Fluorescence in situ hybridization of rDNA, telomeric $(TTAGGG)_n$ and $(GATA)_n$ repeats in the red abalone *Haliotis rufescens* (Archaeogastropoda: Haliotidae)

CRISTIAN GALLARDO-ESCÁRATE[1,2], JOSUÉ ÁLVAREZ-BORREGO[2], MIGUEL ÁNGEL DEL RÍO-PORTILLA[1], ISMAEL CROSS[3], ALEJANDRO MERLO[3] and LAUREANA REBORDINOS[3]

[1]*Departamento de Acuicultura. División de Oceanología*
[2]*Dirección de Telemática, Centro de Investigación Científica y de Educación Superior de Ensenada, Ensenada, B.C. México*
[3]*Laboratorio de Genética, Facultad de Ciencias del Mar, Universidad de Cádiz., Cádiz, España*

Gallardo-Escárate, C., Álvarez-Borrego, J., Del Río-Portilla, M. A., Cross, I., Merlo, A. and Rebordinos, L. 2005. Fluorescence in situ hybridization of rDNA, telomeric $(TTAGGG)_n$ and $(GATA)_n$ repeats in the red abalone *Haliotis rufescens* (Archaeogastropoda: Haliotidae). — *Hereditas 142*: 73–79. Lund, Sweden. ISSN 0018-0661. Received January 26, 2005. Accepted April 7, 2005

The physical location of 18S-5.8S-28S rDNA, telomeric sequences with $(TTAGGG)_n$ DNA probe and $(GATA)_n$ microsatellites were performed by fluorescence in situ hybridization in chromosomes of red abalone *Haliotis rufescens*. The karyotype of red abalone showed a diploid number of 36 (8M+9SM+1ST). FISH performed with rDNA probe, showed the location of major ribosomal clusters in the terminal region of the large arms of two submetacentric pairs (chromosome 4 and 5). Localization of heteromorphisms of FISH-rDNA was found between chromosome homologues and sister chromatids in all metaphases analyzed. This indicates that rDNA clusters are variable within the red abalone genome. The variability in the NOR-bearing reported using silver staining in other gastropods and our result are discussed. In addition, the presence of microsatellite $(TTAGGG)_n$ and $(GATA)_n$ was demonstrated after FISH treatment by DNA probes. The telomeric sequence occurred at the ends of all mitotic chromosomes, while the $(GATA)_n$ repetitive was found on chromosomal interstitial zones as well as at the telomeres in abalone chromosomes.

Josué Álvarez-Borrego, Dirección de Telemática, Centro de Investigación Científica y de Educación Superior de Ensenada, Km. 107 Carretera Tijuana – Ensenada, Código Postal 22860, Ensenada, B.C. México. E-mail: josue@cicese.mx

The red abalone *Haliotis rufescens* (Swainson, 1822) is the largest species of abalone in the world and the most desirable commercially for size and light meat color (Oakes and Ponte 1996). Red abalone supports an important fishery on the California coast, and it is well suited for farming, as both land based and ocean based operations. Natural populations have been reported from Sunset Bay, Oregon in United States to Turtle Bay, Baja California in Mexico (Cox 1962; Lindberg 1992).

Because of its commercial importance, several intensive studies have been carried out in abalones, including population genetic analysis, seed production, and ecology (Lindberg 1992; González and Scoresby 1996; Hamm and Burton 2000; Zúñiga et al. 2000; Del Río-Portilla and González-Avilés 2001). However, the abalone fishery have has undergone a major decline since the 1960s (Tegner et al. 1989), and overexploitation, pollution, habitat degradation and epidemic disease have probably been the main factors to these declines (Burton and Tegner 2000).

To date, few studies have been carried out in order to know genetic markers for population structure in the red abalone. Traditional allozyme studies have not detected any significant population differences, therefore molecular tools like microsatellites and mitochondrial markers have been used in order to detect such differences (Kirby et al. 1998). In addition, cytogenetic analyses are also useful tools in population genetic studies. In this context, Ag-NORs and rDNA-FISH technique have been applied in *Crassostrea angulata* chromosomes, revealed high Ag-NORs and rDNA-FISH polymorphisms between natural populations. Differences found in localization of chromosomal rDNA could be related to the plasticity in molluscs to survive under a wide range of environmental conditions (Cross et al. 2003). Moreover, these results are in agreement with the NOR-bearing chromosome variability found with other molluscs (Pascoe et al. 1996; Martínez-Expósito et al. 1997; Torreiro et al. 1999; Martínez et al. 2002). In reference to gastropod molluscs, few studies have been carried out, mainly because of the low number of species analyzed with cytogenetic molecular markers. In fact, available karyological data in gastropods indicate that less than 20 species have been analyzed cytogenetically using molecular techniques. However,

to date, more than 300 species have been examined by conventional cytogenetics (Vitturi et al. 2002). In reference to abalones, nowadays there are not available karyological studies by FISH. In fact, 65 species have been reported for Haliotidae (Lindberg 1992), while only 15 species have been cytogenetically described (Gallardo-Escarate et al. 2004). Moreover, karyological studies belonging from California abalones have only been performed on the black abalone *H. cracherodii* (Minkler 1977) and recently on the red abalone *H. rufescens* (Gallardo-Escárate et al. 2004).

In order to apply fluorescence in situ hybridization (FISH) in the red abalone, and thus contribute with new cytogenetic information, we organized the present paper with the followings aims: a) to examine the localization of 18S-5.8S-28S rDNA, b) to detect all rDNA-FISH clusters, c) to find telomeric sequences with $(TTAGGG)_n$ DNA probe and d) to ascertain the presence of $(GATA)_n$ microsatellites.

MATERIAL AND METHODS

Chromosome preparation

Chromosome preparation was performed according to Gallardo-Escarate et al. (2004). Briefly, trochophore larvae at 20 h postfertilization were maintained in colchicine treatment (0.005%) for 3 h. After that the larvae were rinsed in clean sea water and immersed in a hypotonic solution (sea water: distilled water, 1:1) for 45 min. Finally, larvae were fixed in modified Carnoy solution (methanol: acetic acid, 3:1). Chromosome spreads were obtained by dissociating larval tissue in acetic acid (50%), pipetting suspension drops onto slides preheated to 45°C and air dried. Chromosomal measurements were performed by image analysis using Image Pro-Plus software (Copyright© 1993–1998 Media Cybernetics). Total length, relative length and centromeric index were obtained for each chromosome pair. The best metaphase plates were used for fluorescence in situ hybridization analyses.

Fluorescence in situ hybridization

The chromosomal localization of rDNA sequence was performed using a probe containing 18S-5.8S-28S genes plus intergenic spacer of *D. melanogaster* (pDm238) (Roiha et al. 1981) and cloned in pBR322 plasmid of *E. coli* (strain JM109). The plasmid purification was performed with a NucleoSpin®Plasmid kit (Macherey-Nagel) and it was labeled with Dig-Nick Translation Mix kit (Roche Molecular Biochemicals) according to manufacture's instructions. After obtaining the labeled probe, a hybridization solution was added at 72°C for 7 min to denature the DNA probe and chromosomal DNA. FISH was performed at 37°C overnight, followed by post-hybridization washes: three times for 5 min each in 50% formamide in $2 \times$SSC at 44°C, three times for 5 min each in $0.1 \times$SSC at 60°C, 5 min in $2 \times$SSC at RT, 5 min in TNT at RT and 30 min in TNB at RT. Telomeric $(TTAGGG)_n$ and $(GATA)_n$ DNA probes were obtained by PCR in absence of DNA template. The primers $(TTAGGG)_5$, $(CCCTAA)_5$ and $(GATA)_7$, $(TATC)_7$ were used according to Ijdo et al. (1991). Nick translation labelling and FISH were performed in conditions mentioned above and followed by post-hybridization at 42°C (for telomeric probe) or 37°C (for GATA probe). Signals of hybridization were detected with mouse anti-digoxigenine (0.5 μg ml^{-1}), FITC-conjugated Rabbit anti-Mouse (0.2 μg ml^{-1}), and FITC-conjugated Goat anti-Rabbit (0.1 μg ml^{-1}). Chromosome slides were mounted with propidium iodide plus Vectashield antifading medium (500 ng ml^{-1}). Fluorescence images were obtained using an epifluorescence microscope (Axioskop 2 plus, Zeiss) equipped with a cooled CCD camera (CoolSnap, Photometrics® Inc.).

RESULTS

All the metaphase plate analyzed from larvae of *Haliotis rufescens* showed a diploid complement of $2n = 36$ chromosomes. The karyotype is composed of eight metacentric pairs (1, 3, 6, 7, 14, 15, 17 and 18), nine submetacentric pairs (2, 4, 5, 8, 9, 10, 11, 12 and 13), and one pair of subtelocentric chromosomes (16) (Fig. 1). The mean values and standard deviations of the total lengths, relative lengths and centromeric index were estimated from chromosome arm lengths. The maximum length of the chromosomes was 6.29 ± 0.08 μm and the minimum was 3.97 ± 0.27 μm (Table 1). The karyotypic arrangements of red abalone chromosomes jointly with rDNA-FISH, allowed visualization of several sites of 18S-5.8S-28S rDNA hybridization. The rDNA probe produced strong signals on the telomere of the long arms of two pairs of submetacentric chromosomes (pairs 4 and 5). Metaphase chromosomes from larvae showed heteromorphisms of rDNA-FISH between sister chromatids (chromosome 7) and low interstitial signals were presented in submetacentric as well as subtelocentric chromosomes (chromosomes 8 and 16 respectively) (Fig. 1). However, the specific localization of regions with low intensity showed a high cytogenetic variability in the all analyzed metaphases. In addition, heteromorphisms with strong signal of rDNA-FISH

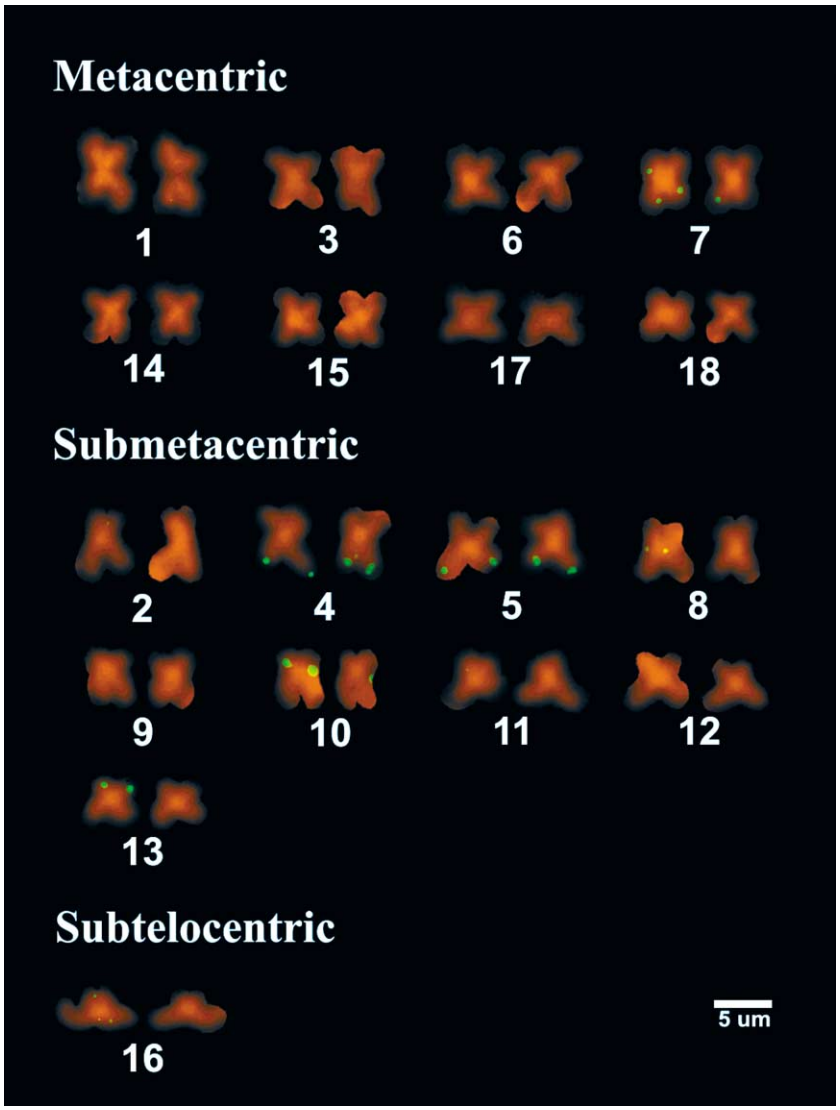

Fig. 1. Karyotypic array of *Haliotis rufescens* chromosomes after fluorescence in situ hybridization with 18S-5.8S-28S rDNA probe.

were observed between homologues of both chromosomes 12 and 13 (Fig. 1).

Fluorescence in situ hybridization applied with the telomeric DNA probe showed that red abalone chromosomes were positive to the hexamer repeat sequence $(TTAGGG)_n$. The hybridization signals were found at the ends of all analyzed chromosome types (Fig. 2a). The visualization of interphase nucleus labelled with telomeric probe allowed evidence the distribution of telomeres at the nuclear periphery (Fig. 2b). The presence of the $(GATA)_n$ sequence was observed in chromosomes of *H. rufescens* (Fig. 2c). The localization of these regions was found in chromosomal interstitial zone as well as at the ends of some chromosomes, this was observed after FISH onto interphase nucleus (Fig. 2d).

DISCUSSION

The chromosome number of *Haliotis rufescens* found in the present study was $2n = 36$. The karyological conformation was observed as $8M + 9SM + 1ST$. This result is in agreement with the previously karyotype reported for the red abalone (Gallardo-Escárate et al. 2004). Moreover, the chromosome complement of 36 is the most frequent among abalone species from North Pacific including *H. discus discus* (Arai et al. 1982), *H. discus hannai* (Okumura et al. 1999), *H. modaka* (Nakamura 1986), and California abalones including *H. cracherodii* (Minkler 1977). However, there are some differences among the karyotypes of North Pacific species (Japanese abalones) and California abalones, mainly by an increase of the sub-

Table 1. *Chromosomal measurements of Haliotis rufescens (2n = 36).*

Chromosome Pair no.	Total length (μm) (mean ± std)	Relative length (%) (mean ± std)	Centromeric index (mean ± std)	Chromosome type*
1	6.29 ± 0.08	7.13 ± 0.14	49.500.29	M
2	5.91 ± 0.04	6.70 ± 0.05	33.350.11	SM
3	5.78 ± 0.10	6.56 ± 0.12	47.160.25	M
4	5.58 ± 0.06	6.33 ± 0.20	37.460.40	SM
5	5.49 ± 0.03	6.22 ± 0.11	37.610.21	SM
6	5.42 ± 0.01	6.15 ± 0.04	49.420.07	M
7	5.30 ± 0.07	6.01 ± 0.07	49.750.15	M
8	5.05 ± 0.11	5.72 ± 0.17	37.960.34	SM
9	4.94 ± 0.18	5.60 ± 0.17	38.640.34	SM
10	4.80 ± 0.09	5.45 ± 0.07	34.080.14	SM
11	4.54 ± 0.20	5.15 ± 0.19	35.410.39	SM
12	4.46 ± 0.03	5.06 ± 0.12	36.380.23	SM
13	4.30 ± 0.03	4.87 ± 0.06	34.330.13	SM
14	4.25 ± 0.02	4.82 ± 0.06	48.660.11	M
15	4.19 ± 0.03	4.75 ± 0.05	48.750.10	M
16	4.09 ± 0.07	4.64 ± 0.02	27.400.05	ST
17	3.95 ± 0.03	4.47 ± 0.07	49.770.14	M
18	3.87 ± 0.27	4.39 ± 0.27	49.970.55	M

* M, metacentric; SM, submetacentric; ST, subtelocentric.

metacentric as well as subtelocentric chromosome types.

To date, the most accurate technique to locate specific DNA sequences is the fluorescence in situ hybridization. In reference to rDNA-FISH, in the eukaryotic genomes genes of ribosomal RNA (rDNA) are organized in two distinct gene classes: the major rRNA gene cluster encoding 18S-5.8S and 28S rRNAs; and the minor rRNA gene cluster encoding 5S rDNA. The major rRNAs are usually found involving the nucleolar organizing region (NOR) on the chromosome in multiple-copy cluster in several molluscs studied (Torreiro et al. 1999; Zhang et al. 1999; González-Tizón et al. 2000; Vitturi et al. 2000b; Xu et al. 2001; Cross et al. 2003). However, the 5S loci have been found separate from those rDNAs and, unlike major rDNA is not involved in NOR formation (Insua et al. 2001). Other results, according to Colomba et al. (2002) showed that the 5S loci have been found co-localized in the periwinkle *Melarhaphe neritoides*. In addition, few studies rDNA-FISH have been applied in gastropod molluscs. According to Vitturi (2000a, 2002), results of rDNA-FISH in *Cerithium vulgatum* and in the neogastropod *Fasciolaria lignaria* showed that the major ribosomal sites are located at the terminal regions of one chromosome pair and four chromosome pair respectively, corresponding at two and eight NORs sites per spread. In abalones, to our knowledge NOR-bearing regions have only been reported in *Haliotis discus hannai*, by silver staining. According to Okumura et al. (1999), NORs were terminally located on the long arms of two chromosome pairs. However, the specific localization showed to be variable among submetacentric chromosomes as well as metacentric chromosomes. These results, could be due because Ag-NORs can only be visualized in transcriptional active state. The silver nitrate staining only stains those NORs which have expressed themselves during the last interphase, because silver binds to a complex of acidic protein associated with the nucleolus and nascent pre-RNA (Jordan 1987). The fluorescence in situ hybridization allows specific identification of all 18S-5.8S-28S rDNA genes clusters independently their transcriptional state. In this context, our results in the red abalone showed rDNA-FISH on the telomere of the long arms of two pairs of submetacentric chromosomes. This result was in accord with the previous report for *Haliotis discus hannai*. In addition, heteromorphisms of rDNA-FISH between sister chromatids and low interstitial signals were presented in submetacentric as well as subtelocentric chromosomes. Moreover, heteromorphisms with strong signal of rDNA-FISH was observed between both homologues chromosomes. This indicates that the copy number of rDNA is variable and occurrence of mistakes of either deleting or duplicating the rDNA clusters during the replication state.

In the present study, we used in addition to the rDNA FISH, in situ hybridization with $(TTAGGG)_n$ and $(GATA)_n$ probes to test for the presence of these sequences in the red abalone *H. rufescens* chromosomes. The results demonstrated that telomeric sequence occurred at the ends of all mitotic

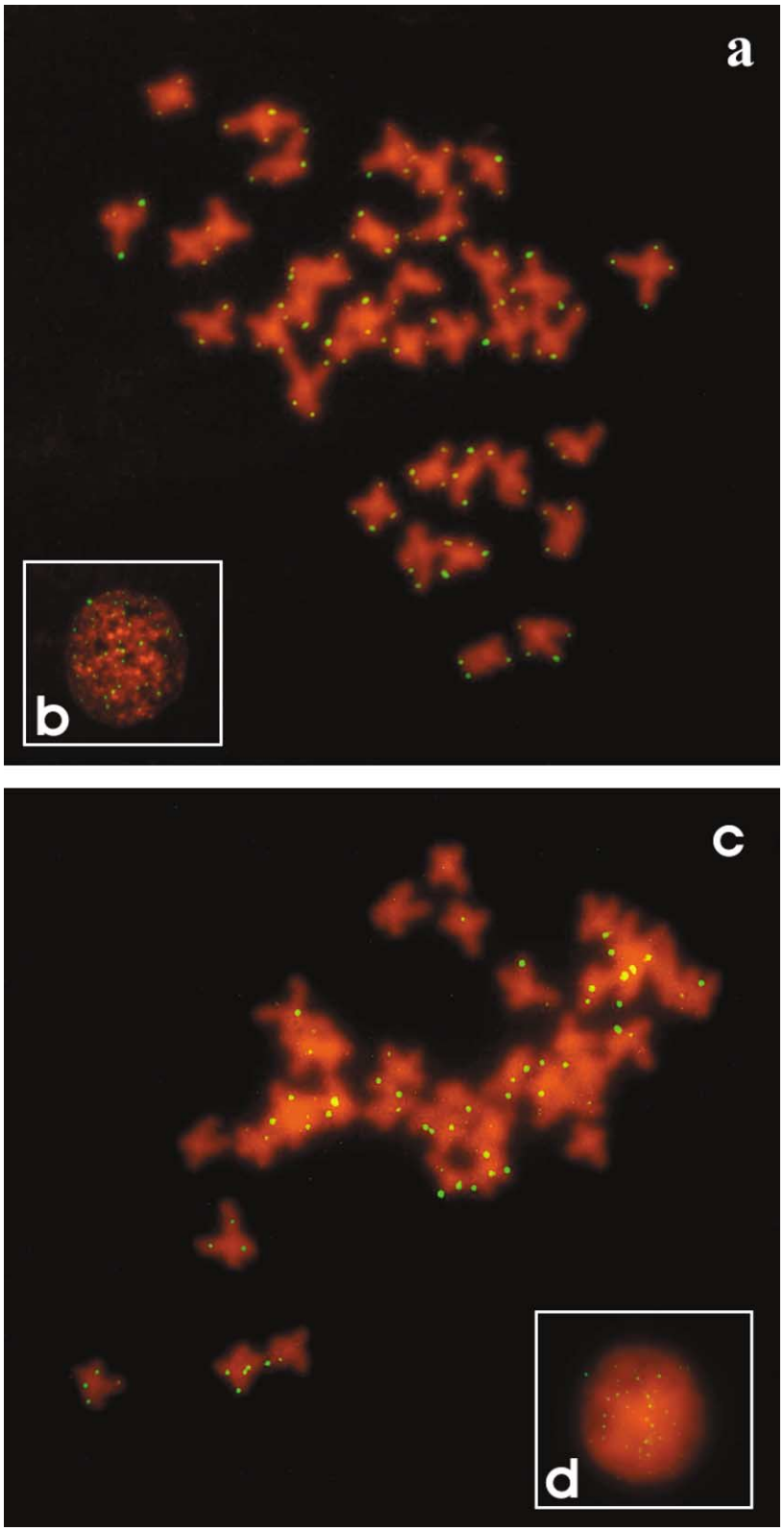

Fig. 2a–d. (a) Mitotic metaphase chromosomes after FISH treatment with the telomeric sequence $(TTAGGG)_n$. (b) Interphase nucleus with telomeric probe. (c) Mitotic chromosomes after being labelled with the (GATA)n probe. (d) Interphase nucleus labelled with $(GATA)_n$ sequence.

chromosomes in abalones. In molluscs, conservation of the telomeric region has been reported in bivalves (Guo and Allen 1997; Plohl et al. 2002) and gastropods (Vitturi et al. 2002) and therefore, it can be considered that telomere repeat is widespread within the Mollusca. Likewise, the genome of the red abalone, revealed $(GATA)_n$ microsatellite sequence. This result was according to that reported in other gastropods species (Vitturi et al. 2000a; Colomba et al. 2002). Finally, future studies by FISH using other DNA probes in more abalone species would allow localizing other genes of interest as well as characterizing the chromosomes of the karyotype of *Haliotis*.

Acknowledgements – This study was supported by CONACYT (Mexico) project 36075-B and Project 33018-B, project OPAM from the INTERREG IIIA (EEC) and project CVI219 from the Junta de Andalucía (Spain). The first author is a CONICYT-BID (Chile) PhD. fellow.

REFERENCES

Arai, K., Tsubaki, H., Ishitani, Y. et al. 1982. Chromosome of *Haliotis discus hannai* Ino and *H. discus* Reeve. – Bull. Jpn Soc. Sci. Fish. 48: 1689–1691.

Burton, R. and Tegner, M. 2000. Enhancement of red abalone *Haliotis rufescens* stocks at San Miguel Island: reassessing a success story. – Mar. Ecol. Progr. Ser. 202: 302–308.

Colomba, M., Vitturi, R., Castriota, L. et al. 2002. FISH mapping of 18S-28S and 5S ribosomal DNA, (GATA)n and (TTAGGG)n telomeric repeats in the periwinkle *Melarhaphe neritoides* (Prosobranchia, Gastropoda Caenogastropoda). – Heredity 88: 381–384.

Cox, K. 1962. California abalones, family Haliotidae. – Calif. Fish Games Fish. Bull., p. 133.

Cross, I., Vega, L. and Rebordinos, L. 2003. Nucleolar organizing region in *Crassostrea angulata*: chromosomal location and polymorphism. – Genetica 119: 65–74.

Del Río-Portilla, M. A. and González-Avilés, J. G. 2001. Population genetics of the yellow abalone, *Haliotis corrugata*, in Cedros and San Benito islands: a preliminary survey. – J. Shellfish Res. 20: 765–770.

Gallardo-Escarate, C., Álvarez-Borrego, J., Del Río-Portilla, M. A. et al. 2004. Karyotype of Pacific red abalone *Haliotis rufescens* (Archaeogastropoda: Haliotidae), using image analysis. – J. Shellfish Res. 23: 205–209.

González, J. and Scoresby, S. 1996. Growth and survival of the blue abalone *Haliotis fulgens* in barrels at Cedros Island, Baja California, with a review of abalone barrel culture. – Aquaculture 140: 169–176.

González-Tizón, A., Martínez-Large, A., Rego, I. et al. 2000. DNA content, karyotypes, and chromosomal location for 18S-5.8S-28S ribosomal loci in some species of bivalve molluscs from Pacific Canadian coast. – Genome 43: 1–8.

Guo, X. and Allen, S. 1997. Fluorescence in situ hybridization of vertebrate telomere sequence to chromosome ends of the pacific oyster, *Crassostrea gigas* Thunberg. – J. Shellfish Res. 16: 87–89.

Hamm, D. and Burton, R. 2000. Population genetics of black abalone, *Haliotis cracherodii*, along the central California coast. – J. Exp. Mar. Biol. Ecol. 254: 235–247.

Ijdo, J., Wells, R., Baldini, A. et al. 1991. Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. – Nucleic Acids Res. 19: 4780.

Insua, A., Freire, R., Ríos, J. et al. 2001. The 5S rDNA of mussels *Mytilus galloprovincialis* and *M. edulis*: sequence variation and chromosomal location. – Chromosome Res. 9: 495–505.

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

Kirby, V., Villa, R. and Powers, D. 1998. Identification of microsatellites in the california red abalone, *Haliotis rufescens*. – J. Shellfish Res. 17: 801–804.

Lindberg, D. 1992. Evolution, distribution and systematics of Haliotidae. – In: Guzmán, S (ed.), Abalone of the word, biology, fisheries and cultured. Fishing News Books, Oxford, USA, p. 3–18.

Martínez, A., Mariñas, L., González-Tizón, A. et al. 2002. Cytogenetic characterization of *Donax trunculus* (Bivalvia: Donacidae) by means of karyotyping, fluorescence banding and fluorescence in situ hibridization. – J. Molluscan Stud. 68: 393–396.

Martínez-Expósito, M., Méndez, J. and Pasantes, J. 1997. Analysis of NORs and NOR-associated heterochromatin in the mussel *Mytilus galloprovincialis* Lmk. – Chromosome Res. 5: 268–273.

Minkler, J. 1977. Chromosomes of the black abalone (*Haliotis cracherodii*). – Experientia 33: 1143.

Nakamura, H. 1986. Chromosomes of Archaeogastropoda (Mollusca: Prosobranchia), with some remarks on their citotaxonomy and phylogeny. – Publ. Seto Mar. Biol. Lab. 31: 191–297.

Oakes, F. and Ponte, R. 1996. Abalone market: opportunities for cultured abalone. – Aquaculture 140: 187–195.

Okumura, S., Kinugawa, S., Fujimaki, A. et al. 1999. Analysis of karyotipe, chromosome banding, and nucleolus organizer region of pacific abalone, *Haliotis discus hannai* (Archaeogastropoda: Haliotidae). – J. Shellfish Res. 18: 605–609.

Pascoe, P., Patton, S., Critcher, R. et al. 1996. Robertsonian polymorphism in the marine gastropod, *Nucella lapillus*: advances in karyology using rDNA loci and NORs. – Chromosoma 104: 455–460.

Plohl, M., Prats, E., Martínez-Large, A. et al. 2002. Telomeric localization of the vertebrate-type hexamer repeat, (TTAGGG)n, in the wedgeshell clam *Donax trunculus* and other marine invertebrate genomes. – J. Biol. Chem. 277: 19839–19846.

Roiha, H., Miller, J., Woods, L. et al. 1981. Arrangements and rearrangements of sequences flanking the two types of rDNA insertion in *D. melanogaster*. – Nature 290: 743–749.

Tegner, M., DeMartini, J. and Karpov, K. 1989. The California red abalone fishery: a case study in complexity. – In: Guzmán, S. (ed.), Abalone of the world: biology, fisheries and culture. Blackwell Fishing News Books, p. 370–383.

Torreiro, A., Martínez-Expósito, M., Trucco, M. et al. 1999. Cytogenetics in *Brachidontes rodriguezi* d'Orb (Bivalvia, Mytilidae). – Chromosome Res. 7: 49–55.

Vitturi, R., Colomba, M., Gianguzza, P. et al. 2000a. Chromosomal location of ribosomal DNA (rDNA), (GATA)n and (TTAGGG)n telomeric repeats in the

neogastropod *Fasciolaria lignaria* (Mollusca: Prosobranchia). – Genetica 108: 253–257.

Vitturi, R., Gianguzza, P., Colomba, M. et al. 2000b. Cytogenetic characterization of *Brachidontes pharaonis* (Fisher P., 1870): karyotype banding and fluorescent in situ hybridization (FISH). – Ophelia 52: 213–220.

Vitturi, R., Colomba, M., Castriota, L. et al. 2002. Chromosome analysis using different staining techniques and fluorescent in situ hybridization in *Cerethium vulgatum* (Gastropoda: Cerithiidae). – Hereditas 137: 101–106.

Xu, Z., Guo, X., Gaffney, P. et al. 2001. Chromosomal location of the major ribosomal RNA genes in *Crassostrea virginica* and *Crassostrea gigas*. – Veliger 44: 79–83.

Zhang, Q., Yu, G., Cooper, R. et al. 1999. Chromosomal location by fluorescence in situ hybridization of the 28s ribosomal RNA gene of the eastern oyster. – J. Shellfish Res. 18: 431–435.

Zúñiga, G., Guzmán, S., Cisneros, R. et al. 2000. Population genetic analysis of the abalone *Haliotis fulgens* (Mollusca: Gastropoda) in Baja California, México. – J. Shellfish Res. 19: 853–859.