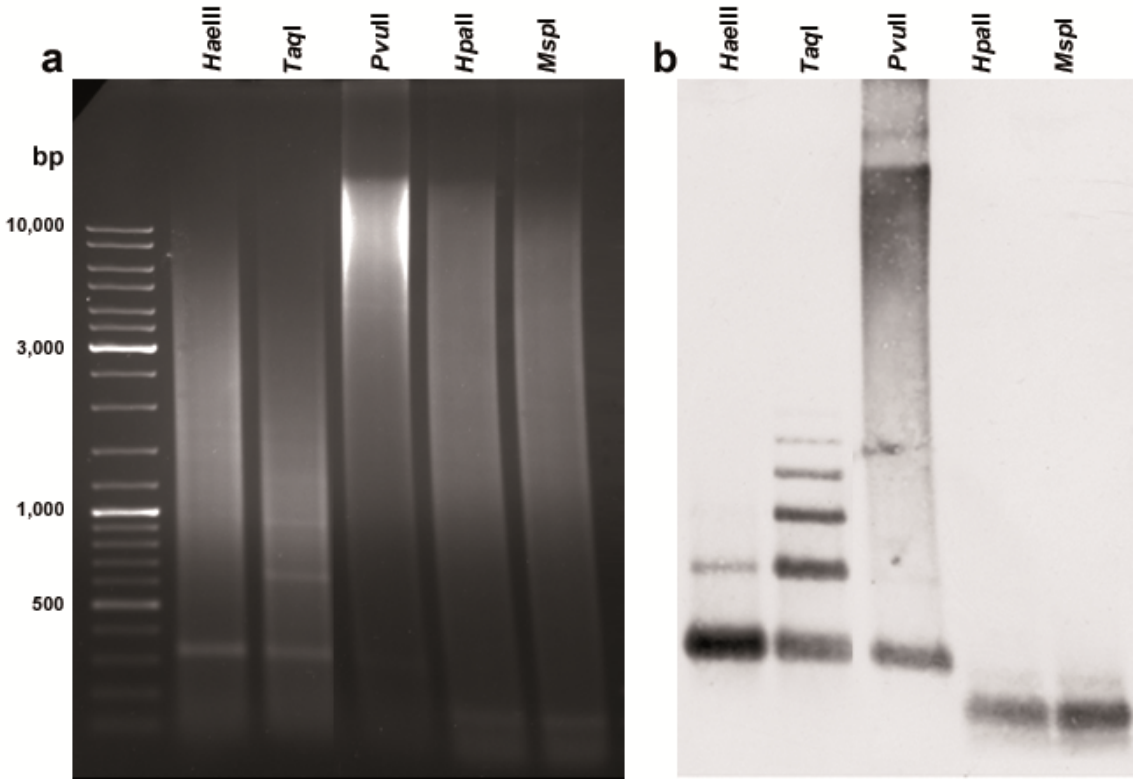
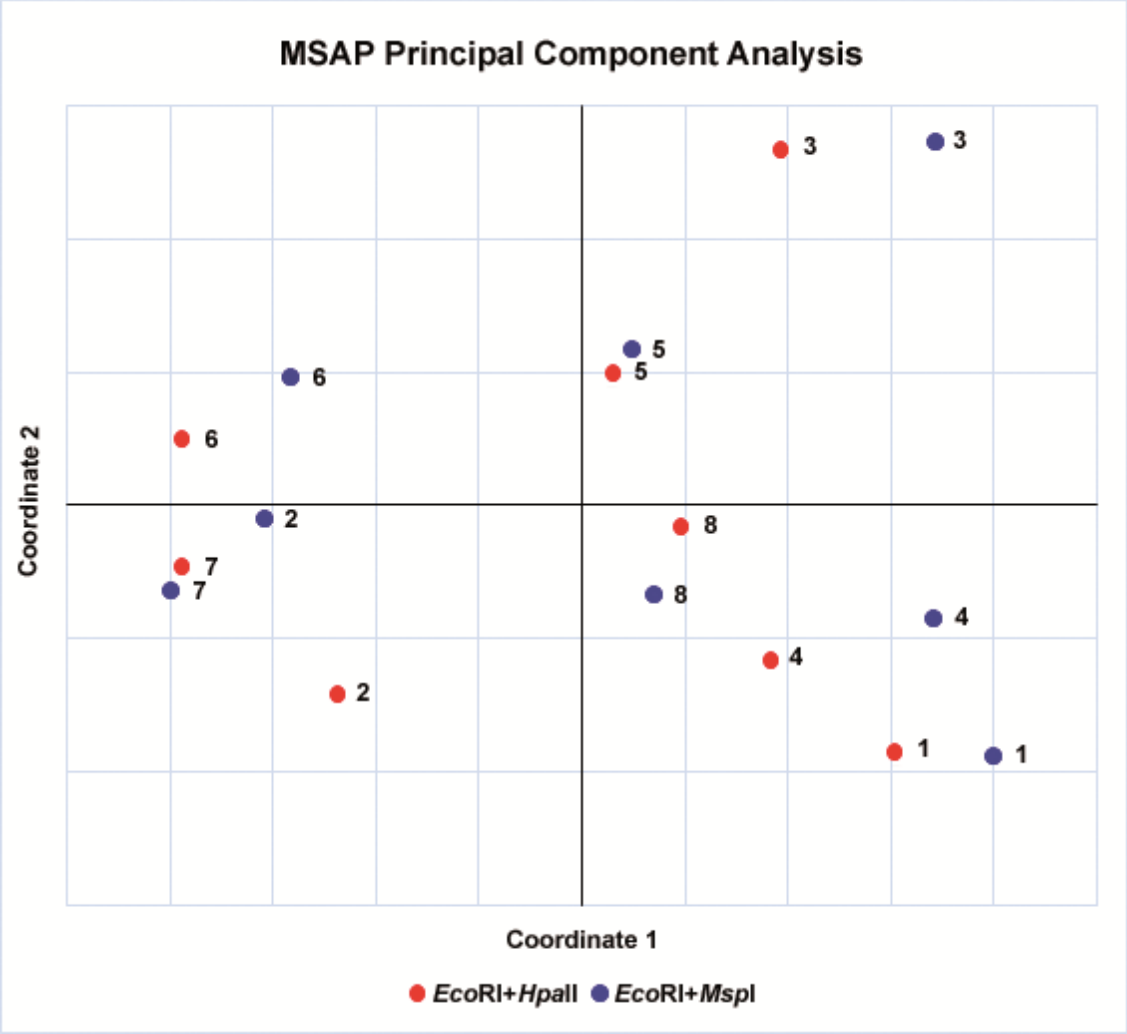
Supplementary Figure 3

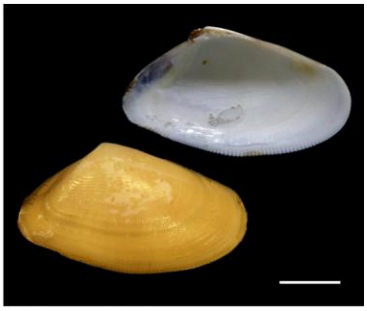


Supplementary Figure 4



DONACIDAE

Donax trunculus
(Linnaeus, 1758)
Coquina



Donax vittatus
(Linnaeus, 1758)
Coquina

Are pericentric inversions reorganizing wedge shell genomes?

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Abstract

Chromosomes are largely fluid structures displaying a plethora of reorganizations among which chromosomal inversions are paramount as they can confer varying fitness in different habitats and intensify reproductive isolation. The wedge shells of the family Donacidae are the dominant bivalves in exposed beaches almost worldwide. Usually, two or more sympatric species of wedge shells differentially occupy intertidal, sub littoral and offshore coastal waters in any given locality. A molecular cytogenetic analysis of two sympatric, closely related wedge shell species inhabiting European exposed sandy beaches, *Donax trunculus* and *Donax vittatus*, was performed. The karyotypes of the two species were at the same time strikingly different and closely alike; whilst metacentric and submetacentric chromosome pairs are the main components of the karyotype of *D. trunculus*, 10-11 of the 19 chromosome pairs are telocentric in *D. vittatus*, most likely as results of different pericentric inversions. GC-rich heterochromatic bands were present in both species and they also presented coincident 45S and 5S rDNA and H3 histone gene clusters at conserved chromosomal locations although *D. trunculus* displayed an additional 45S rDNA. Furthermore, intraspecific pericentric inversions were also detected in both *D. trunculus* and *D. vittatus*.

Keywords: *Donax*; Fluorescent *in situ* hybridization; Histone genes, Ribosomal RNA genes; CG-rich heterochromatin; Pericentric inversions

Introduction

Genomic data obtained along the last two decades have revealed that chromosomes are largely fluid structures displaying a plethora of reorganizations (Kirkpatrick 2010). The patterns of chromosomal rearrangements are, in many cases, lineage-specific (Coghlan et al. 2005). Chiefly among those rearrangements, chromosomal inversions can confer varying fitness in different habitats and intensify reproductive isolation (Ayala and Coluzzi 2005; Hoffmann and Rieseberg 2008; Kirkpatrick 2010).

The wedge shells of the family Donacidae (Bivalvia, Heterodonta) are the dominant bivalves in exposed beaches from tropical and temperate coastal waters (Ansell 1983). The systematic classification of this family presents several unsolved problems (Huber 2010), among which are the assignation of 88 of the 107 species to the genus *Donax* (Huber 2010) and the taxonomic status of many of its taxa (Carstensen et al. 2009). Although some wedge shell specimens are easily identifiable attending to shell morphology, others can only be recognized after observing highly variable shell characteristics showing intermediate states between taxa (Salas-Casanova 1987; Tirado and Salas 1999); this is further complicate by the presence of two or more geographically-sympatric species of *Donax* differentially occupying intertidal, sub littoral and offshore regions in almost any given locality (Ansell 1983).

Even though different kinds of genetic methodologies have been used for solving taxonomic and identification troubles (Adamkewicz and Harasewych 1994; Carstensen et al. 2009; Pereira et al. 2012; Nantón et al. 2014, 2015) and for characterizing repetitive DNA sequences (Plohl and Cornudella 1996, 1997; Petrović and Plohl 2005; Petrović et al. 2009; Šatović and Plohl 2013) in wedge shells, chromosome studies are almost fully neglected. But for the determination of a diploid chromosome number of 2n=38 in *Donax variabilis* (Menzel 1968), karyological data on the species of the family Donacidae are restricted to the abrupt wedge shell *Donax trunculus* (Cornet and Soulard 1990; Martínez et al. 2002; Plohl et al. 2002; Petrović et al. 2009). The karyotype of *Donax trunculus* (2n=38) is entirely (Cornet and Soulard 1990; Petrović et al. 2009) or almost entirely (Martínez et al. 2002) composed of bi-armed chromosome pairs, many of which bear GC-rich heterochromatic bands (Martínez et al. 2002; Petrović et al. 2009). In this wedge shell species, the chromosomal locations of major rDNA (Martínez et al. 2002; Petrović et al. 2009), telomeric sequences (Plohl et al. 2002; Petrović et al. 2009) and a GC-rich satellite DNA (Petrović et al. 2009) are also known.

As part of our ongoing bivalve cytogenetic research program, we discovered that in another species of wedge shell, *Donax vittatus*, metaphase plates displayed a high proportion of chromosome pairs with short arms barely detectable, i.e. telocentric chromosomes. *D. trunculus* and *D. vittatus* are closely related taxa, whose status as separated species is still in question (Huber 2010), that live in sympatry, although occupying intertidal and sub littoral habitats, in NW Spain exposed beaches. As these features make them excellent candidates to gain knowledge on the chromosome diversification patterns in bivalves, we collected *D. trunculus* and *D. vittatus* specimens from sympatric and allopatric populations and analyzed their chromosomes by means of 4',6-diamidino-2-phenylindole (DAPI) / chromomycin A3 (CMA) and DAPI / propidium iodide (PI) fluorescence staining and fluorescent *in situ* hybridization (FISH) with 28S rDNA, 5S rDNA, core histone gene and telomeric probes.

Materials and methods

Biological material

Wedge shells specimens were collected (Figure 1) in exposed beaches of Galicia (NW Spain). The specimens were identified as the abrupt wedge shell *Donax trunculus* Linnaeus, 1758 or the banded wedge shell *Donax vittatus* (da Costa, 1778) through their shell characteristics. Taking into account the reported differences in the karyotype composition of Mediterranean (Cornet and Soulard 1990) and Atlantic (Mártinez et al. 2002) populations of *D. trunculus*, we also included in our study abrupt wedge shell specimens collected in the Gulf of Valencia (E Spain). In all cases, wedge shells were transported to the laboratory, maintained in tanks of 5 L filtered seawater at 18 ± 1°C and fed on microalgae to promote somatic growth (Pasantes et al. 1990).

Mitotic chromosome preparation and fluorochrome staining

Chromosome preparations were obtained following the technique described by Martínez-Expósito et al. (1994a). Wedge shell specimens were housed in 0.5 L beakers and exposed to colchicine (0.005%) for 12 h. Gill and mantle tissues were excised and immersed in 50% (20 min) and 25% (20 min) seawater and fixed with ethanol/acetic acid for 1 h. Chromosome spreads were obtained by dissociating small pieces of tissue in 60% acetic acid and dropping the cellular suspension onto clean slides heated to 50 ºC.

Fluorochrome staining was performed as described by Pérez-García et al. (2010b). After controlling the quality of the chromosome preparations by phase contrast microscopy (Nikon), selected slides were stained for 2 h with CMA (0.25 mg/mL), counterstained with DAPI (0.14 μg/mL) for 8 min, washed in tap water, air-dried and mounted with antifade (Vectashield, Vector). Slide visualization and photography were performed using a Nikon Eclipse-800 microscope equipped with an epifluorescence system. Separated images for each fluorochrome were obtained with a DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon). Merging of the images was performed with Adobe Photoshop. Following visualization and photography, chromosome preparations were re-stained with a combination of DAPI and PI (0.07 μg /mL), washed in tap water, air-dried, mounted in antifade and photographed again.

DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from ethanol-preserved adductor muscles using the EZNA Mollusc DNA Kit (OMEGA). A fragment of the mitochondrial COI gene was amplified by PCR employing the standard barcoding primers *LCO1490* and *HCO2198* (Folmer et al. 1994). Amplification of a fragment of the 28S rDNA used universal primers *LR10R* and *LR12* (Vilgalys lab website, http://www.biology.duke.edu/fungi/mycolab/primers.htm). The amplifications of the entire 5S rDNA repeat and the H3 histone genes were performed using the primers described by Pérez-García et al. (2010a) and Giribet and Distel (2003), respectively. PCR products were examined by electrophoresis on 2% agarose gels.

DNA sequences were amplified in a GeneAmp PCR system 9700 (Applied Biosystems) in 50 μL solutions containing 125 ng of genomic DNA, 50 μM each dNTP, 50 μM each primer, 1xPCR buffer, 15 μM $MgCl_2$ and 5 U of JumpStart™ Taq DNA Polymerase (Sigma). Amplifications included an initial denaturation step at 95 ºC (2 min), 35 amplification cycles (Table 1) and a final extension at 72 °C (5 min). PCR products were examined by electrophoresis on a 2% agarose gels.

The amplified mitochondrial COI gene was purified (FavorPrep™ GEL/PCR Purification Kit, Favorgen) and sequenced (CACTI, University of Vigo) in both directions in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were edited with BioEdit v. 7.1.11 (Hall 1999) and aligned with Muscle set to default parameters using MEGA7 (Kumar et al. 2016). Sequence similarity searches were performed using the Basic

Local Alignment Search Tool algorithm (BLAST), available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The MegaBLAST algorithm set to default parameters was employed against both NCBI nucleotide collection and NCBI nucleotide collection and Barcode of Life Data System (BOLD) databases.

As the analysis of the restriction fragment length polymorphism of COI gene PCR products (PCR-RFLP) has been proposed as a tool to identify the European species of *Donax* (Nantón et al. 2015), we also compared the expected patterns of fragment sizes generated by *Alu*I, *Hae*III and *Msp*I on our sequences with those obtained by Nantón et al. (2015).

Fluorescent *in situ* hybridization (FISH)

Metaphase chromosome spreads obtained from wedge shells collected at all localities were single, double and sequentially hybridized using 5S and 28S rDNA and H3 histone gene probes (Pérez-García et al. 2011; García-Souto et al. 2015, 2016). 28S rDNA probes were labeled with biotin-16-dUTP (Roche Applied Science) and/or digoxigenin-11-dUTP (10x DIG Labeling Mix, Roche Applied Science) using a nick translation kit (Roche Applied Science). H3 histone gene and 5S rDNA probes were directly labeled by PCR either with biotin-16-dUTP (20 μM) or digoxigenin-11-dUTP (5 μM).

Chromosome preparations were digested with RNase and pepsin before denaturation (70 ºC, 2 min) and hybridized overnight at 37 ºC. Biotin was detected with fluorescein isothiocyanate (FITC) conjugated avidin and biotinylated anti-avidin (Vector) whereas digoxigenin was detected with anti-digoxigenin antibodies conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Sigma). Chromosome preparations were counterstained with DAPI, mounted with antifade and examined by fluorescence microscopy. Separated images for each fluorochrome were recorded, pseudo-colored and merged as indicated above. In addition, we also performed FISH with a telomeric $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) following the protocol indicated by the supplier.

Chromosome counting and karyotype analysis were performed in 40 specimens, 20 per species (10 females, 10 males). For each species, 20 complete metaphase plates showing FISH signals were used to construct karyotypes. Chromosome and arm lengths were carefully measured and relative lengths and centromeric indices calculated.

Results

All partial COI gene sequences obtained in this work were independently compared using BLAST with those stored in GenBank and BOLD databases. The nucleotide sequences for all wedge shell specimens morphologically assigned to *D. trunculus* (GenBank accession numbers KY951431 to KY951446) displayed a high degree of similarity (> 98%) with the single *D. trunculus* sequence stored in GenBank (accession number KC429143). Concordantly, all our *D. vittatus* sequences (GenBank accession numbers KY951416 to KY951430) also displayed similarities higher than 98% with the two *D. vittatus* GenBank sequences (accession numbers KR084728 and KR084687). Moreover, the positions occupied by the restriction targets for *Alu*I, *Hae*III and *Msp*I on the COI gene sequences obtained in this work would generate RFLP patterns totally compatible with those predicted by Nantón et al. (2015) for *D. trunculus* and *D. vittatus*.

In agreement with previous reports (Cornet and Soulard 1990; Martínez et al. 2002; Petrović et al. 2009), *D. trunculus* showed a diploid chromosome number of 2n = 38. The chromosome complement of 2n = 38 for *D. vittatus* is described here for the first time. Representative metaphase plates and karyotypes, presenting chromosome pairs in decreasing order of size, of these species appear on Figures 2 and 3. The karyotype of *D. trunculus* (Figure 2e,j) is composed of bi-armed (metacentric, submetacentric and subtelocentric) chromosome pairs whereas 10 or 11 of the 19 chromosome pairs are undoubtedly telocentric in *D. vittatus* (Figure 3e,j). Both wedge shells exhibit DAPI negative (Figures 2 and 3: a,e,f,j), CMA positive (Figures 2 and 3: b,g), PI positive (Figures 2 and 3: c,h), GC-rich heterochromatic bands in a number of chromosome pairs, 14 in *D. trunculus* and 6 in *D. vittatus*. Telomeric sequence signals were restricted to the terminal regions of the chromosomes in both *D. trunculus* (Figure 2c,h) and *D. vittatus* (Figure 3c,h), without any evidence of signals at other chromosomal regions.

Single, double and sequential hybridization experiments were performed to locate H3 histone and 28S and 5S rRNA gene clusters on the chromosomes of the two wedge shell species. H3 histone gene clusters were intercalary to the long arms of chromosome pair 17 in the two wedge shells, this chromosome was submeta/subtelocentric in *D. trunculus* (Figure 2d,e,h,i) and telocentric in *D. vittatus* (Figure 3d,e,h,i). In concordance with previous reports (Martínez et al. 2002; Petrović et al. 2009), major rDNAs map to a single locus intercalary to the short arms of subtelocentric chromosome pair 6 in all Atlantic specimens of *D. trunculus* (Figure 2d,e). In contrast, major rDNA is

subcentromeric to the long arms of chromosome pair 6, metacentric, in all Mediterranean *D. trunculus* (Figure 2h,i). All *D. vittatus* specimens also displayed major rDNAs in the short arms of chromosome pair 6, telocentric (Figure 3d,e,h,i). The number of 5S rDNA clusters displayed by *D. trunculus* and *D. vittatus* was different. Whilst the subterminal signals on the long arms of submetacentric (*D. trunculus*) or subtelocentric (*D. vittatus*) chromosome pair 10 were present in the two wedge shells (Figures 2 and 3: d,e,h,i), *D. trunculus* presented an additional signal intercalary to the short arms of metacentric chromosome pair 3 (Figure 2d,e,h,i). A schematic, comparative representation integrating all cytogenetic results appears in Figure 4.

Atlantic and Mediterranean *D. trunculus* karyotypes are somewhat different. Chromosome pair 6, subtelocentric in the Atlantic specimens, is clearly metacentric in Mediterranean abrupt wedge shells; chromosome pair 11 also differs in morphology, subtelocentric in Atlantic specimens and submetacentric in Mediterranean (Figures 2 and 4), most probably as results of pericentric inversions. A fairly similar situation was detected for three chromosome pairs in *D. vittatus*; chromosome pair 4 is telocentric and pairs 11 and 13 metacentric in Samil specimens; in all other banded wedge shells chromosome pair 4 is metacentric while pairs 11 and 13 are telocentric (Figures 3 and 4).

Interspecific karyotype comparison (Figure 4) clearly shows that the telocentric chromosome pairs numbers 6, 7, 8, 9, 11, 13, 14, 15, 16, 17 and 18 found in most *D. vittatus* specimens are bi-armed pairs, meta and submetacentric (7, 8, 13, 15, 16, 18) or subtelocentric (6, 9, 11, 14, 17), in Atlantic *D. trunculus*. Again, these differences are most likely the consequences of pericentric inversions.

Discussion

The 2n = 38 diploid chromosome numbers of the two wedge shell species studied here coincide with those previously reported for *Donax variabilis* (Menzel 1968) and *D. trunculus* (Cornet and Soulard 1990; Martínez et al. 2002; Petrović et al. 2009). This is also the usual diploid number in most bivalve species belonging to the subclass Heterodonta (Nakamura 1985; Thiriot-Quiévreux 1994, 2002; Leitão and Chaves 2008; Arias-Pérez et al. 2013; González-Tizón et al. 2013; Pérez-García et al. 2014).

In contrast, the karyotypes of the wedge shells studied here present some peculiarities unusual for the subclass Heterodonta. Those bivalve karyotypes are usually poor in telocentric chromosomes (reviewed in González-Tizón et al. 2013) and species with undetectable short arms in those telocentric pairs are uncommon; the best known

examples are the dwarf surf clam *Mulinia lateralis* (Wang and Guo 2008) and three razor shells (Fernández-Tajes et al. 2008; González-Tizón et al. 2013). This is also the case for *D. vittatus* karyotypes in which, of a total of 10 or 11 telocentric chromosome pairs, only the 45S rDNA-bearing chromosome pair 6 displays clearly detectable short arms. In contrast, all chromosome pairs in *D. trunculus* display unmistakably visible short arms. These results are compatible with previous works in *D. trunculus* showing none (Cornet and Soulard 1990; Petrović et al. 2009) or one (Martínez et al. 2002) telocentric chromosome pairs.

Although a certain degree of divergence is usually detected when comparing karyotypes proposed by different researchers for the same or different populations of any given bivalve species (i.e. see the diversity in karyotype formulas given in the classical revisions by Thiriot-Quiévreux 1994, 2002), those are usually a consequence of divergent chromosome measurement methodologies and degrees of condensation. This is clearly not the case for the marked intraspecific karyotypic diversity found in populations of both *D. trunculus* and *D. vittatus*, for which the most parsimonious explanation implies pericentric inversion events. For instance, in coincidence with previous reports (Martínez et al. 2002; Petrović et al. 2009), Atlantic *D. trunculus* present a single 45S rDNA cluster on the short arms of a subtelocentric chromosome pair; in contrast, in Mediterranean specimens the signals for the 45S rDNA cluster are subcentromeric to the long arms of a metacentric chromosome pair. The simplest explanation for this discrepancy is a pericentric inversion that either transformed a subtelocentric pair in a metacentric or the other way round; in any case, one of the inversion breakpoints had to be distal, but not far away, from the 45S rDNA cluster in a chromosome arm and the other far away from the centromere in the other arm. A single pericentric inversion with breakpoints close to the centromere on the short arms and proximal from the heterochromatic band on the long arms can also explain the differences in chromosome pair 11. In a similar way, pericentric inversions can also parsimoniously explain the interpopulational differences in chromosome morphologies found in *D. vittatus* pairs 4, 11 and 13.

The close proximity of these two wedge shell species (Huber 2010) together with the striking conservation of the 45S rDNA, 5S rDNA and H3 histone gene clusters and the degree of maintenance of the GC-rich heterochromatic bands in these taxa also point to pericentric inversions as the main evolutionary force contributing to the karyotype divergence in wedge shells. In a similar way to the reasoning employed above, to most

parsimoniously explain the differing chromosome shapes in *D. trunculus* and *D. vittatus* is resort to pericentric inversions again.

Pericentric inversions, as well as other chromosome reorganizations, may be of considerable importance in parapatric or partly sympatric speciation (Ayala and Coluzzi 2005), facilitating genetic differentiation during speciation (Hoffmann and Rieseberg, 2008; Lowry and Willis 2010). Although it is still not clear if chromosome rearrangements are just a nuisance for the genome or have functional significance (Noor et al. 2001), the fact is that pericentric inversions are widespread and that, in many eukaryotes, the rearrangement breakpoints are strongly associated to repetitive sequences (Coghlan et al. 2005; Knebel et al. 2011; Lange et al. 2013), probably as a consequence of use of ectopic homologous sequences as template for recombination repair (Schubert and Lysak 2010). Whether the relative abundance of repetitive sequences in wedge shells (Plohl and Cornudella 1996, 1997; Petrović and Plohl 2005; Petrović et al. 2009; Šatović and Plohl 2013) is related to the high amount of pericentric inversions is a question to solve in the future.

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Figure Legends

Figure 1: Collection localities and pictures of representative wedge shells analyzed.

Figure 2: Mapping of telomeric sequence, 5S rDNA, 28S rDNA and H3 histone gene clusters to *Donax trunculus* chromosomes. Sequential fluorochrome staining of mitotic

metaphase plates shows DAPI negative regions (**a**, **f**) that fluoresce yellow after CMA (**b**, **g**) and bright red after DAPI/PI (**c**, **h**) staining in both Atlantic (**a-c**) and Mediterranean (**f-h**) specimens of *Donax trunculus*. Hybridization of the same metaphase plates (**c**, **h**) with a telomeric PNA probe discloses signals (TEL, green) at both ends of every chromosome. Sequential FISH experiments using major and minor rDNA and H3 histone gene probes on the same metaphase plates (**d**, **i**), and the corresponding karyotypes (**f**, **h**), show H3 histone gene signals (H3, green) intercalary to the long arms of chromosome pair 17. Minor rDNA probes (5S, red) map to two locations, intercalary to the short arms of chromosome pair 3 and subterminal to the long arms of chromosome pair 10. Major rDNA signals (28S, magenta) are intercalary to the short arms of subtelocentric chromosome pair 6 in Atlantic specimens (**d**, **f**) and subcentromeric to the long arms of metacentric pair 6 in Mediterranean specimens. Note that none of the chromosome pairs is telocentric. Scale bars, 5 μm.

Figure 3: Mapping of telomeric sequence, 5S rDNA, 28S rDNA and H3 histone gene clusters to *Donax vittatus* chromosomes. Sequential fluorochrome staining of mitotic metaphase plates shows DAPI negative regions (**a**, **f**) that fluoresce yellow after CMA (**b**, **g**) and bright red after DAPI/PI (**c**, **h**) staining in *Donax vittatus* specimens from both Samil (**a-c**) and all the other Galician (**f-h**) populations. Hybridization of the same metaphase plates (**c**, **h**) with a telomeric PNA probe discloses signals (TEL, green) at both ends of every chromosome. Sequential FISH experiments using major and minor rDNA and H3 histone gene probes on the same metaphase plates (**d**, **i**), and the corresponding karyotypes (**f**, **h**), show H3 histone gene signals (H3, green) intercalary to the long arms of telocentric chromosome pair 17. Minor rDNA signals (5S, red) are subterminal to the long arms of subtelocentric chromosome pair 10. Major rDNA signals (28S, magenta) overlap the short arms of telocentric chromosome pair 6. Note the presence of 10 telocentric chromosome pairs in specimens from Samil (4, 6, 7, 8, 9, 14, 15, 16, 17 and 18) and 11 in those from the remaining Galician populations (6, 7, 8, 9, 11, 13, 14, 15, 16, 17 and 18). Scale bars, 5 μm.

Figure 4: Schematic representation of the wedge shell haploid chromosome complements. On comparative purposes, the schemas of chromosomes 1 to 19 in *Donax trunculus* (DTR) Mediterranean (M) and Atlantic (A) and *Donax vittatus* (DVI) most Galician (G) and Samil (S) populations are represented in quartets. DAPI-/CMA+ bands (yellow), 5S rDNA

(red), 45S rDNA (magenta) and H3 histone gene (green) are also represented. The chromosomes showing the most remarkable intraspecific differences are shadowed in light green for *Donax trunculus* and light pink for *Donax vittatus*.

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Table 1. Parameters used in the PCR

Sequence	Denaturation	Annealing	Elongation
COI	95 ºC, 30 s	42 ºC, 30 s	72 ºC, 30 s
28S rDNA	95 ºC, 30 s	48 ºC, 30 s	72 ºC, 20 s
5S rDNA	95 ºC, 20 s	48 ºC, 20 s	72 ºC, 20 s
h3	95 ºC, 20 s	48 ºC, 20 s	72 ºC, 30 s

Figure 1

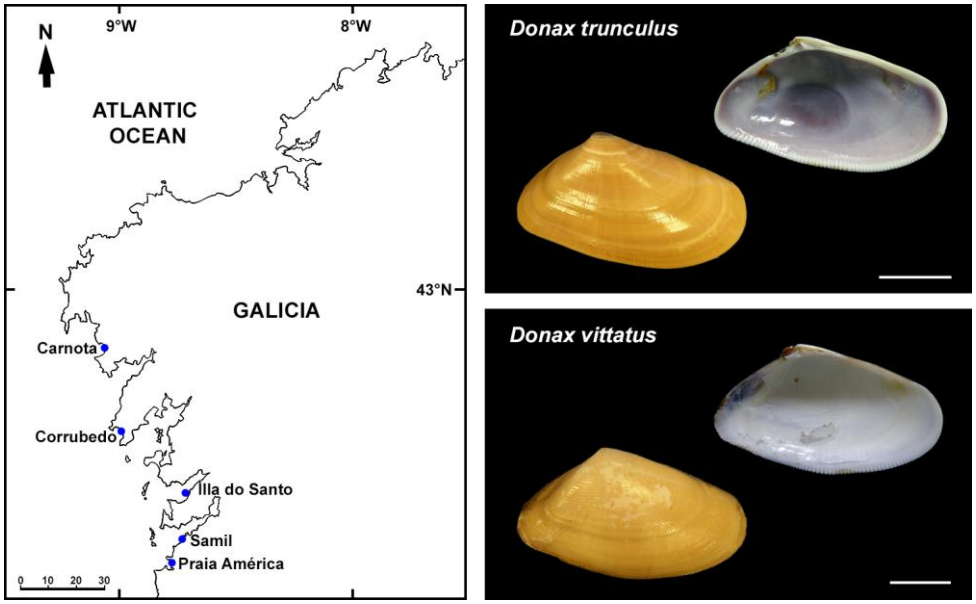


Figure 2

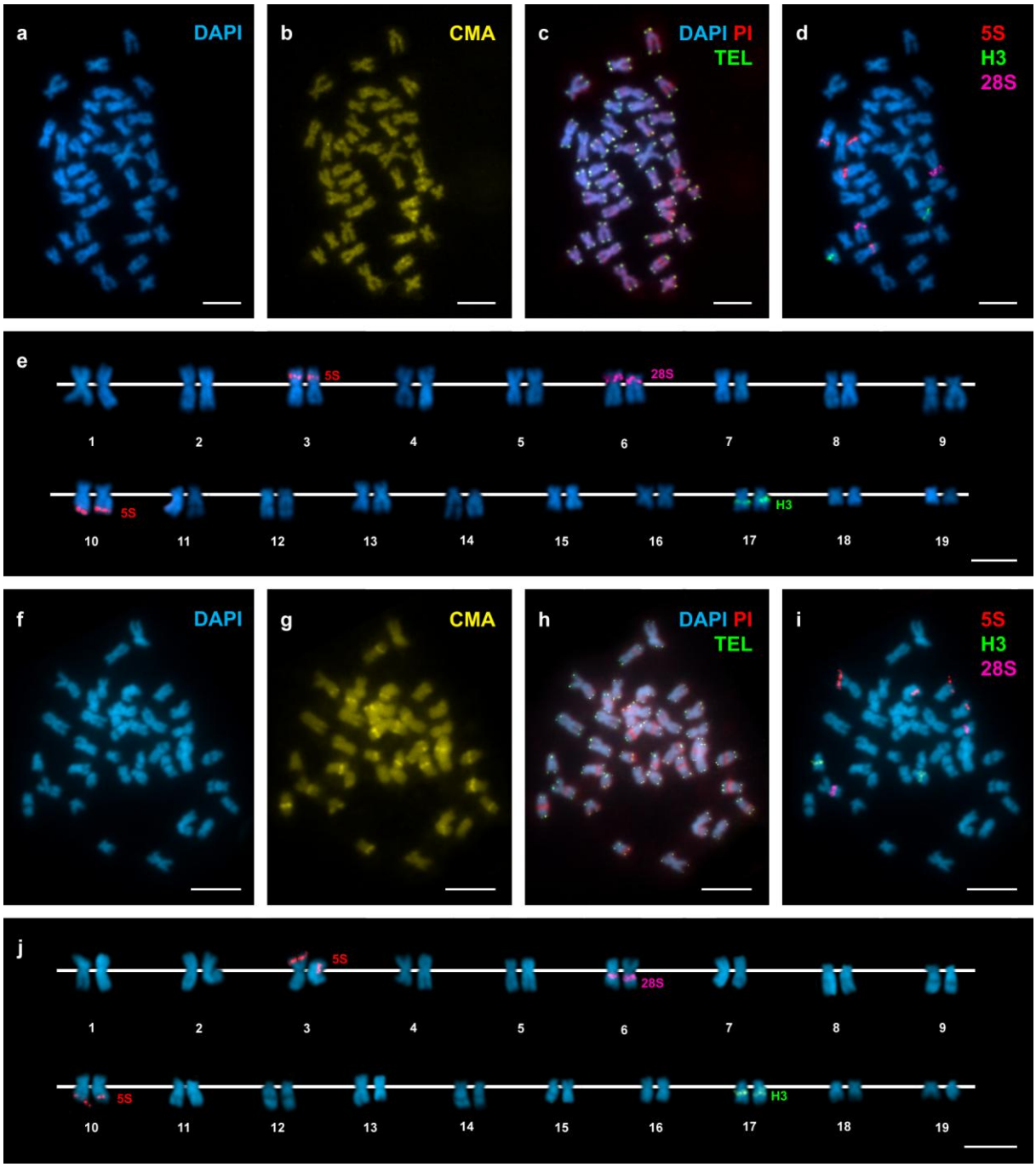

a
DAPI
b
CMA
c
DAPI PI
TEL
d
5S
H3
28S
e
5S
28S
1
2
3
4
5
6
7
8
9
5S
H3
10
11
12
13
14
15
16
17
18
19
f
DAPI
g
CMA
h
DAPI PI
TEL
i
5S
H3
28S
j
5S
28S
1
2
3
4
5
6
7
8
9
5S
H3
10
11
12
13
14
15
16
17
18
19

Figure 3

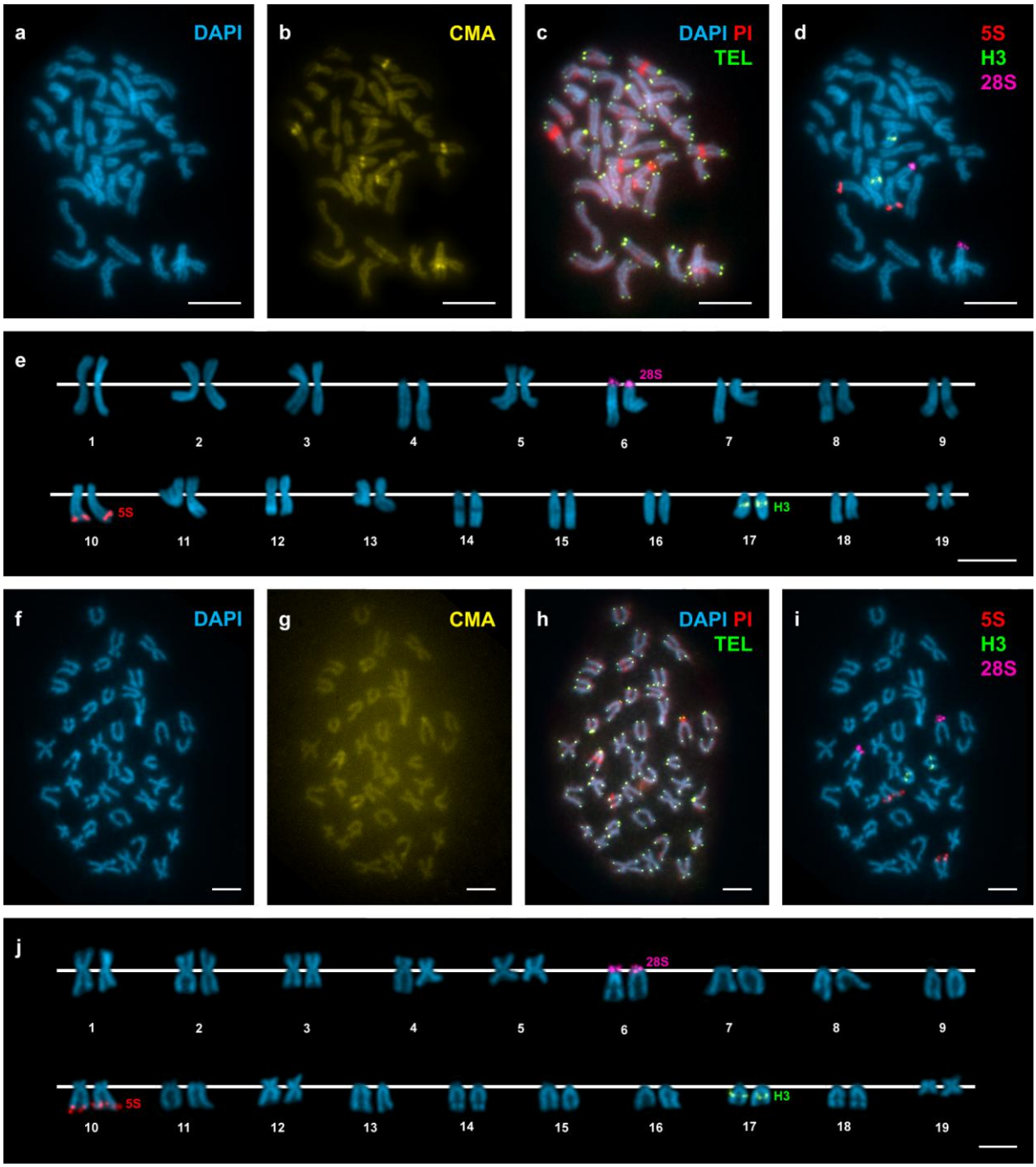
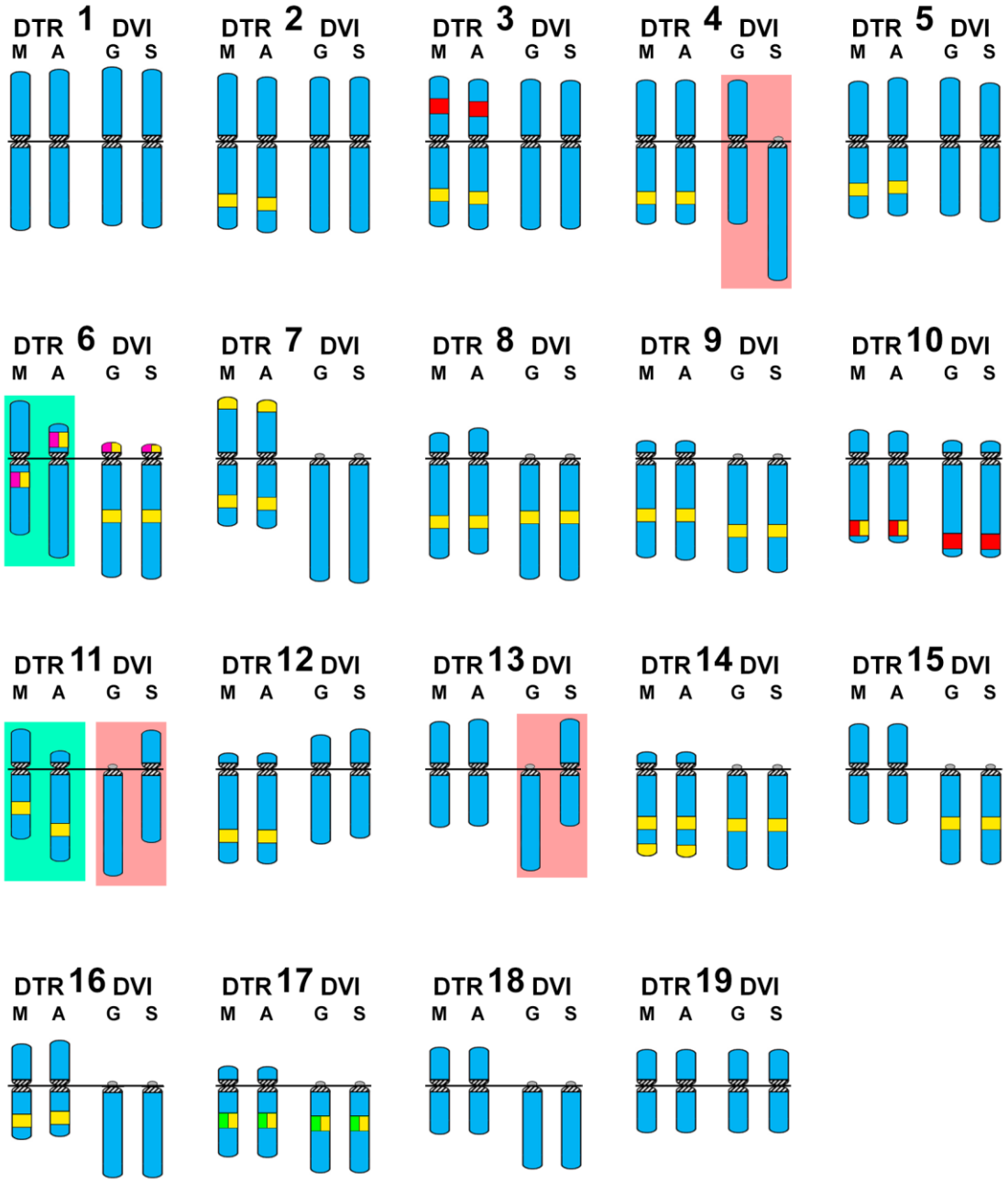

a
DAPI
b
CMA
c
DAPI PI
TEL
d
5S
H3
28S
e
28S
1
2
3
4
5
6
7
8
9
5S
H3
10
11
12
13
14
15
16
17
18
19
f
DAPI
g
CMA
h
DAPI PI
TEL
i
5S
H3
28S
j
28S
1
2
3
4
5
6
7
8
9
5S
H3
10
11
12
13
14
15
16
17
18
19

Figure 4

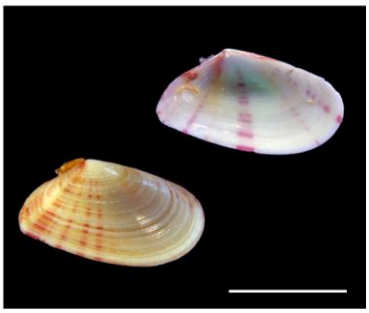

DTR 1 DVI
M A G S
DTR 2 DVI
M A G S
DTR 3 DVI
M A G S
DTR 4 DVI
M A G S
DTR 5 DVI
M A G S
DTR 6 DVI
M A G S
DTR 7 DVI
M A G S
DTR 8 DVI
M A G S
DTR 9 DVI
M A G S
DTR 10 DVI
M A G S
DTR 11 DVI
M A G S
DTR 12 DVI
M A G S
DTR 13 DVI
M A G S
DTR 14 DVI
M A G S
DTR 15 DVI
M A G S
DTR 16 DVI
M A G S
DTR 17 DVI
M A G S
DTR 18 DVI
M A G S
DTR 19 DVI
M A G S

TELLINIDAE

Bosemprella incarnata
(Linnaeus, 1758)

Macomangulus tenuis
(da Costa, 1778)

Serratina serrata
(Brocchi, 1814)



Moerella donacina
(Linnaeus, 1758)

Karyotype differentiation in tellin shells (Bivalvia, Tellinidae)

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Abstract

Although Tellinidae is one of the largest and most diverse families of bivalves, its taxonomy is utterly chaotic. This is mainly due to the morphological diversity and homoplasy displayed by their shells and to the scarcity of the molecular phylogenetic studies performed in them. A molecular cytogenetic analysis of four tellin shell species, *Bosemprella incarnata*, *Macomangulus tenuis*, *Moerella donacina* and *Serratina serrata*, was performed. The karyotypes of the four species were composed of different amounts of bi-armed and telocentric chromosomes. The chromosomal mapping of 45S and 5S rDNA and H3 histone gene clusters by fluorescent *in situ* hybridization also demonstrated that the patterns of distribution of these DNA sequences on their karyotypes showed conspicuous differences. Vertebrate type telomeric sequences were located exclusively on both ends of each chromosome in all four tellin shells. The sequence of a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) was also studied.

Keywords: Tellin shells; Chromosome; Fluorescent *in situ* hybridization; Histone genes, Ribosomal RNA genes;

Introduction

Tellin shells are fast-burrowing bivalves inhabiting marine and estuarine soft bottom ecosystems; they distribute worldwide and are particularly abundant at tropical latitudes (Simone and Wilkinson 2008). With over 550 living species organized in nine subfamilies and 107 genera, Tellinidae (Blainville, 1814) is one of the largest and most diverse families of bivalves (Huber 2015). In spite of that, the taxonomy of tellin shells is utterly chaotic, mainly as a consequence of the morphological diversity and homoplasy displayed by their shells (Simone and Wilkinson 2008; Huber 2015). The situation is further aggravated by the scarcity of molecular phylogenetic studies on this family (Huber 2015). The few molecular analyses including *Limecola* and *Serratina* sequences (Taylor et al. 2007; Saunier et al. 2014; Sun et al. 2016) have also challenged traditional systematics and demonstrated that some of the analyzed specimens were improperly placed and/or misidentified. Therefore, to overcome these difficulties and clarify the classification of tellin shells further identification criteria are required in order to integrate traditional cladistic and molecular phylogenetic studies.

Although chromosome analyses have helped in both characterizing and solving identification problems in some groups of bivalves (Pérez-García et al. 2014b, García-Souto et al. 2017a, 2017b), cytogenetic data in tellin shells is rather scarce and mostly limited to the description of diploid chromosome numbers ($2n = 38$) and karyotypes in three species, *Macomangulus tenuis* (Cornet and Soulard 1990), *Limecola balthica* (Cornet and Soulard 1990; Wolowicz and Thiriot-Quiévreux 1997) and *Macoma nasuta* (González-Tizón et al. 2000), and to the location of the nucleolar organizing regions (NORs) by silver staining in *L. balthica* (Smolarz et al. 2003) and by fluorescent *in situ* hybridization (FISH) in *M. nasuta* (González-Tizón et al. 2000). Additionally, chromosome analysis has also been applied to study neoplastic cells in *L. balthica* (Thiriot-Quiévreux and Wolowicz 1996, 2001; Smolarz et al. 2003, 2005)

To verify if a cytogenetic approach can help in solving some of the identification problems in tellin shells, in this work we characterized the chromosomes of four Tellinidae taxa by means of chromomycin A3 (CMA), 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) fluorescence staining and FISH mapping telomeric sequences and 45S rDNA, 5S rDNAs and H3 histone gene clusters. A fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was also amplified and sequenced in the same tellin shell specimens.

Materials and methods

Biological material

Tellin shell specimens were collected in Ría de Pontevedra (NW Spain). The specimens were identified as *Moerella donacina* (Linnaeus, 1758), *Serratina serrata* (Brocchi, 1814), *Macomangulus tenuis* (da Costa, 1778) and *Bosemprella incarnata* (Linnaeus, 1758) attending to shell morphology criteria. The nomenclature used for the taxa follows the World Register of Marine Species database (http://www.marinespecies.org/). To promote somatic growth, tellin shells were maintained in adequate laboratory conditions for a week (Pasantes et al. 1990).

Chromosome preparation and fluorochrome staining

Following an overnight colchicine (0.005%) treatment, tellin shells were euthanized and gill and gonadic tissues dissected and immersed in 50% (20 min) and 25% (20 min) sea water prior fixation with ethanol/acetic acid following previously described methods for cytogenetic studies in bivalves (Méndez et al. 1990). Chromosome spreads were obtained by disaggregating small pieces of tissue in 60% acetic acid and dropping the resulting cellular suspension onto preheated slides (Martínez-Expósito et al. 1994a, 1994b)

After verifying the quality of the preparations by phase contras microscopy, sequential fluorochrome staining was performed (Pérez-García et al. 2010a, 2010b; García-Souto et al. 2016). Briefly, chromosomes were stained with CMA (0.25 mg/mL, 2 h) and DAPI (0.14 µg/mL, 8 min), mounted with antifade (Vectashield, Vector) and examined by fluorescent microscopy. For every single metaphase plate, images for each fluorochrome were acquired, pseudocolored and merged. After washing and staining with DAPI and PI (0.07 µg/mL, 8 min), the same metaphase plates were recorded again.

DNA isolation, PCR amplification and probe labeling

Genomic DNA was extracted with the EZNA Mollusc DNA Kit (Omega) following manufactures instruction. A BioDrop µLITE (Biodrop) was employed to assess the purity and the concentration of the genomic DNA samples.

FISH probes were obtained by amplifying a 28S rDNA fragment with primers *LR10R* and *LR12* (Vilgalys lab, http://www.biology.duke.edu/fungi/mycolab/primers.htm), the entire 5S rDNA repeat with primers described by Pérez-García et al. (2010a) and the

H3 histone gene with those reported by Giribet and Distel (2003). On barcoding purposes, a fragment of the mitochondrial COI gene was amplified using the primers developed by Nantón et al. (2015) for Donacidae species.

PCRs were carried out in a GeneAmp PCR system 9700 (Applied Biosystems) in 20 μL volumes containing 50 ng DNA, 1x reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM each dNTP (Thermo Fisher Scientific), 1 μM each primer and 1 U BIOTAQ DNA polymerase (Bioline). The amplification of the 28S rDNA included 30 cycles of 20 s denaturation at 95 °C, 20 s annealing at 48 °C and 30 s extension at 72 °C. For 5S rDNA, 40 cycles (95 °C, 44 °C and 72 °C, 20 s each) of amplification were employed. H3 histone gene amplification used 30 cycles (95 °C, 48 °C and 72 °C for 15 s each). For COI gene 35 amplification cycles (95 °C, 48 °C and 72 °C, 30 s each) were employed. All PCR reactions included an initial denaturation at 95 °C (10 min) and a final extension at 72 ºC (5 min).

28S rDNA fragments were labeled either with biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche) by nick translation. Labeling of 5S rDNA and H3 histone gene probes was directly performed by supplementing the PCR mixture with either biotin-16-dUTP (20 μM) or digoxigenin-11-dUTP (5 μM).

DNA sequencing and phylogenetic analysis

COI PCR products were purified (FavorPrep™ GEL/PCR Purification Kit, Favorgen) and sequenced in both directions using a BigDye terminator kit v3.1 (Applied Biosystems) in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) by the genomic service (CACTI) of the University of Vigo. DNA sequences were edited with BioEdit v. 7.1.11 (Hall 1999), aligned with MUSCLE in MEGAv7 (Kumar et al. 2016) and trimmed to 545 bp after excluding primers. The best-fit nucleotide substitution model (HKY+G+I) was selected according to the Akaike's information criterion employing JModelTest 2. A molecular phylogeny was inferred by Maximum likelihood from a combined dataset including both our sequences and those representatives of previously karyotyped Tellinidae available at the NCBI nucleotide collection database. Robustness of nodes was assessed by 1000 bootstrap replicates. All phylogenetic analyses were performed using MEGAv7 (Kumar et al. 2016).

Fluorescent *in situ* hybridization (FISH)

Single, double and sequential FISH were performed following previously described protocols (Pérez-García et al. 2011; García-Souto et al. 2015, 2016). Chromosome spreads were digested with RNase and pepsin before denaturating them in 70% formamide (70 °C, 2 min). Hybridization was performed overnight at 37 °C. Biotin detection was carried out with fluorescein isothiocyanate (FITC) conjugated avidin and biotinylated anti-avidin (Vector), whilst probes labeled with digoxigenin were detected with mouse anti-digoxigenin and anti-mouse tetramethylrhodamine (TRITC) antibodies. Chromosome preparations were counterstained with DAPI. After employing a commercial $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) for FISH mapping vertebrate telomeric repeats, chromosome preparations were counterstained with DAPI and PI. As indicated above, separated images for each fluorochrome were recorded, pseudo-colored and merged. On karyotyping and mapping purposes, at least 10 specimens per taxa (5 female, 5 male) and 20 metaphase plates per specimen were surveyed.

Results

All specimens of *Bosemprella incarnata*, *Macomangulus tenuis*, *Moerella donacina* and *Serratina serrata* are 2n = 38. As shown in Figure 1, these tellin shells present diverse karyotype compositions. *Bosemprella incarnata* exhibits three metacentric, nine submetacentric, two subtelocentric and five telocentric chromosome pairs. *Macomangulus tenuis* karyotype is composed of one metacentric, eight submetacentric and ten subtelocentric pairs. In *Moerella donacina* seven metacentric, five submetacentric, six subtelocentric and one telocentric chromosome pairs appear. Lastly, *Serratina serrata* showed a karyotype composed of four metacentric, six submetacentric, three subtelocentric and six telocentric chromosome pairs.

The combined use of AT-specific (DAPI), GC-specific (CMA) and unspecific fluorochromes revealed DAPI-/CMA+, GC-rich regions close to the centromeres in a single chromosome pair in *Bosemprella incarnata*, *Macomangulus tenuis* and *Moerella donacina*, and in two chromosome pairs in *Serratina serrata*. FISH experiments employing a 28S rDNA probe (Figure 1) demonstrated that these GC-rich regions are coincident with the location of the 45S rDNA clusters.

A single H3 histone gene cluster was present in all four tellin shell species analyzed (Figure 1). This cluster was subcentromeric to the short arms of both telocentric chromosome pair 15 in *Bosemprella incarnata* and submetacentric

chromosome pair 3 in *Serratina serrata*, and to the short arms of telocentric pair 17 in *Moerella donacina*. In contrast, H3 histone genes were subterminal to the long arms of subtelocentirc pair 6 in *Macomangulus tenuis*.

Single 5S rDNA clusters mapped (Figure 1) to subterminal positions on the long arms of metacentric pair 12 in *Bosemprella incarnata* and telocentric pair 9 in *Serratina serrata*. The remaining two tellin shells presented two 5S rDNA clusters. Subterminal to the long arms of telocentric pair 9 and subcentromeric to the long arms of subtelocentric pair 17 in *Macomangulus tenuis*; subterminal to the long arms of subtelocentric pair 5 and subcentromeric to the long arms of metacentric pair 7 in *Moerella donacina*.

Double FISH experiments using 28S and 5S rDNA probes labeled differently demonstrated that subtelocentric chromosome pair 9 of *Macomangulus tenuis* bears both subcentromeric 45S rDNA and subtelomeric 5S rDNA clusters in its long arms.

FISH analysis employing a telomeric $(C_3TA_2)_3$ PNA probe revealed terminal signals at both ends of every chromosome in the four tellin shells.

A summary of the cytogenetic results, together with those previously published for Tellinidae species, is presented in Table 1.

The mitochondrial COI gene was successfully amplified in the four Tellinidae taxa and the corresponding sequences stored in GenBank under accession numbers KY951457 and KY951458 for *Bosemprella incarnata*, KY951461 and KY951462 for *Macomangulus tenuis*, KY951455 and KY951456 for *Moerella donacina* and KY951459 and KY951460 for *Serratina serrata*. All partial COI gene sequences were independently compared using BLAST with those stored in NCBI GenBank and BOLD databases. Out these, high similarity levels (> 99%) were only found among our *Macomangulus tenuis* sequences (Genbank accession numbers KY951461 and KY951462) and a single sequence (Genbank accession number KR084511) from a North Sea specimen stored under the same specific assignation. The same comparison approaches using all the other sequences obtained in this work always displayed levels of similarity (< 93%) well below those corresponding to the same taxa.

Figure 2 shows Tellinidae chromosome data together with a maximum likelihood tree (HKY+G+I) derived from a dataset including our tellin shell sequences and those corresponding to the same and other karyotyped Tellinidae available at NCBI database, the single sequences of *Macomangulus tenuis* (GenBank accession number KR084511) and *Macoma nasutta* (GenBank accession number KF643646) and representative sequences of

Limecola balthica (GenBank accession numbers KP977633, KP977691, KP977733, KP977750 and KP977944).

Discussion

The diploid chromosome numbers, 2n = 38, found for the four tellin shell species studied here coincide with those previously reported for *Macomangulus tenuis* (Cornet and Soulard 1990) and the other two tellin shells previously described, *Limecola balthica* (Cornet and Soulard 1990; Wolowicz and Thiriot-Quiévreux 1997) and *Macoma nasuta* (González-Tizón et al. 2000). This is also the usual diploid number in most Heterodonta bivalve species (Nakamura 1985; Thiriot-Quiévreux 1994, 2002; Leitão and Chaves 2008; Arias-Pérez et al. 2013; González-Tizón et al. 2013; Pérez-García et al. 2014a; García-Souto et al. 2015, 2016).

In regards to karyotype composition, the main difference with previous data for Tellinidae (Table 1) is the occurrence of telocentric chromosome pairs in three of the four tellin shells studied here, a relatively unusual situation in Heterodonta (González-Tizón et al. 2013). This is not the case for *Macomagulus tenuis* whose karyotype, in concordance with previous results (Cornet and Soulard 1990), is exclusively composed of bi-armed chromosomes; the minor differences between the karyotype composition reported here and that previously published (Table 1) can be attributed to methodological variation.

As in most other bivalves studied to date (Thiriot-Quiévreux 2002; Leitão and Chaves 2008; Pérez-García et al. 2010a, 2010b, 2011, 2014a, 2014b; Carrilho et al. 2011; González-Tizón et al. 2013; García-Souto et al. 2015, 2016, 2017a, 2017b), the application of diverse combinations of base-specific fluorochromes to the staining of tellin shell chromosomes demonstrated that GC-rich regions are scarce, two in *Serratina serrata* and a single one in the other three species. As is also the rule for most bivalve species, further FISH using 28S rDNA probes confirmed that these GC-rich regions are coincident with the chromosomal regions bearing 45S rDNA clusters, the NORs.

Tandemly repeated DNA sequences are helpful markers for identifying chromosomes. This is also the case in bivalves in which FISH mapping made possible establishing more reliable karyotypes and identifying some of the chromosomal changes accompanying bivalve evolution (Pérez-García et al. 2014b). As shown in Table 1, this kind of approach has been barely applied to tellin shells. Only 45S rDNAs has been previously located either by silver staining (*Limecola balthica*, Smolarz et al. 2003) or FISH (*Macoma nasuta*, González-Tizón et al. 2000) in Tellinidae. The presence a single

45S rDNA subterminal in a chromosome pair in *Macoma nasuta* (González-Tizón et al. 2000) coincided in number with our results for *Bosemprella incarnata*, *Macomangulus tenuis*, and *Moerella donacina* and differed with the two clusters displayed by *Serratina serrata*. In regards to location, the situation is quite different because most of the 45S rDNA clusters found in this work were subcentromeric; this was also the case for one of the three silver-stained NORs found in *Limecola balthica* (Smolarz et al. 2003).

Regarding 5S rDNA and histone gene clusters, no previous data was available for any of the species of Tellinidae. The presence of one or two 5S rDNA clusters has also been described in another Heterodonta family, Veneridae, in which subcentromeric, intercalary and subtelomeric locations were also found (García-Souto et al. 2015, 2017). The occurrence of single minor histone gene cluster, either subterminal or subcentromeric, in all tellin shells is similar to the situation found in Mactridae (García-Souto et al. 2016) but differs with that in Veneridae in which 1 to 4 clusters have been described (Carrilho et al. 2011; García-Souto et al. 2015, 2017).

Additionally, the simultaneous presence of both 45S and 5S rDNA clusters on the same chromosome pair in *Macomangulus tenuis* is rather scarce in bivalves; *Ruditapes decussatus*, showing overlapping 45S and 5S rDNA signals (Hurtado et al. 2011, Pérez-García et al 2014a), is the only other Heterodonta species showing this particularity that was also found in three Mytilidae, *Perumytilus purpuratus* (Pérez-García et al. 2010b), *Brachidontes rodriguezi* (Pérez-García et al. 2010b) and *Mytilus trossulus* (Pérez-García et al. 2014b).

Concerning telomeric sequences, their presence exclusively at chromosome ends in *Bosemprella incarnata*, *Macomangulus tenuis*, *Moerella donacina* and *Serratina serrata* is the most usual result found in bivalves (Leitão and Chaves 2008; Pérez-García et al. 2010a, 2010b, 2011, 2014a, 2014b; Carrilho et al. 2011; González-Tizón et al. 2013; García-Souto et al. 2015, 2016).

Although no obvious relation between the chromosomal location of these repeats and the clustering of the species on the ML tree (Figure 2) was detected, this is probably due to the low proportion of karyotyped species of Tellinidae (6 out of a total of around 550) and their relative separation.

In summary, we have presented clear evidence of the valuable information provided by FISH signals in both analyzing chromosome evolution in Tellinidae and as a further tool in identifying tellin shell specimens for molecular phylogenies.

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Figure Legends

Figure 1: Chromosomal location of rDNA and histone gene clusters in tellin shells. FISH mapping of 5S rDNA (5S, red), 28S rDNA (28S, magenta) and H3 histone gene (H3, green) probes to mitotic metaphase plates, and corresponding karyotypes, of *Bosemprella incarnata* , *Macomangulus tenuis*, *Moerella donacina* and *Serratina serrata*. Scale bars, 5 μm.

Figure 2: Maximum likelihood tree based on partial mitochondrial COI gene sequences of species of Tellinidae with described karyotypes. The schematic representation of the chromosomal mapping results shows 5S rDNA in red, 45S rDNA in magenta and H3 histone genes in green.

Table 1 Karyotype composition and chromosomal mapping of rDNAs and histone genes in Tellinidae

		karyotype							
	n	(m)	(sm)	(st)	(t)	45S rDNA	5S rDNA	*h3*	References
Bosemprella incarnata	19	3	9	2	5	1p ic (m)	12q ter (m)	15p cen (t)	This work
Limecola balthica	19	13	1	5					Cornet and Soulard 1990
	19	11	2	6					Wolowicz, Thiriot-Quiévreux 1997
	19	11	2	6		1q cen (m) 4q ter (m) ?q ter (st)			Smolarz et al. 2003
Macoma nasuta	19	8	6	5		15q ter (st)			González Tizón et al. 2000
Macomangulus tenuis	19	2	5	12					Cornet and Soulard 1990
	19	1	8	10		9q cen (st)	9q ter (st) 17p cen (st)	6q ter (st)	This work
Moerella donacina	19	7	5	6	1	6q cen (st)	5q ter (st) 7q ic (m)	17p cen (t)	This work
Serratina serrata	19	4	6	3	6	12p cen (t) 17p cen (t)	9q ter (t)	3q cen (m)	This work

(m): metacentric; (sm): submetacentric; (st): subtelocentric; (t): telocentric; ?: not described
p: short arm; q: long arm; cen: subcentromeric; ic: intercalary; ter: subterminal

Figure 1

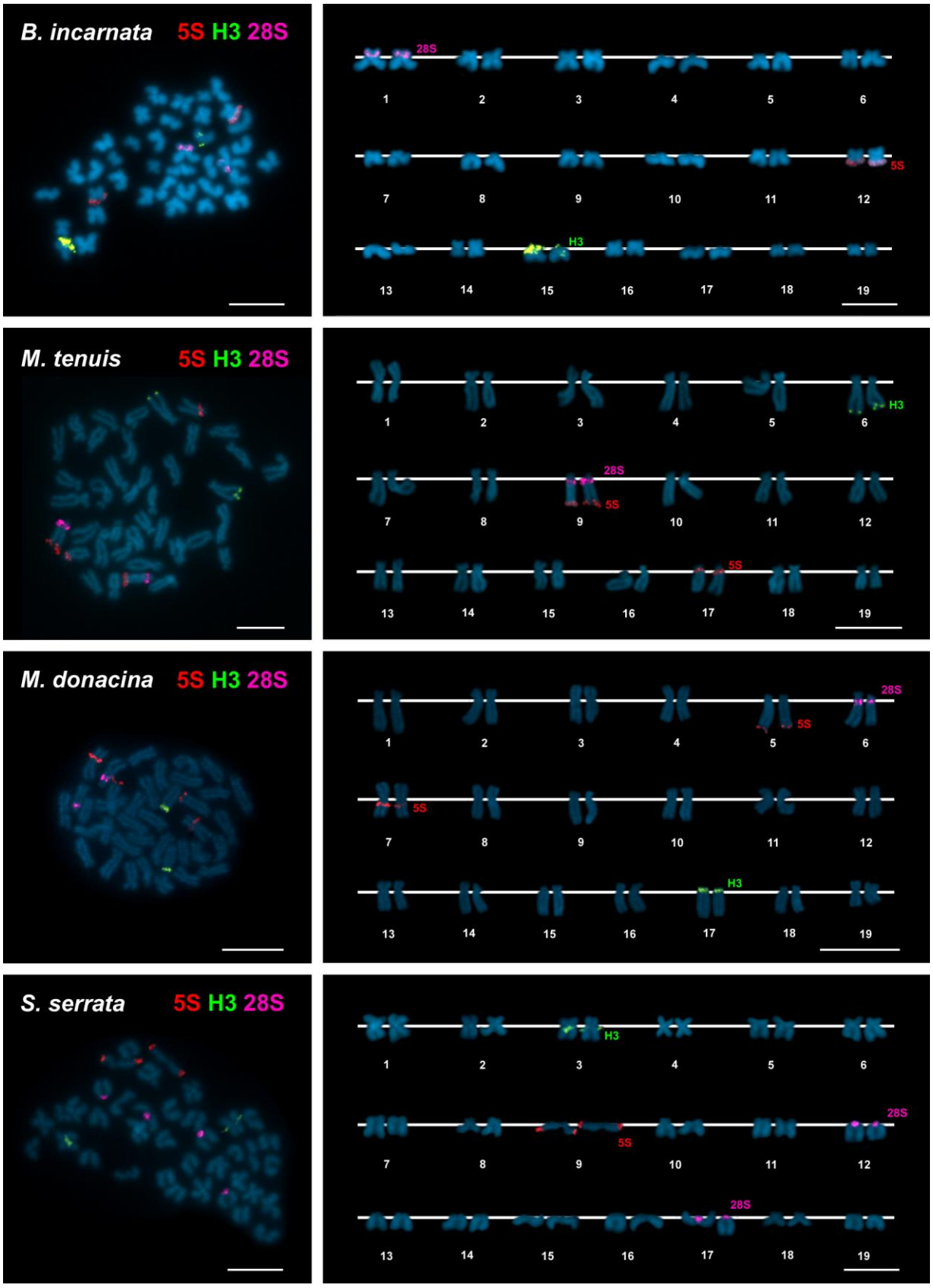
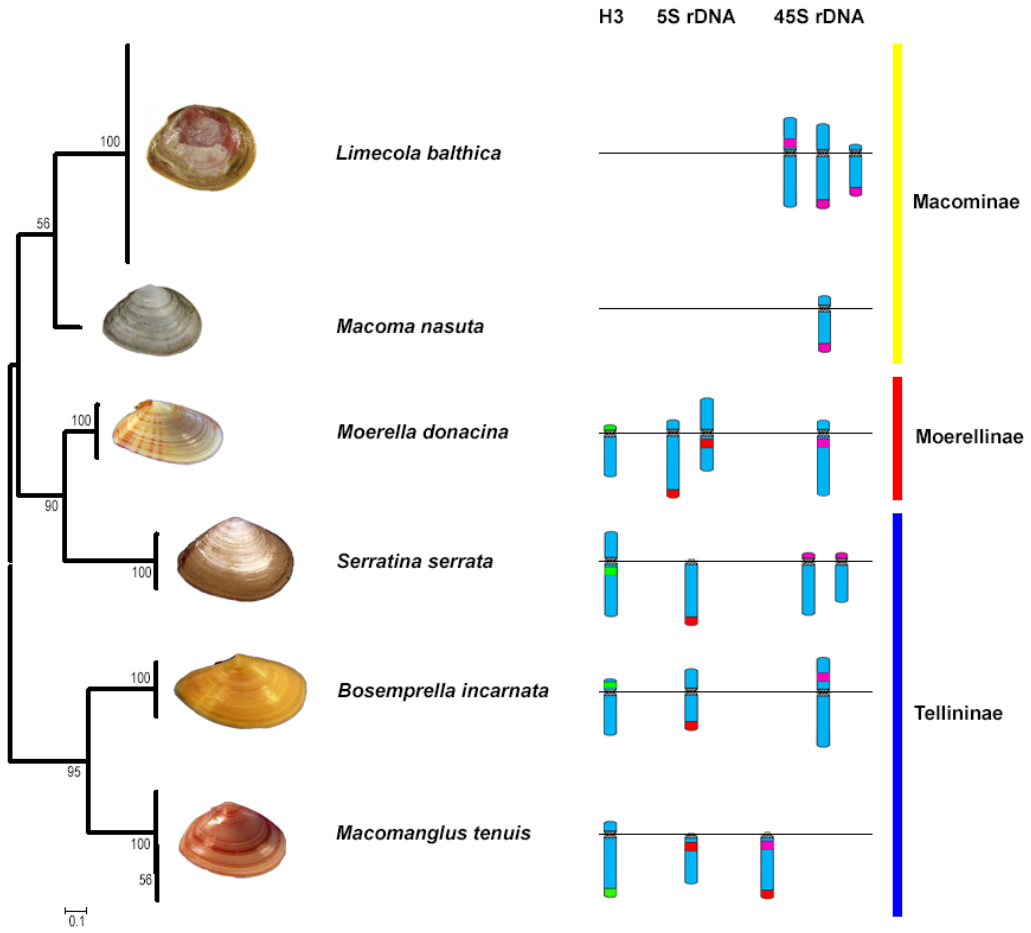

B. incarnata 5S H3 28S
28S
1 2 3 4 5 6
5S
7 8 9 10 11 12
H3
13 14 15 16 17 18 19
M. tenuis 5S H3 28S
H3
1 2 3 4 5 6
28S
5S
7 8 9 10 11 12
5S
13 14 15 16 17 18 19
M. donacina 5S H3 28S
28S
5S
1 2 3 4 5 6
5S
7 8 9 10 11 12
H3
13 14 15 16 17 18 19
S. serrata 5S H3 28S
H3
1 2 3 4 5 6
28S
5S
7 8 9 10 11 12
28S
13 14 15 16 17 18 19

Figure 2:


H3
5S rDNA
45S rDNA
100
56
100
90
100
100
95
100
56
0.1
Limecola balthica
Macoma nasuta
Moerella donacina
Serratina serrata
Bosemprella incarnata
Macomanglus tenuis
Macominae
Moerellinae
Tellininae

DIGENEA

Bucephalus minimus
(Stossich, 1887)

Parorchis acanthus
(Nicoll, 1906)

Cercaria longicaudata
(Bartoli, 1966)

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Molecular Cytogenetics in Digenean Parasites: Linked and Unlinked Major and 5S rDNAs, B Chromosomes and Karyotype Diversification

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Key Words
Digenea · Flukes · Trematodes · Parasites · rDNA clusters

Abstract
Digenetic trematodes are the largest group of internal metazoan parasites, but their chromosomes are poorly studied. Although chromosome numbers and/or karyotypes are known for about 300 of the 18,000 described species, molecular cytogenetic knowledge is mostly limited to the mapping of telomeric sequences and/or of major rDNA clusters in 9 species. In this work we mapped major and 5S rDNA clusters and telomeric sequences in chromosomes of *Bucephalus minimus*, *B. australis*, *Prosorhynchoides carvajali* (Bucephaloidea), *Monascus filiformis* (Gymnophalloidea), *Parorchis acanthus* (Echinostomatoidea), *Cryptocotyle lingua* (Opisthorchioidea), *Cercaria longicaudata*, *Monorchis parvus* (Monorchioidea), *Diphterostomum brusinae,* and *Bacciger bacciger* (Microphalloidea). Whilst single major and minor rDNA clusters were mapped to different chromosome pairs in *B. minimus* and *P. acanthus*, overlapping signals were detected on a single chromosome pair in the remaining taxa. FISH experiments using major rDNA and telomeric probes clearly demonstrated the presence of highly stretched NORs in most of the digenean taxa analyzed. B chromosomes were detected in the *B. bacciger* samples hosted by *Ruditapes decussatus*. Although the cercariae specimens obtained from *Donax trunculus*, *Tellina tenuis,* and *R. decussatus* were in agreement with *B. bacciger*, their karyotypes showed striking morphological differences in agreement with the proposed assignation of these cercariae to different species of the genus *Bacciger*. Results are discussed in comparison with previous data on digenean chromosomes.

Digenetic trematodes, the largest group of internal metazoan parasites [Cribb et al., 2001; Bray et al., 2008], are characterized by the use of 2–4 hosts during their lifespan. This fact makes it difficult to know the stages of their life cycles and complicates their systematic classification [Cribb et al., 2001, 2003; Gibson et al., 2002; Jones et al., 2005; Bray et al., 2008; Kostadinova and Pérez-del-Olmo, 2014]. Although molecular phylogenies, in combination with morphological data, have substantially contributed to solve some of these problems, many questions still remain unanswered [Olson et al., 2003; Bray et al., 2008; Kostadinova and Pérez-del-Olmo, 2014].

Recent molecular cytogenetic studies have enabled us to understand the evolutionary history of chromosome rearrangements in many invertebrate groups [Cabral-de-Mello et al., 2011; Pérez-García et al., 2014a, b; García-

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Souto et al., 2015]; however, this approach has been scarcely applied to Digenea, and chromosome analyses have been performed on only about 300 of the 18,000 described species [Baršiené, 1993; Bell et al., 1998; Petkevičiūtė et al., 2003; Špakulová and Casanova, 2004; Reblánová et al., 2011; Zadesenets et al., 2012a, b; Petkevičiūtė et al., 2012, 2014, 2015; Sofi et al., 2015]. Most of them were restricted to the description of diploid chromosome numbers and karyotypes obtained from Giemsa-stained metaphase plates [Baršiené, 1993; Špakulová and Casanova, 2004]. Although some of the aforementioned studies allowed the detection of both alterations in the ploidy level [Petkevičiūtė et al., 2014] and the presence of supernumerary B chromosomes [Špakulová and Casanova, 2004; Petkevičiūtė et al., 2012], most structural chromosome reorganizations would remain unidentified after Giemsa staining. As in many other invertebrate species, accurate chromosome identification in digeneans requires sets of specific chromosome markers detectable by FISH, and tandemly repeated multigene families are the best candidates.

In eukaryotes, tandem arrays of ribosomal RNA genes (rDNA) are usually organized in 2 different multigene families, major and minor rDNA. Major rDNA comprises the genes expressing the 18S, 5.8S and 26S–28S rRNAs, constituting the nucleolus organizing regions (NORs), whereas minor rDNA only includes the genes expressing the 5S rRNA. The 2 types of rDNA repeats are located on different chromosomes in most species, but some of them present clusters containing both major and 5S rDNA [Drouin and de Sá, 1995; Hurtado et al., 2011; Wicke et al., 2011; García and Kovařík, 2013; Pérez-García et al., 2014a]. With the exception of *Schistosoma* spp. [Hirai and Lo Verde, 1996; Hirai et al., 1989, 2000; Hirai, 2014], FISH experiments in digeneans have only been applied to locate telomeric sequences and/or major rDNA clusters in *Fasciola hepatica*, *Fascioloides magna,* and 5 opistorchiid flukes [Reblánová et al., 2011; Zadesenets et al., 2012a, b]. Major rDNA has also been detected in a member of the non-digenetic trematode *Aspidogaster limacoides* [Bombarová et al., 2015].

Taking into account that changes in both number and chromosomal position of the rDNA clusters detected by FISH have helped to solve some uncertainties in the phylogenies of other organisms, here we report a molecular cytogenetic analysis of 10 taxa of digeneans, namely *Bucephalus minimus* (Stossich, 1887), *B. australis* (Szidat, 1961), *Prosorhynchoides carvajali* (Muñoz and Bott, 2011), *Monascus filiformis* (Rudolphi, 1819), *Parorchis acanthus* (Nicoll, 1906), *Cryptocotyle lingua* (Creplin, 1825), *Cercaria longicaudata* (Bartoli, 1966), *Monorchis parvus* (Looss, 1902), *Diphterostomum brusinae* (Stossich, 1889), and *Bacciger bacciger* (Rudolphi, 1819) isolated from 12 mollusk intermediate hosts.

Materials and Methods

Biological Material

The digenean taxa studied, their mollusk hosts, and the collecting localities are shown in table 1. Mollusk specimens were maintained in seawater at 18 ± 1°C for 1 week. For digeneans with previously described cercarial emergence, mollusks were screened twice a day in search of infested specimens. Mollusk specimens were also dissected searching for parasites with passive transmission. Mollusk specimens were identified according to Tebble [1966], Graham [1988], and Huber [2010]. Digeneans were identified on the basis of their mollusk hosts and their morphological features [Stunkard, 1930; Ress, 1939; Bartoli, 1966; Yamaguti, 1975; Køie, 1979; Kinne, 1983; Ramón et al., 1999; Bartoli et al., 2000; Etchegoin et al., 2005; Ben Abdallah et al., 2009; Pina et al., 2009a, b; Muñoz et al., 2013]. The nomenclature used for these taxa follows the World Register of Marine Species database (http://www.marinespecies.org/).

Chromosome Spreading

Mollusk specimens were exposed overnight to colchicine (0.005%) before dissection. Mollusk tissues and isolated digenean redieae and/or sporocysts were immersed in 50% and 25% seawater for 1 h and fixed in ethanol/acetic acid [Martínez-Expósito et al., 1994a, b]. Fixed samples were disaggregated in 60% acetic acid, and the resulting cellular suspensions were dropped onto slides heated to 50°C [Martínez-Expósito et al., 1994a, b]. The quality of the chromosome preparations was checked by phase-contrast microscopy.

DNA Extraction, PCR Amplification, and Probe Labeling

Isolated digenean larvae were homogenized in hexadecyltrimethylammonium bromide buffer (CTAB) and digested with pronase (1.5 mg/ml) at 60°C for 3 h. DNA was extracted with chloroform/isoamyl alcohol [Winnepenninckx et al., 1993].

The expressing sequence of the 5S rDNA was amplified by PCR (30 cycles: 95°C, 10 s; 48°C, 10 s; 72°C, 10 s) using primers (EXP5SF: 5′-GTCTACGRCCTATCACGTTG-3′; EXP5SR: 5′-GTCTACRCAYYCGGTATTCC-3′) designed from expressing sequences of rainbow trout and Atlantic salmon 5S rDNAs [Morán et al., 1996]. 5S rDNA probes were directly labeled by PCR with digoxigenin-11-dUTP (GeneAmp, Applied Biosystems). A fragment of the 28S rDNA was also amplified by PCR (30 cycles: 95°C, 30 s; 48°C, 30 s; 72°C, 30 s) using universal primers retrieved from the Vilgalys Lab website (LR10R, LR12; http://www.biology.duke.edu/fungi/mycolab/primers.htm) and labeled by nick translation with biotin-16-dUTP (10× DIG Labeling Mix, Roche). PCR reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems) using a mixture containing 25 ng DNA, 5× PCR buffer, 50 μM of each dNTP, 12.5 mM $MgCl_2$, 1 μM of each primer, and 5 U Green Taq DNA Polymerase (GenScript) in a final volume of 20 μl. The labeled PCR products were precipitated before FISH.

Table 1. Chromosomal location of major and 5S rDNA in digenean trematodes

Species	Host	Infected specimens/ total	Locality	2n	Major rDNA	5S rDNA	Reference
Diplostomata							
Schistosomatoidea, Schistosomatidae							
Schistosoma mansoni	*Biomphalaria* sp.			16	2p cen (sm)		Hirai et al. [1989]
Bucephalata							
Bucephaloidea, Bucephalidae							
Bucephalus minimus	*Spisula solida*	11/184	Aveiro (Portugal)	14	7p ic (sm)	3q ic (sm)	this study
Bucephalus australis	*Brachidontes rodriguezii*	5/218	Mar del Plata (Argentina)	14	7q cen (m)	7q cen (m)	this study
Prosorhynchoides carvajali	*Perumitylus purpuratus*	6/317	Valparaiso (Chile)	14	5q cen (m)	5q cen (m)	this study
Gymnophalloidea, Fellodistomidae							
Monascus filiformis	*Nucula nitidosa*	8/20	Pontevedra (Spain)	18	4q ic (m)	4q ic (m)	this study
Echinostomata							
Echinostomatoidea, Fasciolidae							
Fasciola hepatica	*Bos taurus*		Bardejov (Slovakia)	20	5q cen (st)		Reblánová et al. [2011]
Fascioloides magna	*Bos taurus*		Gabčíkovo (Slovakia)	22	10q cen (sm)		Reblánová et al. [2011]
Echinostomatoidea, Philophthalmidae							
Parorchis acanthus	*Nucella lapilus*	16/201	Pontevedra (Spain)	22	2q ic (st/t)	10q cen (st)	this study
Opisthorchiata							
Opisthorchioidea, Heterophyidae							
Cryptocotyle lingua	*Littorina littorea*	8/114	Aveiro (Portugal)	14	5q cen (m)	5q cen (m)	this study
Opisthorchioidea, Opisthorchiidae							
Opisthorchis felineus				14	small pair		Zadesenets et al. [2012a]
Opisthorchis viverrini				12	small pair		Zadesenets et al. [2012a]
Metorchis xanthosomus				14	small pair		Zadesenets et al. [2012a]
Metorchis bilis				14	small pair		Zadesenets et al. [2012a]
Clonorchis sinensis				14	small pair		Zadesenets et al. [2012a]
Monorchiata							
Monorchioidea, Monorchiidae							
Cercaria longicaudata	*Polititapes rhomboides*	10/98	Pontevedra (Spain)	16	6p ter (sm)	6p ter (sm)	this study
Monorchis parvus	*Cerastoderma edule*	5/903	Pontevedra (Spain)	20	4q cen (m/sm)	4q cen (m/sm)	this study
Xiphidiata							
Microphalloidea, Zoogonidae							
Diphterostomum brusinae	*Nassarius reticulatus*	7/105	Pontevedra (Spain)	20	6q cen (st)	6q cen (st)	this study
Microphalloidea, Faustulidae							
Bacciger bacciger	*Donax trunculus*	19/101	Gandía (Spain)	12	3q cen (m)	3q cen (m)	this study
	Tellina tenuis	5/523	Pontevedra (Spain	12	2q cen (sm)	2q cen (sm)	this study
	Ruditapes decussatus	2/4500	Pontevedra (Spain)	12	3q cen (m)	3q cen (m)	this study

p = Short arm; q = long arm; cen = subcentromeric; ic = intercalar; ter = subterminal; m = metacentric; sm = submetacentric; st = subtelocentric.

Fluorescence in situ Hybridization

Single, double, and sequential FISH experiments were performed as described by Pérez-García et al. [2010a, b, 2011]. Chromosome preparations were pretreated with RNase and pepsin, denatured in 70% formamide (2 min at 69°C), and hybridized overnight at 37°C. Biotin-labeled probes were detected with avidin-fluorescein and biotinylated anti-avidin. Digoxigenin-labeled probes were detected with mouse anti-digoxigenin, goat anti-mouse tetramethylrhodamine isothiocyanate (TRITC) and anti-goat TRITC antibodies. Slides were counterstained with DAPI

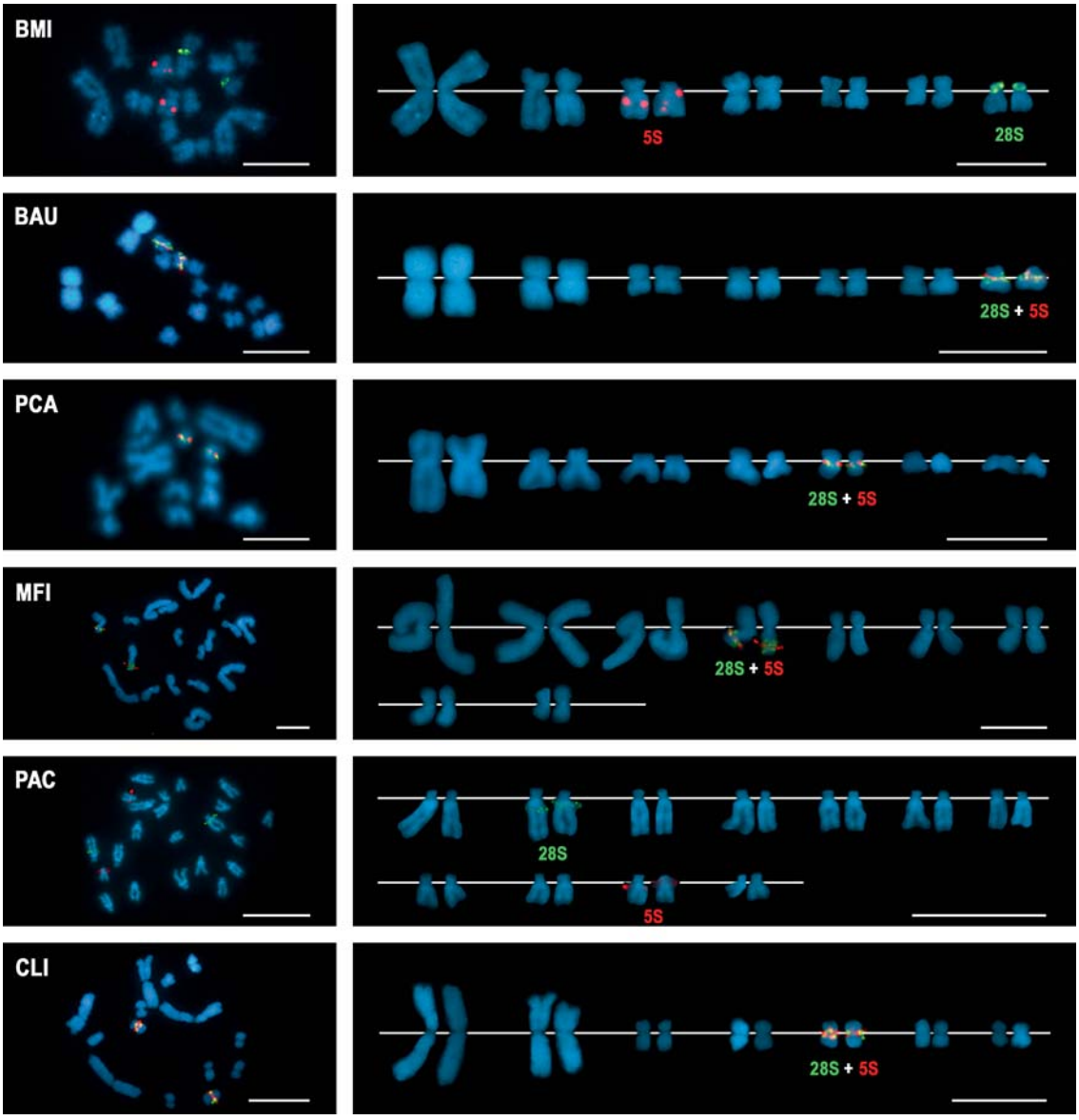

Fig. 1. Chromosomal location of major and 5S rDNA on mitotic chromosomes of digeneans. Mitotic metaphase plates and the corresponding karyotypes show single separated 5S rDNA (5S) and major rDNA (28S) signals in *B. minimus* (BMI) and *P. acanthus* (PAC). In contrast, signals clearly overlap in *B. australis* (BAU), *P. carvajali* (PCA), *M. filiformis* (MFI), and *C. lingua* (CLI). Scale bars = 5 μm.

(0.14 μg/ml in 2× SSC) and mounted with antifade (Vectashield, Vector). Telomeric sequences were also mapped by FISH using a vertebrate telomeric $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems). Slides were counterstained with DAPI and propidium iodide (0.7 μg/m in 2× SSC).

Slides were photographed using a Nikon Eclipse-800 microscope equipped with an epifluorescence system. Separated images were acquired with a DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon). The images were merged with Adobe Photoshop. Chromosomes were measured using Micromeasure 3.3 [Reeves and Tear, 2000] and classified according to their centromeric indices and relative lengths [Levan et al., 1964].

Relative lengths and centromeric indices corresponding to karyotypes of *B. bacciger* infecting different bivalve hosts were analyzed through a principal component analysis by using R vers. 3.0.1 [R Development Core Team, 2013] and the package FactoMineR [Lê et al., 2008]. Significance for the first 2 components was checked through a randomized-block ANOVA [Conde-Padín et al., 2007].

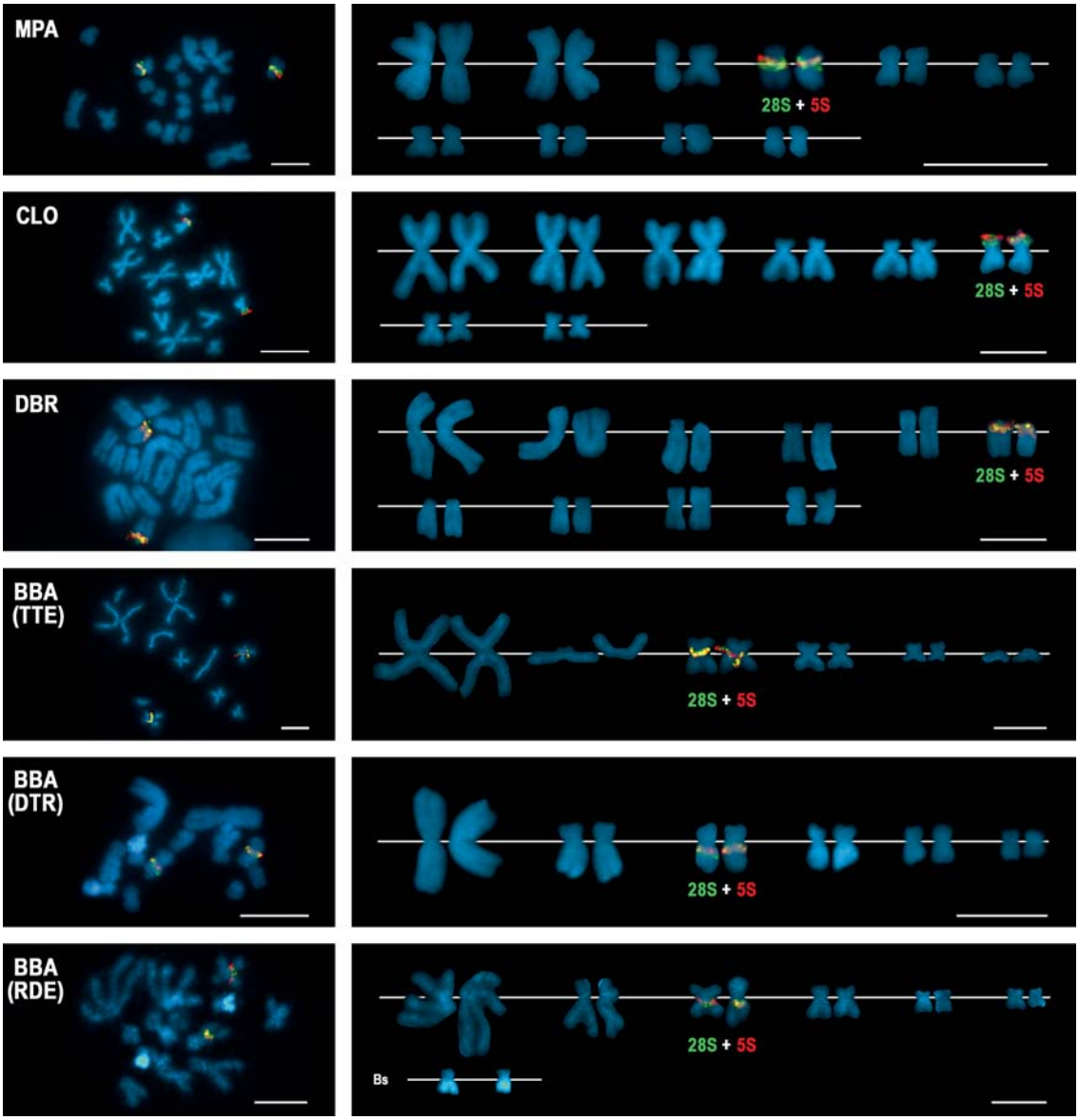

Fig. 2. Chromosomal location of major and 5S rDNA on mitotic chromosomes of digeneans. Single overlapping 5S rDNA (5S) and major rDNA (28S) signals appear in mitotic metaphase plates and the corresponding karyotypes of *M. parvus* (MPA), *C. longicaudata* (CLO), *D. brusinae* (DBR), and *B. bacciger* (BBA). The presumably BBA cercariae were obtained from 3 different bivalve species, *T. tenuis* (TTE), *trunculus* (DTR), and *R. decussatus* (RDE). Scale bars = 5 μm.

Results

Representative metaphase plates and karyotypes of the studied digenean taxa are presented in figures 1 and 2. Diploid chromosome numbers varied from a minimum of 2n = 12 for *B. bacciger* to a maximum of 2n = 22 for *P. acanthus* (table 1). Karyotypes showed clear differences in chromosome composition (figs. 1, 2). A summary of the relative chromosome lengths and centromeric indexes obtained after measuring 10 DAPI-stained metaphase plates per taxon is given in table 2. Whereas *C. lingua* and *P. acanthus* showed karyotypes exclusively composed of metacentric and subtelocentric chromosomes, respectively, the remaining taxa presented diverse combinations of chromosome types.

Single- and double-color FISH experiments demonstrated the presence of both major and minor rDNA clusters at a single locus in all taxa (figs. 1, 2; table 1). In

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Table 2. Relative lengths, centromeric indices, and classification of digenean chromosomes

Species		Chromosome pair										
		1	2	3	4	5	6	7	8	9	10	11
B. minimus	RL	34.14±0.55	17.44±0.39	12.32±0.32	11.16±0.29	9.19±0.25	8.71±0.20	8.03±0.16				
	CI	47.66±0.65	31.37±1.71	27.33±1.83	44.90±1.50	36.47±1.89	29.87±1.72	42.86±1.50				
	C	m	sm	sm	m	sm/m	sm	m				
B. australis	RL	32.64±1.71	18.63±0.83	11.08±1.57	10.78±1.56	9.00±1.81	8.99±0.93	8.73±1.34				
	CI	47.56±0.77	31.09±1.24	42.29±1.35	31.23±1.50	35.31±1.22	45.12±1.34	41.44±1.72				
	C	m	sm	m	sm	sm	m	m				
P. carvajali	RL	35.35±2.97	17.00±1.01	13.50±0.68	10.15±0.75	8.77±1.25	7.85±0.76	7.38±0.79				
	CI	42.73±0.87	21.09±0.76	23.46±0.81	18.07±1.35	37.69±2.45	39.29±1.00	25.67±0.94				
	C	m	st	st	st	m/sm	m	sm/st				
M. filiformis	RL	19.93±0.78	17.96±0.61	16.06±0.03	8.69±0.42	8.97±0.62	8.19±0.74	7.50±0.02	6.69±0.80	6.00±0.41		
	CI	45.31±3.00	47.17±0.21	32.55±1.44	48.00±1.92	42.38±3.64	29.47±2.66	38.61±4.77	31.56±4.57	44.26±0.47		
	C	m	m	sm	m	m	sm	m	sm	m		
P. acanthus	RL	13.46±0.27	12.39±0.29	11.99±0.20	10.91±0.18	10.45±0.13	9.91±0.16	9.09±0.23	7.56±0.23	7.15±0.23	6.91±0.24	6.23±0.21
	CI	12.65±0.53	13.00±0.50	13.95±0.71	14.95±0.84	13.55±0.54	14.35±0.48	15.95±0.81	19.75±0.54	19.55±0.79	20.60±0.99	20.50±0.61
	C	st/t	st/t	st	st	st	st	st	st	st	st	st
C. lingua	RL	29.52±3.46	23.17±1.26	11.38±1.58	10.49±1.39	9.29±0.83	8.62±0.74	7.53±0.52				
	CI	46.74±1.74	43.54±1.47	41.79±3.39	44.24±4.46	43.05±1.60	43.26±3.18	45.16±3.10				
	C	m	m	m	m	m	m	m				
C. longicaudata	RL	21.28±0.35	19.09±0.22	16.57±0.25	11.73±0.23	10.48±0.18	8.21±0.24	7.09±0.29	5.55±0.14			
	CI	44.99±0.70	44.87±0.70	43.93±0.69	27.89±1.20	27.44±1.03	29.65±1.38	41.01±0.84	42.36±1.09			
	C	m	m	m	sm	sm	sm	m	m			
M. parvus	RL	18.66±0.42	16.77±0.25	10.32±0.57	8.78±0.21	8.07±0.16	7.91±0.60	7.90±0.41	7.47±0.19	7.45±0.93	6.67±0.23	
	CI	45.18±0.17	42.77±0.35	36.85±1.11	37.87±0.22	45.43±0.43	31.94±1.20	42.30±0.15	38.35±0.62	41.16±0.45	42.88±0.74	
	C	m	m	sm/m	m/sm	m	sm	m	m	m	m	
D. brusinae	RL	18.57±1.21	15.75±0.32	10.78±0.44	9.48±0.43	8.58±1.36	8.10±0.49	7.76±0.98	7.60±0.58	7.25±0.50	6.08±0.72	
	CI	43.76±1.79	27.81±1.48	9.12±1.26	7.36±1.99	44.22±1.53	16.17±1.79	11.84±1.10	44.75±0.74	27.70±1.37	46.00±1.29	
	C	m	sm	t	t	m	st	t/st	m	sm	m	
B. bacciger (DTR)	RL	34.16±0.50	19.10±0.29	14.56±0.20	12.55±0.16	10.88±0.15	8.74±0.13					
	CI	46.32±0.47	28.92±0.80	42.20±0.84	42.90±0.59	42.88±0.66	41.80±0.76					
	C	m	sm	m	m	m	m					
B. bacciger (TTE)	RL	37.45±0.91	16.43±0.36	15.00±0.30	13.84±0.36	10.34±0.41	6.93±0.26					
	CI	45.46±0.56	35.15±1.06	0.80±0.57	38.21±0.96	41.22±1.12	0.74±0.62					
	C	m	sm	T	m/sm	m	T					
B. bacciger (RDE)	RL	43.00±0.93	21.27±1.47	15.26±0.76	8.80±0.54	8.59±0.40	6.28±0.24					
	CI	45.63±0.64	25.86±1.61	40.80±1.60	26.85±1.00	43.50±1.09	32.73±1.74					
	C	m	sm/t	m	sm	m	sm					

RL = Relative length; CI = centromeric indices; C = classification (m = metacentric; sm = submetacentric; st = subtelocentric; t = telocentric; T = telocentric senso stricto); DTR = *D. trunculus*; RDE = *R. decussatus*; TTE = *T. tenuis*.

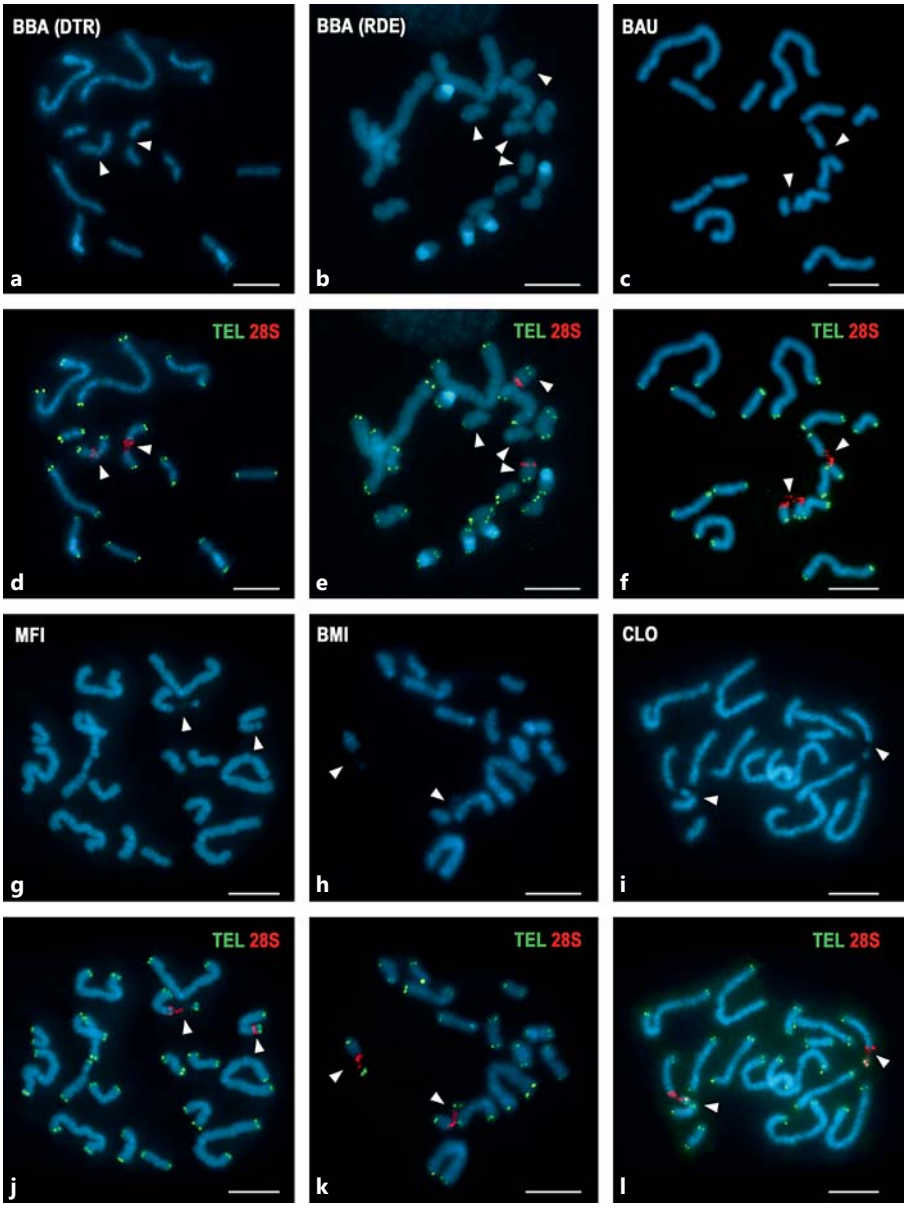

Fig. 3. Secondary constrictions, NORs, and telomeric DNA in digenean chromosomes. DAPI-stained mitotic metaphases of *B. bacciger* cercariae (**a**, BBA DTR; **b**, BBA RDE), *B. australis* (**c**, BAU), *M. filiformis* (**g**, MFI), *B. minimus* (**h**, BMI), and *C. longicaudata* (**i**, CLO) showing seemingly separated large (arrowheads in **a–c**) and small (arrowheads in **g–i**) chromatin fragments. FISH using major rDNA (28S) and telomeric (TEL) probes demonstrated that secondary constrictions of different size still link fragments similar in length (arrowheads in **d–f**) or long fragments to small ones (satellites, arrowheads in **j–l**). Note the lack of telomeric signals at one of the ends of those segments. Scale bars = 5 μm.

B. minimus and *P. acanthus*, major and minor rDNA clusters were located on different chromosome pairs (fig. 1; table 1). In contrast, the remaining digeneans showed overlapping signals for both major and minor rDNA at a single chromosome pair (figs. 1, 2; table 1). The chromosomal position of these clusters also presented variation among the digeneans. Although most of the double signals appeared at a subcentromeric location on the long arm of a metacentric chromosome pair, subterminal signals were detected in *C. longicaudata* and intercalary ones in *M. filiformis*. For the taxa showing separated major and 5S rDNA clusters, the situation was markedly different. Major rDNA clusters were intercalary to the short arms of a submetacentric chromosome pair in *B. minimus* and to the long arm of a subtelo/telocentric one in *P. acanthus*. Minor rDNA clusters were intercalary to the long arms of a submetacentric chromosome pair in *B. minimus* and subcentromeric to the long arms of a subtelocentric pair in *P. acanthus* (fig. 1; table 1).

As shown in figure 3, FISH using $(C_3TA_2)_3$ PNA probes revealed fluorescence signals at the end of every single

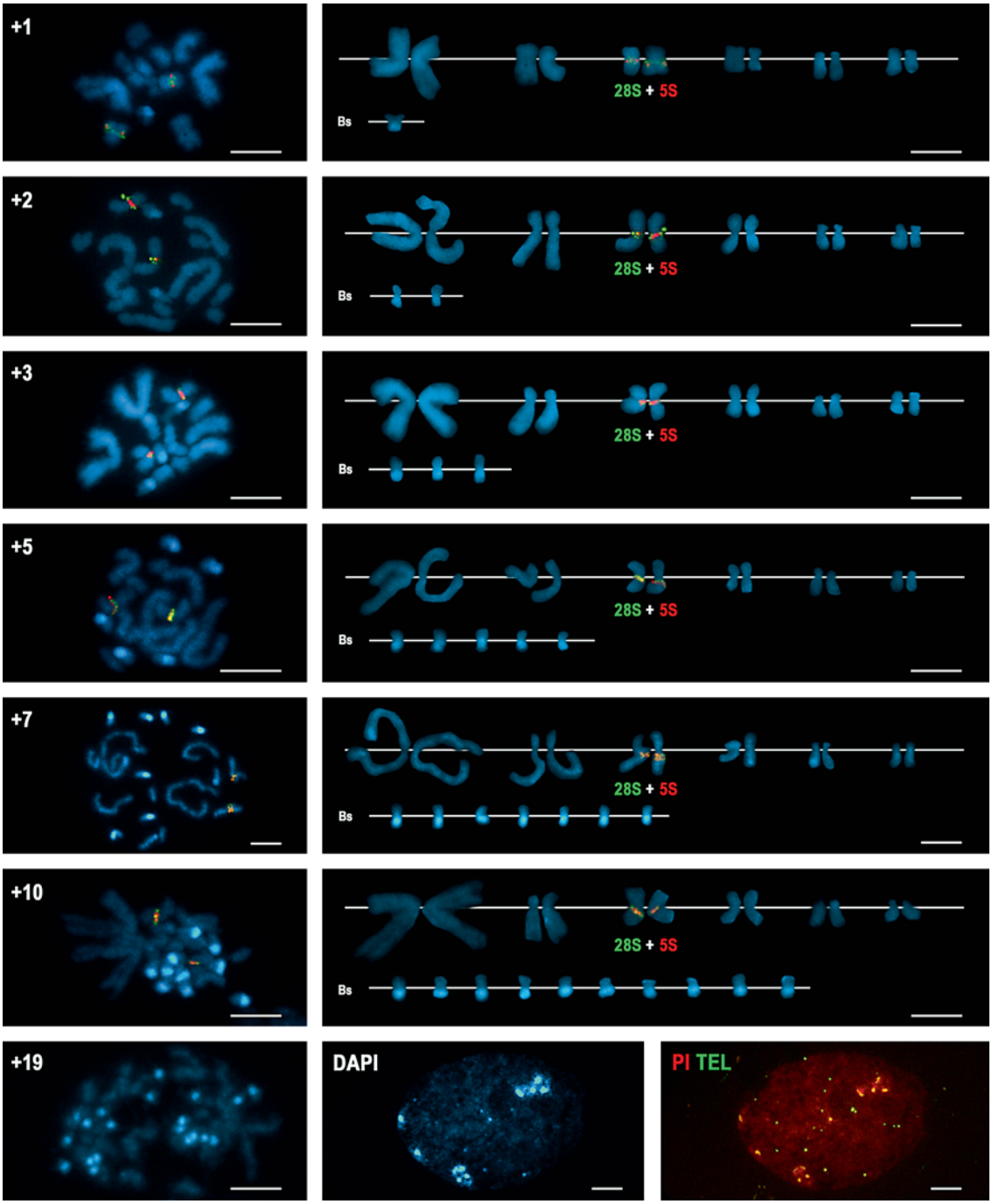

Fig. 4. B chromosomes in *B. bacciger*. Examples of the variation in the number of Bs displayed by *B. bacciger* cercariae hosted by *R. decussatus*. All Bs are characterized by a bright DAPI-stained region on their long arms, also visible in interphase nuclei stained with either DAPI or propidium iodide (PI). Scale bars = 5 μm.

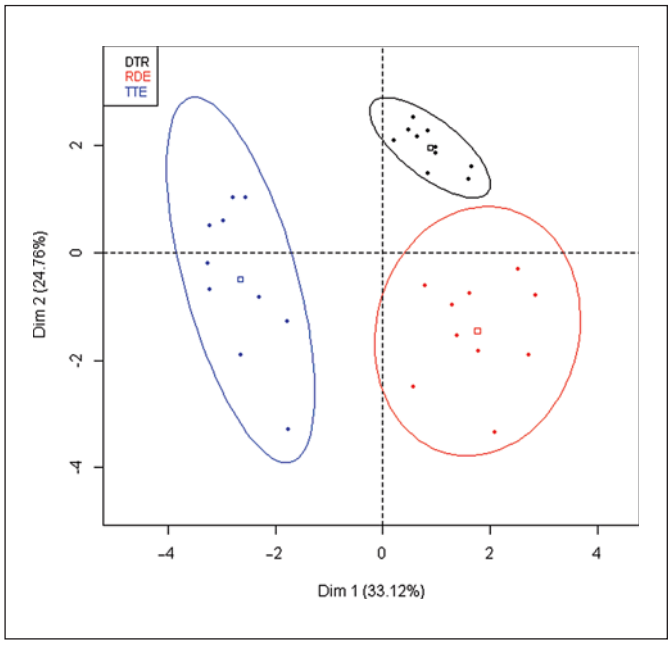

Fig. 5. Principal component analysis of relative chromosome lengths and centromeric indices for 30 karyotypes of *B. bacciger*. The 2 principal dimensions are shown. The percentage of the variance explained by each principal component is shown on the axis. Color dots, grouped by 95% confidence ellipses, represent the karyotypes obtained from cercariae hosted by *T. tenuis* (TTE, blue), *R. decussatus* (RDE, red), and *D. trunculus* (DTR, black).

chromosome for all species, with no evidence for the existence of internal telomeric signals.

FISH mapping of major rDNA and telomeric probes to metaphase plates of most of the digenean taxa analyzed clearly demonstrated the presence of highly uncondensed, DAPI-unstained regions covered by major rDNA signals (fig. 3). The condensed chromatin at both sides of the uncondensed regions also showed major rDNA signals but did not display any trace of telomeric signals. These regions were subcentromeric in *B. australis* (fig. 3f), *P. carvajali*, *P. acanthus*, *C. lingua*, *M. parvus,* and *B. bacciger* (fig. 3d, e), thus yielding apparently higher chromosome numbers than the modal number recorded for their respective species after DAPI (fig. 3a–c) or Giemsa (not shown) staining. In the remaining taxa, *B. minimus* (fig. 3h, k), *M. filiformis* (fig. 3g, j), *C. longicaudata* (fig. 3i, l), and *D. brusinae*, many of the metaphase plates showed clear DAPI-negative secondary constrictions that separated relatively small pieces of chromatin from the chromosome.

Additional chromosomes were detected in the samples of *B. bacciger* isolated from the 2 only *Ruditapes decussatus* venerid clam specimens infected. In contrast with the results acquired for the other taxa, all mitotic plates obtained from these cercariae showed, besides the standard chromosomal complement of 2n = 12, mitotically unstable accessory chromosomes in a variable number, from a minimum of 1 to a maximum of 19 (fig. 4). These supernumerary metacentric chromosomes presented telomeric signals at both ends and were characterized by showing long arms strongly stained with DAPI. Such heterochromatic regions were also detected in nuclei (fig. 4).

As shown in figure 2 and table 2, the karyotypes of *B. bacciger* infecting different clam species have some morphological differences. The cercariae hosted by *Tellina tenuis* showed a karyotype containing 2 telocentric chromosome pairs that did not appear in cercariae hosted by *Donax trunculus* and *R. decussatus*. The application of a principle component analysis to the relative chromosome lengths and centromeric indices of 30 *B. bacciger* karyotypes displayed 3 separated groups, corresponding to the different clam host species (fig. 5). The first dimension explains 33.12% of the differences and strongly correlates with shape and length of chromosome pair 2 and shape of chromosome pair 6. The second dimension that explains 24.76% of the differences is mostly due to the relative chromosome length of chromosome pair 5. Further randomized-block ANOVA showed highly significant differences ($p < 0.001$) among the means.

Discussion

Overlapping and Separated Major and 5S rDNA Signals

The presence of a single major rDNA cluster in the 10 digenean taxa studied is in agreement with previous results in other trematodes. Major rDNA clusters were located by FISH in only 9 species of flukes [Hirai et al., 1989; Reblánová et al., 2011; Zadesenets et al., 2012b; Hirai, 2014; Bombarová et al., 2015]. Although single clusters were present in all of them, their chromosomal locations differed. Intercalary signals were detected in *Schistosoma mansoni* [Hirai et al., 1989], whereas *F. hepatica* presented subterminal signals [Reblánová et al., 2011] and *F. magna* subcentromeric ones [Reblánová et al., 2011]. Subcentromeric signals were also detected in the Aspidogastrea *A. limacoides* [Bombarová et al., 2015]. In the remaining 5 species, *Opisthorchis felineus*, *O. viverrini*, *Metorchis xanthosomus*, *M. bilis,* and *Clonorchis sinensis*, rDNA signals were assigned to 'small chromosome pairs' [Zadesenets et al., 2012b]. The major rDNA signals detected in this study

also displayed variation in their chromosomal location (table 1). Although most signals were subcentromeric, intercalary and subterminal signals were also found.

All flukes analyzed showed a single 5S rDNA cluster (table 1). While in most taxa the 5S rDNA clusters were coincident with the major rDNA clusters, independent signals located on different chromosome pairs were found in *P. acanthus* and *B. minimus*. As this study is the first description of the chromosomal location of 5S rDNA sequences in trematodes, it is unwarranted to assume that linked major and 5S rDNA is the ancestral character within this group. The availability of the draft genome sequences for *S. mansoni* and *S. japonicum* [Copeland et al., 2009] allowed for the search of 5S rDNA sequences within the major rDNAs of those species. As no trace of them was found, it was inferred that 5S and major rDNA clusters were also separated in those taxa and that additional species must be analyzed before proposing evolutionary trends for these sequences in trematodes. Furthermore, the presence of linked and unlinked signals in species belonging to the family Bucephalidae is in accordance with results obtained from other organisms. The presence of 5S rDNA linked to other multigene families, including major rDNA, is a common situation in fungi, nematodes, and crustaceans [Drouin and de Sá, 1995; Vierna et al., 2013]. The study of the molecular organization of the linked tandem arrays in those groups demonstrated that, at least in some cases, closely related species showed differences in both tandem arrangement and gene orientation and led to suggestions that those 5S rRNA gene linkages were repeatedly established and lost during the evolution of their genomes [Drouin and de Sá, 1995; García and Kovařík, 2013].

Highly-Stretched Secondary Constrictions and B Chromosomes

The chromosomal regions occupied by major rDNA repeats (NORs) sometimes appear as secondary constrictions on Giemsa- or DAPI-stained chromosomes in a wide variety of eukaryotes. In these regions the chromatin does not fully condense at metaphase, thus showing a lesser degree of compaction relative to the surrounding chromatin. Although the ability to condense relies upon the maintaining of the major rDNA transcription machinery still assembled during mitosis in active NORs and its absence in inactive ones [Roussel et al., 1996; O'Sullivan et al., 2002], the detection of secondary constrictions also depends on their relative positions on the chromosomes. Satellite chromosomes usually display small separated segments as a consequence of bearing decondensed, active subterminal NORs. On the other hand, a chromosome bearing a highly decondensed, active intercalary or subcentromeric NOR may appear as 2 different chromosomes upon microscopic examination, therefore giving chromosome counts higher than the diploid number. This explains the presence of additional elements in *B. australis*, *P. carvajali*, *P. acanthus*, *C. lingua*, *M. parvus,* and *B. bacciger* and satellite chromosomes in *B. minimus*, *M. filiformis*, *C. longicaudata,* and *D. brusinae*. After FISH using major rDNA probes, signals covered both the, sometimes very long, unstained section and the terminal part of 2 separated fragments of chromatin. These apparently terminal segments were not actual chromosome ends as demonstrated by the absence of telomeric signals on them. The presence of large decondensed regions has been previously reported for some flukes [Hirai et al., 1989; Bomvarová et al., 2015] and may well be the cause of some of the discrepancies in chromosome numbers reported for digenean species [Baršiené, 1993; Sofi et al., 2015].

B chromosomes (Bs) are non-essential chromosomes, supernumerary to the standard complement of a species, that show non-Mendelian inheritance [Jones and Rees, 1982; Camacho et al., 2000]. These chromosomes have been detected in many organisms and show extensive differences in size, shape, and molecular composition. Bs have been described in 12 digenean species belonging to 7 families [Špakulová and Casanova, 2004; Petkevičiūtė et al., 2012]. In most of these species, the number of Bs detected in metaphase plates predominantly ranged from 0 to 2 [Špakulová and Casanova, 2004; Petkevičiūtė et al., 2012], but *Diplodiscus subclavatus* [Petkevičiūtė et al., 1989] and *Notocotylus* sp. [Baršiené et al., 1990] displayed 0–10 Bs. In this regard, the presence of 1–19 mitotically unstable Bs in metaphase plates of the cercariae hosted by *R. decussatus* is anomalous both in the prevalence of Bs (all metaphase plates showing them) and their incidence (many plates showing more than 5 Bs). Heterochromatic regions have previously been described on the Bs of the fluke *Trichobilharzia regenti* [Spakulová et al., 2001].

Karyotype Diversification

Chromosome numbers and karyotypes have been previously described for 10 species of the superfamily Bucephaloidea, suborder Bucephalata [Baršiené, 1993; Petkevičiūtė et al., 2014]. Most of them presented 2n = 14 chromosomes, and their karyotypes were characterized by both the presence of a large metacentric pair and the predominance of metacentric and submetacentric chromosomes. The karyotypes of *B. minimus*, *B. australis,* and *P. carvajali* presented here also shared these characteristics. Our results showed remarkable differences on the chromosomal location of

major and 5S rDNA clusters among these taxa. Whereas *B. australis* and *P. carvajali* presented overlapping major and 5S rDNA signals at subcentromeric positions, *B. minimus* displayed separated signals at intercalary locations. Although the phylogenetic tree proposed by Olson et al. [2003] grouped the superfamilies Bucephalloidea and Gymnophalloidea in the suborder Bucephalata, the karyotype of *M. filiformis*, the only species of Gymnophalloidea studied to date, showing 2n = 18 chromosomes and overlapping major and 5S rDNA signals at a subterminal location, was clearly different from those of the 3 bucephalids.

With regard to the suborder Echinostomata, in accordance with Ress [1939], *P. acanthus* presented a diploid chromosome number of 2n = 22 and a karyotype composed of subtelocentric chromosomes. The other species of the family Philophthalmidae (Echinostomatoidea) studied to date showed 2n = 20, and their karyotypes included a large submetacentric chromosome pair [Baršiené, 1993] not present in *P. acanthus*. Taking into account the similarities among these karyotypes, the simplest explanation for the divergence is the occurrence of a chromosomal fusion in an ancestral karyotype similar to the one of *P. acanthus*. Another possibility is that the ancestral karyotype presented the large metacentric chromosome pair still appearing in the extant 2n = 20 species, and a fission event originated the 2 subtelocentric chromosomes. A similar situation is present in the closely related families Echinostomatidae and Fasciolidae (Echinostomatoidea), in which reported diploid chromosome numbers are mostly 2n = 20 and 2n = 22 [Baršiené, 1993; Reblánová et al., 2011; Sofi et al., 2015], the most conspicuous difference being the presence or absence of a large metacentric chromosome pair [Reblánová et al., 2011].

The Monorchioidea *C. longicaudata* and *M. parvus* (suborder Monorchiata) presented differences in both chromosome number and karyotype morphology. The diploid chromosome number, 2n = 20, and the karyotype of *M. parvus* is concordant with those reported for some representatives of the genus *Asymphylodora* [Baršiené, 1993] and one unidentified monorchid species [Baršiené et al., 1995]. On the other hand, neither the diploid chromosome number 2n = 16 nor the karyotype of *C. longicaudata* resemble any of those reported for other species of this group. Although major and 5S rDNA signals overlap in both species, their chromosomal locations differ, being subcentromeric in *M. parvus* and subterminal in *C. longicaudata*. These results further confirm the karyological heterogeneity of the species of the Monorchiidae [Baršiené et al., 1995] and give additional support to consider this clade to be polyphyletic [Jousson and Bartoli, 2002].

In contrast with a previous report of a haploid chromosome number of n = 6 (2n = 12) for *C. lingua* [Cable, 1931], we obtained a diploid number of 2n = 14 for this species and a karyotype very similar to those presented by the other representatives of the family Heterophyidae, superfamily Opisthorchioidea, suborder Opisthorchiata [Baršiené et al., 1995]. Furthermore, the presence of 2 large metacentric chromosome pairs constituting more than 50% of the total length of the karyotype is in accordance with the reported results for those species [Baršiené et al., 1995] and also conserved in the related families Opisthorchiidae and Cryptogonimidae [Baršiené, 1993; Zadesenets et al., 2012a].

The families Zoogonidae and Faustulidae have been illustrated as a paraphyletic clade within the superfamily Microphaloidea, suborder Xiphidiata [Olson et al., 2003]. In contrast to previous reports of 10 or 12 chromosomes in the only other species of Zoogonidae analyzed to date, *Zoogonus mirus* [Walton, 1959], *D. brusinae* presented 2n = 20. The species with known chromosome numbers in sister clades (as proposed by Olson et al. [2003]) displayed mostly 2n = 22 chromosomes in the families Lecithodendriidae and Prosthogonimidae and 2n = 18 in Microphalliidae [Baršiené, 1993; Birstein and Mikhailova, 1999]. The only described case of 2n = 20 is *Transversotrema patialense* (Eucotylidae), which showed a karyotype composed exclusively of metacentric and submetacentric chromosomes [Mad Havi and Ramanjaneyulu, 1986].

The morphologies of the cercariae isolated from *D. trunculus*, *T. tenuis,* and *R. decussatus* greatly resemble *B. bacciger*, as first described by Palombi [1933]. Nevertheless, when taken into account the strict first-host specificity shown by many digeneans and their isolation from species belonging to 3 different bivalve families (Donacidae, Tellinidae, and Veneridae), these cercariae most likely correspond to different *Bacciger* taxa [Kinne, 1983]. In fact, on the basis of morphologic characteristics of the cercariae, they were already reported as different species [Ramón et al., 1999; Ben Abdallah et al., 2009], therefore providing further evidence for cryptic speciation among digeneans infecting sympatric hosts [Jousson et al., 2000]. Our cytogenetic results were found to be consistent with cryptic speciation. While these presumably cryptic species presented the same chromosome number (2n = 12), striking differences in chromosome morphologies were recorded among their karyotypes.

In conclusion, our results support evidence for important chromosome divergence among closely related digeneans that can help to understand evolutionary patterns in these organisms.

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Statement of Ethics

The experimental procedure was performed with the approval of the Ethics Committee of the University of Vigo, complying with the current laws of Spain. All institutional and national guidelines for the care and use of laboratory animals were followed.

Disclosure Statement

The authors confirm that they have no conflicts of interest to declare.

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DISCUSIÓN GENERAL

La realización de estudios citogenéticos en bivalvos siempre ha sido una tarea complicada; debido a ello, los resultados obtenidos tras más de un siglo de estudios son ciertamente escasos. Aunque el número estimado de taxones que componen la clase Bivalvia es del orden de 8500 (Huber 2010, 2015), no llegan a 300 las especies con dotaciones cromosómicas conocidas y a la mitad aquéllas en las que se han descrito los cariotipos (revisado por Nakamura 1985; Thiriot-Quiévreux 1994, 2002; Leitão y Chaves 2008; Arias-Pérez et al. 2013). La situación es incluso peor en lo que se refiere a los análisis citogenéticos de trematodos digeneos en los que, pese a constituir el mayor grupo de parásitos internos de metazoos con más de 18000 taxones conocidos, sólo se han descrito los cariotipos de unos 300 (Hirai et al. 1989, 2000; Baršiené 1993, Hirai y LoVerde 1996; Bell et al. 1998; Petkevičiūtė et al. 2003, 2006, 2012, 2014, 2015; Špakulová y Casanova 2004; Reblánová et al. 2011; Zadesenets et al. 2012a, 2012b; Hirai 2014; Sofi et al. 2015).

Además de escasos, los estudios citogenéticos se han realizado fundamentalmente en bivalvos de interés comercial y en digeneos causantes de enfermedades humanas. Así, la mayoría de bivalvos analizados pertenecen a cuatro familias, tres incluidas en la subclase Pteriomorphia (Mytilidae, Pectinidae y Ostreidae) y una en la subclase Heterodonta (Veneridae). De este modo, de las 45 especies en las que se han publicado estudios cariotípicos en lo que va de siglo, 13 son mitílidos, 8 pectínidos, 6 ostreidos y 8 venéridos mientras que las 10 restantes pertenecen a otras 8 familias (Thiriot–Quiévreux 2002; Leitão y Chaves 2008; Arias-Pérez et al. 2013; González-Tizón et al. 2013; Feng et al. 2014; Pérez-García et al. 2014a, 2014b; Li et al. 2016; Yang et al. 2016; García-Souto et al. 2017). Por lo que se refiere a los trematodos digeneos, el conocimiento disponible es mucho más fragmentario y se centra en los que tienen impacto médico y/o económico, como es el caso del género *Schistosoma* (Hirai et al. 1989, 2000; Hirai y Lo Verde 1996; Hirai 2014) y, en menor medida, las familias Fasciolidae (Reblánová et al. 2011) y Opisthorchiidae (Zadesenets et al. 2010).

La escasez de datos cariológicos en estos grupos puede ser atribuida a una serie de causas íntimamente relacionadas. A las dificultades para obtener preparaciones cromosómicas en las que aparezcan metafases mitóticas en suficiente cantidad, y con calidad mínima para este tipo de estudios, se agregan el poco interés que las directrices

de política científica marcan para todo lo que no sea investigación aplicada, la supuesta ausencia de "problemas interesantes" a resolver y la escasez, cada vez mayor, de grupos de investigación dedicados a la citogenética. Los resultados obtenidos en esta tesis, aunque confirman las dificultades de este tipo de trabajo, tanto en bivalvos como en digeneos, también ponen de manifiesto que un estudio más profundo de estos organismos no solo permite responder a algunas preguntas de interés científico sino que, todavía más interesante, plantea nuevas cuestiones.

Números cromosómicos y cariotipos

En la subclase Heterodonta, que incluye almejas, berberechos y arcas, sólo se conoce la dotación cromosómica de unas 80 especies, siendo la inmensa mayoría de ellas 2*n* = 38 (Nakamura 1985; Thiriot–Quiévreux 1994, 2002; Leitão y Chaves 2008; Arias-Pérez et al. 2013; Pérez-García et al. 2014a). Las veinte especies de bivalvos analizadas en el presente trabajo no son una excepción. Aunque esto era ya conocido para 10 de ellas, es descrito por vez primera para los venéridos *Clausinella fasciata*, *Chamelea striatula*, *Venus casina*, *Dosinia lupinus* y *Petricola litohpaga*, para la mactra *Spisula subtruncata*, la coquina *Donax vittatus* y las tellinas *Bosemprella incarnata*, *Moerella donacina* y *Serratina serrata*. Si bien la uniformidad de los números cromosómicos en las familias Veneridae, Mactridae, Donacidae y Tellinidae pudiera ser reflejo de un alto grado de conservación del número cromosómico, la todavía escasa proporción de especies analizadas hace necesario ser cauteloso en la generalización puesto que otras familias de bivalvos muestran comportamientos diferentes. Por ejemplo, una de las 17 especies de la familia Ostreidae es 2n = 18 mientras que las 16 restantes son 2*n* = 20 y la restante (Thiriot-Quiévreux 1994, 2002; Leitão y Chaves 2008); en la familia Pteriidae una de las ocho especies analizadas es 2*n* = 26 y las siete restantes 2*n* = 28 (Thiriot-Quiévreux 1994, 2002). Las especies de Mytilidae presentan números diploides que van de 2*n* = 22 a 2*n* = 32 (Thiriot-Quiévreux 2002; Leitão y Chaves 2008; García-Souto et al. 2017), comportamiento similar al que se encuentra en la familia Pectinidae en la que las dotaciones cromosómicas van de 2*n* = 26 a 2*n* = 38 (Thiriot-Quiévreux 2002; Leitão y Chaves 2008).

Por lo que a la morfología del complemento cromosómico se refiere, hasta la actualidad se han descrito los cariotipos en una 50 especies de la subclase Heterodonta, incluyendo quince de la familia Veneridae, cuatro de la Mactridae, tres de la Tellinidae y una de la Donacidae (Nakamura 1985; Thiriot-Quiévreux 1994, 2002; Leitão y Chaves 2008; Arias-Pérez et al. 2013; González-Tizón et al. 2013; Pérez-García et al. 2014a). Como es habitual en la mayoría de los bivalvos, los cariotipos de estas especies se caracterizan por mostrar pares cromosómicos en los que morfología y tamaño muestran pequeñas diferencias que se distribuyen de forma casi continua (Thiriot-Quiévreux 2002; González-Tizón et al. 2013). Como consecuencia de ello, la identificación de pares cromosómicos por tamaño y morfología es dificultosa y se ve agravada por la utilización de metafases mitóticas que suelen estar en estadios de condensación muy avanzados. Dadas estas dificultades de tipo técnico, es posible que algunas de las presuntas diferencias cariológicas entre bivalvos pertenecientes a poblaciones de la misma especie sean más artefactos técnicos que realidades. Por ello, la obtención de buenos marcadores cromosómicos es un requisito imprescindible para la elaboración de cariotipos fiables.

En lo que se refiere a los digeneos, los números cromosómicos descritos varían desde un mínimo de $2n = 10$ a un máximo de $2n = 56$ aunque predominando las especies $2n = 20$ y $2n = 22$ (Baršiené 1993; Petkevičiūtė et al. 2012). En la mayoría de los casos los cariotipos difieren enormemente en los tipos de cromosomas que los componen. Puesto que, por una parte, las diferencias en los números cromosómicos y en las composiciones de los cariotipos detectadas entre especies próximas son bastante menores que las que aparecen entre taxones más alejados y, por otra, que las relaciones filogenéticas en la clase Digenea no están del todo claras, la información citogenética es de enorme relevancia. Asimismo, la presencia de cromosomas B en algunas especies (Petkevičiūtė et al. 2012) y de cromosomas sexuales en otras (Hirai 2014) hace que los digeneos sean un grupo de organismos atractivos para analizar la evolución cromosómica. Los resultados obtenidos en esta tesis doctoral tras analizar metafases mitóticas correspondientes a diez taxones, aislados de doce moluscos intermediarios, corroboran la enorme variabilidad citogenética existente en este grupo.

Heterocromatina constitutiva, DNA satélites y metilación

La mayoría de los bivalvos estudiados presentan regiones ricas en GC poco abundantes y coincidentes con las regiones organizadoras de los nucleolos (Martínez-Expósito et al. 1997; Torreiro et al. 1999; Pérez-García et al. 2011). Estas regiones suelen ser, además, las únicas que responden positivamente a los tratamientos de bandas C y, por tanto, pueden ser calificadas de heterocromáticas. La mayoría de las especies de bivalvos estudiadas en este trabajo, 16 de un total de 20, mostraron también este comportamiento. En ellas la heterocromatina, rica en GC, se encuentra asociada a las regiones en las que se localizan los rDNA 45S y, dada su escasez, es poco útil en la identificación de pares cromosómicos. Por el contrario, las cuatro especies restantes (*Spisula subtruncata*, *Mactra stultorum*, *Donax trunculus* y *Donax vittatus*) presentan regiones heterocromáticas, generalmente intercalares, con un contenido en GC marcadamente superior a la media de sus respectivos genomas. Aunque una o dos de estas regiones coinciden con los NORs, no es así en las restantes. La existencia de este tipo de bandas sólo había sido descrita previamente en *Donax trunculus* (Martínez et al. 2002; Petrović et al. 2009) y *Dreissena polymorpha* (Woznicki y Boron 2003; Boron et al. 2004), Los resultados obtenidos en esta tesis indican que es factible que este tipo de bandas también exista en otros bivalvos pero que el tamaño de las regiones heterocromáticas o su contenido en GC no haya permitido su detección.

En el DNA de las regiones heterocromáticas es bastante frecuente encontrar secuencias repetidas, muchas de ellas DNA satélites. Los análisis moleculares realizados en distintos grupos han constatado que algunos de estos satélites, o ciertas fracciones de los mismos, presentan un grado de conservación relativamente elevado, atribuido a una función posiblemente estructural. Asimismo, grupos de especies filogenéticamente relacionadas comparten un conjunto de DNA satélites homólogos, diferencialmente representados en sus genomas debido a procesos de contracción o expansión desigual entre linajes. Esta *library hypothesis* (Fry y Salser 1977) explica que algunas especies de bivalvos presenten una sobrerrepresentación de ciertos DNA satélites que en taxones muy relacionados no aparecen o lo hacen en un bajo número de copias (Martínez-Lage et al. 2005; López-Flores et al. 2010; Plohl et al. 2010; Petraccioli et al. 2015). La expansión desigual de estos satélites está relacionada, en

algunos casos, con una heterocromatinización diferencial en las especies implicadas. El satélite identificado en esta tesis doctoral, SSUsat, está poco representado en las mactras *Spisula solida* y *Mactra stultorum* pero, como demostramos mediante hibridación *in situ*, es el constituyente principal de la heterocromatina intercalar en otra especie de mactras, *Spisula subtruncata*. Aunque todavía no identificados, es posible que otros satélites sean los principales constituyentes de la heterocromatina en las otras tres especies, *Mactra stultorum Donax trunculus* y *Donax vittatus*, estudiadas en esta tesis doctoral y que también se caracterizan por presentar un número relativamente elevado de bandas C; en el futuro pretendemos investigar más profundamente este aspecto.

Durante años se ha especulado sobre las relaciones entre reorganización cromosómica y especiación. Las opiniones son variadas y van desde las que suponen que estas reorganizaciones son una mera molestia para el genoma a las que conjeturan que estas reorganizaciones pudieran tener un significado funcional tanto a corto plazo, posibilitando la adaptación a condiciones ambientales cambiantes, como a largo plazo, facilitando la especiación (Noor et al. 2001). Los resultados obtenidos en los últimos años parecen demostrar que las inversiones cromosómicas facilitan la diferenciación durante la especiación (Ayala y Coluzzi 2005; Hoffmann y Rieseberg 2008). Las inversiones pericéntricas que diferencian poblaciones atlánticas y mediterráneas de *Donax trunculus* y poblaciones gallegas de *Donax vittatus* indican que este tipo de reorganizaciones pudieran jugar un papel fundamental en los cambios cariotípicos que han acompañado la evolución en Donacidae. Esto es reforzado por la comparación cariológica entre ambas especies pues mientras la posición ocupada por diversos tipos de secuencias repetidas es generalmente mantenida, la morfología de muchos de los pares de cromosomas es distinta. Puesto que los puntos de rotura requeridos en cualquier reorganización cromosómica estructural están íntimamente relacionados con la presencia de secuencias repetidas (Noor et al. 2001; Coghlan et al. 2005; Krikpatrick 2010; Schubert y Lysak 2010) y en estas especies, en comparación con otros bivalvos, existe una gran cantidad de heterocromatina, no es desmesurado especular con que ambos fenómenos pudieran estar relacionados.

La metilación es un mecanismo epigenético, comúnmente asociado a la represión génica en vertebrados, que juega un papel crucial en el desarrollo y

mantenimiento del ciclo celular, en la pluripotencialidad de la línea germinal y en la impronta genómica (Albalat et al. 2012). En contraposición, la mayoría de invertebrados muestra niveles de metilación escasos o moderados (Schübeler 2015). Esto mismo sucede en moluscos, en los que la metilación se sitúa en un 2-5% en adultos (Regev et al. 1998; Fneich et al. 2013; Gavery y Roberts 2013) y afecta preferentemente a genes y, en menor medida, a secuencias repetidas (Wang et al. 2014). Puesto que tanto en *Donax trunculus* (Petrović et al. 2009) como en *Crassostrea gigas* (Wang et al. 2014) se había detectado metilación en satélites, en este trabajo analizamos la metilación de SSUsat en relación al resto del genoma, incluidas las regiones heterocromáticas. En vertebrados las regiones cromosómicas metiladas detectadas mediante inmunofluorescente suelen coincidir con regiones heterocromáticas (Covelo-Soto et al. 2015). Por el contrario, muy pocas especies de invertebrados presentan perfiles de metilación cromosómica bien definidos. Los resultados obtenidos en *Spisula subtruncata* muestran que la metilación de la eucromatina es uniformemente escasa y que algunas de las regiones heterocromáticas están claramente hipometiladas. Si este hecho es de por sí sorprendente, pues sólo hemos encontrado resultados parcialmente similares en plantas (Schmidt et al. 2014), todavía lo es más dado que los monómeros SSUsat, componentes primarios de la heterocromatina, presentan un grado de metilación que triplica la media del genoma. Aunque este comportamiento pudiera ser atribuido a muy diversas causas, una de las más atractivas sería que los monómeros SSUsat metilados y no metilados se acumulasen de forma diferencial en diferentes regiones heterocromáticas y que este fenómeno estuviese relacionado con una función específica de las mismas.

Mapas físicos de familias multigénicas

La identificación inequívoca de pares cromosómicos por tamaño y morfología es en bivalvos una tarea prácticamente imposible. Puesto que estos cromosomas tampoco presentan patrones de bandas longitudinales claros, resulta de gran interés la localización de secuencias repetidas conservadas mediante hibridación *in situ*. Incluso en comparación con otros bivalvos como mitílidos (Thiriot-Quiévreux 2002; Pérez-García et al 2014b; García-Souto et al. 2017), ostreidos (Leitao y Chaves 2008; Bouilly

et al. 2008, 2010; López-Flores et al. 2010) y pectínidos (Leitao y Chaves 2008; Hu et al. 2011; Petraccioli et al. 2015; Li et al. 2016), este tipo de análisis es muy escaso en las especies de la subclase Heterodonta (Tabla 1).

Tabla 1 Mapeo cromosómico en especies de la subclase Heterodonta

Especies	n	45S rDNA	5S rDNA	*h3*(core)	Otras	References
Veneridae						
Ruditapes philippinarum					sat	Passamonti et al. 1998
Ruditapes philippinarum	19	? ter	? ic		tel	Hurtado et al. 2011
Ruditapes philippinarum	19	8p ter (m)	5q ic (st) 6q ic (st)		tel	Pérez-García et al. 2014a
Ruditapes philippinarum	19	8p ter (m)	5q ic (st) 6q ic (st)	4q ter (m)		Este trabajo
Ruditapes decussatus	19	? ic	? ic		tel	Hurtado et al. 2011
Ruditapes decussatus	19	3q ic (sm)	3q ic (sm) 8q ter (st)		tel	Pérez-García et al. 2014a
Ruditapes decussatus	19	3q ic (sm)	3q ic (sm) 8q ter (st)	4p cen (st)		Este trabajo
Polititapes aureus	19	5p ic (m)	17q ter (st)	2p ter, 2q ter (m) 3q ter (m) 8q ter (m)	tel	Carrilho et al. 2011
Venerupis corrugata	19	10q ic (m)	9q ter (st)		tel	Pérez-García et al. 2014a
Venerupis corrugata	19	10q ic (m)	9q ter (st)	2q ter (m) 4q ter (sm)		Este trabajo
Polititapes rhomboides	19	17q ter (st)	9p ter (m)	5q ic (m) 12q cen (st)	tel	Carrilho et al. 2011
Clausinella fasciata	19	10q cen (sm)	5p ic (sm)	10q ter (m) 12p ter (sm)	tel	Este trabajo
Chamelea gallina	19	19p cen (sm)	5p ic (sm) 9q ic (m)	15q ter (m) 17q ter (st)	tel	Este trabajo
Chamelea striatula	19	8q ter (sm) 19p cen (sm)	5p ic (sm) 9q ic (m)	5q ter (m) 6q ter (m) 15q ter (m) 17q ter (m)		Este trabajo
Venus verrucosa	19	12p ter (sm)	9q ic (m)		tel	Pérez-García et al. 2014a
Venus verrucosa	19	12p ter (sm)	9q ic (m)	13q ter (m)		Este trabajo
Venus casina	19	16q ter (st)	6p ic (sm)	9q ter (m)	tel	Este trabajo
Mercenaria mercenaria	19				tel	Wang y Guo 2001
Mercenaria mercenaria	19	10q ic (sm) 12p ter (st)				Wang y Guo 2007
Dosinia exoleta	19	3p ter (m)			tel	Hurtado y Pasantes 2005
Dosinia exoleta	19	3p ter (m)	13q ic (sm) 15p ter (sm)			Pérez-García et al. 2014a
Dosinia exoleta	19	3p ter (m)	13q ic (sm) 15p ter (sm)	2q ic (m)		Este trabajo
Dosinia lupinus	19	12q ic (m)	14q ter (sm)	9q ic (m)	tel	Este trabajo
Petricola litophaga	19	19p cen (m)	5q ic (m) 12q ic, 12q ic (st)	3p ter (m) 14q ter (m)	tel	Este trabajo
Cardiidae						
Cerastoderma edule	19	?p ter	5 pairs: q ter			Insua et al.1999

p: short arm; q: long arm; cen: subcentromeric; ic: intercalary; ter: subterminal
(m): metacentric; (sm): submetacentric; (st): subtelocentric; (t): telocentric; ?: not described

Tabla 1 (continuación) Mapeo cromosómico en especies de la subclase Heterodonta

Especies	n	45S rDNA	5S rDNA	*h3*(core)	Otras	References
Donacidae						
Donax trunculus						Plohl et al. 2002
Donax trunculus	19	9p ic (sm)				Martínez et al. 2002
Donax trunculus	19	6p ic (sm)			tel sat	Petrovic et al. 2009
Donax trunculus	19	6p ic (sm) [6q cen (m)]	3p ic (m) 10q ter (st)	17q ic (st)	tel	Este trabajo
Donax vittatus	19	6p ic (t)	10q ter (st)	17q ic (st)	tel	Este trabajo
Mactridae						
Mulinia lateralis	19				tel	Wang y Guo 2001
Mulinia lateralis	19	15q ter (t) 19q ter (t)				Wang y Guo 2008
Mactra stultorum	19	3q ter (st) 4q ter (st)	15p cen (sm)	12q ic (st)	tel	Este trabajo
Spisula solida	19	17p ter (sm) 19p ter (st)	5p ter (sm)	8q ic (st)	tel	Este trabajo
Spisula subtruncata	19	18q ter (m)	3q ter (m)	14q cen (m)	tel	Este trabajo
Pharidae						
Ensis magnus	19	12q cen (sm)				Fernández-Tajes et al. 2008
Ensis directus	19	2p cen (m)			tel	González-Tizón et al. 2013
Ensis minor	19	2p ic (sm)	?q ter (t)		tel	González-Tizón et al. 2013
Ensis siliqua	19	7q ter (sm)				Fernández-Tajes et al. 2008
Psammobiidae						
Nutallia nuttallii	19	8p ter (m)				González-Tizón et al. 2000
Solenidae						
Solen marginatus	19	9q cen (st) 12p ter (st)				Fernández-Tajes et al. 2003
Tellinidae						
Macoma nasuta	19	15q ter (st)				González Tizón et al. 2000
Bosemprella incarnata	19	1p ic (m)	12q ter (m)	15p cen (t)	tel	Este trabajo
Macomangulus tenuis	19	9q cen (st)	9q ter (st) 17p cen (st)	6q ter (st)	tel	Este trabajo
Moerella donacina	19	6q cen (st)	5q ter (st) 7q ic (m)	17p cen (t)	tel	Este trabajo
Serratina serrata	19	12p cen (t) 17p cen (t)	9q ter (t)	3q cen (m)	tel	Este trabajo

(m): metacentric; (sm): submetacentric; (st): subtelocentric; (t): telocentric; ?: not described
p: short arm; q: long arm; cen: subcentromeric; ic: intercalary; ter: subterminal

En esta tesis doctoral se ha localizado la posición cromosómica ocupada por las agrupaciones rDNA 5S y 45S, los genes de la histona H3 y las secuencias teloméricas en 20 especies pertenecientes a cuatro familias de veneroideos y, por tanto, constituye un muy importante avance en el estudio de estos organismos. En conjunto, la diversidad detectada en los patrones de distribución de estas secuencias, y las diferencias de comportamiento con respecto a otros taxones de invertebrados, sugieren que la propagación de estas familias multigénicas en los genomas depende de la acción

conjunta de diversos mecanismos evolutivos que operan de manera diferente para cada familia multigénica y cada taxón considerados.

En Heterodonta, 27 de las 33 especies estudiadas presentan una única agrupación para el rDNA 45S, mientras que en las seis restantes aparecen dos (Tabla 1). La presencia de una mayoría de especies con una única agrupación se repite en todas las familias excepto en Mactridae en la que tres de las cuatro especies analizadas presentan dos agrupaciones. En cuanto a su posición cromosómica, existe una gran variedad, detectándose en regiones subterminales, intercalares o subcentroméricas. Este comportamiento se repite en todas las familias en las que se han estudiado varias especies salvo en Mactridae, en la que todas las señales son subterminales. Tal dinámica evolutiva es similar a la hallada en polillas de la familia Tortricidae, que poseen una única agrupación para el rDNA 45S pero variando su localización cromosómica (Šíchová et al. 2010). En cualquier caso, estas secuencias presentan comportamientos dispares en invertebrados, con taxones en los que hay divergencia en localización y número de agrupaciones, por ejemplo mitílidos (Insúa et al. 2001; Pérez-García et al. 2010a, 2010b, 2011, 2014), saltamontes (Cabrero et al. 2009; Cabral-de-Mello et al. 2011a) y coleópteros (Cabral-de-Mello et al. 2011b, 2011c), y otros en los que las agrupaciones rDNA 45S están más conservadas, como por ejemplo las ostras (Cross et al. 2003; Wang et al. 2004).

Con respecto al rDNA 5S, de un total de 24 especies de Heterodonta estudiadas (Tabla 1), 13 mostraron una única agrupación, nueve presentaron dos, *Petricola lithophaga* tres y el berberecho *Cerastoderma edule* cinco (Insua et al. 1999). A excepción de la familia Mactridae, en la que las tres especies poseen una única agrupación rDNA 5S, el resto de las familias estudiadas presentan variación en número. En todas ellas la posición cromosómica puede ser subterminal, intercalar o subcentromérica. En otros bivalvos la situación es más variable, mientras que en la familia Mytilidae aparecen de dos a cinco agrupaciones en distintas posiciones (Insúa et al. 2001; Pérez-García et al. 2010a, 2010b, 2011, 2014), en los pectínidos las agrupaciones rDNA 5S, una o dos, son mayoritariamente subterminales (Insua et al. 1998; Wang y Guo 2004; López-Piñón et al. 2005). Por el contrario, otros invertebrados

muestran gran variación tanto en número como en posición cromosómica (Cabrero et al. 2009; Cabral-de-Mello et al. 2011a, 2011b, 2011c).

Por lo que se refiere a los genes de la histona H3, de un total de 22 especies de Heterodonta analizadas (Tabla 1), 15 presentan una única agrupación, cinco mostraron dos y *Venerupis aureus* y *Chamelea striatula* cuatro. Pese a ello y al igual que en mitílidos (Pérez-García et al. 2010a, 2010b, 2011, 2014; García-Souto et al. 2017) y pectínidos (Zhang et al. 2007), la variación en el número de señales no muestra ningún patrón específico de familia. En otros invertebrados como polillas (Šíchová et al. 2013), saltamontes (Cabrero et al. 2009; Cabral-de-Mello et al. 2011a, 2011b, 2011c) o áfidos (Mandrioli y Manicardi 2013) el número de agrupaciones de genes de histonas suele estar conservado. Por lo que se refiere a la posición que ocupan y en contraposición a su variabilidad numérica, en Heterodonta el 75% de las agrupaciones detectadas (21 de un total de 28) son subterminales, este valor roza el 80% en la familia Veneridae (19 de 24). Esto es inusual tanto en bivalvos como en otros invertebrados puesto que la localización subterminal sólo ha sido descrita en un 24% (6 de 25) de las agrupaciones en mitílidos (Pérez-García et al. 2010a, 2010b, 2011, 2014; García-Souto et al. 2017) y en un 40% (2 de 5) en pectínidos (Zhang et al. 2007) mientras que en otros invertebrados esta posición sólo ha sido descrita en tres especies de saltamontes (Cabrero et al. 2009).

Aunque la sintenia de rDNA y genes de histonas es relativamente frecuente en otros organismos (Cabrero et al. 2009), no lo es tanto en bivalvos puesto que sólo se había descrito en algunos mitílidos (Pérez-García et al. 2010a, 2010b, 2011, 2014; García-Souto et al. 2017). Dado que el número de especies estudiadas es todavía bajo, sería imprudente considerar esto como una situación generalizada. En las especies de Heterodonta estudiadas en esta tesis doctoral solo hemos encontrado sintenia entre genes de histonas y rDNA 5S en *Chamelea striatula* y entre ambos rDNAs en *Clausinella fasciata* y *Macromagnus tenuis*. También se ha confirmado la colocalización de ambos tipos de rDNA en *Ruditapes decussatus*. La existencia de sintenia en una especie pero no en otra próxima facilita los estudios comparativos como el realizado en especies del género *Chamelea*, que ha permito demostrar que *C. gallina* y *C. striatula* son especies diferentes.

En cuanto a los digeneos, los resultados obtenidos en esta tesis doctoral indican que en todos ellos existe una única agrupación tanto para el rDNA 45S como para el 5S y que en la mayoría estas agrupaciones colocalizan en posición generalmente subcentromérica. Las excepciones son Cercaria longicaudata, subterminal, y *Monascus filiformis,* intercalar. Por el contrario, tanto en *Bucephalus minimus* como en *Parorchis acanthus* los rDNAs 45S y 5S están en diferentes cromosomas, siendo el primero intercalar en ambas especies y el rDNA 5S intercalar en *B. minimus* y subcentromérico en *P. acanthus*. La presencia de una única agrupación rDNA 45S es coincidente con la situación en otros digeneos en los que ocupa posiciones intercalares (Hirai et al. 1989), subterminales (Reblánová et al. 2011) o indeterminadas (Zadesenets et al. 2012b). Dada la todavía escasa cantidad de datos, sería imprudente deducir que la presencia de ambos tipos de secuencias ligadas sea un carácter ancestral pues en otros organismos se ha postulado que ambas secuencias pueden asociarse o separarse en repetidas ocasiones (Drouin y de Sá 1995; Vierna et al. 2013).

CONCLUSIONS

1. The FISH mapping results obtained in this work demonstrate that 45S rDNA, 5S rDNA and H3 histone genes are helpful markers for identifying chromosomes and, therefore, establishing more reliable karyotypes in species of the families Veneridae, Mactridae, Donacidae and Tellinidae.

2. Far from the apparent stability denoted by the conservation of diploid chromosome numbers, 2n = 38, the degree of karyotype diversification in the families Veneridae, Mactridae, Donacidae and Tellinidae is high.

3. As the FISH-mapped karyotypes of all the species analyzed are clearly different, this character can constitute a further taxonomic criterion for species delimitation and cryptic and alien species identification in the order Veneroida.

4. The divergence in both the chromosome distribution of 45S rDNA, 5S rDNA and H3 histone genes and the clustering of samples on the phylogenetic trees provide clear evidence that *Chamelea gallina* and *Chamelea striatula* are different species.

5. Pericentric inversions seem to be the main force reshaping karyotypes in Donacidae. Although the occurrence of lineage-specific patterns of chromosome rearrangements in other families cannot be ruled out, they were not as obvious.

6. The abundance, the sequence conservation and the relative high level of methylation of the satellite DNA constituting the heterochromatin of *Spisula subtruncata*, together with the chromosome-specific differences in the distribution of over- and under-methylated monomers, highlights the complexity of the methylation processes that shape repetitive genome compartments.

7. The significant chromosomal divergence detected among closely related digeneans is key to understanding evolutionary processes in these organisms.

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ANNEX I

PERSONAL INFORMATION

Daniel García Souto

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+34 986 883 491 +34 605 462 581

danielgarciasouto@gmail.com

Sex Male | Date of birth 02/18/1988 | Nationality Spanish

WORK EXPERIENCE

From 2012 to present

PhD student in METHODOLOGY AND APPLICATIONS IN LIFE SCIENCES

University of Vigo, Vigo, Spain. FPU grant, Spanish Ministry of Education till December 2016

PhD thesis title: *Cytogenetic characterization of veneroid bivalves and some of their parasites*

Sector Research

EDUCATION AND TRAINING

From 2011 to 2012

Master in METHODOLOGY AND APPLICATIONS IN MOLECULAR BIOLOGY

University of Vigo, Vigo, Spain

- Master thesis: "Cytogenetic location of rRNA and histone gene clusters and telomeric sequences in *Chamelea (Venus) striatula*"

From 2007 to 2011

Degree in BIOLOGY

University of Vigo, Vigo, Spain

- Degree thesis: "Chromosome characterization in two Venerid species: *Chamelea (Venus) striatula* and *Clausinella fasciata*"

PERSONAL SKILLS

Mother tongue: Spanish

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1

Levels: A1/2: Basic user - B1/2: Independent user - C1/2 Proficient user
Common European Framework of Reference for Languages

ADDITIONAL INFORMATION

Publications and papers (PhD thesis)

García-Souto D, Rios G, Pasantes JJ (**2017**) Karyotype differentiation in tellin shells (Bivalvia, Tellinidae). In preparation

García-Souto D, Pérez-García C, Pasantes JJ (**2017**) Are pericentric inversions reorganizing wedge shell genomes? In preparation

García-Souto D, Mravinac B, Šatović E, Plohl M, Morán P, Pasantes JJ (**2017**) Methylation profile of a satellite DNA constituting the intercalary CG-rich heterochromatin of the cut trough shell *Spisula subtruncata* (Bivalvia, Mactridae). **Scientific Reports** (under review)

García-Souto D, Qarkaxhija V, Pasantes JJ **(2017)** Resolving the taxonomic status of *Chamelea gallina* and *C. striatula* (Veneridae, Bivalvia): A combined molecular cytogenetic and phylogenetic approach. **Biomed Research International** (in press)

García-Souto D, Pérez-García C, Kendall J, Pasantes JJ (**2016**) Molecular cytogenetics in trough shells (Mactridae, Bivalvia): Divergent GC-rich heterochromatin content. **Genes** (Basel). 7(8):47. http://dx.doi.org/10.3390/genes7080047

García-Souto D, Pasantes JJ (**2015**) Molecular cytogenetics in digenean parasites: Linked and unlinked major and 5S rDNAs, B chromosomes and karyotype diversification. **Cytogenetics and Genome Research**, 147(2-3):195-207. http://dx.doi.org/10.1159/000442504

García-Souto D, Pérez-García C, Morán P, Pasantes JJ (**2015**) Divergent evolutionary behavior of H3 histone gene and rDNA clusters in venerid clams. **Molecular Cytogenetics** 8:40. http://dx.doi.org/10.1186/s13039-015-0150-7

Publications and papers (Additional)

García-Souto D, Sumner-Hempel A, Fervenza S, Pérez-García C, Torreiro A, González-Romero R, Eirín-López JM, Morán P, Pasantes JJ (**2017**) Detection of invasive and cryptic species in marine mussels (Bivalvia, Mytilidae): A chromosomal perspective. **Journal for Nature Conservation** (under review)

García-Fernández P, **García-Souto D**, Almansa E, Morán P, Gestal C (**2017**) Epigenetic DNA methylation mediating the *Octopus vulgaris* early development: effect of essential fatty acids enriched diet. **Frontiers in Physiology** (in press)

Gonzalez-Romero R; Suárez-Ulloa V; Rodríguez-Casariego J; **García-Souto D**; Diaz G; Smith A; Pasantes JJ; Rand G; Eirin-Lopez JM (**2017**) Effects of Florida Red Tides on histone variant expression and DNA methylation in the Eastern oyster *Crassostrea virginica*. **Aquatic Toxicology** 186: 196-204. doi: http://doi.org/10.1016/j.aquatox.2017.03.006

García-Souto D; Troncoso T; Pérez M; Pasantes JJ (**2015**) Molecular cytogenetic analysis of the European Hake *Merluccius merluccius* (Merlucciidae, Gadiformes): U1 and U2 snRNA gene clusters map to the same location. **PLOS one** 10(12):e0146150. http://dx.doi.org/10.1371/journal.pone.0146150

Muinelo-Romay L; **García-Souto D;** Alonso-Alconada L; Vieito M; Carmota M; Martínez N (**2013**) Zoledronic acid as an antimetastatic agent for different human tumor cell lines, **Anticancer Research** 33(12):5295-5300. PMID: 24324062

Seminars

Gonzalez-Romero R; Suarez-Ulloa V; Rodriguez-Casariego JA; **Garcia-Souto D**; Diaz G; Smith A; Pasantes JJ; Rand G; Eirin-Lopez J. Epigenetic mechanisms mediating responses to Florida Red Tides in the Eastern oyster *Crassostrea virginica*. 7th SETAC World Congress, Orlando, Florida, USA (**2016**). Oral communication.

García-Souto D; Sumner-Hempel A; Fervenza S; González-Romero, G; Eirín-López J; Pérez-García C; Morán P; Pasantes JJ. Mytilidae: a cytotaxonomic approach. I International symposium on advances in marine mussel research, Vigo, Spain (**2016**). Poster.

García-Souto D; Pérez-García C; Morán P; Diz AP; Pasantes JJ. Chromosomal analysis of hybrid mussels. I International symposium on advances in marine mussel research, Vigo, Spain (**2016**). Poster.

Sumner-Hempel A; Fervenza S; **García-Souto D**; González-Romero R, Eirín-López JM; Pasantes JJ; Morán P. Cytogenetic analysis on the invasive mussel *Perna perna* and *Brachidontes sp*. ALERTOOLS 2016, Avilés, Spain (**2016**). Oral communication.

García-Souto D; Qarkaxhija V; Pérez-García C; Morán P; Pasantes JJ. Insights into the *Chamelea gallina/striatula* complex evolution: A cytogenetic and population genetics approach. 22nd International colloquium on animal cytogenetics and gene mapping, Toulouse, France (**2016**). Poster.

García-Souto D; Fernández-Rodríguez J; Fuller N; Galindo J; Pasantes JJ. Analysis of the chromosome pairing in the dog whelk *Nucella lapillus*. 22nd International colloquium on animal cytogenetics and gene mapping, Toulouse, France (**2016**). Oral communication.

Fernández-Rodríguez J; Fuller N; **García-Souto D**; Galindo J, Pasantes JJ. Polimorfismo cromosómico en *Nucella lapillus* en la costa gallega. IX seminario de citogenética, Toledo, Spain (**2016**). Oral communication.

García-Souto D; Fervenza S; Pasantes JJ. Caracterización cariotípica de mitílidos de los géneros *Perna* y *Brachidontes*. IX seminario de citogenética, Toledo, Spain (**2016**). Oral communication.

García-Souto D; Pasantes JJ. Citogenética de digeneos, primeras aproximaciones. VIII Seminario de Citogenética, Alcalá de Henares, Spain (**2014)**. Oral communication.

Covelo-Soto L; **García-Souto D**; Pérez-García C; Pasantes JJ; Morán. Evidences of DNA methylation changes in the early development and metamorphosis of the sea lamprey. 21st International colloquium on animal cytogenetics and gene mapping, Ischia (Naples), Italia (**2014**). Oral communication.

García-Souto D; Pérez-García C; Pasantes JJ; Morán P. Comparative chromosome mapping of repetitive sequences in two species of Mactridae (Bivalvia). 21st International colloquium on animal cytogenetics and gene mapping, Ischia (Naples), Italia (2014). Oral communication.

García-Souto D; Pérez-García C; Pasantes JJ; Morán P. Caracterización cromosómica de dos especies de venéridos: Chamelea (Venus) Striatula y Clausinella fasciata. VII Seminario de Citogenética, Pontevedra, Spain (**2012**). Oral communication.

de Miguel Villegas E; Barja Fernández S; Debenedetti López A; **García-Souto D**; Murgarella Ciuraneta M; Álvarez Otero R. Las plataformas docentes como fuente de ayuda al estudio: Visión del alumno y del profesor. II Congreso Internacional de Docencia Universitaria (CIDU2011), Vigo, Spain (2011). Poster presentation.

de Miguel Villegas E; Barja Fernández S; Debenedetti López A; **García-Souto, D**; Murgarella Ciuraneta M; Álvarez Otero R. Las plataformas docentes como fuente de ayuda al estudio: Visión del alumno y del profesor. TicEDUCA2010 (I Encontro Internacional TIC e Educação: Inovaçao Curricular com TIC), Lisbon, Portugal (2010). Poster presentation.

Research stays

Pre-doctoral research stay,, 3 months, **Florida international University**, Florida, USA. (FPU research stay grant, Spanish Ministry of Education) (**2016**). Main theme: Molecular characterization of the histone H1 gene on marine invertebrates. Supervisor: Eirín-López JM.

Pre-doctorate research stay, 3 months, **Rudjer Boskovic Institute**, Zagreb, Croatia. (FPU research stay grant, Spanish Ministry of Education) (**2015**). Main theme: Molecular characterization of non coding DNA satellites on mollusks. Supervisor: Plohl M.

Summer laboratory stay, 2 months, **University Hospital of Santiago de Compostela,** Santiago de Compostela, Spain. (Summer stay grant, Spanish Asociation Against Cancer AECC) (**2011**). Main theme: Medical Translational Oncology. Supervisor: López-López R

Undergraduate research stay, 8 months part time, **University of Vigo**, Vigo, Spain. (Undergraduate research grant, Spanish Ministry of Education) (**2010/2011**). Main theme: Chromosomal location of repetitive sequences in Venerid clams. Supervisor: Pasantes JJ.

Courses

Xornada técnica de Citometría e Sorting. CITEXVI-CACTI. Vigo, Spain (**2016**)

Curso de formación en estatística con "R" para investigadores. Vigo, Spain (**2015**)

Seminario de citogenética para clínica: Análisis con arrays CGH de oligos. Santiago de Compostela, Spain (**2014**)

XIII curso práctico de iniciación ás análises clínicas, A Coruña, Spain (**2010**)

I curso de cultivos celulares, Málaga, Spain (**2010**)

Curso de extensión universitaria: Enfermería radiolóxica. Procedementos e coidados nos servizos de radiodiagnóstico, radioterapia e medicina nuclear, Vigo, Spain (**2010**)

Course in English as a foreign language at advanced I level, **The Language Centre of Ireland**, Dublin, Ireland (**2007**).