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Banding for Chromosomal Identification in Bivalves: A 20-year History

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ABSTRACT

Bivalves include some of the world's best known invertebrates and several species are very economically important for aquaculture production. This is reflected in the increasing amount of data on chromosomal characterization in this group compared to other aquatic invertebrates. This paper presents a review on banding for chromosomal identification in bivalves during the last two decades, which highlights the continuously development in the last years of the banding technique of fluorescence in situ hybridization (FISH), which represents nowadays more than three quarts of all chromosomal banding studies in bivalves. Our intention was to provide an exhaustive bibliographic review useful not only to marine cytogeneticists, but also to marine biologists, marine taxonomists and also aquaculture producers, among others. An overview of the main application of chromosomal banding, both from a more fundamental evolutionary point of view, as well as from a more practical production point on view are also presented.

Keywords: bivalve, chromosome, chromosomal banding
Abbreviations: FISH, Fluorescent *in Situ* Hybridization; **NOR**, Nucleolar Organizer Region

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INTRODUCTION

Due to the economic and ecological importance of a large number of marine bivalve species, genetic investigations are of special interest. The identification of structural chromosomal features can be very useful in gene mapping, hybrid breeding or stock conservation programs.

According to White (1978), it is fairly certain that in many species of animals various types of chromosomal rearrangements are directly adaptive to certain types of habitats and ecological niches. Cytogenetic analyses are a useful tool for phylogenetic comparisons, chromosomal rearrangements and karyotype differentiation can be important mechanisms for reproductive isolation and speciation in some taxa (White 1978; King 1993).

The first studies, on bivalve chromosomes, mainly concerned data on chromosome number and gross morphology. Later, the application of "classical" cytogenetic banding techniques allowed the identification of whole chromosome or chromosomal specific parts (see for review Thiriot-Quiévreux 2002). The "classical" banding can be longitudinal, producing alternating light and dark bands that appear along the length of the chromosome (e.g. G- and R-banding), or, specifical banding where certain chromosome subregions are highlighted (e.g. NOR-banding). In the last 20 years, the development of molecular banding techniques, has allowed the fine characterization of individual chromosomes.

In this paper, we intend to provide an up-to-date review on banding for chromosomal identification in marine bivalves over the last 20 years. By complementing the previous reviews by Patterson 1969 (mostly on chromosome number), Nakamura 1985 and Thiriot-Quiévreux 2002 it reflects the progress of the different techniques used through out the time for bivalve chromosome studies, from the simple karyotpe formulae description to the more recent use of molecular cytogenetic techniques. We also present an overview of the main applications of banding for chromosomal identification in this group both from a fundamental evolutionary point of view but also from a more practical one (e.g. aquaculture production).

"Classical" cytogenetic banding

Chromosomal Ag-NORs (Nucleolar Organizer Regions) can be used as characters for inferring phylogenetic relationships (e.g. Amemiya and Gold 1990). The silver staining method, which detects NORs that were active at the precedent interphase (e.g. Howell 1977), has been applied to 31 bivalve species belonging to eight different families (see **Table 1** for details and references). Most of the Ag-

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Invited Mini-Review

NORs banding studies have been performed in the Mytilidae, Ostreidae and Pectinidae families (Insua *et al*. 2001, **Table 1**).

A substantial proportion of the higher eukaryote genome consists of constitutive heterochromatin. This genomic fraction includes, among other repetitive sequences, satellite DNAs (very highly repetitive, tandemly repeated sequences) that usually reside in the pericentric regions of chromosomes (D'Aiuto *et al*. 1997; Chaves *et al*. 2000). Sequence analysis of these repeats suggests that the sequences are rapidly evolving, and hence they are valuable as evolutionary markers (Saffery *et al*. 1999; Chaves *et al*. 2000). The evolutionary significance of the heterochromatin has been previously discussed in vertebrates (e.g. Chaves *et al*. 2003, 2004). Constitutive heterochromatin regions (C-bands) banding was applied to 16 species (see **Table 1** for details and references), and similarly to the Ag-NOR banding mainly in Mytilidae, Ostreidae and Pectinidae families, which can be easily justified by the large economical relevance of these three families. The existence of constitutive heterochromatin in centromeres was generally common in oysters, but not in mussels or scallops.

The comparison of Ag-NORs and C-band location has allowed the analysis of the cytotaxonomical relationships within certain families of marine bivalves such as the Mytilidae (Martinez-Lage *et al*. 1995), and the Ostreidae, both for the Ostreinae flat oysters' sub-family (Leitão *et al*. 2002) and also the Crassostreinae cupped oysters' sub-family (Leitão *et al*. 1999a). Nevertheless these two classical cytogenetic banding presents several weaknesses, for instance silver staining detects only the transcriptional active NORs in the precedent interphase (e.g. Howell 1977). Moreover, the number and location of NOR is also often variable not allowing an accurate location of the major rRNA genes (Wang *et al*. 2004). Additionally, and although they allow the identification in optimal conditions of some of the chromosomal pairs in the karyotype, they do not allow the individual identification of all chromosomal pairs, not allowing in consequence the establishment of precise karyotypes.

The G-banding technique can be defined as a system of alternating dark and light bands throughout the length of the euchromatic parts of the chromosome (Sumner 1990), and allows the identification of each individual chromosome pair. Among marine bivalves, a first attempt on the application of the G-banding technique was performed by Rodriguez-Romero in 1979 in *Crassostrea virginica*, however G-banding patterns were only obtained latter in the mussel *Mytilus galloprovincialis* (Martinez-Lage *et al*. 1994) and in the cupped oysters *C. virginica* (Leitão *et al*. 1999b), *C. angulata* (Leitão *et al*. 1999b) and *C. gigas* (Leitão *et al*. 1999b, 2001). However, this technique presents some disadvantages such as limited reproducibility, large time investment required, and the fact that the banding is often lost during the Fluorescent *in Situ* Hybridization (FISH) procedure.

Molecular cytogenetic banding

Reliable techniques were then a major requirement for genetic research in bivalves. Recently, the molecular cytogenetic technique of *in situ* digestion with restriction endonucleases (REs), which cleave DNA at specific target sequences, producing consistent banding patterns in fixed mammalian and insect chromosomes, has already been successfully applied to eight marine bivalve species belonging to the Mytilidae, Ostreidae, Pectinidae, Cardiidae and Veneridae families (see **Table 1** for details and references). In all cases, specific longitudinal chromosomal banding patterns were obtained after digestion with REs, allowing the individual identification of all chromosome pairs as well as the establishment of precise karyotypes. This technique has been applied in chromosomal evolution studies as for example in the Ostreidae family, where it supported the closest relationships of the oyster species studied within Ostreinae and Crassostreinae than between the two subfamilies.

RE banding, as it has been recently shown in mammals, presents the major advantage of being compatible with FISH (Chaves *et al*. 2002). The use of this chromosomal banding technique can then also provide a fundamental step in genome mapping in bivalve families, since chromosome banding with restriction enzymes will facilitate gene mapping. This technique has been increasingly applied to bivalve chromosomal studies; indeed until 2002 (see for review Thiriot-Quiévreux 2002) it had been applied in only one of the 48 studied species, the mussel *M. galloprovincialis* (Martinez-Lage *et al*. 1994). However, in the last seven years, it has already been successfully applied to seven other bivalve species (see **Table 1** for details and references).

Fluorescent *in Situ* Hybridization (FISH) studies

FISH is a rapid and reliable technique for chromosomal investigations that is presently used for a large diversity of molecular cytogenetic studies, such as chromosome identification, gene mapping, localization of gene expression, and analysis on chromosome rearrangements in a wide variety of genomes. This molecular cytogenetic method has been continuously developed in the last years and different kinds of fluorescently labeled probes have been introduced to optimize the detection of DNA and RNA (Lakatosova and Holeckova 2007). In bivalves, it represents the great majority of recent studies in chromosomal banding. Indeed, between 1992-2002 (see for review Thiriot-Quiévreux 2002) FISH banding represented 32% of all chromosomal banding studies in marine bivalves, this percentage has increased to 77% from 2002 until now (see **Table 1** for details and references). In total, this technique has already been successfully applied to 31 bivalve species (see **Table 1** for details and references), with probes such as telomeric sequences, satellite DNA, histones genes, microsatellites and ribosomal RNA genes (rDNA probes).

The major and minor rRNA genes are two gene families of ribosomal RNA genes which are fairly independent of each other and often structured into separate loci on one or different chromosomes (e.g. Liu *et al*. 2002). Both genes are present in large numbers of tandem repeats, making them ideal targets for FISH (Wang *et al*. 2004). In bivalves, the major (18S-5.8S-28S) ribosomal RNA genes (rDNA), which correspond to NORs, have already been mapped by FISH in (see **Table 1** for details and references): a) five Mytilidae; b) six Ostreidae; c) three Pectinidae; d) two Mactridae; e) one Psammobidae; f) one Veneridae; g) two Pharidae; h) one Donacidae and i) one Tellinidae species. The physical location of the minor (5S) ribosomal RNA genes (rDNA) has been determined in the mussel *M. galloprovincialis* and in the two cupped oyster species *C. angulata* and *C. virginica.* Both major and minor ribosomal RNA genes have been assigned to the chromosomes of the mussel *M. edulis*, the pectinids *Aequipecten opercularis*, *Chlamys farreri*, *Patinopecten yessoensis*, *Argopecten irradians*, *Hinnites distortus*, *Pecten maximus* and *Mimachlamys varia*, and in the cockle *Cerastoderma edule* (see **Table 1** for details and references).

The use of FISH with ribosomal probes has proven useful to infer phylogenetic relationships in several bivalve species. For example, in Pectinidae, Wang and Guo (2004) found unexpectedly that *A. irradians* with a haploid number of 16, had three rRNA bearing chromosomes, whilst *C. farreri* with an haploid number of 19 had only one rRNA bearing chromosomes, suggesting that this last karyotype would be plesiomorphic. In view of these results, the authors proposed that the ancestral karyotype of the Pectinidae, which have a majority haploid number of 19, would have originated through duplication from an ancestral bivalve with an haploid number of 10 (number that is the most common in the Ostreidae family). Wang and Guo (2004) also suggested that species with haploid numbers between 13-16 would represent triploid states.

Table 1 Published data on banding for chromosomal identification in bivalves. 2n: diploid number, NORs: silver staining, C: C-banding, Q: Q-banding, G: G-banding, N: N-banding, Flrc: fluorochrome staining, RE: restriction enzyme banding, FISH: Fluorescent *In Situ* Hybridization, rDNA: ribosomal RNA genes (rDNA probes).

Family/Species	2n	Banding technique								References
		NORs	C	Q	G	N	Flrc	RE	FISH	
Mytilidae										
Brachidontes minimus	28	x					x		rDNA (major)	Vitturi *et al.* 2000
Brachidontes rodriguezi	32	x					x		rDNA (major)	Torreiro *et al.* 1999
Mytilus californianus	28	x					x			Martinez-Lage *et al.* 1997
									rDNA (major)	Gonzalez-Tizon *et al.* 2000
									3 satellite DNA	Martinez-Lage *et al.* 2002
Mytilus edulis	28	x								Cornet 1993
		x								Insua *et al.* 1994
		x	x				x			Martinez-Lage *et al.* 1995
									rDNA (minor and major)	Insua *et al.* 2001
		x	x				x			Martinez-Lage *et al.* 1996
									3 satellite DNA	Martinez-Lage *et al.* 2002
Mytilus galloprovincialis	28				x					Mendez *et al*. 1990
		x								Insua *et al.* 1994
			x		x		x	x		Martinez-Lage *et al.* 1994
		x								Martinez-Exposito *et al*. 1994
		x	x				x			Martinez-Lage *et al.* 1995
		x	x				x			Martinez-Lage *et al.* 1996
			x							Pasantes *et al.* 1996
		x	x			x	x		rDNA and Telomeric	Martinez-Exposito *et al*. 1997
		x							rDNA (major)	Insua and Mendez 1998
									rDNA (minor)	Insua *et al.* 2001
									3 satellite DNA	Martinez-Lage *et al*. 2002
									Telomeric	Phlol *et al*. 2002
									rDNA (minor) and histone H1	Eirin-Lopez *et al.* 2004
Mytilus trossulus	28	x								Insua *et al.* 1994
		x	x				x			Martinez-Lage *et al.* 1995
		x	x				x			Martinez-Lage *et al.* 1996
		x								Martinez-Lage *et al.* 1997
									rDNA (major)	Gonzalez-Tizon *et al.* 2000
									3 satellite DNA	Martinez-Lage *et al.* 2002
Perna viridis	30	x	x							Iqbal *et al*. 2008
Ostreidae										
Subfamily Ostreinae										
Ostrea angasi	20	x	x							Li and Hanvenhand 1997
Ostrea denselamellosa	20	x	x							Insua and Thiriot-Quiévreux 1991
Ostrea edulis	20	x								Thiriot-Quiévreux and Insua 1992
								x		Leitão *et al.* 2004
Ostrea puelchana	20	x								Insua and Thiriot-Quiévreux 1993
Ostrea conchaphila	20	x	x							Leitão *et al.* 2002
								x		Leitão *et al.* 2004
Tiostrea chilensis	20	x								Ladron de Guevara *et al.* 1994
Subfamily Crassostreinae										
Crassostrea angulata	20	x								Leitão *et al.* 1999a
					x					Leitão *et al.* 1999b
								x		Leitão *et al.* 2004, 2007
		x					x		rDNA (major)	Cross *et al.* 2003
			x					x	GATA, Telomeric and rDNA (minor)	Cross *et al.* 2005
Crassostrea ariakensis	20	x								Leitão *et al.* 1999a
									rDNA (major)	Wang *et al.* 2004
Crassostrea gasar	20	x								Leitão *et al.* 1999a
Crassostrea gigas	20	x								Thiriot-Quiévreux and Insua 1992
		x								Leitão *et al.* 1999a
					x					Leitão *et al.* 1999b, 2001
								x		Leitão *et al.* 2004, 2007
									Satellite DNA	Clabby *et al.* 1996
									Telomeric	Guo and Allen 1997
									rDNA (major)	Xu *et al.* 2001
									Centromeric Satellite sequence	Wang *et al.* 2001
									rDNA (major)	Wang *et al.* 2004
								x		Bouilly *et al.* 2005
									GGAT, GT and TA	Bouilly *et al.* 2008
Crassostrea sikamea	20	x								Leitão *et al.* 1999a
Crassostrea plicatula	20								rDNA (major)	Wang *et al.* 2004
Crassostrea rhizophorae	20								rDNA (major)	Wang *et al.* 2004
									Telomeric	Wang and Guo 2001
Crassostrea virginica	20	x								Leitão *et al.* 1999a
					x					Leitão *et al.* 1999b

Table 1 (Cont.)

Family/Species	2n	Banding technique								References
		NORs	C	Q	G	N	Flrc	RE	FISH	
									rDNA (major)	Zhang *et al.* 1999
									bacteriophage P1clones	Wang *et al.* 2005a
									rDNA (ITS1 and ITS2)	Xu *et al.* 2001
									Telomeric	Wang and Guo 2001
									rDNA (major)	Wang *et al.* 2004
									rDNA (minor)	Wang *et al.* 2005b
Saccostrea commercialis	20	x								Leitão *et al.* 1999a
Pectinidae										
Adamussium colbecki	38	x		x			x	x	rDNA (major)	Odierna *et al.* 2006
Aequipecten opercularis	26	x	x						rDNA (minor and major)	Insua *et al.* 1998
Argopecten irradians	32								rDNA (minor and major)	Wang and Guo 2004
									Histone H3	Zhang *et al.* 2007a
			x				x		rDNA (major) and histone H3	Zhang *et al.* 2007b
		x	x				x		rDNA (minor and major) and telomeric	Huang *et a.l* 2007b
Argopecten purpuratus	32	x		x			x			Gajardo *et al.* 2002
Chlamys farreri	38								rDNA (minor and major)	Wang and Guo 2004b
									Histone H3	Zhang *et al.* 2007a
									19 fosmid clones	Zhang *et al.* 2008
Chlamys nobilis	32								Histone H3	Zhang *et al.* 2007a
Hinnites distortus	38	x					x		rDNA (minor and major)	López-Piñón *et al.* 2005
Mimachlamys varia	38								rDNA (minor and major)	Insua *et al.* 2006
Nodipecten nodosus	38	x	x							Pauls and Afonso 2000
Patinopecten yessoensis							x		rDNA (minor and major) and Telomeric	Huang *et al.* 2007a
									Histone H3	Zhang *et al.* 2007a
			x				x		rDNA (major) and histone H3	Zhang *et al.* 2007b
Pecten maximus	38								rDNA (minor and major)	Insua *et al.* 2006
Unionidae										
Anodonta anatina	38	x					x			Woznicki and Jankun 2004
Hyriopsis cumingii	38		x							Wang *et al.* 2000
Cardiidae										
Cerastoderma edule	38		x						rDNA (minor and major)	Insua *et al.* 1999
								x		Leitão *et al.* 2006
Cerastoderma glaucum	38	x								Thiriot-Quiévreux and Wolowicz 1996
Mactridae										
Mulinia lateralis	38								Telomeric	Wang and Guo 2001
									rDNA (major)	Wang and Guo 2008
Tresus capax	34								rDNA (major)	Gonzalez-Tizon *et al.* 2000
Solenidae										
Solen marginatus	38						x		rDNA (major)	Fernández-Tajes *et al.* 2003
Pharidae										
Ensis arcuatus	38	x							rDNA (major)	Fernández-Tajes *et al.* 2008
Ensis siliqua	38	x							rDNA (major)	Fernández-Tajes *et al.* 2008
Tellinidae										
Macoma nasuta									rDNA (major)	Gonzalez-Tizon *et al.* 2000
Psammobiidae										
Nuttalia nuttallii	38								rDNA (major)	Gonzalez-Tizon *et al.* 2000
Sinonovacula constricta	38	x	x							Wang *et al.* 1998
Veneridae										
Dosinia exoleta	38						x		rDNA (major) and Telomeric	Hurtado and Pasantes 2005
Mercenaria mercenaria									Telomeric	Wang and Guo 2001
									rDNA (major)	Wang and Guo 2007
Ruditapes decussatus	38							x		Leitão *et al.* 2006
Donacidae										
Donax trunculus	38						x		rDNA (major)	Martinez *et al.* 2002
									Telomeric	Plhol *et al.* 2002
Dreissenidae										
Dreissena polymorpha	32	x	x				x			Boron *et al.* 2004
Myidae										
Mya arenaria	34	x								Thiriot-Quiévreux *et al.* 1998

Also in Pectinidae the evidence of a non telomeric NOR location in *A. colbecki* (Odierna *et al*. 2006) and *H. distortus* (Lopez-Piñon *et al*. 2005), together with different karyotype formulae of the 2n=38 Pectinidae, suggested that chromosomal inversion might be implicated in karyotype evolution (Odierna *et al*. 2006). In the cupped oyster subfamily Crassostreinae, the rDNA chromosomal location and the size and morphological classification of the bearing chromosmes (as well as the percentage of sub-metacentric chromosomal pairs in the karyotype), allowed the division between Asian-Pacific and Atlantic *Crassostrea* species (Wang *et al*. 2004).

CONCLUDING REMARKS

Chromosomal rearrangements and karyotype differentiation are important mechanisms for reproductive isolation and speciation in some taxa (Navarro and Barton 2003). Major chromosomal divergence can, in fact, cause reproductive isolation and speciation, by altering normal gene expression and regulation or causing problems for meiosis or fertility in hybrids (White 1978; King 1993).

Clearly, significant changes in chromosome number and structure must have occurred during the evolution of Bivalves. As showed in this review, the application of banding for chromosomal identification may offer a different viewpoint on the evolution of marine bivalves. The applications of chromosomal banding offer then new approaches to specific problems in bivalve taxonomy through the understanding of the evolutionary relationships not only between but also within each major economic important bivalve family studied.

Moreover chromosomal studies of commercially important bivalve species are also important to achieve genetic improvement of bivalve production through, among others, chromosome set manipulation and genetic selection. Indeed, the individual identification of chromosomes and the establishment of precise karyotypes are essential, for instance, in studies on the economical important phenomena of aneuploidy, triploidy, and tetraploidy but also in bivalve aquaculture interspecifc hybridization programs.

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