U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Caracterização e mapeamento de marcadores moleculares em espécies da família Veneridae de interesse comercial em Portugal e Espanha. Estudo da hibridação entre *Ruditapes decussatus* e *Ruditapes philippinarum*

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Tese de Doutoramento em Ciências Biomédicas

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Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientadora – **Prof. Doutora Maria Isabel da Silva Nogueira Bastos Malheiro**

Categoria – Professora Associada (com Nomeação Definitiva)

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientadores:

Doutora Alexandra Maria Bessa Ferreira Leitão-Ben Hamadou

Categoria – Investigadora Auxiliar

Afiliação – Instituto Nacional de Recursos Biológicos (INRB/L-IPIMAR)

Prof. Doutor Juan José Pasantes Ludeña

Categoria – Professor Titular Universidade

Afiliação – Dpto. de Bioquímica, Xenética e Inmunoloxía, Universidade de VIgo

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De acordo com o disposto no ponto nº 2, alínea a, do Art.º 31º do Decreto-Lei nº 230/2009, nesta tese foram utilizados resultados das publicações abaixo indicadas. No cumprimento do disposto no referido Decreto-Lei, a autora deste tese declara que interveio na concepção e na execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados sob o nome de **Carrilho, J.**

Carrilho J., Pérez-García, C., Leitão, A., Malheiro, I., Pasantes J.J. 2011. Cytogenetic characterization and mapping of rDNAs, core histone genes and telomeric sequences in *Venerupis aurea* and *Tapes rhomboides* (Bivalvia: Veneridae). Genetica 139 (6): 823-831

"The most beautiful experience we can have is the mysterious. It is the fundamental emotion which stands at the cradle of true art and true science"

Albert Einstein

Contents

Resumo

Este estudo teve o intuito de desenvolver ferramentas para a caracterização e identificação a nível genético de espécies de bivalves marinhos de interesse comercial.

Através da localização cromóssomica das sequências de DNA ribossomal (*cluster* de genes 28S-ITS1-5.8S-ITS2-18S e unidade de repetição do gene 5S) e das histonas nucleossomais, nomeadamente H3, descrevemos os cariótipos das espécies *Ruditapes decussatus, Ruditapes philippinarum, Venerupis aurea, Venerupis (= Tapes) rhomboides, Venerupis corrugata (= senegalensis = pullastra)* e *Dosinia exoleta*.

Os resultados destes trabalhos permitiram-nos, por um lado criar padrões de marcação cromossómica que nos permitem hoje em dia identificar qualquer uma das espécies referidas, e por outro lado propor algumas relações filogenéticas entre estas espécies baseadas em padrões de rearranjo cromossómico evidenciados pelas diferenças nas localizações das sequências.

Com o intuito de proceder ao mesmo tipo de identificação em situações em que não era possível obter metafases e/ou as amostras eram vendidas separadamente da sua concha, frescas ou congeladas, desenvolvemos também métodos de identificação moleculares baseados nas diferenças de tamanho das moléculas amplificadas por PCR referentes às sequências do rDNA 5S (e o seu espaçador não transcrito) e dos espaçadores internos do rDNA principal.

No entanto, necessitamos de melhorar ainda esta metodologia, de modo a aplica-la com sucesso a amostras que tenham sido sujeitas a processamento a altas temperaturas (cozedura e apertização, por exemplo).

O nosso estudo incluiu também uma aplicação prática ao campo da genotoxidade (capítulo 4), com a descrição de dois casos de possível processo neoplásico através das alterações cromóssicas observadas, nas espécies *Venus verrucosa* e *Venerupis aurea*.

A segunda vertente do nosso trabalho concentrou-se no estudo da magnitude do processo de hibridação inter-específica entre *Ruditapes decussatus* e *Ruditapes philippinarum,* que havia sido descrito anteriormente. O nosso estudo incidiu sobre a indução *in vitro* de cruzamentos entre indivíduos destas duas espécies de modo a determinar as condições sob as quais tal fenómeno ocorreria.

No entanto, este estudo não foi completado por ter sido impossível proceder à desova dos indivíduos de uma das espécies no nosso conjunto de condições experimentais. Assim, fica em aberto a questão de existir efectivamente um processo de hibridação em condições naturais.

Um conhecimento mais aprofundado das características genéticas das espécies permite não só utilizar os dados obtidos no estabelecimento de relações filogenéticas, como também contribuir para aplicações mais práticas, quer do ponto de vista da monitorização ambiental, quer em termos de apoio à produção e comercialização destas espécies. Em última instância, os estudos de caracterização genética como aquele que apresentamos neste trabalho, podem servir como ponto de partida para futuros programas de melhoramento de espécies.

Abstract

The aim of this study was the development of tools for the genetic characterization and identification of marine bivalve species of economical interest.

Through the chromosomal physical mapping of the sequences for ribosomal DNA (28S-ITS1-5.8S-ITS2-18S gene cluster and 5S repeat unit) and nucleosomal histones, namely H3, we described the karyotypes of *Ruditapes decussatus, Ruditapes philippinarum, Venerupis aurea, Venerupis (= Tapes) rhomboides, Venerupis corrugata (= senegalensis = pullastra)* and *Dosinia exoleta*.

The results from these works allowed us, on one hand, to create the specific standards for chromosomal hybridization sites that enable us to identify any of the referred species. On the other hand, the data allows us to establish phylogenetic relationships based on chromosomal rearrangement patterns shown by the differences in gene locations.

In order to do the same type of identification in situations where metaphases are not possible to obtain and/or the samples are sold separately from the shell, either fresh or frozen, we developed molecular identification methods based on the differences in size between PCR amplified fragments for the 5S rDNA (and its non-transcribed spacer) and for the internal transcribed spacers of major rDNA.

Nevertheless, we need to improve this technique in order to successfully apply it to samples that have undergone high temperature processing (as cooking and canning, for example).

Our study also included a practical application of these techniques in the field of genotoxicity (chapter 4), with the description of two cases of possible neoplasia in the species *Venerupis aurea* and *Venus verrucosa*, through the observed chromosomal alterations.

The second part of our work focused on the study of the magnitude of the inter-specific hybridization process between *Ruditapes decussatus* and *Ruditapes philippinarum* that had been previously described by other authors. Our study had as a primary objective the *in vitro* induction of crosses between these two species, in order to determine the conditions under which this phenomenon would occur.

Unfortunately, this work was not completed because we were unable to obtain spawning from one of the species, under our experimental conditions.

Thus, the hypothesis of hybridization under natural conditions is still to be proved.

A more comprehensive knowledge of the genetic characteristics of species allows us to use the data not only to establish phylogenetic relationships but also contributes to more specific aplications, in terms of environmental monitoring, production and commercialization of these species. Ultimately, genetic characterization studies like the one we present can serve as a stariting point for future species enhancement projects.

Chapter 1. Introduction

The Class Bivalvia includes a wide range of aquatic species, mostly marine, which descend from an ancestral mollusc that lost its cephalic structures (Giribet & Distel, 2003).

Reduced to its simplest dimensions, the mollusc body plan may be said to consist of a head-foot portion and a visceral mass portion. The head-foot is the more active area, containing the feeding, cephalic sensory and locomotor organs. It depends primarily on muscular action for its function. The visceral mass is the portion containing digestive, circulatory, respiratory and reproductive organs, and it depends primarily on ciliary tracts for its functioning.

Bivalves have very little cephalisation and a characteristic laterally compressed body and shell consisting of two asymmetrically rounded halves called valves that are mirror images of each other, joined at one edge by a flexible ligament called the hinge. The shell is typically bilaterally symmetrical, with the hinge lying in the sagittal plane (Gosling, 2003).

Two folds of skin, outgrowths of the dorsal body wall, make up a protective mantle, or pallium, which encloses a space between the mantle and shell called the mantle cavity (pallial cavity). This cavity communicates with the exterior through inhaling and exhaling siphons. The mantle lobes secrete the valves and the mantle crest secretes the

ligament and hinge teeth. The mantle is attached to the shell by the mantle retractor muscles at the pallial line.

The foot is adapted for locomotion or for attachment to a substratum. It is usually a ventral, sole-like structure in which waves of muscular contraction affect a creeping locomotion. Blood is pumped into the foot, causing it to swell and to act as an anchor in the mud or sand, then longitudinal muscles contract to shorten the foot and pull the animal forward.

In the pallial cavity, we find the gills. Respiratory currents bring both oxygen and organic materials to the gills where ciliary tracts direct them to the tiny pores of the gills. Gland cells on the gills and labial palps secrete copious amounts of mucus, which entangles particles suspended in water going through gill pores. These mucous masses slide down the outside of the gills toward food grooves at the lower edge of the gills.

Unlike other Mollusca, they have lost their odontophore and radula in their transition to filter feeding (Hickman, Roberts & Larson, 2001).

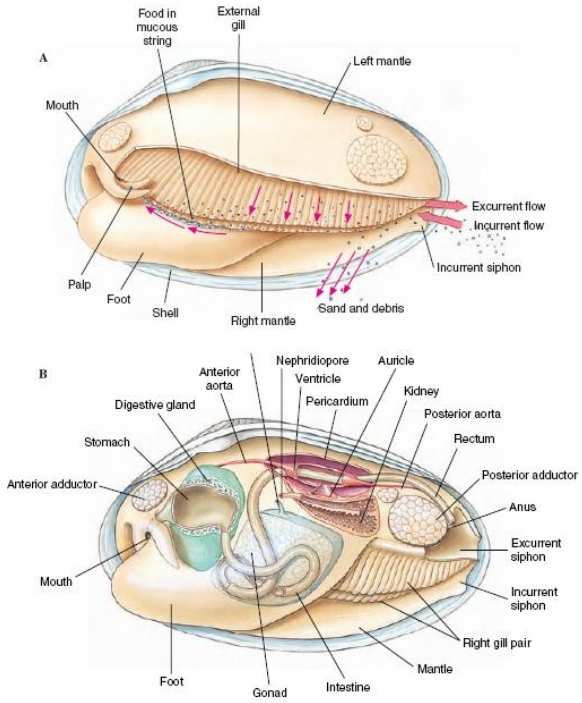


Figure 1 A, Feeding mechanism of freshwater clam. Left valve and mantle are removed. Water enters the mantle cavity posteriorly and is drawn forward by ciliary action to the gills and palps. As water enters the tiny openings of the gills, food particles are sieved out and caught in strings of mucus that are carried by cilia to the palps and directed to the mouth. Sand and debris drop into the mantle cavity and are removed by cilia. **B, Clam anatomy** (according to Hickman, Roberts & Larson, 2001)

Sexes are usually separate. Under optimal environmental conditions, usually in the late spring, the gonads have a quick maturation process and the gametes are discharged into the suprabranchial chamber to be carried out with the excurrent flow.

The fecundated egg gives birth to a trocophor larva and then to a veliger larva. The later leaves pelagic life and settles at the sediment, where the metamorphosis occurs (Gosling, 2003).

Bivalves are a main component of the benthic fauna of many marine and estuarine areas. Some bivalves are epifaunal (attached to surfaces), while others are infaunal (buried in the sediment). These forms typically have a strong digging foot. Some bivalves such as scallops can swim. Due to their high abundance, they are an important food source for many species of gastropods, fish and shorebirds (Kabat, 1990; Bowers & Szalay, 2007; Stempien, 2007). Evidences of human consumption report back to the Palaeolithic.

Bivalve exploitation has a very big impact in the economy and social characteristics of Portugal and the Galiza region in Spain. Artisanal collection of bivalves is an industry of approximately 30 million Euros each year, with about 8.000 tonnes of specimens harvested, mostly clams from the intertidal areas of sandy beaches (FAO, 2008).

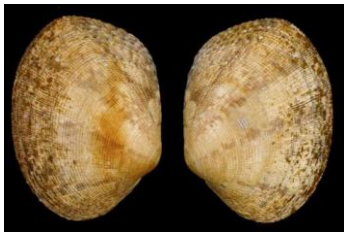
Clam breeding in the Iberian Peninsula is reduced to basic care – dragging of grounds for oxygenation, removal of algae, predator control, recollection in case of overpopulation and seeding of juveniles. This process occurs in grounds with sandy/silt bottoms, with good water flow and variable depth, depending on the species.

Ongrowing experiments in trays have been performed, whether suspended or grounded. Larvae can not be caught in collectors, like in oysters or mussels, and thus the production depends on natural offspring or hatcheries.

The family Veneridae gathers approximately 500 living species, distributed in 50 genera and 12 sub-families (Canapa *et al*. 1996).

Studied species and their characteristics

The three species with the higher economic value, in the Iberian Peninsula due to their quality and price are the **Grooved Carpet Shell** ("amêijoa boa", in Portuguese / "almeja fina", in Spanish), the **Pullet Carpet Shell** ("amêijoa macha", in Portuguese / "almeja babosa", in Spanish) and the **Banded Carpet Shell** ("amêijoa vermelha", in Portuguese / " almeja rubia", in Spanish).



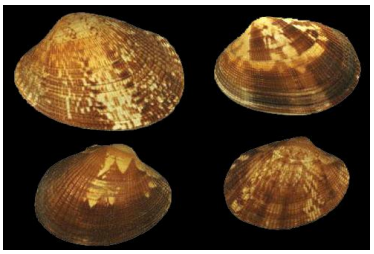
The Grooved Carpet Shell (*Ruditapes decussatus*) is a high quality clam. The shell has fine concentric striae and bolder radiating lines, giving a characteristic squared appearance. Colour may vary from white to brown depending on the production area. Its siphons are long and separated in their entire length. This clam can sustain long periods outside of the water, which grants it a higher market value, because its shelf-life is longer than in other species.

The Pullet Carpet Shell (*Venerupis corrugata* = *pullastra* = *senegalensis*) is usually pale gray or cream coloured with light and dark brown fine radiating ridges. The siphons are attached along their whole length. Its organoleptic quality approaches the one from *Ruditapes decussatus* but its resistance outside the water is lower.

The Banded Carpet Shell (*Tapes* = *Venerupis rhomboides*) presents a shiny shell with unclear growth stages. Radiating lines are very thin and the colour can go from white-yellowish to pink and brown. It presents distinguishing zigzag radial lines and red-brown *flames*. The interior is white, presenting a pink colour near the edges.

For their economical interest (translated in sheer volume of trade) and/or for presenting ecological characteristics of relevance, other clam species were also examined.

At the same time, we were interested in studying the phylogenetic relation between the species above and other species of the same or close genera.



The **Japanese Carpet Shell** ("amêijoa japonesa", in Portuguese / "almeja japonica", in Spanish), *Ruditapes philippinarum*, is originally from the Pacific, and was introduced initially in the coasts of France and rapidly spread in Spain and Italy, where it is largely produced because of its high growth rates and resistance.

Its shell has very well marked lines, both radial and concentric, and although its siphons are attached until half their length it is sometimes very hard to distinguish from *Ruditapes decussatus*. This leads to a frequent mislabelling of the products and the sale of Japanese carpet shell, which has inferior quality and consequent lower price, as grooved carpet shell, better and more expensive.

The **Golden Carpet Shell** ("amêijoa cão", in Portuguese / "almeja bicuda", in Spanish), *Venerupis aurea*, has a sculpture of numerous, fine, concentric ridges and grooves, and faint radiating striations. It presents variable colour: off-white, cream, yellow, or light brown, usually with deeper reddish or purplish-brown markings, in a variety of patterns. Inner surfaces are glossy, white or yellow, often with orange or purple markings.

The **Rayed Artemis** ("amêijoa relógio", in Portuguese / "almeja reloj", in Spanish), *Dosinia exoleta,* has a solid and almost circular shell. It is white, yellowish, or light brown in colour with irregular rays, streaks, or blotches of darker brown or pinkish brown.

The **Warty Venus** ("pé-de-burro", in Portuguese / "escupiña grabada", in Spanish), *Venus verrucosa,* is beige to brown in colour with a white internal surface. The warty venus is characterized by a series of 20 or more prominent concentric ridges intersected by radiating grooves resulting in wart-like spines.

(All images in this section belong to the Natural History Museum Rotterdam's website [http://www.nmr-pics.nl/Veneridae/album/index.html] and were used with the consent of their author)

Studied locations

There were two sampling broad regions: South of Portugal – Ria Formosa, Algarve; and North-western Spain, in several Rías of the province of Pontevedra, in Galiza.

According to Newton and Mudge (2003), the Ria Formosa region can be described by: *water temperature in the channels ranged from 12 °C in winter to 27 °C in summer and salinity from 13 to 36.5, although much higher values are observed in saltpans. Conditions in the Ria Formosa are not homogeneous despite a large tidal exchange of water; the inner channels of the Ria Formosa are brackish in winter but hypersaline in summer. Water in inner areas the lagoon has significantly different temperature and salinity characteristics compared to the inflowing coastal water, both in winter and in summer. Areas with these differences in temperature and salinity are detectable both at low water and at high water neaps.*

In what concerns nutrient availability and phytoplankton, Martins *et al.* (2004) describe average annual concentrations of phytoplankton of 1.73 E^{-01} mg/L and average annual concentrations of nitrates of 8.29 E^{-02} mg/L. The same authors claim that the Ria Formosa estuary has a low residence time, which means that *"the biologic processes do not have enough time to complete inside the estuary. In these systems cells are flushed out of the system before they can divide, inhibiting blooms. The nutrients are also flushed out and the uptaking and recycling occurs in the coastal zone."*

In Portugal, we mainly collected specimens from *Ruditapes decussatus*, as it is an indigenous species of the region. The introduction of *Ruditapes philippinarum,* as a foreign species, is not allowed in Portuguese shores, according to national law (Decreto-Lei nº 565/99, de 21 de Dezembro).

In what concerns Galiza, the Ría de Vigo behaves as an estuary with positive residual circulation, which is enhanced in summer by **coastal upwelling** (Prego & Fraga, 1992). In the Rias Baixas, upwelling events last from April to October. This upwelling is commonly attributed to the action of northerly winds along the shelf producing, by Coriolis Effect, an Ekman transport offshore, which displaces surface water seaward. This water is replaced by colder nutrient-rich deeper water (Wooster *et al.* 1976).

Recent data point out that the hydrodynamics depend on input of continental runoff, water masses at the adjacent shelf, solar radiation and wind stress regime.

Sousa *et al.* 2011 studied the seasonal variations in salinity and temperature, at both the North and the South mouth of the Ria and concluded that: *During winter the surface salinity decreases, synchronized with the high Minho river discharge. In the summer, the surface-to-bottom salinity difference increases. This surface-to-bottom difference may be related to the upwelling events. No seasonality in salinity was observed at the bottom. The water temperature was similar on the surface and near the bottom, in both mouths. In general, at the southern mouth, the influence of the upwelling events and of the Minho River discharge is more frequent. At the northern mouth, the air temperature is the major forcing. In summary, it was found that temperature is weakly dependent on the river discharge and related closely to the air temperature pattern. Salinity is related closely to wind variability and to the Minho River discharge.*

Overall, temperatures ranged from 12 °C to 17 °C (surface) / 13 °C to 15 °C (bottom), and salinities from approximately 32.8 to 35.5 (surface) / 34.5 to 35.5 (bottom).

Nitrates ranged from 0.6 to 7.7 µmol/kg (López-Sandoval *et al.* 2010), depending on the time of the year (downwelling or upwelling movements), leading to phytoplankton concentrations of 12.5 to 22.1 mg Chl/m^3 [measure in regard of chlorophyll present] (Cebrian & Valiela, 1999).

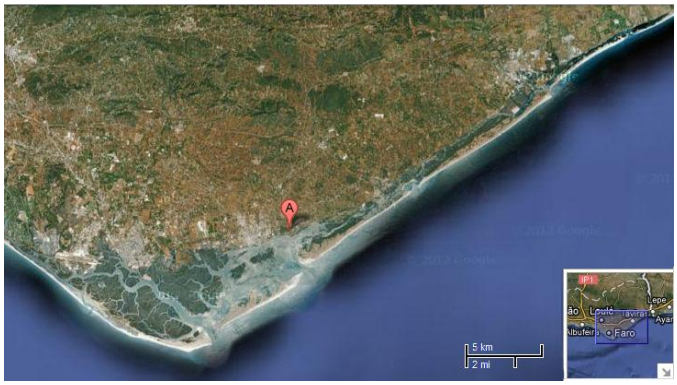


Figure 2. Map of the region of Ria Formosa, in Portugal. A was the main sampling site

Figure 3. Map of the region of the Rias in Galiza

APPROACH

Characterization and physical mapping of molecular markers

A. Classical cytogenetics

The field of cytogenetics can be broadly defined as the study of chromosomes, the structures responsible for the transmission of genetic data from cell to cell, through mitosis, and from individual to individual, through meiosis. The main concerns of cytogeneticists are the structure, composition and functions of chromosomes, which simultaneously account, on the one hand, for the variability – between cells, tissues, organs, individuals, species, and so on – and on the other hand for the stability of characteristics within each of these groups.

The first step in cytogenetic studies is to describe, as detailed as possible, the standard karyotype of the species we are focusing on. By karyotype we mean the ordered arrangement of the chromosomes, as first described by Levitsky in 1931. Nowadays, with the conjoined use of several techniques, we arrange the chromosomes on basis of size, morphology, presence/absence of certain sequences or structures.

Once this has been accomplished, studies of deviations from normal characteristics of the chromosomes (structural and numerical changes) can be made, from a clinical diagnosis to an environmental assessment point of view.

Differences in karyotype between species can also be used as tools to obtain insights regarding the evolution of the different species (as an example, see Wang & Guo, 2004)

Moreover, cytogenetics can also be used to support animal production, by testing the quality of *stocks*, helping in the selection of breeders, and ultimately as a way of creating genetically modified individuals.

Cytogenetical studies in clams have begun in the 1970's, with the establishment of the representative diploid chromosome number in several species. The first Veneridae species to have their chromosome number published were *Ruditapes decussatus* and *Ruditapes philippinarum,* both in an article by Gérard from 1978.

With time, we witnessed a development of the techniques which allowed a clearer view of the morphology of the chromosomes. Thus, the number of studies increased greatly with the karyotype for *Ruditapes philippinarum* published in 1985 by Ieyama; *Ruditapes decussatus* – with additional data on *R. philippinarum* – and *Ruditapes aureus* (now *Venerupis aureus*) published by Borsa and Thiriot-Quiévreux in 1990; *Venerupis pullastra* (then *V. senegalensis*, now *V. corrugata*) and *Tapes* (= *Venerupis*) *rhomboides* published by Insua and Thiriot-Quiévreux in 1992; *Venus verrucosa*, in 2004 by Ebied and Aly and *Dosinia exoleta* by Hurtado and Pasantes in 2005.

The next step in describing a karyotype is the employment of banding techniques, in order to distinguish the chromosome pairs. Structural banding techniques (G, R and Q bands) don't always produce reproducible patterns in invertebrates. Experiments with these techniques in bivalves have been made in Pectinidae (Odierna *et al.* 2006; Gajardo *et al.* 2002) – Q bands – and in Ostreidae (Rodríguez-Romero *et al.* 1978; Leitão *et al.* 1999, 2001) and Mytilidae (Mendéz *et al.* 1990; Martínez-Lage *et al.* 1994) – G bands. Bernardi (1995) presents an explanation to the difficulty in obtaining good quality G-bands in invertebrates with the lack of compartmentation of these genomes in regions of variable abundance in GC. Later articles say this may reflect differences in mitotic chromosome substructure, for example, tight compaction of the chromatin compared to vertebrates (Lorite *et al.* 1996; Appels *et al.* 1998; Baldanza *et al.* 1999; Sumner, 2003).

As a consequence, to date, the only way to somehow overcome this problem was to apply functional banding methods. Nevertheless, very few studies were made to improve this technique, with only some results for replication banding in Mytilidae (Cambeiro, 2002), due to some previous experiments with *in vivo* incorporation of bromodeoxyuridine (BrdU) and cell proliferation kinetics in the gills through fluorescence plus Giemsa (FPG) techniques (Martínez-Expósito *et al.* 1994).

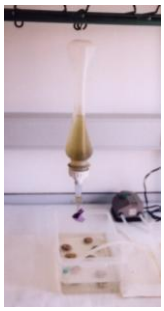
In what concerns structural selective banding, C banding is not a usual procedure for bivalves in general and was never successfully employed in venerids, mostly for technical obstacles in its use and because of the small amount of detected heterochromatic regions. As for the detection of nucleolar organizing regions (NORs) through silver nitrate precipitation (Ag-NOR), this technique is based on the interaction between the reactive and the proteins surrounding the NORs that have been transcribed in the previous interphase (Howell, 1977), which means this technique depends on the expression of the genes. Furthermore, Dobigny *et al.* (2002) shows that sometimes there are positive Ag-NOR results that do not correspond to the location of the rRNA genes. We would like to point out that there are 33 species of bivalves with Ag-NOR banding patterns already identified, but none of them belong to the Veneridae family.

Finally, molecular banding techniques using Fluorescent *in situ* Hybridization (FISH) or the use of restriction enzymes that create a banding pattern as a result of their selective digestion can be applied for the unequivocal identification of the chromosome pairs beyond size and morphology. The only case where endonuclease digestion banding was applied, so far, in venerid clams is Leitão *et al.* (2006). FISH will be further described later in this work.

Chromosome spreads

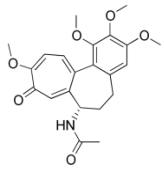
Although the study of metaphases provides the most comprehensive information, there are major limitations to their obtaining in marine invertebrates because of the lack of *in vitro* cell lines. Furthermore, a lack of well-defined meristematic regions and a low cell turnover rate, especially under field conditions (Dixon, 1983; Dixon *et al*. 2001), leads us

to the need to recreate in a controlled environment the conditions of Spring/Summer, stimulating somatic growth of the individuals.



In the several sampling groups, the animals were kept in the lab for about two weeks, in filtered sea water with constant aeration and fed with a suspension of microalgae from the species *Tetraselmis suecica* and *Isochrysis galbana*. Natural daylight exposure was maintained and water temperature was kept around 20 °C at all times.

After the feeding period, the animals were treated with colchicine.



Colchicine is a tryciclic alkaloid that inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to mitosis, and therefore colchicine effectively functions as a "mitotic block". The mitosis-inhibiting function of colchicine has been of great use in the study of cytogenetics. For a better comprehension of their number and morphology, chromosomes should be viewed near the point in the cell cycle in which they are most condensed. This occurs near the middle of mitosis, therefore mitosis must be stopped before it completes. This technique was first used in 1956 by Ford and Hamerton.

It is at this point that the individuals are sacrificed, and the organs dissected, according to the aim of the study. For the observance of chromosomes, the gills or the gonads may be used. The gills are chosen preferably because, on the one hand they have a higher mitotic index than most organs all around the year, and on the other hand, in opposition to the gonads, only mitotic diploid cells are found.

Considering we intend to achieve the optimal observation of the chromosomal number and morphology, the next step in our technique is the application of a hypotonic shock to the cells.

In this step, the cells are immersed in a **hypotonic solution**, which by the principle of osmosis, forces the entrance of water through the cell membrane and its turgescence will make the referred membrane frail, ultimately causing its burst. As the nuclear

envelope has dissolved in this stage of the cell cycle, the chromosomes are now free and can spread more widely, helping their visualisation.

The use of the hypotonic solution was firstly described by Hsu and Pomerat in 1953, but the simultaneous use with the colchicine treatment was what led to the correct observation of chromosome spreads, thus enabling, for example the first accurate description of the human karyotype in 1956 by Tijo and Levan.

The next step needed for obtaining chromosome spreads is the **fixation** of the chromosomes. This is made with a mixture of 3:1 ethanol: acetic acid and intends to preserve the structure of the chromosome, preventing as most as possible, any breaks due to manipulation.

Up until this point, we have dealt with whole gill or gonad samples. These can be maintained for long periods in the fixative solution, stored at -20 °C. When necessary, the samples are used to obtain cell suspensions, through a process of mechanic and chemical dissociation, using a 50-60% acetic acid solution.

The spread of the **cell suspension** in slides by the "air-drying technique" was made following Martínez-Expósito *et al.* (1997). The cell suspension is dropped onto a heated slide and then gradually aspirated in order to create a series of concentric rings where the material will be distributed. As the name says, the slides are then left to dry and afterwards are dehydrated in an increasing series of ethanol dilutions.

Meiotic SC spreads

Meiotic synaptonemal complexes (SC) are protein structures that form between homologous chromosomes (two pairs of sister chromatids) during meiosis and that are thought to mediate chromosome pairing, synapsis, and recombination (crossing-over). These structures were first described by Fawcett in 1956, and Wettstein and Sotelo claimed, in 1967, that their number corresponded to the haploid number of the chromosomes for the species.

Sequential colouring with dyes specific for DNA (for example DAPI) will make the chromatin in the homologous chromosomes visible, not the SC itself. Nevertheless, for

simplicity in description here forth, the expression SC will be used to describe this technique. In these preparations, bivalents in paquitene appear as masses of chromatin anchored to the SC. Given that each bivalent corresponds to a chromosome pair, differences in length are still visible and meiotic karyotypes can be used to study the species.

The use of SC in cytogenetics has two main advantages. On the one hand it's especially useful when applied to species in which chromosomes are difficult to characterize with classic techniques (Zickler & Kleckner, 1999), as they are in most bivalve species. On the other hand, the fact that we now have both the homologous chromosomes closer together is a great aid in the detection of the FISH signals, as there are the double amount of target sequences in one place, and so the intensity of the signals is exponentiated (Zickler & Kleckner, 1999).

Nevertheless, these spreads are harder to obtain, technically wise; are more dependent on the condition of the individuals (even though bivalves have asynchronous gonadal development, they have specific fertile periods throughout the year) and more importantly, we have to take in consideration the difficulty in distinguishing individual chromosomes (Belonogova *et al.* 2006).

B. DNA extraction and amplification techniques

Studied genes/gene clusters

Histone genes

Histones are a small set of basic proteins found in all eukaryotic organisms, involved in DNA packaging in nucleosomes and also in the regulation of gene expression. There are five histone classes which can be classified in two major groups according to structural and functional criteria: core histones (H2A, H2B, H3, H4), which form multiprotein complexes and interact with DNA to constitute the nucleosome structure, and linker histones (H1), which interact with DNA stretches between nucleosomes

giving rise to the chromatosome structure and participate in nucleosome positioning (Simpson, 1978).

Besides their structural role, histones also participate in DNA replication, reparation, gene expression regulation, recombination in meiosis and chromosome condensation (with relevance in the formation of heterochromatin). These later functions are associated mostly to non-allelic variants of the histones and act through post-translational changes in their structure (Ausió, 2006).

The number of copies of the histone genes is very variable among different taxa, with no particular similarity between closely related species. Hays *et al.* (2002) reported only three copies of these genes in fungal species, while some sea urchins show several hundred copies (Marzluff *et al*. 2006).

Broadly speaking, species with higher copy number tend to have organized quintets, with each type of histone, tandemly repeated. On the other hand, species with low gene copy number tend to have only one to three major clusters on the same or in different chromosomes, as it happens on birds or mammals (Ahn & Gruen, 1999). Thus, it is usually said that the tandem organization of the histone gene clusters tends to be lost with the ascent in the evolutionary scale.

The H3 histone is among the most conserved eukaryotic proteins (Miller *et al.* 1993). In bivalvia, H3 histone genes are frequently found in repeated clusters (Albig *et al.* 2003), usually along with the rest of the core histones (the location of linker histones is variable among the different organisms) (Eirín-Lopéz *et al.* 2004, for example). For this reason, we used the published primers for both the H3 and the H2B genes in order to locate this gene cluster. It is unknown for Veneridae species the exact organization of the histone genes, but table 4 shows the organization in other bivalvia.

Table 1. Organization of the histone genes and number of copies per haploid genome

Species	Organization	N copies/n	Reference
Chlamys farreri	→ ← → → H4 – H2B – H2A – H3	---	Li *et al.* 2006
Mytilus edulis	→ ← ← → → → → H4 – H2B – H2A – H3 – H1 – 5S – 5S	200	Albig *et al.* 2003
	H1 independent	100	Drabent *et al.* 1999
Mytilus galloprovincialis	→ ← → → H4 – H2B – H2A – H3	224	Eirín-Lopéz *et al.* 2004
	H1 independent	100-110	Eirín-Lopéz *et al.* 2002

It has also been shown that these genes are rather conservative in their chromosome location among relatively close species (Hankeln *et al.* 1993; Ranz *et al.* 2003). Thus, they are a very good chromosomal marker of historical and ongoing karyotypic repatterning. In Veneridae, this gene has not been located by FISH in any species before this work. Among other bivalves the location of this gene has been performed in Mytilidae (Eirín-Lopéz *et al.* 2004) and in Pectinidae (Zhang *et al.* 2007).

Studied genes/gene clusters

rRNA genes

There are two types of rRNA genes – the major rRNA gene family and the 5S rRNA gene family (Long & Dawid, 1980). The major rRNA gene family encodes the ribosomal subunits components, and corresponds to the nucleolar organizing regions. As shown in Figure 4, in eukaryotes, major rDNA consists of a tandem repeat of a unit segment composed of 18S, 5.8S and 28S rDNA tracts, flanked by an external transcribed spacer (ETS) and ITS1 and ITS2 – noncoding regions located in the rDNA between 18S and 5.8S rRNA genes and between 5.8S and 28S rRNA genes, respectively. Between each segment, we find a NTS – non transcribed spacer.

Figure 4. Schematic diagram of the nuclear major ribosomal DNA cluster with its internal transcribed spacers in eukaryotes. ITS1: first internal transcribed spacer; ITS2: second internal transcribed spacer; ETS: external transcribed spacer

The different coding regions of the rDNA repeats usually show distinct evolutionary rates. As a result, this DNA can provide phylogenetic information of species belonging to wide systematic levels (Hillis & Dixon, 1991).

For the rDNA coding sequences, polymorphisms between repeat units are very low, indicating that rDNA tandem arrays are evolving through concerted evolution of intra and inter-chromosomal loci (Cheng *et al.* 2006; Richard *et al.* 2008). This low rate of polymorphism, allows interspecific comparison to elucidate phylogenetic relationship, especially between remote species.

On the other hand, ITS regions are variable due to insertions, deletions, and point mutations (Sumida *et al.* 2004). For this reason, comparison of the ITS tracts has shown more effective in the analysis of several close-related species of Bivalvia.

To quote only a few examples, observations of sequence size or restriction patterns of the complete ITS region were employed to differentiate *Mytilus* mussels (Heath *et al.* 1996; Toro, 1998), Veneridae clams (Fernández *et al.* 2001) and Pectinidae scallops (Insua *et al.* 2003). In the same way, ITS1 or ITS2 separately were used to distinguish several freshwater mussels (White *et al.* 1994) and *Mytilus* and *Modiolus* mussel species (Dixon *et al.* 1995).

The chromosomal location of the major rRNA gene family can be determined using the Ag-NOR technique, but as stated previously, this is not a very reliable method.

In recent years, FISH has been used to describe the number and position of these gene clusters. We can find data for nine species of Mytilidae (for example, Insua *et al.* 1994; Pérez-Garcia *et al.* 2010), six Ostreidae (for example, Wang *et al.* 2004), seven Pectinidae (for example, Wang & Guo 2004), two Mactridae (Wang & Guo 2008; González-Tizón *et al.* 2000), one Psamobidae (González-Tizón *et al.* 2000), two Pharidae (Fernandéz-Tajes *et al.* 2008), one Donacidae (Martínez *et al.* 2002), one Cardidae (Insua *et al.* 1999), one Tellinidae (González-Tizón *et al.* 2000), one Solenidae (Fernandéz-Tajes *et al.* 2003) and two Veneridae, namely *Dosinia exoleta* (Hurtado & Pasantes, 2005) and *Mercenaria mercenaria* (Wang & Guo, 2007).

In all these species the number of major rDNA loci varies between 1 and 4. This higher number of NORs, which possibly is the result of movements (transpositions) of rDNA between different chromosomes, might represent a derived (apomorphic) condition, according to authors (Thiriot-Quiévreux & Insua, 1982; Pascoe *et al.* 1996) who assume that a single pair of NORs per cell is primitive (plesiomorphic) at the phylogenetic level.

The second type of rDNA is the 5S rDNA. 5S rRNA is a conserved component of the large ribosomal subunit that is thought to enhance protein synthesis by stabilizing ribosome structure.

Long and Dawid, in 1980, stated that 5S rRNA genes are usually in tandem repeats of hundreds or thousands of copies. The repeat unit is made up by a coding sequence of 120bp and a non-transcribed spacer (NTS) of variable length (Figure 5).

Figure 5. Schematic diagram of the nuclear 5S ribosomal DNA cluster with its non transcribed spacers in eukaryotes.
NTS: non-transcribed spacer

These gene clusters can be dispersed all over the genome, as in some fungus (Selker *et al.* 1981; Mao *et al.* 1982), or associated with gene families, namely the major rRNA genes (Bergeron & Drouin, 2008), histone genes, in *Mytilus galloprovincialis* (Eirin-López *et al.* 2004), snRNA, in *Crassostrea angulata* and *C. gigas* (Cross & Rebordinos, 2005) and the ubiquitine genes (Guerreiro *et al.* 1993).

The 5S coding sequences seem to have suffered concerted evolution too, with low intra-individual and intra-specific variation. Comparison of 5S tandem repeat sequences in several *Drosophila* species revealed that insertions and deletions were very frequent between species and were often flanked by conserved nucleotides, suggesting that they could occur by slippage of the newly synthesized strand during DNA replication or alternatively by gene conversion (Pâques *et al.* 1998)

Nevertheless, similar to the major rRNA genes, the spacer sequences seem to have fixated mutations over time, presenting a high variability, both in sequence and in length. Thus, comparison of these sequences has been used for phylogeny

determinations and species identification in closely related species (Cross *et al.* 2006; López-Piñón *et al.* 2008).

The chromosomal location of this gene family has been determined in six species of Mytilidae (for example, Insua *et al.* 2001; Pérez-Garcia *et al.* 2010), two Ostreidae (Cross *et al.* 2005, 2006; Wang *et al.* 2005), seven Pectinidae (for example, Wang & Guo, 2004) and one Cardidae (Insua *et al.* 1999). In what concerns the Veneridae family, Hurtado *et al.* 2011 shows, in synaptonemal complexes, the relative position of the 5S rRNA gene cluster to the major rRNA in *Ruditapes decussatus* and *Ruditapes philippinarum*, but since it is impossible to determine which chromosome pair is the signal-bearer, further studies are required to determine the exact position in these species.

Methodology

We followed two different methods for DNA extraction. The first method used Phenol-Chloroform-Isoamyl Alcohol and was based, with minor modifications, on the work of Winnepenninckx *et al.* (1993). The second method was described by Estoup *et al.* (1996), using a chelating resin called Chelex 100 (Bio-Rad).

Both methods start with cell lysis using CTAB Buffer (hexadecyltrimethylammonium bromide), which disrupts the hydrophilic nature of the cell membrane, effectively breaking it open so that DNA can enter the aqueous solution. Hence, DNA strands and cell remnants are dissolved in the aqueous solution. Proteinase K and RNAse are also added to the incubation mix.

DNA must then be purified – removal of digested and non-digested proteins and RNA, cell matter and enzymes such as DNAse, which would otherwise hydrolyze DNA – and retrieved. Both methods rely on the formation of a dual-phase solution, aqueous/organic, with the purified DNA on one of them and the discarded substances on the other.

The Chelex method is a one-step process, in which the sample (after a first centrifugation to eliminate larger tissue portions) is incubated with the resin. The exact role of Chelex is uncertain. It appears to protect the DNA, perhaps through

sequestering divalent heavy metals that would otherwise damage the DNA (Walsh *et al.* 1991). The polar resin beads bind polar cellular components while DNA remains in water solution above Chelex.

The Phenol-Chloroform-Isoamyl Alcohol method is more complex, involving a series of sequential steps. In order to obtain a more pure DNA, the purification with this compound is repeated twice. The compound removes proteins from the nucleic acid through interactions between phenol and water which cause proteins to undergo a conformational change, exit the aqueous and enter the organic solution. As a consequence, **nucleic acids** remain in the **aqueous phase**, while **protein** appears in the **organic phase.** The aqueous phase will then be composed of a purified DNA solution. The two layers are partitioned and the aqueous solution can be isolated.

Finally, DNA is precipitated by the addition of ethanol and sodium acetate to the solution. Sodium acetate is added because it facilitates the pelleting of DNA after precipitation. The added ethanol facilitates the interaction between Na^+ (from NaCl) and PO_4^{3-} on the DNA, causing the nucleic acid to become less negatively charged and hence less hydrophilic - leading it to leave the aqueous solution and enter the ethanol.

The precipitated DNA was resuspended in TE (10:1 Tris:EDTA) buffer 0.1%. This buffer maintains a suitable basic medium (pH 8.0) that reduces the activity of the nucleases. The EDTA in the buffer chelates cations like Mg^{2+}, further contributing to the inactivation of the nucleases, by binding to metal ions required by these enzymes.

After the extractions, certain sequences of this DNA were amplified, whether to undergo electrophoresis and analysis of their relative inter-specific differences in length (chapter 5) or to produce probes for FISH experiments (chapters 2, 3 and 4).

Amplification by Polimerase Chain Reaction (PCR) is a cyclic process with 3 main stages or steps, as seen on the following figure.

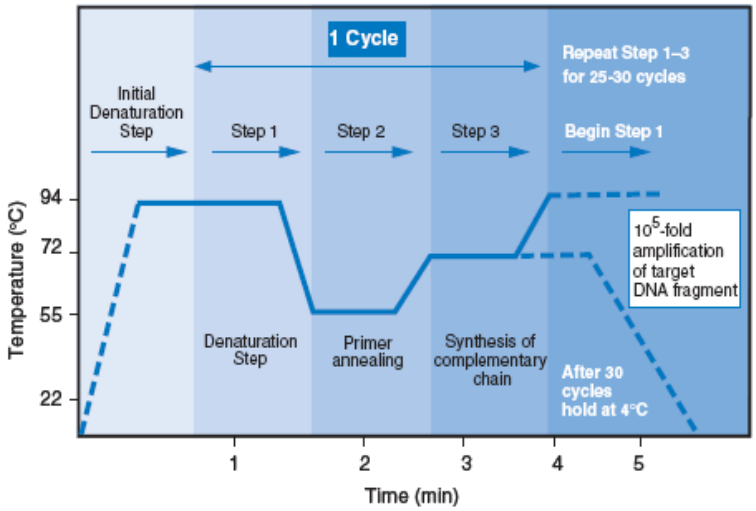


Figure 6. Profile of Routine PCR Reaction (from TaKaRa Bio USA. Successful PCR Guide. 3rd Edition)

Step 1 refers to **Denaturation**. In this step the double-stranded DNA fragment is denatured in a reaction mixture containing primers, dNTPs and polymerase.

Step 2 is the **Annealing**, where primers are annealed (binded) to denatured single-stranded DNA.

Finally, step 3 is the **Extension**. DNA polymerase uses the original single strand of DNA as a template to add complementary dNTPs to the 3' ends of each primer and generate a section of double-stranded DNA in the region of interest. Primers that have annealed to DNA sequences that are not an exact match do not remain annealed at annealing temperatures, thus limiting elongation to the gene of interest.

The following table shows the primer sequences used in this study for the amplification of different rRNA and histone gene clusters.

Table 2. Combinations of primers used

	Primers		References
H3	Direct: 5′ ATGGCTCGTACCAAGCAGACVGC 3′ Reverse: 5′ ATATCCTTRGGCATRATRGTGAC 3′		Giribet & Distel, 2003
H2AB	Direct: 5′ TCCCGAGATGTGATGGTAGA 3′ Reverse: 5′ AGTACAGCCTGGATGTTTGGTAA 3′		Albig *et al.* 2003
ITS	ITS5: 5′ GGAAGTAAAAGTCGTAACAAGG 3′ ITS4: 5′ TCCTCCGCTTATTGATATGC 3′		White *et al.* 1990
28S	LR12: 5′ GACTTAGAGGCGTTCAG 3′ LR8: 5′ CACCTTGGAGACCTGCT 3′	LR10R: 5′ GACCCTGTTGAGCTTGA 3′ LR17R: 5′ TAACCTATTCTCAAACTT 3′	
5S	Direct: 5′ CAACGTGATATGGTCGTAGAC 3′ Reverse: 5′ AACACCGGTTCTCGTCCGATC 3′		Fang *et al.* 1982

ITS = internal transcribed sequence of major rDNA, 28S = 28S rDNA, 5S = 5S rDNA, H3 = H3 histone, H2AB = H2B and H2A histone

The amplification of different sequences requires different PCR conditions. The requirement of an optimal PCR reaction is to amplify a specific locus without any unspecific by-products. Therefore, annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA-DNA matches to occur in the reaction. For any given primer pair, the PCR conditions can be selected based on the **composition (GC content) of the primers** and the **length of the expected PCR product**.

Table 3. Conditions used in the PCR amplification

Region	Cycles	Denaturation	Annealing	Extension
ITS		95 °C, 30 s	48 °C, 30 s	72 °C, 30 s
28S		95 °C, 20 s	48 °C, 20 s	72 °C, 30 s
5S	30	95 °C, 30 s	48 °C, 30 s	72 °C, 60 s
H3		95 °C, 15 s	48 °C, 15 s	72 °C, 15 s
H2BA		95 °C, 30 s	48 °C, 30 s	72 °C, 40 s

ITS = internal transcribed sequence of major rDNA, 28S = 28S rDNA, 5S = 5S rDNA, H3 = H3 histone, H2BA = H2B and H2A histone

As stated earlier, FISH probes must be labelled with some known molecule (in our case the haptens biotin and digoxigenin) in order to be detected later.

This labelling is obtained by the introduction of conjugated nucleotides in the DNA sequence, namely digoxigenin-UTP and biotin-UTP. The introduction of these labelled nucleotides can be direct or indirect.

In the direct method, the labelled nucleotides are added during the PCR amplification and incorporated in the "original" DNA replication.

The indirect method is known as "Nick translation". In this method, the PCR products are unlabelled probes which are submitted to a treatment with deoxyribonuclease I (DNAse I) and DNA Polimerase I. The first catalyzes the hydrolytic cleavage[5] of phosphodiester bonds in the DNA backbone, thus cutting or "nicking" the DNA molecule in random locations, creating free 3' ends. The DNA Polimerase I has three activities: as an exonuclease that removes individual base pairs from the nick in the 5' to 3' direction, as a polymerase that adds new nucleotides from the 3' nick copying the template in the opposite DNA strand (and thus introducing the labelled dNTPs in the probe sequence) and a 3' to 5' proof-reading activity. This method creates probe segments with variable length, which will hybridise complementarily with the target sequences (Maniatis *et al*. 1975).

C. Fluorescent *in situ* hybridization (FISH)

As stated earlier, classical banding techniques have been unsuccessful in producing clear and unequivocal identifications of the chromosomes in bivalves. Thus, the need to use techniques based on the sequence itself, rather than just the relative abundance of the nucleotides, are required to further develop the cytogenetics of bivalves.

Specifically *in situ* hybridization using fluorescent probes or fluorescent detection molecules (antibodies) is one of the methods that meet this demand.

FISH can be used in a variety of situations. The first is the detection of specific genes or gene families. This method allows us to distinguish the carrier chromosome pair(s) from the rest of the chromosomes in the karyotype. In what concerns the application of this technique to bivalvia, we highlight the studies which include venerid species: Wang and Guo (2001, 2007); Hurtado and Pasantes (2005) and Hurtado *et al*. (2011). Although useful, this kind of technique provides a limited amount of insight into the karyotype.

The second type of FISH is the one where we detect specific chromosomes in their full length – whole chromosome probes or chromosome painting (for a review on this, see Chowdhary & Raudsepp, 2001).

Unfortunately, to date, no article on the application of this methodology in invertebrates (animals, since there are some works in plants) was found. In the case of probes for specific genes, we rely on the knowledge of the sequences of the target genes to build *in vitro* our probes. Whole chromosome probes, on the other hand, are most commonly obtained by chromosome flow-sorting and micro dissection. In flow-sorting, chromosomes are sorted using a laser system that distinguishes chromosomes depending on their size and fluorochrome affinity (AT, GC base content) while in chromosome microdissection chromosomes or chromosomal segments are literally scraped and collected (Zhou & Hu, 2007; Henning *et al.* 2008). In the application of flow-sorting techniques, specifically concerning genetic studies in clams, a new difficulty arises, since most species present a highly uniform karyotype regarding chromosomal size and base content. On the other hand, microdissection requires the collection of between five to twenty chromosome copies. As it has been referred earlier, unequivocal chromosome identification is yet to be achieved in clams, and so the application of this technique has been deemed unfeasible for the time being.

In this work we performed FISH experiments, using probes to locate rRNA (major and 5S) gene clusters and H3 histone genes location. We followed, with minor modifications, the protocol described by Torreiro *et al.* (1999). The FISH technique is divided in a series of sequential steps, which we will now describe briefly.

a) Pre-treatment of the preparations – This stage is intended to: reduce the background interference of unwanted biological material in the chromosome spreads; enable the access of probes to the DNA – permeabilizing the target material and fix the preparation so chromosomes and nuclei are not lost during the procedure. Thus, we submit the slides to a digestion with **RNAse**, followed by a digestion with **Pepsin**, to remove extraneous proteins (such as remaining cytoplasm). The digestion time of Pepsin must be rigorously controlled to avoid the removal of chromosomal structural proteins, and

consequent lost of morphology. Finally, we fix the preparations with **paraformaldehyde** and dehydrate them.

b) Denaturation – This stage is intended to turn both the studied sample and the probe into a single-strand DNA molecule, so they can link and hybridize. In both cases, we rely on the action of **Formamide** to help in the process. Formamide is an organic solvent that impairs the DNA thermal stability and allows us to reduce the denaturation temperatures, preventing high temperatures from destroying the chromatin structure. To the probe solution we also had a detergent that interferes with the base-pair covalent links, making the denaturation more effective.
c) Hybridization – In this step we have the link, by complementarity of the basis, between the DNA in the chromosomes of our samples and the DNA in the probes. In order to create the appropriate environment for the reaction to occur, the preparations were sealed and maintained in a humid chamber while incubating at the optimal annealing temperature overnight.

d) Stringency washes – In this step, we wash our preparations in a series of solutions of variable concentration and temperatures. **Stringency** is the condition that allows a DNA sequence to hybridize with a non-specific/non-complementary sequence. High temperatures (who favour denaturation) and low saline concentration (which increase the electro-static repulsion forces between DNA chains) increase the stringency, avoiding the occurrence of non-specific pairing. Thus, the higher the stringency, the lower the background interference, but also the lower hybridization rate.

e) Detection of the probes – The probes used were not fluorescent for themselves. On the one hand because emission of fluorescence has a limited duration and it's not renewable and on the other hand because areas with a low copy number of the target sequences would hybridise with a reduced number of probes, thus emitting a faint signal that might not be detectable

with the resolution enabled by our microscopes. To solve this problem, we use fluorescent-labelled antibodies, specific for non-fluorescent molecules incorporated in our probes. Specifically, our probes were either labelled with **Biotin** or **Digoxigenin**. Both of these molecules are haptens (small molecules that can elicit an immune response only when attached to a large carrier such as a protein). In general, antibodies generated against haptens have higher affinities for their targets than other antibodies, so haptens are conjugated to other biological molecules as all-purpose immuno-tags.

The use of antibodies allows us to renew the emission of fluorescence, at any moment, simply by repeating the immuno-detection of the probes, and through the use of sequential steps of detection, it allows us to amplify the signal we are searching. In detail, the amplification occurs as follows: there are several different antibodies being used to detect the target hapten. For biotin-labelled probes we chose a combination of avidin, a naturally occurring biotin-binding protein, conjugated with **FITC** (Fluorescein Isothiocyanate), with an emission maximum at about 520 nm [green] and an antibody against avidin, also conjugated with the hapten. Because it is biotinylated, this antibody also acts as a target for avidin. In this way, we can repeat the first step of the detection. For digoxigenin-labelled probes we used an antibody against digoxigenin, generated in mouse; followed by an antibody against mouse, generated in goat, conjugated with **TRITC** (tetramethylrhodamine isothiocyanate), with an emission maximum between 570-580 nm [orange-red], and finally an antibody against goat, also conjugated with the fluorochrome.

This method allows us to have two fluorescent molecules per hapten molecule, and thus amplify the hybridization signal.

Table 4 and figure 7 explain the process.

Table 4. Sequence of antibodies for probe detection; BIO – biotin label; coloured cells correspond to antibodies with fluorescent molecules.

hapten	***Antibody 1***	***Antibody 2***	***Antibody 3***
Biotin	avidin-FITC	BIO anti-avidin	avidin-FITC
Digoxigenin	mouse anti-DIG	goat anti-mouse-TRITC	rabbit anti-goat-TRITC

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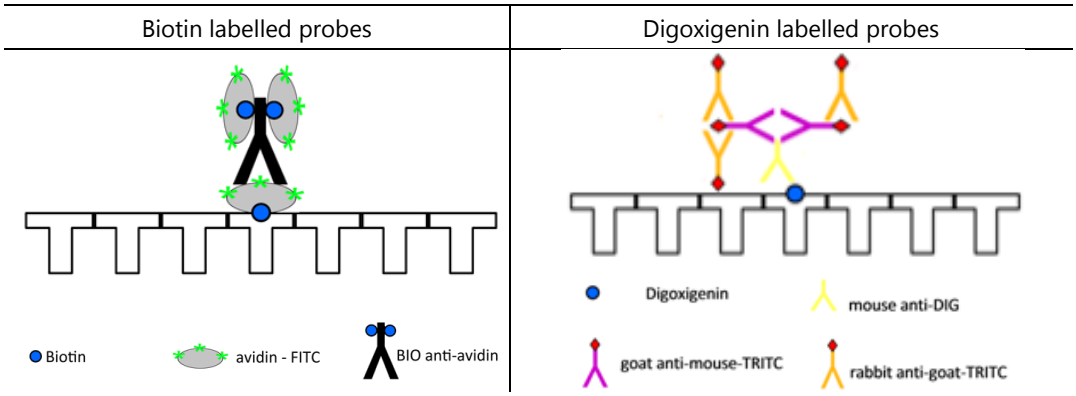

Figure 7. Schematic representation of the detection method

f) Counterstaining and mounting – For the visualization of the chromosomes, DNA is counterstained with adequate fluorochromes. The most widely used are 4', 6-diamidino-2-phenylindole (DAPI) and Propidium iodide (PI).

DAPI has a blue fluorescence in the emission range of ~460nm, which stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures. It appears to associate with AT clusters in the minor groove of the double helix (Kubista *et al.* 1987).

PI is a red dye, emitting fluorescence in the range of ~617nm. It binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA.

The differences in staining affinity make it possible to highlight the regions of the chromosome richer in GC, as they will show mostly (if not exclusively) a red fluorescence, while the AT rich regions show bright blue staining.

This finishes the description of the techniques employed in the obtaining and physical mapping of molecular markers. The combined use of these techniques allowed us to characterize several species of clams, based on the probe-signalling patterns found on their chromosomes.

Leitão and Chaves (2008) have made an extensive review on chromosome banding studies in bivalves, both with classical techniques and with *in situ* hybridization.

Inter-specific hybridization

The second part of this work focused on studying the hypothesis of inter-specific breeding between *Ruditapes decussatus* and *Ruditapes philippinarum*.

Reports from local producers in the area where our studies occurred raised the possibility of the existence of these hybrids.

It is well known that productivity and profit in aquaculture can be increased through the introduction of foreign species. As an example, we can refer to the introduction of the Japanese carpet shell, *Ruditapes philippinarum*, and the consequent increase of importance in European economy of the clam production. This species is native to the Pacific coast of Asia and was deliberately introduced into Europe during the 1970s and 1980s (Gosling, 2003). A very successful process of adaption to the European conditions led to a rapid spread and the establishment of natural populations of Japanese carpet shell. Jensen *et al.* (2004) show that *R. philippinarum* is now growing faster than the native European grooved carpet shell, *R. decussatus*, becoming the major contributor to clam aquaculture in Europe.

Although shell characteristics are generally used to separate species of bivalves, such features may be inconclusive for closely related species.

The similarity of *Ruditapes philippinarum* and *R. decussatus*, the plasticity of their shell morphology and the presence of both species on the same beds make it sometimes very difficult to do the morphological identification of specimens that show intermediate shell characteristics (Hurtado *et al*. 2011). In order to solve this problem, a

genetic approach was used and molecular markers were applied to identify these species and determine if some of the specimens showing intermediate shell characteristics were interspecific hybrids as observed in some other bivalves (for example, Masaoka & Kobayashi, 2005).

The introduced species *Ruditapes philippinarum* shows a spawning season at least partly overlapping the spawning season of the native *R. decussatus*. As described by Delgado and Pérez-Camacho (2007), *R. decussatus* has an oocyte release from April onwards. On the other hand, there is partial gamete emission from April to September by *R. philippinarum* (Rodríguez-Moscoso *et al.* 1992), creating the conditions for interspecific crosses.

From the work of Hurtado *et al.* (2011) we also highlight the detection of mature gametes on the hybrid specimens. This indicates that these individuals are potentially able to cross with other hybrids and/or the parental species. To check this hypothesis it is necessary to obtain interspecific crosses, in order to determine the fertility and the viability of the hybrids.

OBJECTIVE

The main goal of this study was the development of the genetic technology for the identification and characterization of marine bivalve species of commercial interest.

Ultimately, we aimed at developing reliable tools for the identification of specimens from several species, not only in live or fresh individuals but also frozen, canned or pre-cooked samples.

On the other hand, we tried to determine the magnitude of the reported inter-specific hybridization phenomenon between *Ruditapes decussatus* and *Ruditapes philippinarum*, with studies focusing on the *in vitro* crossings of the individuals and subsequent analysis of the embryos.

OUTLINE OF THE THESIS

Similar to our description of the approach, we divide this thesis into several components: Characterization and physical mapping of molecular markers (Chapters 2 to 5) and Inter-specific hybridization studies (chapter 6), followed by some closing remarks on this work (chapter 7).

In further detail, the first three chapters refer to applications of *in situ hybridization* - chapters 2 and 3 describe the cytogenetic features of the studied species, chapter 4 is an applied study to describe genetically damaged individuals – and chapter 5 reports the use of molecular biology methods.

Chapter 2

This work describes the chromosomal location of major rDNA, 5S rDNA, core histone genes and telomeric sequences in the venerid bivalve species *Venerupis aurea* and *Tapes (= Venerupis) rhomboides*.

Major rRNA genes are clustered at a single chromosome pair in *V. aurea* and in *T. rhomboides*. 5S rDNA repeats also constitute a single cluster. On the contrary, different numbers of core histone gene clusters were found in these species. *V. aurea* presents four telomeric histone gene clusters on three metacentric chromosome pairs. Histone genes in *T. rhomboides* appear clustered interstitially on the long arm of metacentric chromosome and close to the centromere on the long arm of a subtelocentric chromosome.

Fluorochrome staining with propidium iodide (PI), DAPI and chromomycin A3 (CMA) showed DAPI dull / CMA bright bands coincident with the chromosomal location of the major rRNA genes in both species.

Double and triple FISH experiments demonstrated that major rDNA, 5S rDNA and H3 histone gene signals appear on different chromosome

pairs in the two clam species. Telomeric signals appear at both ends of every single chromosome in both species.

The information on the location of these three gene families in two species of Veneridae constitute a starting point to further evolutionary studies, in order to infer karyotypic relationships in this commercially important bivalve family.

Chapter 3

We analysed the chromosomal location of H3 histone genes in four species of Veneridae clams, belonging to three different genera (*Dosinia exoleta, Ruditapes decussatus, R. philippinarum* and *Venerupis corrugata*).

FISH results showed that the H3 histone genes were located in only one chromosome pair for three of the species – interstitially in the long arms of a submetacentric chromosome in *Dosinia exoleta;* in the telomeres of the long arms of a submetacentric chromosome in *R. philippinarum*; interstitially in the short arms of a submetacentric chromosome in *R. decussatus* – and in two chromosome pairs, a metacentric and a submetacentric, with signals on the telomeres of the log arms in *V. corrugata*.

We found no co-location of the H3 histone genes with the previously reported locations of the rRNA genes. We also compared our findings with the location of these genes in other species of venerids.

The change in the number of H3 signals, from one signal to four signals, is most parsimoniously explained by common ancestry, i.e. by the involvement of the H3-carrying acrocentric in processes of chromosomal rearrangement, such as isochromosomes and inversions. In conclusion, this work shows, through the location of these genes, the relations between the species studied.

Chapter 4

Bivalvia are present in very different surroundings, with different degrees of ecosystem disturbance. Previous studies have shown that clams are susceptible to a wide range of environmental chemical compounds, including carcinogens, and thus have often been employed as bioindicator species.

Cytogenetic studies are frequently used to determine changes in chromosome number and structure as a consequence of genotoxicity.

In this study, we used standard karyotyping through fluorochrome staining and Fluorescent *in situ* Hybridization (FISH) to describe abnormal cytogenetic constitutions found in apparently otherwise healthy individuals, from areas of commercial exploitation.

Results from standard karyotyping show a high percentage of cells with unidentifiable chromosomal rearrangements among cells with normal karyotypes, along with aneuploid and polyploid cells. Most of the abnormal cells also show the presence of minute/marker chromosomes.

These cases could be the first reports of possible disseminated neoplasia in *Venerupis aurea* and *Venus verrucosa* from this region.

Chapter 5

This work refers to an initial stage of a study showing the potential of rDNA and histone genes as markers to identify clam species from processed products, based on their differences in size between the several studied species.

The study shows that in fresh and frozen samples, DNA can be extracted and target genes amplified. As for cooked and canned samples, the production process, specifically the high temperatures needed for

sterilization, destroy DNA molecules, and so we were unable to amplify and compare the studied genes.

Further studies are required to determine a definite way to check the origin of the samples and to prove the correct species identification in the marketed products.

Chapter 6

Following reports from local producers, an investigation was conducted to characterize a group of individuals which presented intermediate morphological features between *Ruditapes decussatus* and *Ruditapes philippinarum*. Hurtado *et al*. (2011) had previously shown that these individuals were genetical hybrids between these two species, presenting, for example, both species specific 5S rDNA and major rDNA internal transcribed spacer sequences (confirmed by molecular amplification of the sequences and by FISH hybridization).

Our aim was then to induce *in vitro* spawning and produce inter-specific hybrids that could be compared to these naturally occurring individuals, in order to prove they were indeed biological putative hybrids.

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Chapter 2. Cytogenetic characterization and mapping of rDNAs, core histone genes and telomeric sequences in Venerupis aurea and Tapes rhomboides (Bivalvia: Veneridae)

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Cytogenetic characterization and mapping of rDNAs, core histone genes and telomeric sequences in *Venerupis aurea* and *Tapes rhomboides* (Bivalvia: Veneridae)

Joana Carrilho · Concepción Pérez-García · Alexandra Leitão · Isabel Malheiro · Juan J. Pasantes

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Abstract We describe the chromosomal location of GC-rich regions, 28S and 5S rDNA, core histone genes, and telomeric sequences in the veneroid bivalve species *Venerupis aurea* and *Tapes* (*Venerupis*) *rhomboides*, using fluorochrome staining with propidium iodide, DAPI and chromomycin A3 (CMA) and fluorescent in situ hybridization (FISH). DAPI dull/CMA bright bands were coincident with the chromosomal location of 28S rDNA in both species. The major rDNA was interstitially clustered at a single locus on the short arms of the metacentric chromosome pair 5 in *V. aurea*, whereas in *T. rhomboides* it was subtelomerically clustered on the long arms of the subtelocentric chromosome pair 17. 5S rDNA also was a single subtelomeric cluster on the long arms of subtelocentric pair 17 in *V. aurea* and on the short arms of the metacentric pair 9 in *T. rhomboides*. Furthermore, *V. aurea* showed four telomeric histone gene clusters on three metacentric pairs, at both ends of chromosome 2 and on the long arms of chromosomes 3 and 8, whereas histone genes in *T. rhomboides* clustered interstitially on the long arms of the metacentric pair 5 and proximally on the long arms of the subtelocentric pair 12. Double and triple FISH experiments demonstrated that rDNA and H3 histone genes localized on different chromosome pairs in the two clam species. Telomeric signals were found at both ends of every single chromosome in both species. Chromosomal location of these three gene families in two species of Veneridae provides a clue to karyotype evolution in this commercially important bivalve family.

Keywords *Venerupis aurea* · *Tapes rhomboides* · Fluorescent in situ hybridization · Ribosomal genes · Core histone genes · Telomeric sequences

J. Carrilho · C. Pérez-García · J. J. Pasantes (✉)
Department of Biochemistry, Genetics and Immunology, University of Vigo, 36310 Vigo, Spain
e-mail: pasantes@uvigo.es

J. Carrilho · I. Malheiro
Cytogenetics Laboratory, Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Porto, Portugal

J. Carrilho · I. Malheiro
Centro de Estudos de Ciências Animais (CECA/ICETA), University of Porto, Vairão, Porto, Portugal

A. Leitão
Instituto Nacional de Recursos Biológicos (INRB/L-IPIMAR), Olhão, Portugal

Introduction

The number of cytogenetic studies on bivalves has greatly increased in recent years (reviewed by Thiriot-Quiévreux 2002; Guo et al. 2007; Leitão and Chaves 2008). In these studies great emphasis has been made to physically map DNA sequences by using fluorescent in situ hybridization (FISH) in order to better identify chromosome pairs and/or to draw conclusions on bivalve phylogeny (Guo et al. 2007; Zhang et al. 2007). FISH gives a better identification of chromosomes and makes it possible to track chromosomal rearrangements (Guo et al. 2007).

Ribosomal RNA genes in higher eukaryotes are organized in two gene families known as the major and 5S rRNA genes. These two gene families are relatively independent of each other and are usually organized into separate loci on the same or different chromosomes. As the hundreds of copies of these genes are tandemly repeated and constituting one or several clusters, they provide good targets for FISH and have been used in some bivalve

species (reviewed by Thiriot-Quiévreux 2002; Guo et al. 2007; Leitão and Chaves 2008). The knowledge of the chromosomal location of rDNA has already proven useful for chromosome identification as well as for studies of chromosome rearrangements in bivalves (Wang and Guo 2007). Major rDNA has been located in several families of Bivalvia, namely Mytilidae (Martínez-Expósito et al. 1997; Torreiro et al. 1999; Pérez-García et al. 2010a, b), Ostreidae (Zhang et al. 1999), Pectinidae (Wang and Guo 2004; Huang et al. 2007a, b), Cardiidae (Insua et al. 1999), Mactridae (González-Tizón et al. 2000), Solenidae (Fernández-Tajes et al. 2003), Pharidae (Fernández-Tajes et al. 2008), Tellinidae (González-Tizón et al. 2000), Psamobidae (González-Tizón et al. 2000) and Donacidae (Martínez et al. 2002). However, major rDNA has only been located in *Dosinia exoleta* (Hurtado and Pasantes 2005), *Mercenaria mercenaria* (Wang and Guo 2007), *Ruditapes decussatus* and *R. philippinarum* (Hurtado et al. 2011) within the family Veneridae. Furthermore, the chromosomal location of 5S rDNA is only known in a small number of species of Mytilidae (Insua et al. 2001; Pérez-García et al. 2010a, b), Ostreidae (Wang et al. 2005), Pectinidae (Wang and Guo 2004; Huang et al. 2007a, b), Cardiidae (Insua et al. 1999) and Veneridae (Hurtado et al. 2011).

Histone genes in invertebrates are usually organized in tandem arrays clustered in one or more chromosomal positions (reviewed by Eirín-López et al. 2009). This organization makes them an ideal material for chromosome mapping by means of in situ hybridization. In bivalves the location of histone genes is only known in some species of Mytilidae (Eirín-López et al. 2004; Pérez-García et al. 2010a, b) and in Pectinidae (Zhang et al. 2007).

The telomeric repeat sequence, present at the telomeres, also constitutes a good probe to study basic questions related to chromosomal evolution. Telomeric sequences have been characterized and located in several species of bivalves (reviewed by Leitão and Chaves 2008; Pérez-García et al. 2010a, b; Hurtado et al. 2011).

The family Veneridae contains approximately 500 living species from 50 genera of 12 subfamilies (Canapa et al. 1996), which are among the most important commercially exploited bivalves all over the world (FAO 2008). The golden carpet shell *Venerupis aurea* (Gmelin 1791) and the banded carpet shell *Tapes (Venerupis) rhomboides* (Pennant 1777) are amid the many species of clams which are commercially exploited along the Atlantic coast of the Iberian Peninsula. Nevertheless, previous chromosome studies on these species have only been conducted to establish the standard karyotype (Borsa and Thiriot-Quiévreux 1990; Insua and Thiriot-Quiévreux 1992).

This work intends to detect the chromosomal regions with different GC-content and to determine the location of major and 5S rDNAs, histone genes, and telomeric sequences in two species of Veneridae, *Venerupis aurea* and *Tapes rhomboides* by fluorochrome staining and FISH. This would be an initial step towards a better understanding of the evolutionary relationships within this family, and could also be a useful tool in the design of genetic improvement programs.

Materials and methods

Clam samples

Samples of the golden carpet shell *Venerupis aurea* and the banded carpet shell *Tapes rhomboides* were collected from wild and cultured populations in northwest Spain. Male and female juveniles were maintained in the laboratory in 10 L tanks of aerated, filtered sea water at 18 ± 1°C. They were fed on a suspension of algal cells (*Isochrysis galbana*) for at least 5 days in order to promote both somatic growth and gonadal maturation. The nomenclature used for these species followed the CLEMAN database (http://www.somali.asso.fr/clemam/index.php).

Probe preparation

Total genomic DNA was isolated from ethanol-preserved adductor muscles following Winnepenninckx et al. (1993) with slight modifications. Tissue was homogenized in acetyltrimethyl ammonium bromide buffer and digested overnight with proteinase K at 60°C. DNA was extracted with chloroform/isoamyl alcohol (24/1).

Fluorescent in situ hybridization probes were amplified with polymerase chain reaction (PCR) in 20 µL of reaction mixture containing 50 ng template DNA, 1× PCR buffer, 50 µM each dNTP, 2.5 mM $MgCl_2$, 1 µM each primer and 1 U BIOTAQ DNA polymerase (Bioline). Primer combinations are shown in Table 1. Universal primers were used to amplify both the whole internal transcribed spacer (ITS) region and a fragment of the 28S gene of the major rDNA repeats (ITS4, ITS5, LR10R, LR12; White et al. 1990). For the 5S rDNA amplification, primers were designed from the sequence of the 5S rRNA of *Mytilus edulis* (Fang et al. 1982). Amplification of the H2B/H2A and H3 core histone genes was performed using primers designed from the histone gene sequences of *Mytilus edulis* (Albig et al. 2003) and those described by Giribet and Distel (2003), respectively. After 5 min denaturation at 95°C, 30 cycles of amplification were performed using the conditions in Table 2 with post-cycling extension for 7 min at 72°C. All reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems) and PCR products were examined by electrophoresis on a 2% agarose gel.

Table 1 Combinations of primers used in the PCR amplification

Region	Primer sequence (5′–3′)	
ITS	*ITS5*	GGAAGTAAAAGTCGTAACAAGG
	ITS4	TCCTCCGCTTATTGATATGC
28S	*LR10R*	GACCCTGTTGAGCTTGA
	LR12	GACTTAGAGGCGTTCAG
5S	*5SD*	CAACGTGATATGGTCGTAGAC
	5SR	AACACCGGTTCTCGTCCGATC
H3	*H3D*	ATGGCTCGTACCAAGCAGACVGC
	H3R	TATCCTTRGGCATRATRGTGAC
H2BA	*H2BAD*	TCCCGAGATGTGATGGTAGA
	H2BAR	AGTACAGCCTGGATGTTTGGTAA

ITS internal transcribed sequence of the major rDNA, *28S* 28S rRNA gene, *5S* 5S rRNA gene, *H3* histone H3 gene, *H2BA* H2B and H2A histone genes

Table 2 Conditions used in the PCR amplification

Region	Cycles	Denaturation	Annealing	Extension
ITS	30	95°C, 30 s	48°C, 30 s	72°C, 30 s
28S	30	95°C, 20 s	48°C, 20 s	72°C, 30 s
5S	30	95°C, 30 s	44°C, 30 s	72°C, 60 s
H3	30	95°C, 15 s	48°C, 15 s	72°C, 15 s
H2BA	30	95°C, 30 s	45°C, 30 s	72°C, 40 s

ITS internal transcribed sequence of the major rDNA, *28S* 28S rRNA gene, *5S* 5S rRNA gene, *H3* histone H3 gene, *H2BA* H2B and H2A histone genes

Chromosome preparation and fluorochrome staining

Mitotic chromosome preparations were obtained after Martínez-Expósito et al. (1997). Juvenile specimens were housed in 0.5 L beakers and exposed to colchicine (0.005%) for 12 h. Gills were excised and immersed in 50 and 25% sea water for 1 h, fixed with ethanol/acetic acid for 1 h, and dissociated in 60% acetic acid. Resulting cell suspension was dropped onto slides heated to 50°C and air-dried.

Some preparations were stained for 2 h with chromomycin A3 (CMA, 0.25 mg/ml) and counterstained with 4′,6-Diamidino-2-phenylindole (DAPI, 0.14 µg/ml) for 8 min. Once washed with distilled water, slides were air-dried and mounted with antifade (Vectashield, Vector). After visualization and photography, preparations were washed and re-stained with a combination of DAPI (0.14 µg/ml) and propidium iodide (PI, 0.07 µg/ml). The slides were then washed in distilled water, air-dried, mounted in antifade, and photographed again.

Fluorescent in situ hybridization

Single and double FISH experiments using biotin and digoxigenin-labeled major and 5S rDNA and core histone gene probes were performed following the methods by Hurtado and Pasantes (2005) with minor modifications. Chromosome preparations were denatured for 2 min at 70°C. Hybridization was performed overnight at 37°C. Signal detection was carried out with fluorescein avidin and biotinylated anti-avidin for the biotinylated probes, and with mouse antidigoxigenin, anti-mouse rhodamine and anti-goat rhodamine for the digoxigenin-labeled probes. Slides were counterstained with DAPI and mounted in antifade.

In order to map three probes on the same metaphase plates, two consecutive FISH experiments were performed. In the first FISH, biotin-labeled core histone gene probes and digoxigenin-labeled 5S rDNA probe were employed. After visualization and photography, the preparations were re-hybridized using biotin-labeled major rDNA probe, and the same metaphase plates were visualized and photographed again. In addition, we also performed FISH with a vertebrate telomeric $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) following the supplier's protocol.

Slide visualization and photography were performed with a Nikon Eclipse-800 microscope equipped with an epifluorescence system. FISH signals of each probe were examined in a minimum of 150 metaphase plates obtained from five individuals in each species. Separated images for each fluorochrome were obtained with a Sensys CCD camera (Photometrics) connected to the microscope. Camera control and digital image acquisition employed a Power Macintosh computer. Pseudocoloring and merging of the images were performed with Adobe Photoshop.

Results

In accordance with previously published results (Borsa and Thiriot-Quiévreux 1990; Insua and Thiriot-Quiévreux 1992), analysis of 400 metaphases in five male and five female specimens in both *Venerupis aurea* and *Tapes rhomboides* showed $2n = 38$ chromosomes (Fig. 1). In *V. aurea* the karyotype is composed of eight metacentric, nine submetacentric, one subtelocentric and one telocentric chromosome pairs. *T. rhomboides* showed a karyotype composed of four metacentric, eight submetacentric, four subtelocentric and three telocentric pairs.

The possible presence of AT- and/or GC-rich regions was examined with a combination of AT-specific (DAPI), GC-specific (CMA) or unspecific (PI) fluorochromes. The combined DAPI/PI staining revealed a DAPI negative intercalary region on the short arms of the metacentric pair 5 in *Venerupis aurea* (Fig. 1a). A DAPI negative region, subtelomerically located on the long arms of chromosome pair 17, also was seen in *Tapes rhomboides* (Fig. 1d).

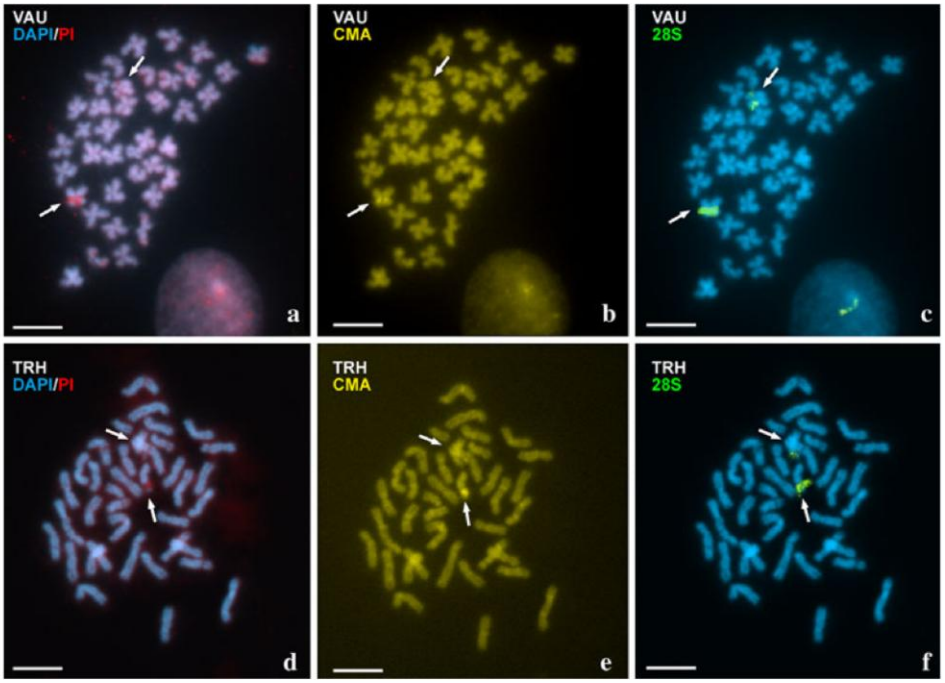

Fig. 1 DAPI/PI and CMA staining followed by fluorescent in situ hybridization (*FISH*) with a 28S rDNA probe on metaphase chromosomes of *Venerupis aurea* (*VAU*, **a**–**c**) and *Tapes rhomboides* (*TRH*, **d**–**f**). Sequential staining of the same metaphase plates with DAPI/PI (**a**, **d**) and CMA (**b**, **e**) shows GC-rich (DAPI−/CMA+) regions interstitial on the short arm of a metacentric chromosome pair in *V. aurea* (*arrows* in **a**, **b**) and subterminal on the long arm of a subtelocentric chromosome pair in *T. rhomboides* (*arrows* in **d**, **e**). FISH on the same metaphase plates (**c**, **f**) using a 28S rDNA probe (biotin, fluorescein, *green*) demonstrates that these GC-rich regions correspond to the NORs (*arrows* in **c**, **f**). *Scale bars* 5 μm

Simultaneous DAPI/CMA staining of the same metaphases detected CMA positive bands in the DAPI negative regions (Fig. 1b, e).

Fluorescent in situ hybridization with the whole ITS region or a fragment of the 28S rDNA as a probe mapped major ribosomal gene clusters in one chromosome pair in both *Venerupis aurea* and *Tapes rhomboides*. In *V. aurea* major rDNA was clustered on the short arms of the metacentric pair 5 (Figs. 1c, 2c, d). In *T. rhomboides* major rDNA was mapped subterminally on the long arms of the subtelocentric pair 17 (Figs. 1f, 2g, h). Sequential DAPI/PI and CMA staining followed by FISH using the major rDNA probe on the same metaphase plates (Fig. 1a–f) clearly demonstrated the occurrence of major rDNA in the DAPI negative/CMA bright bands.

In *Venerupis aurea*, FISH signals of whole 5S rDNA repeat as probe showed one cluster of 5S rDNA repeats subtelomerically located on the long arms of the subtelocentric pair 17 (Fig. 2a, c, d). *Tapes rhomboides* (Fig. 2e, g, h) exhibited a single cluster of 5S rRNA genes subtelomerically located on the short arms of chromosome pair 9. Double-color FISH confirmed the occurrence of major and 5S rDNA signals on different chromosome pairs in both *V. aurea* and *T. rhomboides*.

Histone H3 gene FISH detected four clusters terminally located on three chromosome pairs in *Venerupis aurea* (Fig. 2b, c, d). Two of the clusters were at both ends of the chromosome pair 2, and the other two clusters on the long arms of the chromosome pairs 3 and 8. In *Tapes rhomboides* histone gene clustered interstitially on the long arms of chromosome pair 5 and proximally on the long arms of the chromosome pair 12 (Fig. 2f, g, h). Double-color FISH experiments using differently labeled H3 and H2B/H2A histone gene probes always showed coincident signals (not shown). Double-color FISH and re-hybridization experiments showed that core histone gene clusters and major and 5S rDNA repeats were on different chromosome pairs (Fig. 2c, d, g, h).

Fluorescent in situ hybridization with a telomeric PNA probe detected single distinct terminal signals on both sister chromatids of every mitotic chromosome, without interstitial signals, in the two species (Fig. 3).

Discussion

Mitotic chromosome numbers and karyotypes are known in 11 species of the family Veneridae (Thiriot-Quiévreux

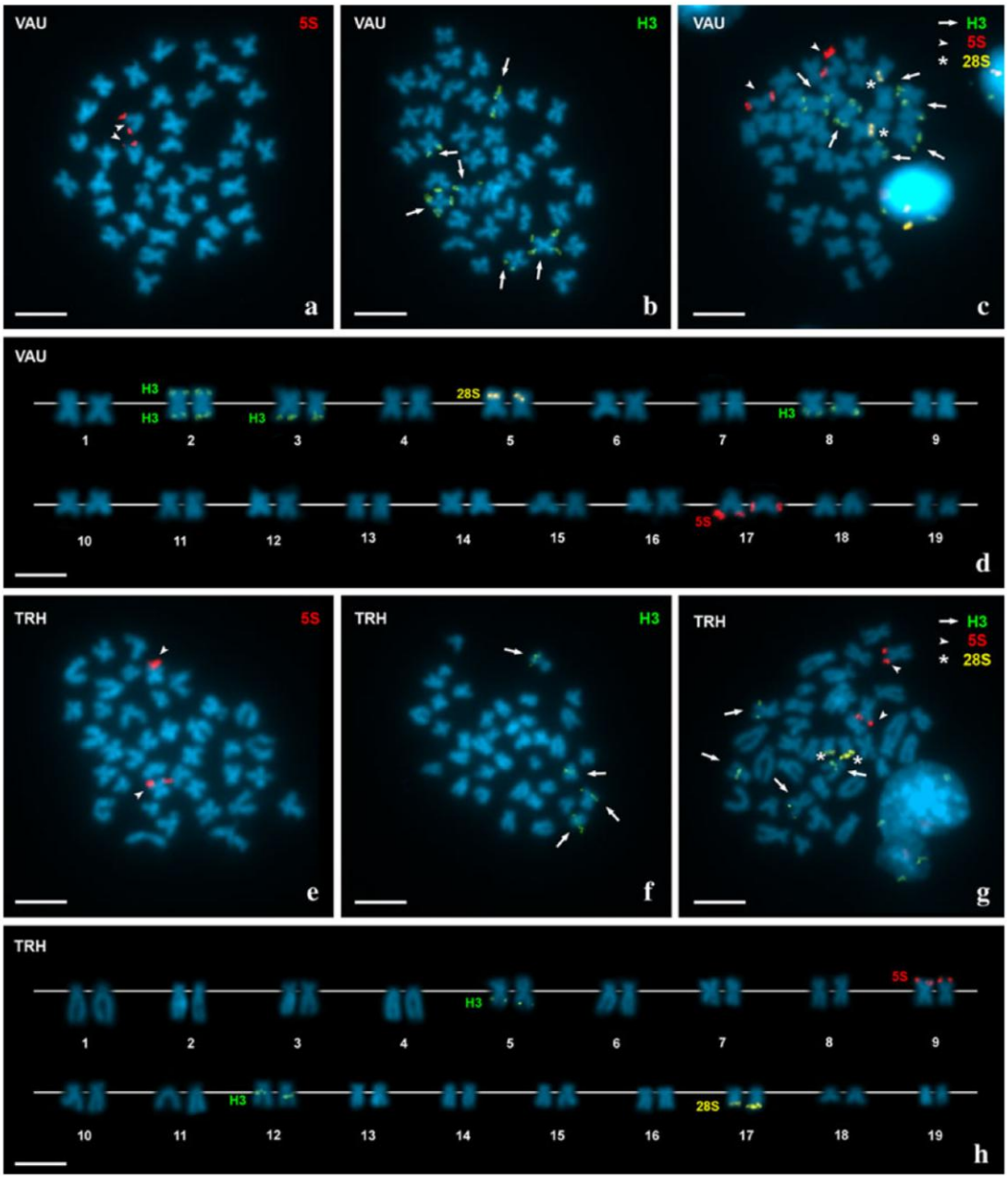

Fig. 2 Chromosomal location of 5S rDNA and histone genes, together with major rDNA, on *Venerupis aurea* (*VAU*, **a**–**d**) and *Tapes rhomboides* (*TRH*, **e**–**h**) chromosomes counterstained with DAPI. Fluorescent in situ hybridization (*FISH*) experiments using 5S rDNA probes (digoxigenin, rodhamine, *red*; **a**, **e**) show minor rDNA signals at a single locus in both *V. aurea* (*arrowheads* in **a**) and *T. rhomboides* (*arrowheads* in **e**). FISH experiments using histone H3 gene probes (biotin, fluorescein, *green*; **b**, **f**) show that histone genes are clustered at four different loci on three chromosome pairs in *V. aurea* (*arrows* in **b**) and at two loci on two chromosome pairs in *T. rhomboides* (*arrows* in **g**). FISH using histone H3 gene probes (H3, biotin, fluorescein, *green*, *arrows*) and 5S rDNA probes (5S, digoxigenin, rodhamine, *red*, *arrowheads*) followed by a second FISH using 28S rDNA probes (28S, *yellow*, *asterisks*) demonstrates the independent location of the three gene families in both *V. aurea* (**c**) and *T. rhomboides* (**g**). This is more clearly shown on the corresponding karyotypes (**d**, **h**). *Scale bars* 5 μm

1994, 2002; Hurtado and Pasantes 2005). The diploid chromosome number of 38 in all the specimens of *Venerupis aurea* and *Tapes* (*Venerupis*) *rhomboides* studied here is in accordance with previous studies (Borsa and Thiriot-Quiévreux 1990; Insua and Thiriot-Quiévreux 1992). The karyotypes determined also are in accordance

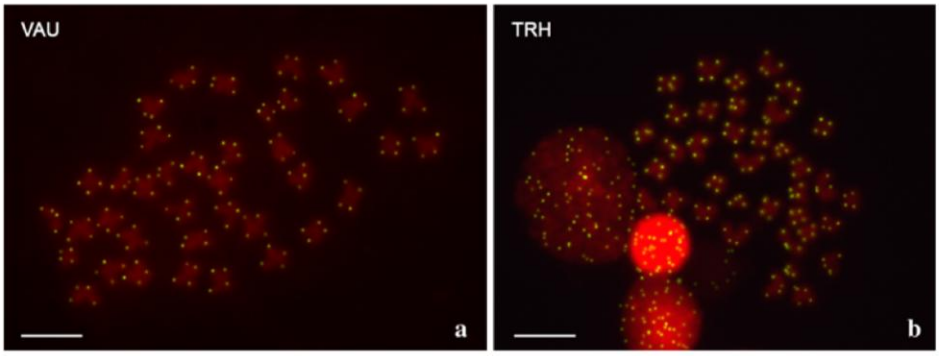

Fig. 3 Chromosomal mapping of telomeric sequences to *Venerupis aurea* (*VAU*) and *Tapes rhomboides* (*TRH*) chromosomes counterstained with PI. Hybridization of mitotic metaphase chromosomes with a telomeric PNA probe (fluorescein, *green*) shows telomeric signals at the ends of every mitotic chromosome in both *V. aurea* (**a**) and *T. rhomboides* (**b**). *Scale bars* 5 μm

with those proposed by Borsa and Thiriot-Quiévreux (1990) for *V. aurea* and by Insua and Thiriot-Quiévreux (1992) for *T. rhomboides*.

Although the use of base-specific fluorochromes has allowed the detection of the presence of many heterochromatic GC-rich regions on the chromosomes of *Donax trunculus* (Martínez et al. 2002; Petrović et al. 2008), these methods in most other bivalves have detected scarce GC-rich regions, usually coincident with major rDNAs (reviewed by Thiriot-Quiévreux 2002; Guo et al. 2007; Leitão and Chaves 2008; Pérez-García et al. 2010b and references therein). This also is the case in both the present *V. aurea* and *T. rhomboides*, showing a single GC-rich region coincident with the single major rRNA gene cluster.

The presence of major rDNA signals on a single chromosome pair in both *V. aurea* and *T. rhomboides* is concordant with the results obtained in other bivalves. NORs have been located by silver staining and/or FISH in around 50 species of bivalves (reviewed by Thiriot-Quiévreux 2002; Guo et al. 2007; Leitão and Chaves 2008). Most of these species show NORs located at terminal positions on one to three chromosome pairs. In the family Veneridae the position of major rDNAs is known in four species. One NOR has been detected in *Dosinia exoleta* (Hurtado and Pasantes 2005), *Ruditapes decussatus* and *R. philippinarum* (Hurtado et al. 2011), whereas two in *Mercenaria mercenaria* (Wang and Guo 2007). The presence of a single major rDNA cluster is quite usual as well in species of other families of the subclass Heterodonta to which the venerids belong. In most species analyzed so far, major rDNAs are found in one chromosome pair (Insua et al. 1999; González-Tizón et al. 2000; Martínez et al. 2002; Fernández-Tajes et al. 2008). The exceptions are *Solen marginatus* (Fernández-Tajes et al. 2003) and *Mulinia lateralis* (Wang and Guo 2008), in which two pairs of NOR-bearing chromosomes were described. The situation in families of the other subclass of Bivalvia (Pteromorphia) is somewhat more variable. Major rDNAs occur in only one chromosome pair in most Ostreidae species analyzed (Cross et al. 2003; Wang et al. 2004) and in half of the species of Pectinidae (Wang and Guo 2004; Insua et al. 2006; Odierna et al. 2006), but not in *Hinnites distortus* (Lopez-Piñon et al. 2005), *Patinopecten yessoensis* (Huang et al. 2007b), *Argopecten irradians* (Wang and Guo 2004; Huang et al. 2007a), and *A. purpuratus* (Gajardo et al. 2002). However, in Mytilidae, major rDNA was found in one (*Brachidontes pharaonis*, Vitturi et al. 2000; *B. puniceus*, Pérez-García et al. 2010b), two (*Mytilus galloprovincialis,* Martínez-Expósito et al. 1997; *M. edulis*, Insua and Mendez 1998; *B. rodriguezi*, Torreiro et al. 1999; *Perumytilus purpuratus*, Pérez-García et al. 2010a) or three chromosome pairs (*M. trossulus* and *M. californianus*, González-Tizón et al. 2000).

The chromosomal location of 5S rDNA is only known in 14 species of bivalves (reviewed by Thiriot-Quiévreux 2002; Guo et al. 2007; Leitão and Chaves 2008; Pérez-García et al. 2010b and references therein). Single 5S rDNA cluster in the present *V. aurea* and *T. rhomboides* is concordant with the results obtained in the other two species of Veneridae (*Ruditapes decussatus* and *R. philippinarum*, Hurtado et al. 2011), but differs from *Cerastoderma edule* (Cardiidae) showing five clusters of 5S rDNA on five different chromosome pairs (Insua et al. 1999). Most species of Pectinidae also show a single cluster of 5S rDNA (Wang and Guo 2004; López-Piñón et al. 2005; Insua et al. 2006; Huang et al. 2007b), but *Aequipecten opercularis* (Insua et al. 1998) shows two clusters. For *Argopecten irradians* one (Huang et al. 2007a) and two clusters (Wang and Guo 2004) have been described. The presence of two clusters of 5S rDNA is also the case in all species of Ostreidae (Cross et al. 2005; Wang et al. 2005). This is the situation as well in two species of Mytilidae, *Brachidontes puniceus* and *B. rodriguezi* (Pérez-García et al. 2010b), but neither in *Perumytilus purpuratus* that shows three clusters

in two chromosome pairs (Pérez-García et al. 2010a) nor in *Mytilus edulis* and *M. galloprovincialis* (Insua et al. 2001) that show four clusters in three chromosome pairs.

The occurrence of major and 5S rDNA clusters on different chromosome pairs in the present *V. aurea* and *T. rhomboides* is in accordance with the results obtained in *Ruditapes philippinarum* (Hurtado et al. 2011) and most of the other species of bivalves analyzed so far (reviewed by Leitão and Chaves 2008), but differs with the presence of both classes of rDNA clusters on the same chromosome pair in *Chlamys farreri* (Wang and Guo 2004), *Brachidontes rodriguezi* (Pérez-García et al. 2010b), and *R. decussatus* (Hurtado et al. 2011).

Histone genes are usually organized as tandem repeats in invertebrate genomes (reviewed by Eirín-López et al. 2009). These clusters can consist in a copy of each of the core histone genes (*h4*, *h3*, *h2b* and *h2a*) or both core and linker (*h1*) histone genes. Histone gene arrangement in bivalves have been studied in the mussels *Mytilus edulis* (Drabent et al. 1999; Albig et al. 2003) and *M. galloprovincialis* (Eirín-López et al. 2002, 2004), and the scallop *Chlamys farreri* (Li et al. 2006). All of them show clusters of core histone genes ordered in the same way, thereby indicating a probable conserved arrangement. Core histone genes are known to form a single cluster in the scallops *Argopecten irradians*, *C. farreri* and *C. nobilis* (Zhang et al. 2007) and the mussel *Perumytilus purpuratus* (Pérez-García et al. 2010a), but two clusters in the mussels *M. galloprovincialis* (Eirín-López et al. 2004), *Brachidontes puniceus* and *B. rodriguezi* (Pérez-García et al. 2010b) and the scallop *Patinopecten yessoensis* (Zhang et al. 2007). Therefore, two histone gene clusters in *T. rhomboides* do not differ from other cases in bivalves, but four histone gene clusters in *V. aurea* are intriguing. Furthermore, unlinked localization of core histone genes and 5S rDNA revealed by double-color FISH and rehybridization experiments in the clam species studied here is different from the situation described in *M. galloprovincialis* (Eirín-López et al. 2004).

Detection of the vertebrate $(TTAGGG)_n$ repeat at chromosome ends in the present *V. aurea* and *T. rhomboides* also is coincident with the results obtained in other species of bivalves (reviewed by Thiriot-Quiévreux 2002; Guo et al. 2007; Leitão and Chaves, 2008), including the venerid clams *Dosinia exoleta* (Hurtado and Pasantes 2005), *Ruditapes decussatus* and *R. philippinarum* (Hurtado et al. 2011). Although in bivalves telomeric sequences have only been isolated and characterized in *Donax trunculus* (Plohl et al. 2002), the FISH results obtained so far seem to indicate that the bivalve telomeres are composed of tandem repeats of the hexanucleotide also found in the vertebrate telomeres.

Molecular phylogenies (Canapa et al. 2003; Kappner and Bieler 2006) show that *Ruditapes decussatus*, *R. philippinarum*, *V. aurea*, and *T. rhomboides* all belong to the subfamily Tapetinae, but also indicate that *R. decussatus* is closer to the species coming from the same geographic area (*V. aurea* and *T. rhomboides*) than to its congeneric *R. philippinarum* (Canapa et al. 2003). The scarcity of chromosome markers in these species still do not allow to support or argue against the molecular and morphological findings. However, the differences in the number and the chromosomal location of the histone gene clusters detected in the present clam species will make them promising chromosome markers in the study of the evolutionary relationships among venerid species. In conclusion, the information on the location of rDNA and histone gene clusters for the first time in the two species of Veneridae examined herein is a starting point for further evolutionary studies in this commercially important bivalve family.

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Chapter 3. Chromosomal mapping of H3 histone genes by fluorescent in situ hybridization in several species of clams (Bivalvia: Veneridae)

(in publication)

Chromosomal mapping of H3 histone genes by fluorescent *in situ* hybridization in several species of clams (Bivalvia: Veneridae)

Carrilho, J.[1,3,4], Malheiro, I.[1], Morán, P.[3], Leitão, A.[2], Pasantes, J.J.[3]

1- Cytogenetics Laboratory, Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Portugal
2- Instituto Nacional de Recursos Biológicos (INRB/L-IPIMAR), Olhão, Portugal
3- Department of Biochemistry, Genetics and Immunology, University of Vigo, E-36310 Vigo, Spain
4- Centro de Estudos de Ciências Animais (CECA/ICETA), University of Porto, Vairão, Portugal

Abstract

We analysed the chromosomal location of H3 histone genes by Fluorescent *in situ* Hybridization (FISH) in four species of Veneridae clams, belonging to three different genera (*Dosinia exoleta, Ruditapes decussatus, R. philippinarum* and *Venerupis corrugata*). The established karyotype was confirmed for all species. FISH results showed the H3 histone genes were located in only one chromosome pair for three of the species – interstitially in the long arms of a submetacentric chromosome in *Dosinia exoleta;* in the telomeres of the long arms of a submetacentric chromosome in *R. philippinarum* and interstitially in the short arms of a submetacentric chromosome in *R. decussatus* – and in two chromosome pairs, a metacentric and a submetacentric, with signals in the telomeres of the long arms in *V. corrugata*. The fact that the 5S rRNA genes seem to be in the same pair of chromosomes in most species may point that these are somehow conservative genes in Veneridae clams. We found no co-location of the H3 histone genes with the locations of the rRNA genes. We also compared our findings with the location of these genes in other species of venerids. We found evidence of a possible involvement of an ancestral H3-carrying acrocentric chromosome in misdivision giving rise to an isochromosome and several phenomena of inversion. In conclusion, through the location of these genes, we settle a starting point to further evolutionary studies, in order to infer karyotypic relationships in this commercially important bivalve family.

Keywords: *D. exoleta, R. decussatus, R. philippinarum, V. corrugata,* FISH, cytogenetics, histones, rRNA genes

Introduction

Bivalves, belonging to the phylum Mollusca, constitute some of the most important marine species. This group includes, among others, clams, mussels and oysters. The family Veneridae gathers approximately 500 living species, distributed by 50 genera and 12 sub-families (Canapa *et al.* 1996). This diversity is associated with the great variety of habitats to which these species are adapted to, from fine sand to silt and coral sandy biotopes (Cantera, 1991). Venerids are among the most important commercially exploited bivalves all over the world (FAO, 2008) and can be found distributed all along the Iberian Peninsula coasts.

The interest in bivalves has greatly boosted in recent years, due to the increase of their role on economy. Thus, cytogenetic studies have increased both in number and complexity. The establishment of the standard karyotype for the species here studied was made in 1990 by Borsa and Thiriot-Quiévreux for *R. decussatus* and *R. philippinarum*, in 1992 by Insua and Thiriot-Quiévreux for *V. corrugata (= V. senegalensis = V. pullastra*) and in 2005 by Hurtado and Pasantes for *D. exoleta*.

Attention has been drawn recently to the physical mapping of DNA sequences, in bivalves, as a way to identify chromosome pairs and/or establish the phylogeny of species (Zhang *et al.* 2007). FISH creates a better resolution for the identification of chromosomes and allows the tracking of chromosomal rearrangements at a minute scale (Ran *et al*. 2001). Earlier studies using FISH in Veneridae include the localization of major rDNA and telomeric sequences in *D. exoleta* (Hurtado and Pasantes, 2005) and major rDNA and telomeric sequences in *Mercenaria mercenaria* (Wang and Guo, 2001; 2007). A study by Hurtado *et al.* (2011) located major and minor rDNA sequences in meiotic synaptonemal complexes of *Ruditapes decussatus* and *R. philippinarum*. This last study gives us information on the number of signals and whether they are co-located or not, but does not provide any information on which chromosome pair is the bearer of the sequences.

Histones are a type of basic proteins found in all eukaryotic organisms. They are responsible for DNA packaging in nucleosomes and are also involved in the regulation

of gene expression. Histones can be divided in two major groups, depending on their structure and function. Core histones (H2A, H2B, H3, H4) associate in complexes and interact with DNA to constitute the nucleosome structure, and linker histones (H1) interact with DNA stretches between nucleosomes, participating in nucleosome positioning (Simpson, 1978).

The H3 histone is among the most conserved eukaryotic proteins (Miller *et al.* 1993). H3 histone genes are frequently found in repeated clusters (Maxson *et al.* 1983), usually along with the rest of the core histones (the location of linker histones is variable among the different organisms) (Eirín-Lopéz *et al.* 2004). It has also been shown that these genes are rather conservative in their chromosome location among relatively close species (Hankeln *et al.* 1993; Ranz *et al.* 2003). Thus, they are a very good chromosomal marker of historical and ongoing karyotypic repatterning. In Veneridae, this gene has been located in *Venerupis aurea* and *Venerupis (= Tapes) rhomboides* (Carrilho *et al.* 2011). Among other bivalves the location of this gene has been performed in Mytilidae (Eirín-Lopéz *et al.* 2004) and in Pectinidae (Zhang *et al.* 2007).

Although the chromosomal number of 2n=38 has been maintained in all the species of venerid clams studied so far, there are important differences in morphology (revealed by different NF), making the karyotypes highly variable and not truly conservative. No study so far has presented a proposal for the evolutionary mechanism responsible for this variation, thus lacking in a tentative ancestral karyotype.

It now becomes necessary to investigate which types of chromosomal rearrangements played a role in the appearing of the modern variability.

The aim of this work was to determine for the first time the location of the H3 histone genes in the Veneridae species *D. exoleta, R. decussatus, R. philippinarum* and *V. corrugata,* using fluorescent *in situ* hybridization. Two-colour FISH and re-hybridization with a third probe was performed to study the position of this gene in relation to the chromosomal location of rRNA genes, both the major rDNA (18S-5.8S-28S) and the 5S rDNA.

This would be an initial step towards the enlightenment of the evolutionary relationships within this family.

Material and methods

Biological material and chromosome preparation

40 individuals of each species (with approximately 2-3 cm diameter) were collected from wild and cultured populations in northwest Spain and fed in the laboratory with the microalgae *Isochrysis galbana* for 10 days before performing chromosome spreads.

The first step of treatment was an incubation of the animals in 0.005% colchicine in seawater for 10–12 h. The metaphases were obtained from dissected gills and gonadic tissue, which suffered a hypotonic shock in 50 and 25% seawater solution for 30 min each. Fixation was made by three incubations of 20 min each in a freshly prepared mixture of absolute ethanol and acetic acid (3:1). The samples were dissociated in 50% acetic acid and the suspension obtained was dropped onto slides heated at 42 °C (Martínez-Expósito *et al.* 1997).

Probe construction and labelling

The three different sequences studied were 5S rDNA, H3 histone and major rDNA clusters. Internal transcribed spacers (ITS) between the 18S and 5.8S (ITS1) and between 5.8S and 28S (ITS2) were amplified and used as major rDNA FISH probes, as well as 28S gene alone. From here forward all references to ITS mean the ITS1-5.8S-ITS2 cluster. Specific probes were generated by PCR. Extraction of genomic DNA was made according to Winnepenninckx *et al.* (1993) from ethanol-preserved adductor muscle.

We used the PCR primers designed by Giribet & Distel (2003) for the histone H3 genes, White *et al.* (1990) for the major rRNA genes and Fang *et al.* (1982) for the 5S rDNA. Probes were labelled with digoxigenin-11-dUTP (alkali-stable) or biotin-16-UTP either by PCR or nick translation. PCR were performed in a 50 µl solution containing 1X PCR buffer with $MgCl_2$, BSA, all the nucleotides dATP, dCTP, and dGTP in the same

proportion, dTTP in a smaller amount, the chosen labelling molecule, Taq DNA polymerase, the primers, and clam genomic DNA. The optimized thermal cycling parameters were 30 cycles of 5 min at 95 °C, 15-30 s at 48 °C, and 15-60 s at 72°C. Amplified products were visualized on 2% agarose gels.

Fluorescent in situ hybridization (FISH)

Hybridization was performed according to Torreiro *et al.* (1999), with minor modifications. Preparations were denatured for 2 min at 70 °C. Hybridization was performed overnight at 37 °C. Signal detection was carried out with Fluorescein avidin and biotinylated anti-avidin for the biotinylated probes and with mouse antidigoxigenin and anti-mouse TRITC for the digoxigenin-labelled probes. Slides were counterstained with DAPI and mounted in antifade. Slide visualization and photography were performed with a Nikon Eclipse-800 microscope equipped with an epifluorescence system. Separated images for each fluorochrome were obtained with a DS-Qi1Mc camera (Nikon) connected to the microscope. Camera control and digital image acquisition employed a computer with NIS-Elements BR 3.0 software. Pseudocolouring and merging of the images were performed with Adobe Photoshop.

Results and discussion

R. decussatus showed 2n=38 chromosomes and the karyotype is composed of 6 metacentric, 3 submetacentric and 10 subtelocentric chromosome pairs. In *R. philippinarum* we found a chromosome number of 2n=38, with 9 metacentric and 10 submetacentric chromosome pairs. This is in accordance with the findings of Borsa and Thiriot-Quiévreux (1990). *D. exoleta* showed 2n=38, with 11 pairs of metacentric chromosomes and 8 submetacentric, in accordance with Hurtado and Pasantes (2005). Finally, in *V. corrugata* we found 2n=38, with 3 metacentric, 8 submetacentric and 8 subtelocentric chromosome pairs, which confirms the results previously described by Insua and Thiriot-Quiévreux (1992).

FISH results were analyzed in a minimum of 150 metaphases per species. Fluorescent *in situ* hybridization of H3 histone genes revealed that *R. decussatus* is labelled interstitially in the short arms of a submetacentric chromosome (Fig.1a), *R. philippinarum* presents labelling telomerically in the long arms of a submetacentric chromosome (Fig.1b), in *D. exoleta* interstitially in the long arms of a submetacentric chromosome (Fig.1c) and *V. corrugata* shows labelling telomerically in the long arms of a submetacentric and a metacentric chromosomes (Fig.1d).

Simultaneous hybridization with rRNA genes, both major and minor, showed that there was no co-location between H3 histone genes and these two gene clusters in any of the species studied.

The location of the H3 histone gene had previously been performed in other species of the family Veneridae. In *V. aurea*, three pairs of chromosomes appeared labelled, one of them presenting two hybridization signals, in a total of eight signals. In *V. rhomboides,* two pairs of chromosomes show hybridization signals (Carrilho *et al.* 2011).

In table 1 we summarized the present data (this study and previously published results) on chromosome mapping of major and minor rDNA and H3 histone genes in Veneridae.

Our first observation is the fact that these data are not in accordance with was published by Hurtado *et al.* (2011) regarding *R. decussatus*. The number of signals was confirmed, but there was no co-location of the major and minor rDNA loci.

The simpler explanation is the misidentification of the specimens, whether in our study or in the previous one. There is even the possibility of our individuals belonging to the group of individuals with the intermediate morphology that Hurtado *et al.* (2011) defines as hybrids.

Another possible explanation is the artefacts of the technique itself, such as low stringency conditions that may be responsible for non-specific probe hybridization. This would produce high background interference and could mask secondary signals of lower intensity.

Table 1. Number and location of signals for rDNA and H3 histone genes in Veneridae

Species	Histone H3 genes			Major rDNA genes			Minor rDNA genes		
	N	Location	References	N	Location	References	N	Location	References
Venerupis aurea	4	Subtelomeric, p, m* Subtelomeric, q, m* Subtelomeric, q, m Subtelomeric, q, m	Carrilho *et al.* 2011	1	Interstitial, p, m	Carrilho *et al.* 2011	1	Subtelomeric, q, st	Carrilho *et al.* 2011
V. rhomboides	2	Interstitial, q, sm Centromeric, q, sm		1	Subtelomeric, q, st		1	Subtelomeric, p, m	
V. corrugata	2	Subtelomeric, q, m Subtelomeric, q, sm	This study	1	Interstitial, q, sm	This study	1	Subtelomeric, q, st	This study
Dosinia exoleta	1	Interstitial, q, sm		1	Subtelomeric, p, m	Hurtado & Pasantes, 2005	1	Interstitial, q, st	
Ruditapes decussatus	1	Interstitial , p, sm		1	Interstitial, q, sm	This study #	1	Subtelomeric, q, st	This study #
R. philippinarum	1	Subtelomeric, q, sm		1	Subtelomeric, q, sm		1	Interstitial, q, st	

N – number of signals in haploidy p – short arm / q – long arm * - same chromosome / m - metacentric / sm – submetacentric / st – subtelocentric / t – telocentric
- number of signals previously published in Hurtado *et al.* 2011

More importantly, the fact that Hurtado *et al*. (2011) described the hybridization of these sequences in meiotical synaptonemal complexes (SC) made their exact location impossible to determine. SCs don't show any morphological landmarks like centromere position, making the determination of the signal-bearer chromosome pair uncertain, as well as the difference between long and short arms for interstitial signals.

It is noticeable that, although the overall karyotype is different, the banding pattern for the 5S rDNA is most commonly located in subtelocentric chromosomes. This may suggest that chromosome location of 5S rRNA genes seem to be somehow conservative between closely related species. The differences in the location in *Venerupis (= Tapes) rhomboides* may be the result of a pericentric inversion that transformed the subtelocentric chromosome in a metacentric chromosome, or vice-versa.

The appearance of the signals for the H3 histone genes on both telomeres of the same long metacentric chromosome of *Venerupis aurea* may be the result of a misdivision of an ancestral signal-bearing telocentric chromosome that gave rise to an isochromosome with signals on both telomeres, as this is the only method of maintaining the same chromosome number while duplicating the information.

We also call attention to the possibility of an inversion justifying the differences in the H3 histone genes hybridization patterns between *Venerupis rhomboides* and *Venerupis corrugata*, namely the interstitial ←→ subtelomeric signal on the smaller submetacentric chromosome. Nevertheless, further studies, namely using longitudinal banding techniques, are necessary to determine which was the ancestral condition and which is the derived.

In conclusion, we find that the differences found in the pattern of location of the H3 histone genes, and its relation to the locations of the rRNA genes are useful tools in the establishment of phylogenetic relationships among closely related species.

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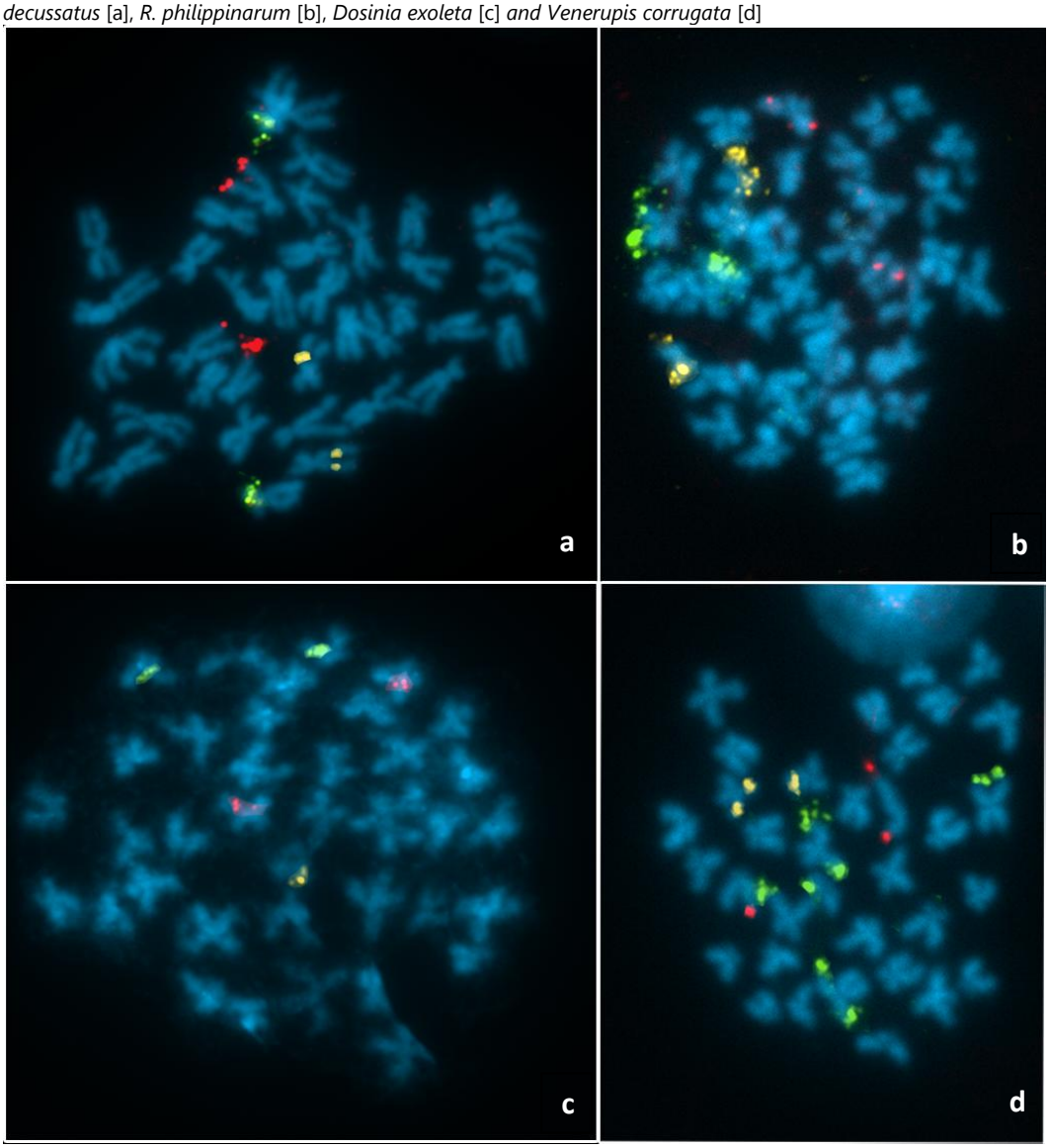
Figures

Figure 1. FISH with histone H3 (green), 18S rDNA (yellow) and 5S rDNA (red) probes in metaphases of *Ruditapes decussatus* [a], *R. philippinarum* [b], *Dosinia exoleta* [c] *and Venerupis corrugata* [d]



Chapter 4. Cytogenetic damage in the tissues of two species of clams, Venerupis aurea and Venus verrucosa: possible evidence of neoplasic processes

(in publication)

Cytogenetic damage in two species of clams, *Venerupis aurea* and *Venus verrucosa*: possible evidence of neoplasic processes

Carrilho, J. [a,b,d], Leitão, A. [c], Pasantes, J.J. [b], Malheiro, I. [a]

a. Cytogenetics Laboratory, Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Portugal
b. Department of Biochemistry, Genetics and Immunology, University of Vigo, Spain
c. Instituto Nacional de Recursos Biológicos (INRB/L-IPIMAR), Olhão, Portugal
d. Centro de Estudos de Ciências Animais (CECA/ICETA), University of Porto, Vairão, Portugal

Abstract

Bivalvia is one of the most widely spread class of animals and can be found in very different surroundings, from practically pristine to heavily anthropogenized areas. Previous studies have shown that clams are damaged by a wide range of environmental chemical compounds, including carcinogens, and thus have often been employed as bioindicator species.

A common approach to genotoxicity assessment is through cytogenetic studies to determine changes in chromosome number and structure.

In this study, we chose standard karyotyping through fluorochrome staining and Fluorescent *in situ* Hybridization (FISH) using probes for the rDNA (major and 5S) and H3 histone genes to describe abnormal cytogenetic constitutions found in otherwise apparently healthy individuals. The samples were collected in northern Spain, in areas of commercial exploitation, and by such should be free from contamination.

Standard karyotyping demonstrated that a high percentage of cells show anomalous karyotypes, including unidentifiable chromosomal rearrangements, aneuploidies and polyploidies, but others present normal karyotypes. Most abnormal cells also show the presence of minute/marker chromosomes. In *V. aurea,* FISH results showed hybridization signals for both rRNA genes in several chromosomes. Hybridization with H3 histone genes only identified two clusters, in two different chromosome pairs. The

telomeric probe showed terminal and intermediate signals, but not in all chromosomes. None of the smaller supernumerary chromosomes showed hybridization signals that were reproducible in all metaphases analysed.

In what concerns *V. verrucosa*, rDNA and histone probes showed faint signals, in variable number between metaphases. None of them appeared to be located on the small supernumerary chromosomes. Telomeric probes gave no consistent information, with only three to six chromosome pairs ever showing signals, but these chromosome pairs would not be the same from metaphase to metaphase.

These cases could be the first reports of possible disseminated neoplasia in *Venerupis aurea* and *Venus verrucosa* from this region.

Keywords: Bivalvia, *Venerupis aurea, Venus verrucosa*, FISH, karyotype, cytogenetic alterations

Introduction

Bivalves have been used as bio-indicator organisms for the last forty years because of their abundance, filter feeding and sessile life at the adult stage, which make them ideal for monitoring local ecosystem exposure to many sorts of natural and anthropogenic mutagenic agents in the near-shore environment (Gosling, 2003).

Proliferative disorders or tumours are among the possible consequences of contamination exposure. According to Sinderman (1990), a tumour is "any swelling or abnormal mass of tissue resulting from 1) non-neoplasic, controlled cell proliferation (hyperplasia); 2) a non-neoplasic increase in cell size (hypertrophy); or 3) an uncontrolled cell proliferation (neoplasia)".

In bivalve molluscs, most identified neoplasms have been diagnosed as sarcomas of haematopoietic origin, which means that they are proliferations of abnormal circulating cells of unknown origin. Gutiérrez & Sarrasquete 1986 – for *Mytilus edulis* – and Rodriguez *et al*. 1997 – for *Cerastoderma glaucum* – are examples of reports on haemocytic sarcomas in Spain, particularly in Galicia. Among venerid clams we find

descriptions of this kind of pathology in *Ruditapes decussatus* (Villalba *et al.* 1995). Gonadal germinomas, which are proliferations of undifferentiated germ cells, have also been reported in marine bivalve molluscs, mainly *Mya arenaria* (Barber, 1996, for example) *Mercenaria spp*. (Hesselman *et al.* 1988), *Mytilus galloprovincialis* (Alonso *et al.* 2001) and *Crassostrea virginica* (Peters *et al.* 1994, for example).

Both types fit the criteria of malignant tumours, including atypical structure (often with pleomorphic, undifferentiated cells); rapid and invasive growth; an abundance of mitotic figures; metastasis; and progressive growth resulting in death of the host (Sparks, 1985).

This work will use the more conservative term "disseminated neoplasia", based on the lack of evidence regarding the origin of proliferating cells and the possibility that there may be more than one tissue of origin for this condition.

There are two main identified origins or factors of increased predisposition to neoplasia in molluscs: environmental chemicals or infectious processes (Farley *et al.* 1991; Farley, 1994).

The infectious process may be derived from an infectious agent such as oncogenic viruses or from a heritable change in the genome of neoplasic cells followed by natural transplantation (Farley, 1994).

As for chemical changes, neoplasic processes can be triggered by induced rapid and uncontrolled cell proliferation. This increase in cell division rate often gives origin to a series of division errors both physical (changes in chromosome number and/or structure) and functional (activation / inactivation of genes).

The latter can even mean the loss or inactivation of tumour-suppressor genes, and thus the development of a fully-malignant tumour (Preston-Martin *et al.* 1990).

Specifically relating to bivalves, disseminated neoplasias are still of unconfirmed aetiology, but may be related to retroviral infections. Representatives of the Reoviridae and Birnaviridae have been isolated by using fish cell lines. One definitive study concludes that at least one example is not infective for bivalves while other studies

claim molluscan infectivity (review by Elston, 1997). Other authors tried to show the effect of biotoxins in the development of this pathology (Landsberg, 1996).

In what concerns chemical induction, in most cases of gonadal neoplasm in bivalves, attempts were made to link occurrence with environmental contamination either from hydrocarbons (Brown *et al.* 1977, for example) or herbicides (Gardner *et al.* 1991b, van Beneden *et al.* 1993).

The work by Ciocan & Rotchell in 2005 describes the sequences of "cancer genes" homologue to vertebrate *ras* proto-oncogene, and *p53* tumour suppressor gene in *Mytilus edulis*.

Previous studies (Reno *et al.* 1994; Sokolowski *et al.* 2004, Smolarz *et al.* 2005, Carrilho *et al.* 2008, for example) support the hypothesis that chromosomal changes may be a rapid response from aquatic organisms to environmental pollutants, as previously stated by Depledge (1997).

Relationships between hyperdiploid metaphases and effects of environmental mutagenic factors, such as industrial pollution were already hypothesized by Giagia *et al.* (1985).

These works commonly identify as features of neoplasia the increase in chromosome number (Smolarz *et al.* 2005, for example, reports a median 2.37 of diploid chromosome number in animals with neoplasic features), increase in mitotic activity, presence of acrocentric or telocentric and microchromosomes and higher activity of AgNORs in diseased animals.

This report refers to two isolated cases in the species *Venerupis aurea* and *Venus verrucosa*. For purposes of routine cytogenetic characterizations, batches of 20 individuals from each species were collected in areas of commercial exploitation, and by such subject to reduced contamination. Among the analysed clams from both sampling batches, we found an individual presenting abnormal karyotype in a high percentage of the cells. In order to access the type of damage suffered and the characteristics of the abnormal cytogenetic features, we chose to apply the following

techniques: standard karyotyping through fluorochrome staining and Fluorescent *in situ* hybridization (FISH).

The combined use of different fluorescent dyes is a tool to access the type of DNA present in certain regions or entire chromosomes, as it gives us information on the composition in base-pairs and therefore shows if these are theoretically rich or poor in genes (Kim *et al.* 2002). In this work we tried to locate the major and minor rRNA genes (the 28S and the 5S subunits of the ribosome, respectively), the H3 histone gene and telomeric sequences.

This is the first report of neoplasia in both these species.

Material and Methods

Biological Material

The studied animals came from cultured populations in the northwest Spain, namely the Ría de Vigo and Ría de Pontevedra. No reports of major contamination have been found recently in the area, and given that these are areas of commercial exploitation for consumption purposes, we expected a tight control of the water and ground quality. They were each part of a group of 20 individuals of the species *Venerupis aurea* and *Venus verrucosa* maintained in 10 L tanks of aerated, filtered, sea water at 18 $\pm$ 1 °C. The entire group was fed on a suspension of algal cells (*Isochrysis galbana*) for at least 5 days in order to promote somatic growth. All external and internal aspects of the animals appeared normal.

Chromosome preparation and fluorochrome staining

Mitotic chromosome preparations were obtained according to Martínez-Expósito *et al.* (1997). The animals were kept in a 0.5 L beaker and exposed to colchicine (0.005%) during 12 h. Gills were excised and immersed in 50% and 25% sea water for 1 h and fixed with ethanol/acetic acid for 1 h.

Chromosome spreads were obtained by dissociating small pieces of tissue in 60% acetic acid, pipetting suspension drops onto slides heated to 50 °C and air-drying.

Fluorochrome staining was performed with DAPI (0.14 µg/mL) and a Propidium Iodide (PI) (0.07 µg/mL) counterstain.

DNA preparation, PCR amplification and sequencing

Total genomic DNA was isolated from ethanol-preserved adductor muscles following Winnepenninckx *et al.* (1993) with slight modifications. Tissue was homogenized in cetyltrimethyl ammonium bromide buffer and digested overnight with proteinase K at 60 °C. DNA was extracted with chloroform/Isoamyl alcohol (24/1).

Amplifications were performed in 20 µl of a solution containing 50 ng DNA, 1xPCR buffer, 50 M each dNTP, 2.5 mM $MgCl_2$, 1 M each primer and 1 U BIOTAQ DNA polymerase (Bioline). Universal primers were used to amplify both the whole internal transcribed spacer (ITS) region and fragments of the 28S and the 18S genes of the major rDNA repeats (ITS4, ITS5, LR10R, LR12; White *et al*., 1990). For the 5S rDNA amplification, primers were designed from the sequence of the 5S rRNA of *Mytilus edulis* (Fang *et al*., 1982). The amplification of the H3 histone genes was performed using primers designed from the histone genes of *Mytilus edulis* described by Giribet & Distel (2003). After 5 min denaturation at 95 °C, 30 cycles of amplification were performed using the conditions that are shown in Table 1. A final extension step of 7 min at 72 °C was applied. All reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems) and PCR products were examined by electrophoresis on a 2% agarose gel.

Table 1. Conditions used in the PCR amplification

Region	Cycles	Denaturation	Annealing	Extension
ITS	30	95 °C, 30 s	48 °C, 30 s	72 °C, 30 s
28S		95 °C, 20 s	48 °C, 20 s	72 °C, 30 s
5S		95 °C, 30 s	48 °C, 30 s	72 °C, 60 s
H3		95 °C, 15 s	48 °C, 15 s	72 °C, 15 s

ITS = internal transcribed sequence of the major rDNA, 28S = 28S rRNA gene, 5S = 5S rRNA gene, H3 = histone H3 gene

Fluorescent in situ *Hybridization*

Single and double FISH experiments using biotin and digoxigenin labelled major rDNA, 5S rDNA and core histone gene probes were performed following the methods published by Hurtado & Pasantes (2005), with minor modifications. Preparations were denatured for 2 min at 70 °C. Hybridization was performed overnight at 37 °C. Signal detection was carried out with Fluorescein avidin and biotinylated anti-avidin for the biotinylated probes and with mouse antidigoxigenin, anti-mouse rhodamine and antigoat rhodamine for the digoxigenin-labelled probes. Slides were counterstained with DAPI and mounted in antifade.

In addition, we also performed FISH with a telomeric $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems) following the protocol indicated by the supplier.

Slide visualization and photography were performed with a Nikon Eclipse-800 microscope equipped with an epifluorescence system. Separated images for each fluorochrome were obtained with a Sensys CCD camera (Photometrics) connected to the microscope. Camera control and digital image acquisition employed a Power Macintosh computer. Pseudocolouring and merging of the images were performed with Adobe Photoshop.

Results

The normal karyotype of *Venerupis aurea*, with data on the location of the rDNA and H3 histone gene clusters and patterning of telomeric sequences, was described by Carrilho *et al.* (2011). In the case analysed, normal diploid cells (about 58% of the total) had a modal chromosome number of 38, with few aneuploid exceptions of 36 and 37 chromosomes. On the contrary, abnormal/polyploid cells (42% of the total analysed cells) had between 52 and 94 chromosomes with a modal number of 76.

Supernumerary chromosomes were not result of aneuploidy of the normal karyotype, as they were not paired with the chromosomes of the normal diploid complement. They were smaller than any other chromosome of the karyotype and in most cases had no visible centromere. (Fig. A). Many rearrangements could be observed as the normal

karyotype could not be reproduced due to the significant differences in chromosome morphology.

The dyeing behaviour of all the chromosomes present was not clear, with no evidence of pattern in DAPI-PI alternate brightness.

In the abnormal cells, FISH showed hybridization signals for the 5S rRNA genes in several chromosomes (in opposition to only one chromosome pair in normal cells).

These signals were present in what could be the "original" 5S rDNA bearing telocentric chromosome pair 17 and in other re-arranged chromosomes that we were unable to identify.

The same findings were observed for the major rRNA genes, with several chromosome pairs showing hybridization signals (Figure B.a). In what concerns the H3 histone genes, only two clusters were identified, in two different chromosome pairs (in opposition to four clusters in three pairs observed in normal cells) and none of them were telomeric, as in normal cells (Figure B.b).

The telomeric probe showed hybridization signals, not only in the telomeres of the chromosomes, but also intermediate signals that are not found in normal metaphases. It was also noticeable that not all chromosomes showed hybridization signals.

None of the smaller supernumerary chromosomes showed hybridization signals that were reproducible in all metaphases analysed.

In what concerns the *V. verrucosa* individual, 62% of the cells showed a normal diploid karyotype (described by Ebied & Aly, 2004), while 17% were polyploid and 21% showed the presence of supernumerary chromosomes, ranging between 46 and 128. In these metaphases, both re-arranged and small supernumerary chromosomes could be found (Figure C).

FISH results in this individual were very inconclusive. rDNA and histone probes showed faint signals, in variable number between metaphases. None of them appeared to be located on the small supernumerary chromosomes. Telomeric probes gave no consistent information, with only three to six chromosome pairs ever showing signals, but these chromosome pairs would not be the same from metaphase to metaphase.

In both individuals, mitotic index appeared elevated in relation to other individuals of the same group.

Discussion

This work described, through cytogenetic techniques, the occurrence of cell proliferation abnormalities, which can be considered neoplasic processes in two species of venerid clams.

Diagnosis and staging of bivalve disseminated neoplasias has been based mostly in the occurrence of an arbitrary proportion of neoplasic cells relative to normal cells, and their level of intrusion in connective tissues and inter-cellular spaces (Barber, 2004).

As the discrimination of neoplasia from atypical but non-neoplasic cells is very difficult, reliable diagnosis of the tumour is a challenging task, especially in early stages of the disease. In this way, we believe more studies are required, including other complementary techniques (such as flow cytometry and histology) to assess more accurately the type of problems these animal populations are suffering.

Nonetheless, the results show clearly that the animals were being submitted to a stressing factor in their natural environment, either chemical or infectious.

As pointed out earlier, Theodorakis *et al.* (2001) stated that examination of DNA damage and repair can give information about exposure of organisms to genotoxic chemicals.

Effects of pollutants are usually displayed first at the biochemical and molecular levels (Veldhuizen-Tsoerkan et al., 1991). This then leads to genetic changes that become cytological visible, especially in the tissues of organisms which are good pollutant bio accumulators, such as molluscs.

In regard with the changes in the karyotype, it wasn't clear whether the smaller supernumerary chromosomes in the abnormal metaphases were mainly heterochromatic or encoded other genes not tested for in this work. Consequently, further studies are required to determine if the response to the stressing factor induced

a higher transcriptional need for a specific gene, which then lead to the appearance of multiple copies in these supernumerary chromosomes (see Albertson, 2006).

In order to do so, we propose the use of FISH with a different set of probes. A search through GenBank shows that several genes with implications in oxidative stress have been sequenced in the Veneridae family, namely thioredoxin proteins, cytochrome subunits and glutathione peroxidases. Genes for defensine proteins (that act by binding to the microbial cell membrane, and, once embedded, forming pore-like membrane defects that allow efflux of essential ions and nutrients) are also sequenced and may be a clue to access infectious processes.

Rearrangements in chromosome morphology can be explained by replication and division errors due to the abnormally high mitotic index, which might have been in such an extent that could not be dealt with in full by the DNA repair system. On the other hand, this same repair system may have been impaired or inactivated by these same chromosome breaks/fusions.

Furthermore, we propose that other cytogenetic studies should be conducted in order to determine if the findings are according to the previous description from several authors that these proliferative disorders in bivalves are linked with presence of more than one nucleoli in the nucleus, the presence of ectopic AgNORs and a high nucleus to cytoplasm ratio (e.g. Elston *et al*. 1992; Krishnakumar *et al*. 1999; Smolarz *et al*. 2003; Mix, 1983).

Furthermore, we only analyzed the gills of the animals, not knowing if this condition was present in other organs, such as the mantle or gonads.

Considering these were fortuitous findings, we believe it would be important to determine, in the first place, the type of agent the animals were exposed to, and in posterior work if there is a significant increase in the appearance of these types of neoplasia in the area.

There have been several exposures in the media of high-profile environmental disasters involving policyclic aromatic hydrocarbons in the coasts of Galicia. In the last 30 years we can list the following oil spills: in 1970 the Norwich tank ‘‘Polycommander’’ with

50,000 tons of heavy fuel oils, in 1976 the Spanish ''Unquiola'' released 100,000 tons of oil, in 1978 the Greek tank ''Andros Patnia'' released 50,000 tons, and in 1992 another Greek tank ''Mar Egeu'' created a massive marine pollution over more than 200 km of the Galician coast. And more recently, the ''Prestige'' oil spill in November 2002 that sunk 270 km of the Galician coast releasing 30,000 tons of oil.

Barsiene (1994), has shown that in 80% of the bivalve specimens studied, there is a positive correlation between heavy metal, polycyclic aromatic hydrocarbon or radionuclide bioaccumulation and chromosome set disturbances.

In what specifically concerns pollution by oil spills, cytogenetic consequences such as higher micronuclei frequencies were found in mussels, following an accidental oil spill in November 2001 (Barsiene *et al*. 2004).

We can then suggest, as a hypothesis, that the extensive chromosomal rearragements and numerical alterations observed could possibly be explained by the important pollution by polycyclic aromatic hydrocarbons due to several major oil spills suffered by the Galician populations.

It has been known for a long time that marine invertebrates living under natural conditions accumulate potent carcinogens. Shimkin *et al.* showed in 1951 that when extracts from apparently healthy barnacles were injected in mice, the latter developed tumours. Following studies (Gardner *et al.* 1991a) support the theory that carcinogenic contaminants may be passed through the food-chain via species resistant to induced toxic change.

In what concerns infectious origins of neoplasia, Meyers showed in 1984 that bivalves are bioaccumulators of viruses from humans and other vertebrates. Generally, these viruses don't infect and replicate in bivalves, but use the molluscs as a transient reservoir for entrained but infectious virions, later transferred to vertebrate hosts through ingestion or contact after discharge into the water column. Farley has already showed in 1994 the multiple relations between viral presence and the development of neoplasia. Moreover, according to Gardner 1994: "Transfection assays suggest chemical

carcinogens induce malignant transformation through activation of cellular oncogenes".

These studies are an alert for the fact that even when the stressing factor (for example, a chemical spill in the area) is no longer present, cytogenetical alterations and consequent neoplasic processes may still occur due to accumulation and vertical transmission in the ecossystem.

Finally, there have been studies linking high population density and restricted water circulation with elevated neoplasia both in the field and in laboratory experiments (Elston *et al.* 1992), leaving the possibility of neoplasic processes triggered by other stimuli.

All data considered, we are left with several open questions:

1. Whether chemical, infectious or populational, the entire sampled populations were exposed to the stressing element. What is the increased predisposition factor in these particular individuals that made them develop the neoplasia?

2. Unlike other reports of neoplasia in bivalvia, our findings seem to be limited to a very specific area of the gill, in both species. Are we dealing with the same kind of pathology described for *Cerastoderma glaucum* (Rodriguez *et al.* 1997), in the same area? In the previous case there was an infiltration of haemocytes in the extravascular spaces in all tissues. Is our case a milder expression of the same pathology?

3. Is the neoplasia caused by:

    a. Changes in cell division determining genes/oncogenes

    b. Changes in the genes responsible for the ability to accumulate chemicals, that might be dysfunctional in these individuals

    c. Mutations in other genes, for example in membrane molecules that change their cell death signalling (see Le Grand *et al.* 2008)

    d. Chemical suppression of the immune system, which allow spontaneously occurring events to proliferate (as suggested by Depledge in 1997)

    e. Mutations that create new viral strains, now able to infect molluscs

In conclusion, we present this study as a first report in which we found of neoplasia in venerids and leave it as a draw of attention to the urgency of determining the factors behind these events. It is also an approach to the characterization of the cytogenetical features of these kinds of pathologies.

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Figures:

Figure A. Mitotic chromosome spreads from the gill of *Venerupis aurea* coloured with DAPI - normal metaphase, 2n =38 [left] and metaphase from a cell with an abnormal chromosome number, 2n = 76 [right]. Abnormal cells showed between 2n=52 and 2n=94, with modal number of 2n=76. Hyperploydy was characterized by the presence of microchromosomes, not polyploidy of normal karyotype.

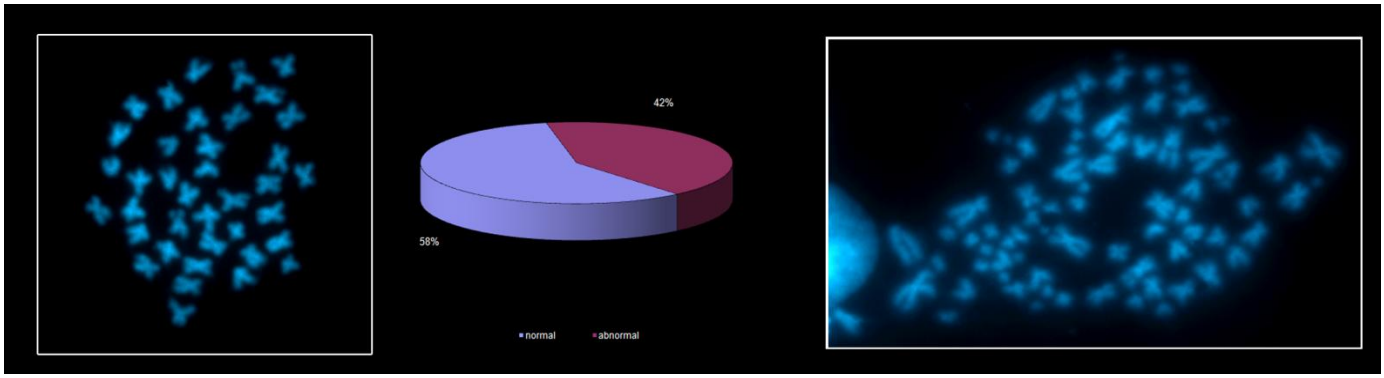


Figure B. FISH in metaphases of *Venerupis aurea* with histone H3 (green), 18S rDNA (yellow) and 5S rDNA (red) probes [a] and with telomeric sequences probe (green) [b]. The smaller images on the left refer to results in normal metaphases.

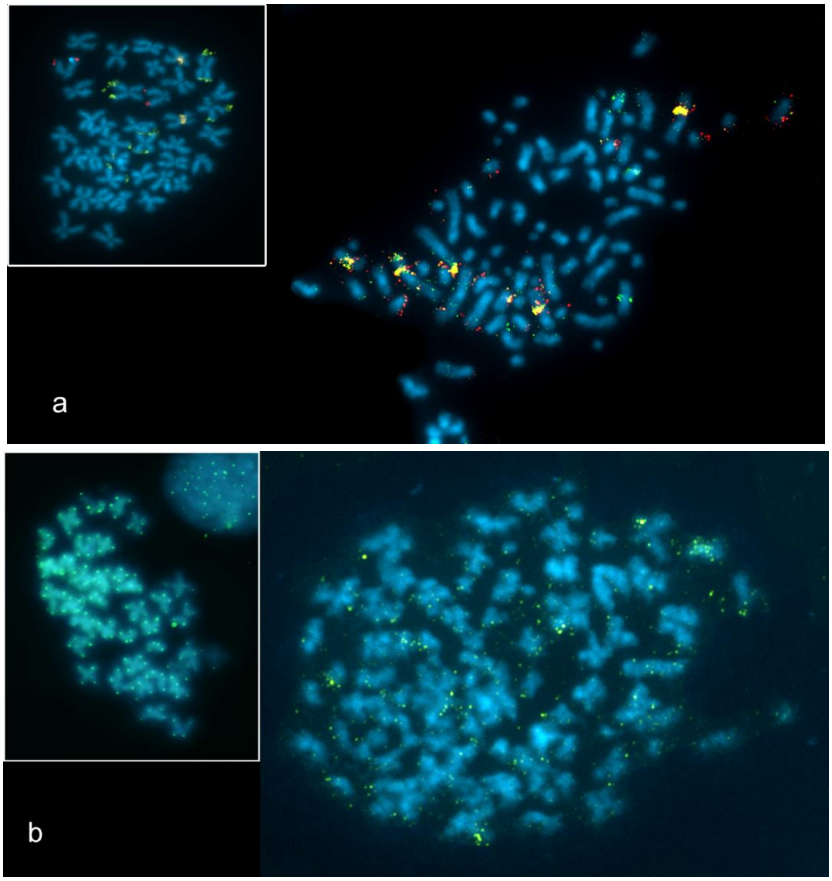
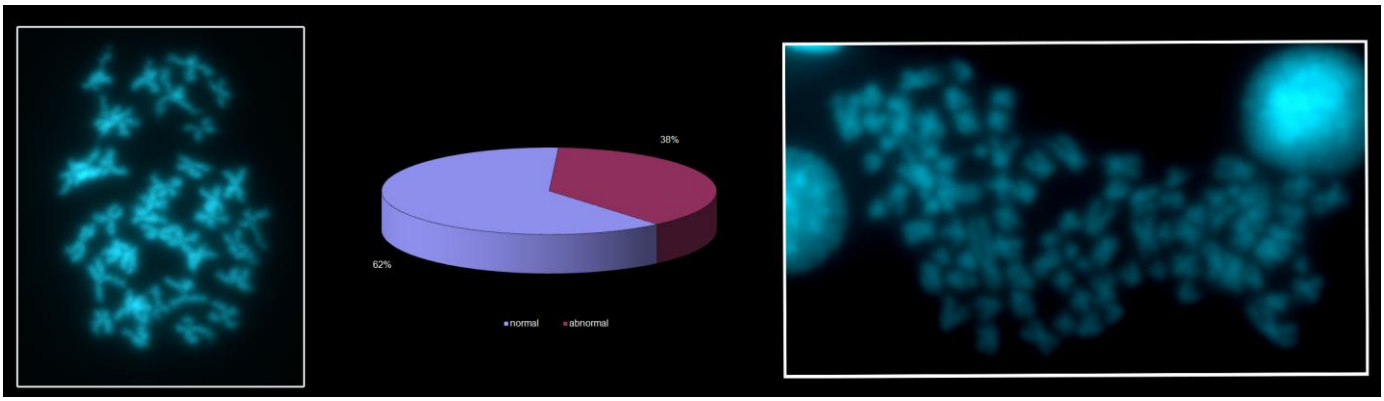


Figure C. Mitotic chromosome spreads from the gill of *Venus verrucosa* coloured with DAPI - normal metaphase, 2n =38 [left] and metaphase from a cell with an abnormal chromosome number, 2n = ~90 [right]. Abnormal cells showed between 2n=46 and 2n=128, with modal number of 2n=84. Hyperploydy was characterized by both the presence of microchromosomes and polyploidy of normal karyotype.



Chapter 5. DNA extractions from fresh, frozen and cooked samples of several species of venerid clams – a new approach to identification methods?

Introduction

Food authenticity is presently a subject of great concern to food authorities, as the incorrect labelling of foodstuffs can represent a commercial fraud. The implication of misleading labelling can be much more important concerning the presence of potentially allergenic foods.

International labelling standards applicable to food products are set down by the *Codex Alimentarius Food Labelling Committee*, part of the FAO (Food and Agriculture Organization of the United Nations) and the World Health Organization. Moreover, it is a legal requirement in Europe (Council Regulation nº 104/2000, 17th December 1999), for the commercialisation of fishery products to show on the label the scientific and commercial name of the species, because even species belonging to different genera can be designated by the same commercial denomination, the production method and the capture zone.

Concerning clams, taste and meat texture, as well as extended "shelf-life", cause that some species, like *Ruditapes decussatus*, have a market value that can reach 4-fold the price of any other clam species. When the clams are sold alive, they can be identified by morphological criteria, such as siphon shape and location as well as shell characteristics (shape, colour, and striations). Unfortunately, processed clams (frozen and cooked) are mostly commercialized without their shells. In this context, commercial fraud is relatively easy, because it is not possible to carry out the assignation of these products to a particular species using morphological traits. Consumers and the fishing industry are concerned about this question, and they demand the correct labelling of seafood products and transparency in the international trade. In order to contribute to solve this problem, we set as our objective to find biological markers within the animals themselves that would allow us to distinguish between the most common commercial species.

The need to support food labelling has provided the development of analytical techniques for the analysis of food ingredients. In the last years, several methods based on polymerase chain reaction (PCR) have been proposed as useful means for species'

identification in food. The use of the nucleic-acid-based analytical method can present a solution for this problem because DNA is a very stable and long-lived biological molecule that is present in all tissues of all organisms. Moreover, PCR has simplified earlier molecular methods that were complicated and time-consuming.

Differences in the length, both in the non transcribed spacer (NTS) of the 5S rRNA genes and in the internal transcribed spacer (ITS) region of the major rRNA genes, were chosen. The method used was the extraction of the DNA, followed by PCR amplification and electrophoresis. This is an innovative methodology because previous works have used PCR associated with complementary techniques such as RFLP – restriction fragments length polymorphism and SSCP – single strand conformation polymorphism, making it more time and resource-consuming. We propose a simplified and more direct methodology, using the variations not in the coding sequences but in their spacers.

Markers based on ITS are one of the most employed in this field (for example, Fernández *et al.* 2001, López-Piñon *et al.* 2002, Freire *et al.* 2011), probably due to the possibility of using universal primers anchored in conserved coding regions. The ITS sequences show more variability than their flanking coding regions (Hillis & Dixon, 1991), displaying polymorphism even among related species, and they are useful to distinguish between different taxa.

The differences in 5S rDNA were only used in two previous works to date: Fernández-Tajes and Méndez (2007) used it in the identification of species of the families Pharidae and Solenidae and Hurtado *et al.* (2011) used it, along with other sequences, to distinguish between *Ruditapes decussatus* and *Ruditapes philippinarum*.

Material and methods

Figure 1 shows the packages of the two brands of pre-cooked canned clams. As can be seen, the species are not identified in the package, so we decided to compare samples from these brands to previously identified control samples from *Ruditapes decussatus,*

Ruditapes philippinarum, Venerupis aurea, Venerupis (= Tapes) rhomboides, Venerupis corrugata and *Dosinia exoleta*.

As for the frozen samples, a brand commercialized without shell was labelled as *Ruditapes philippinarum*. Samples were compared to all control individuals in order to ensure the correct identification.

Total genomic DNA was isolated from ethanol-preserved adductor muscles following Winnepenninckx *et al.* (1993) with slight modifications. Tissue was homogenized in cetyltrimethyl ammonium bromide buffer and digested overnight with proteinase K at 60°C. DNA was extracted with chloroform/Isoamyl alcohol (24/1).

Amplifications were performed in 20 µl of a solution containing 50 ng DNA, 1xPCR buffer, 50 M each dNTP, 2.5 mM $MgCl_2$, 1 M each primer and 1 U BIOTAQ DNA polymerase (Bioline). Universal primers were used to amplify the whole ITS region, including a part of 18S rDNA upstream, the entire ITS1, 5.8S rDNA, ITS2 and part of 28S downstream (White *et al*. 1990). For the 5S rDNA amplification, primers were designed from the sequence of the 5S rRNA of *Mytilus edulis* (Fang *et al*. 1982). After 5 min denaturation at 95 °C, 30 cycles of amplification were performed with 30 sec denaturation at 95 °C, 30 sec annealing at 48 °C and extension at 72 °C for 30 sec for the ITS and 60 sec for 5S rDNA . A final extension step of 7 min at 72 °C was applied. All reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems) and PCR products were examined by electrophoresis on a 2% agarose gel.

Results

We were able to extract DNA from all samples: control, frozen and cooked samples. This was proven empirically by the clear observation of precipitated DNA consequent of the presence of ethanol in the solution. Centrifugation of these solutions led to the formation of relatively large pellets in most of the samples. Electrophoresis of the ressuspended pellets of the control samples showed large bands that proved there was extraction of the DNA (data not shown), but we had no evidence on the quality of this DNA.

Figure 2 shows the electrophoresis gel with the 5S rDNA amplified molecules, for the cooked and the frozen samples.

The frozen samples showed being well labelled, as they correspond to the results expected for *Ruditapes philippinarum*. In figure 2, there is a blue highlighting circle for the 5S amplified molecule, which correspond to the same molecular weight as the amplified molecule for the standard specific sample.

As for the cooked samples we found that no DNA was detected in the electrophoresis after the amplification, even though there was visible precipitation of DNA after the extraction. Repeated PCR experiments, using a higher amount of DNA for the amplification also proved unsuccessful.

Discussion

Our experiments show that this is a viable, safe and accurate method for the control of frozen samples of clams. There doesn't seem to be any loss in DNA structure as a result of the processing method, and in this way, gene amplification and analysis of length differences in variable sequences can be used as a way to identify several species.

On the other hand, the fact that the DNA of the cooked samples didn't amplify lead us to think that it had been damaged by the processing of the samples.

Canning implies the hermetical enclosing of the food and the treatment of the full recipient with high temperatures to inactivate enzymes and microorganisms. Moreover, in the case of clams, there is a previous boiling of the animals, to make them ready to eat.

The sterilization process, through autoclave, is made for 30 to 45 minutes at temperatures between 115 and 121 °C. This temperature is calculated to eliminate the most heat-resistant pathogen – the bacteria *Clostridium botulinum*. This is a common organism in the natural habitat of marine species, it causes botulism, which is a serious and potentially deadly disease, its spores are resistant to very high temperatures and it can grow in anaerobiosis.

Even if we consider the boiling process to occur at temperatures around 100 °C, DNA molecules would be denaturated but, upon returning to room temperature, they would re-anneal and the DNA strands would be intact and functional.

On the other hand, when we reach autoclave temperatures, heat can induce DNA depurination, followed by cleavage of the nearby phosphodiester (Lindahl, 1993). It was reported that DNA in dried seeds could not be retrieved after just one hour of heating at 150°C or above (Chalfoun & Tuross, 1999). Studies have shown that this degradation of DNA produces fragments of about 200 bp (Quinteiro *et al.* 1998).

Oxygen is another factor that influences DNA degradation. It is involved in cellular DNA degradation in dried tissues (Matsuo *et al.* 1995). This is not our major concern, as a good production method should reduce the amount of air inside the cans as a method of conservation. Nevertheless, we have to stay open to the possibility of deficient production methodology.

Oxygen is also involved in the heat-induced formation of singlet oxygen ($^1O_2$), which leads to the appearance of H_2O_2, a substance that causes DNA damage (Bruskov *et al.* 2002).

Both types of DNA damage (base release and oxidative damage to the bases) result in a blockage or a false result in DNA amplification (Zhang & Wu, 2005).

Nevertheless, Freire *et al.* (2011) managed to extract and amplify the ITS region from canned samples of cockles from the genus *Cerastoderma* (Cardidae family). The extracted DNA was degraded to small fragments, but was still appropriate to be used in PCR amplifications.

Espiñeira *et al.* (2009) also present results on canned samples of the family Pharidae, in experiments of species identification through amplification of mitochondrial 18S rRNA sequences.

In both cases, the amplified sequences were under 200bp (185bp for *Cerastoderma edulis* ITS sequence [Freire *et al.* 2011] and between 148 and 196bp for the mitochondrial 18S rRNA [Espiñeira *et al.* 2009]).

This may explain why we have not been able to amplify our target sequences, even following the same methodology as the referred works. It appears that the set of available primers amplifies mostly regions of much greater length. For example see, table 1 with data on the length of 5S rDNA in our species.

Table 1. Amplified sequence length for the 5S rDNA in the studied species

Species name	**Sequence length (bp)**	**Genbank reference**
Ruditapes decussatus	593	HQ634137.1
R. philippinarum	526	HQ634138.1
Venerupis aurea	285	AJ583104.1
V. rhomboides	511	AJ583107.1
V. corrugata	580	AJ583108.1

Note: there is no published data for nuclear gene sequences in Dosinia exoleta

In what concerns ITS, published sequences are only available for *R. decussatus* and *R. philippinarum*, and show that the regions amplified by our primers are, respectively, 1259bp and 1298bp long (Genbank HQ634139.1 and EF035086.1). These data is supported by Fernández *et al.* (2001), that identify the region amplified by another set of primers that was composed by ITS1-5.8S rDNA-ITS2 as a fragment of approximately 1100bp in *R. decussatus, R. philippinarum* and *Venerupis pullastra* (now *V. corrugata*).

According to Hurtado *et al.* 2011, partial ITS sequences for these species are also too large for amplification in the canned samples (see following table for details).

Table 2. Amplified sequence length for ITS1 and ITS2 in *Ruditapes decussatus* and *R. philippinarum*

Species name	**ITS 1 (bp)**	**ITS 2 (bp)**
Ruditapes decussatus	797	482
R. philippinarum	698	565

Altogether, we are lead to conclude that these are not the appropriate genes for species identification in Veneridae, in what concerns samples that have been canned or otherwise processed through high temperatures.

Fernández *et al.* (2000; 2002) present the possibility of the use of α-actins as amplifiable sequences in clams (*Ruditapes decussatus, R. philippinarum* and *Venerupis pullastra* [now *V. corrugata*]). The first work uses primers for a ~500bp fragment, which is of no use for our experiments, but the later work, with a new set of primers presents a partial fragment of 150bp present in *R. decussatus* and *V. pullastra*. Unfortunately, as far as we

can conclude, the amplified sequence has the same length in both species, and thus is not helpful in the methodology we are trying to apply. A more detailed analysis into the actins, shows that these are highly conserved sequences (Kovaleva *et al.* 2005) and so unfit for comparisons between closely related species using this technique.

We intend to pursue further studies in order to discover other sequences that are short enough to be amplified in canned samples and at the same time have enough inter-specific variability to be used as markers. We also intend to explore other possible primers that allow us to amplify shorter fragments of the target sequences.

Finally, we intend to compare our methodology to the one used by Freire *et al.* (2011), as they refer preliminary results of amplifications of 5S rDNA, which indicates that fragments of up to 550 bp could be amplified from canned samples.

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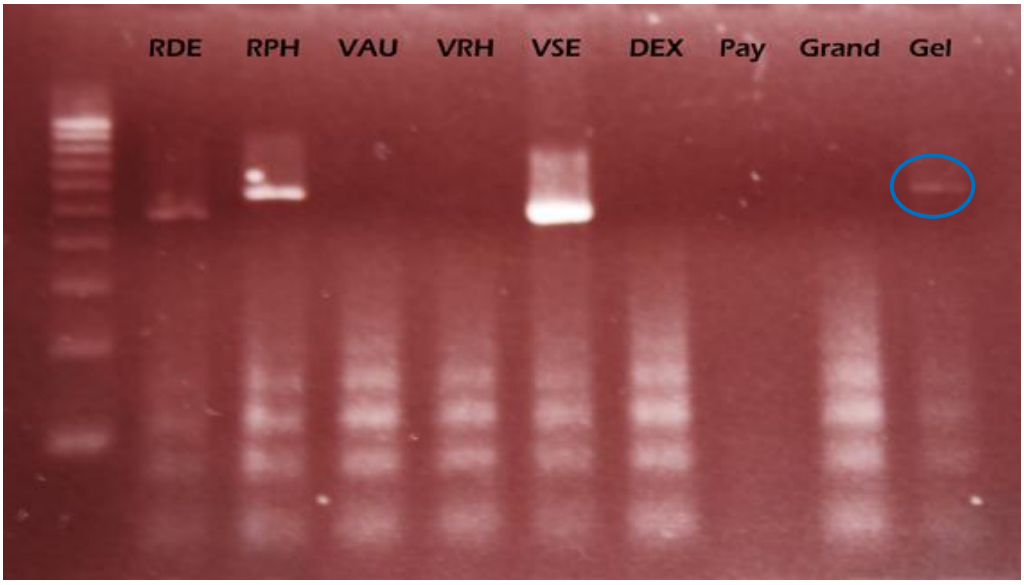
Figures

Fig. 1. Packages of the two brands of pre-cooked canned clams we analysed.

Fig. 2. Agarose gels (2%) showing the amplified PCR products of the 5S rDNA of the cooked and frozen samples. *Ruditapes decussatus* [RDE], *Ruditapes philippinarum* [RPH], *Venerupis aurea* [VAU], *Venerupis rhomboides* [VRH], *Venerupis corrugata* [VSE], *Dosinia exoleta* [DEX], commercial cooked sample 1 [Pay], commercial cooked sample 2 [Grand] and frozen sample [Gel] (blue circle).



Chapter 6. Inter-specific hybridization: Ruditapes decussatus and R. philippinarum

Experimental design

In order to study the hybridization process between the species *Ruditapes decussatus* and *R. philippinarum,* we designed a breeding program that involved the following steps:

1. **Induce spawning in both species.** We tried preferently to cross females of *R. decussatus* and male *R. philippinarum*. The reason we chose this gender separation is the fact that Hurtado *et al.* 2011 found specific *R. decussatus* 16S mitochondrial rDNA sequences in the hybrid individuals.
2. **Maintain a nursery of the larvae and check by PCR (5S and NTS) if the crosses were the ones intended.**
3. **Create cytogenetic spreads of whole larvae.**
4. **Perform FISH of the marker genes in the larvae spreads.**
5. **Maintain in laboratory optimal conditions the hybrid individuals until reproductive maturity**, in order to assess fertility.

Previous articles had stated that the peak in gamete emission periods for these species were, respectively, from April onwards for *R. decussatus* (Delgado and Pérez-Camacho, 2007) and from April to September for *R. philippinarum* (Rodríguez- Moscoso *et al.,* 1992). For this reason we conducted the experiment during the months of May and June.

Following the work by Matias *et al.* (2009), we set a series of tanks for a broodstock conditioning period. During this period, the animals were kept with *ad libitum* feeding, natural photoperiod and water temperatures ranging between 20-22 °C. These tanks were in open circuit, had about 25 L volume, with no substrate and were clear bottomed or white.

After 3 weeks, individuals were sampled and gonadal cell spreads, following Costelo and Hendley (1971), were performed to determine if there was a majority of mature gametes present.

At this point, we induced spawning. Joaquim, Matias & Moreno (2008) identify as determining factors for spawning: temperature, salinity, light, mechanical stimulation, presence of gametes in the water and endogenous neurosecretions.

We chose thermal shock as the induction method. Through this method we submit the animals to alternate temperatures, with an average difference of 10 °C. We started with the higher temperature, raising the water temperature to 25 °C, so the animals would increase their filtration water intake. After about 30 min, the water was replaced with water at 15 °C, for another 30 min, restarting this cycle the number of needed times until spawning began. For this stage the animals were placed in trays, with about 10 cm of water column, and in a black bottom, to facilitate the visualization of gamete emission.

Complementary, we left the animals outside of the water for about 35 min prior to the thermal shock induction to increase the stimuli effect. The intensity of the light was also increased during the induction period.

Development of the work

Spawning of the female *R. decussatus* occurred with no incident after three thermal cycles. As a control group, a small sample of male *R. decussatus* was subject to the same treatment and also spawned.

Regardless all our efforts, none of the individuals of *Ruditapes philippinarum* emitted any gametes. We repeated the experience in July-August, without any results again. The spawning of females *R. philippinarum,* following the same protocol did not work either.

As the assumption for this study was the interaction between the gametes of both species, we tried using the sperm from *R. decussatus* control group to stimulate the spawning of male *R. philippinarum*, but this had no effect.

For this reason, the following steps of our experimental design did not come into place.

Discussion

Our research did not find alternative conditions for induction of spawning in *Ruditapes philippinarum*.

Previous studies have shown variations between geographical populations of the same species in what concerns the response of individuals to conditioning (Iglesias *et al.* 1996; Avendaño & Le Pennec, 1997). In this way, even though our samples were from the same location as described by the earlier studies (Delgado & Pérez-Camacho, 2007), differences in the individuals themselves may account for the lack of response to the induction stimuli.

On the other hand, this may also be an indication that simultaneous spawning under the natural conditions is not so frequent, and these populations require different temperatures or salinity for spawning. This seems contradictory to the evidences of hybridization, because it would largely reduce the chances for inter-specific fertilization.

A more comprehensive experimental design is needed to determine the correct conditions to induce spawning in these populations of *Ruditapes philippinarum*.

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Chapter 7. Concluding remarks

As a conclusion, we would like to relate the several chapters in this thesis. We look upon chapters 2 and 3 as one big description of the cytogenetics of Venerid species, and only when seen as a single work they are understood as an insight on the phylogeny of these species.

Moreover, this complete analysis of the most important commercial clam species of the Iberian Peninsula, meets the objective we had defined of creating a set of markers that would allow us to identify clam species based on their genetic characteristics. We are now able to, through the hybridization pattern on the karyotype, identify specimens of each of the species.

The same objective was fulfilled in chapter 5, in this case based not on the chromosomal location of the genes but on the identification of the PCR amplified fragments' size in electrophoresis gel. This second method came as way to respond to the need of analysing samples in situations when we can't obtain metaphases and/or the samples are sold separate from their shells, fresh or frozen. The advantage of the presented method over previous approaches is its simplicity because it is based only on the differences between fragment sizes. Nevertheless, we still need to improve our methodology in order to apply it successfully to samples that have undergone high temperature processing, such as canned/cooked samples.

Having established this standard for the karyotypes on our target species, we were able to develop a specifically applied study on the field of genotoxicity and chromosomal alterations in two of these species, as presented on chapter 4. The knowledge of not only the specific chromosome number but also of the number of normal hybridization signals for each of the marker sequences, allowed us to see that the mutation process, whichever it was, induced the appearance of new chromosomes and that these were carriers of specific sequences (and not just accumulation of nontranscribed heterochromatic DNA).

However, chapters 2/3 and 4 left a series of open hypothesis that still need proving. On the one hand, the hypothesis of an inversion justifying the differences in H3 histone gene location between two species of the same genus (*Venerupis rhomboides* and *Venerupis corrugata*), the hypothesis of an isochromosome giving origin to *Venerupis aurea* chromosome 2 or the similarity in the hybridization pattern for 5S rRNA genes in all but two of the species, with the possibility of an inversion explaining the difference in these two. On the other hand, the high number and lack of definition of some of the hybridization signals on the metaphases of the individuals described in chapter 4, that didn't allow us to establish the origin of the supernumerary chromosomes.

Armed with the tools we developed in the previous chapters, we were now in conditions of designing a study concerning the hybridization phenomenon between *Ruditapes decussatus* and *Ruditapes philippinarum*. Once again, knowing the specific characteristics, on a cytogenetic and a molecular level, of both parental species we could study the hybrids produced and describe them, as well as their biological features (such as viability, fitness and fertility).

This would then complete the objectives we had set for this thesis. Unfortunately, as referred earlier, we were unable to pursue this objective to end because we could not induce spawning in one of the parental species.

Altogether, we see this work not as closed set of experiments but as the laying of foundations for further investigations in this area.

We leave a series of open questions that only the time restrictions and technical difficulties unabled us to pursue.

We believe that it is fundamental to develop further studies to understand the causes of the cytogenetical alterations described in chapter 4, as this may be an alarming sign of contamination and possible public health hazard.

In the same way, the physical mapping of the rDNA and H3 histone sequences seems to be a promising work for other sequences localization, as the H1 histone gene, for example. A larger number of sequences would allow us to identify even more chromosome pairs and to look in deeper detail into the relationships between these species.

In sum, we believe we have accomplished most of the objectives set for this thesis in what concerns the characterization and definition of genetic markers for the commercially important species of clams and we are now looking forward to continue developing our work in the field of cytogenetics.

Acknowledgements

Finally, the longly expected, but still stressful moment of my PhD has arrived. On the one hand the relief of finishing the thesis, on the other hand the uncertainty about the future. Either way, I wouldn't have been able to reach this point without the help, support and friendship of some people I would like to acknowledge now.

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I truly hope I have made you proud.

I'm doing it for music

I'm doing it for love

I'm doing it for everyone around me

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