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Chromosome analysis using different staining techniques and fluorescent in situ hybridization in *Cerithium vulgatum* (Gastropoda: Cerithiidae)

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In the present paper one population of the "large" subtidal mollusc *Cerithium vulgatum* Bruguière, 1792 (Gastropoda: Cerithiidae) from the Northwestern coast of Sicily was investigated from a karyological point of view. The chromosome complement was Giemsa stained, conventionally karyotyped in 18 homomorphic chromosome pairs (10 bi-armed and 8 mono-armed), and subsequently analysed using silver, CMA_3 and DAPI staining, and fluorescent in situ hybridization (FISH) with three repetitive DNA probes [ribosomal DNA (rDNA), $(TTAGGG)_n$ and $(GATA)_n$]. FISH with the rDNA probe consistently mapped major ribosomal sites (18S-28S rDNA) in the terminal region of the short arms of one small sized mono-armed chromosome pair. Ribosomal DNA was transciptionally active as indicated by its preferential impregnation with silver nitrate (Ag-NOR) and did not contain a high amount of GC base pairs as suggested by the lack of a bright CMA_3 fluorescence. The $(TTAGGG)_n$ telomeric probe was hybridized to the termini of nearly all chromosomes, thus demonstrating that, in *C. vulgatum*, this sequence has been conserved during the genomic evolution. The finding of the telomeric hexanucleotide in six species belonging to the three high taxa of Gastropoda supports the notion that this sequence is widespread within this class. The $(GATA)_n$ probe did not label any chromosome regions except for a minute terminal area of a single bivalent at pachytene stage.

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Modern cytological methods such as chromosome banding and fluorescent in situ hybridization (FISH) proved to be very useful for understanding the genomic organization of numerous vertebrate species (SUMNER 1990; TRASK 1991). The same methods have sporadically been used to examine the chromosomes in the phylum Mollusca. In particular, available karyological data on the class Gastropoda indicate that more than 300 species have been analysed cytogenetically (PATTERSON 1969; PATTERSON and BURCH 1978; NAKAMURA 1986; THIRIOT-QUIEVREUX 1994), while less than 20 of them have been examined using banding techniques.

Several families of this class have remained poorly characterized from a karyological point of view. One of this is the family Cerithiidae, where the haploid (n) and/or diploid (2n) chromosome numbers of eight species of four different genera have been determined from analysis of Giemsa stained preparations (NISHIKAWA 1962; VITTURI and CATALANO 1984; YASEEN et al. 1995; EBIED et al. 2000).

The Cerithiidae, order Neotaenioglossa, belong to the superfamily Cerithioidea (PONDER and WARÉN 1988), a group whose origins can be traced back to Jurassic-Cretaceous. According to HOUBRICK (1988) and BOISSELLER-DUBAYLE and GOFAS (1999), it includes organisms whose diversity, in terms of morphology, size, habitat, feeding methods, reproductive biology and ecophenotypic plasticity is notable.

The present paper was designed with several aims: 1) to describe the karyotype of the "large" *Cerithium vulgatum* Bruguière, 1792; 2) to examine the nucleolar organizer regions (NORs) using both conventional (silver, CMA_3, and DAPI staining) and molecular (rDNA FISH) techniques; 3) to test for the presence of telomeric $(TTAGGG)_n$ and $(GATA)_n$ repeats; and 4) to compare results of this study with those reported upon other molluscan species.

MATERIALS AND METHODS

According to the classical view, in the Mediterranean sea the genus *Cerithium* is represented by one "small" littoral species, called *C. rupestre* Risso, 1826 and one "large" subtidal species called *C. vulgatum* Bruguière, 1792 (BUCQUOY et al. 1882). In turn, in a more recent revision (GIANNUZZI-SAVELLI et al. 1996) the state of the art of Mediterranean *Cerithium* is confusing. In fact, this genus, whose taxonomy is currently uncertain due to high intraspecific variability and low

interspecific differentiation, includes many taxa whose classification at the species level is extremely problematic (see also BOISSELLER-DUBAYLE and GOFAS 1999).

Specimens of the "large" *Cerithium vulgatum* employed for this study were collected by hand at low depth (0.5–1 m) from September 1999 to March 2002 in a Zoostera-Caulerpa meadow in the Gulf of Castellammare (S Tyrrhenian Sea; Trapani, NW Sicily).

Collections were made every two weeks and samples were transported to the laboratory at the Department of Animal Biology, University of Palermo, in plastic bags with seawater. Data reported herein were gathered from 15 specimens with testes in the proper sexual condition to give good chromosome spreads.

Chromosome preparations were obtained by the air-drying method after immersing the molluscs in a seawater colchicine solution (0.1 %) for 12–16 hrs and removing male gonads under a stereomicroscope at a magnification of 20 ×.

The air-drying method was performed as follows: testes were treated in a distilled water hypotonic solution for 20 min and then fixed in an ethyl alcohol/acetic acid solution (3:1) for 20 min. Small pieces of testes were crushed with dissection needles in 1–2 ml of 50 % acetic acid and, finally, the cell suspension was dropped on warm precleaned slides.

Slides were processed for Giemsa staining (VITTURI et al. 2000a), Ag-NOR staining (HOWELL and BLACK 1980), Chromomycin A_3 (CMA_3) and DAPI staining (SCHMID et al. 1983), and fluorescent in situ hybridization (FISH) (VITTURI et al. 2000a).

FISH was carried out on spermatocyte chromosomes using three different probes: a sea urchin (*Paracentrotus lividus*) rDNA probe consisting of sequences of the 18S rDNA; a telomeric hexanucleotide $(TTAGGG)_n$; and a $(GATA)_n$ sequence. Both telomeric and $(GATA)_n$ sequences were generated by PCR (PCR DIG-Probe Synthesis kit, Roche) in the absence of template (IJDO et al. 1991) using $(TTAGGG)_5$ and $(CCCTAA)_5$ and $(GATA)_7$ and $(TATC)_7$ as primers, respectively. Nick translation labelling with digoxigenin of 18S rDNA was performed according to the manufacturer's (Roche) instructions, while the remaining two probes were DIG-labelled following the random priming Roche protocol. In situ hybridization was performed as described by VITTURI et al. (2000a). Following hybridization, the slides were washed twice in 50 % formamide/2 × SSC at 40°C (5 min each), twice in 2 × SSC at 40°C (5 min each), once (5 min) in 4 × SSC/0.1 % Tween at room temperature (RT) and finally, once (5 min) in PBS/0.1 % Tween/0.5 % skimmed milk powder at RT. Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and viewed under a Leica I3 filter set (BP 450-490, LP 515). Chromosomes were observed with a Leica microscope and photographed with a Kodak Ektacolor 800 ASA film.

Chromosome morphology was described according to the criteria proposed by LEVAN et al. (1964).

RESULTS

In all 15 specimens examined in this study, the diploid chromosome number was 2n = 36. By arranging into pairs the chromosomes from ten Giemsa stained spermatogonial metaphases according to dimension and arm-ratio, a karyogram consisting of 10 bi-armed (M + SM) pairs (no. 1,3,5,8,11,13,14,16, 17,18) and 8 mono-armed (A + ST) pairs (all others) was traced (Fig. 1). Dimensions of these chromosomes decreased uniformly from 4 μm of the longest element to 1.5 μm of the shortest one.

As previously reported (VITTURI and CATALANO 1984), analysis of Giemsa stained metaphase-I chromosomes indicated that the haploid number of *C. vulgatum* was n = 18 and that bivalents had ring-, cross- and rod morphologies (Fig. 2).

After silver staining carried out on 8 specimens, the pattern obtained consisted of argentophilic dots located at the terminal regions of two mono-armed chromosomes per spread at spermatogonial metaphase (Fig. 3). On the basis of morphology and size, these chromosomes were assigned to pair no. 15. Small silver positive signals varying from one to four per bivalent, presumably located at the centromeric

Fig. 1–9. 1. Karyotype of *Cerithium vulgatum* obtained from a Giemsa stained spermatogonial metaphase. **2.** Giemsa stained metaphase-I bivalents of *C. vulgatum*. **3.** Silver stained spermatogonial chromosomes of *C. vulgatum* (arrows indicate Ag-NORs). **4.** Silver stained metaphase-I bivalents of *C. vulgatum*. **5.** CMA_3 stained spermatogonial chromosomes of *C. vulgatum*. **6.** DAPI stained metaphases-I bivalents of *C. vulgatum*. **7.** rDNA FISH chromosomes of *C. vulgatum*. Spermatogonial metaphase plate (A). Pachytene bivalents (B). Metaphase-I bivalents (C). **8.** Pachytene bivalents of *C. vulgatum* after FISH treatment with the telomeric sequence $(TTAGGG)_n$. **9.** Pachytene bivalents of *C. vulgatum* after FISH treatment with the $(GATA)_n$ sequence (arrow indicates a small FISH positive region). Bar = 10 μm.

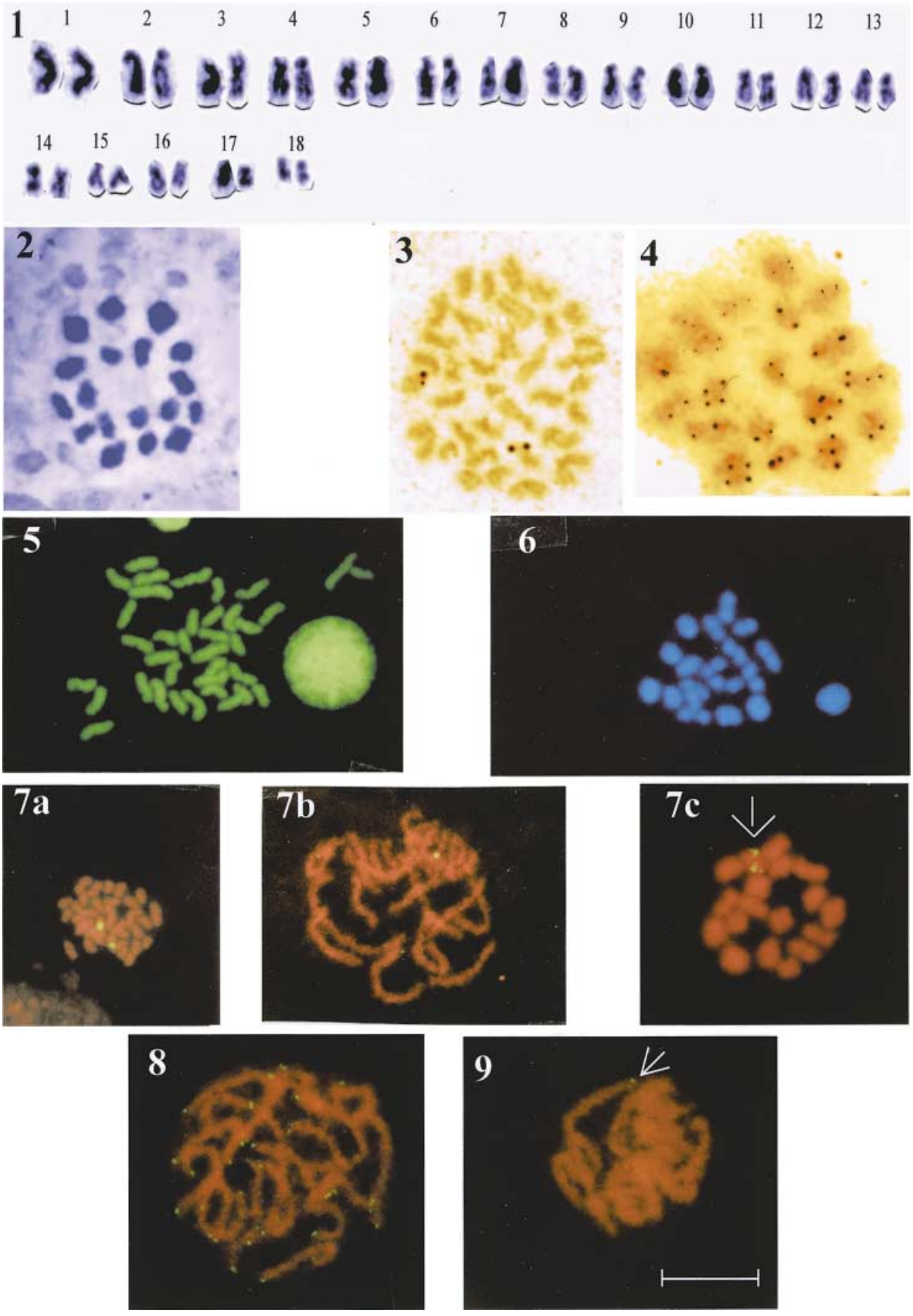

Fig. 1–9.

region, were visible on all chromosomes at metaphase-I (Fig. 4).

CMA_3 (Fig. 5) and DAPI (Fig. 6) treatments revealed that all chromosomes fluoresced with a uniform intensity, thus being uninformative in the detection of any repeated and/or compartmentalized DNA sequences including the nucleolus organizers.

18S rDNA FISH on 25 spermatogonial metaphases of 5 different specimens always showed hybridization signals on two small sized chromosomes (Fig. 7a). A repetitive rDNA pattern was also observed in FISH-treated meiotic spreads. It consisted of a single hybridization site at pachytene stage (Fig. 7b) and of two distinct sites, one for each homologue, at metaphase-I (Fig. 7c). The finding of a single labelled bivalent per spread after ribosomal FISH treatment conclusively established the homology of the two rDNA-bearing chromosomes.

After FISH, the $(TTAGGG)_n$ telomeric repeat was hybridized to the ends of each pachytene bivalent (Fig. 8), whereas the microsatellite $(GATA)_n$ did not show any labelled area on the bivalents at the same stage, except for a minute hybridization site at the terminal region of one element (Fig. 9, see arrow).

DISCUSSION

The "large" *Cerithium vulgatum* shows a diploid number of 36, which is in line with the finding of 18 bivalents in the corresponding spermatocytes.

This result is not consistent with those reported by YASEEN et al. (1995) for *C. adustum* ($2n = 18$) and *C. nodulosum* ($2n = 16$) and by EBIED et al. (2000) for *C. coeruleum* ($2n = 14$). However, the occurrence of different chromosome numbers between species of the genus *Cerithium* is not to be considered an unusual finding since cytological data on Gastropoda show that inter-specific variations in chromosome numbers occur within other genera of this class. Two examples are the prosobranch genera *Semisulcospira* ($2n = 14$, 24, 26, 28, 36 and 40) and *Viviparus* ($2n = 14$, 18, 20, 24 and 26) (PATTERSON 1969).

rDNA FISH results of this study show that, in *C. vulgatum*, major ribosomal sites are located at the terminal regions of two small mono-armed chromosomes at spermatogonial metaphase. Corresponding to the number of two rDNA sites per spread after FISH, two NORs per spread were detected after silver staining, demonstrating that, as observed in other molluscan species (VITTURI et al. 2000b,c), in *C. vulgatum* as well, no discrepancies occur among NORs and rDNA mapping.

The finding of NORs, the fluorescence of which was not enhanced by the GC-specific CMA_3 indicates that these regions do not contain GC-rich DNA. Such an absence is in agreement with results obtained from the Mediterranean tulip shell *Fasciolaria lignaria* (VITTURI et al. 2000a), but disagrees with results reported for other molluscs. In fact, in two bivalve species, *Mytilus galloprovincialis* (MARTÍNEZ-LAGE et al. 1994) and *Brachidontes pharaonis* (VITTURI et al. 2000c), NOR regions were brightly highlighted by the GC-specific fluorochrome. Moreover, since in *C. vulgatum*, the DNA fluorescence was enhanced neither by CMA_3 nor by DAPI, it may be supposed that GC and AT base pairs are interspersed in the genome of this species.

A diffuse silver stainability of metaphase-I bivalents – a feature which is quite unusual in vertebrates – was regularly observed in *C. vulgatum* spermatocyte chromosome preparations. This finding can not be attributed to technical reasons since firstly, in the same chromosome preparation, we found a single NOR-bearing pair at spermatogonial metaphase, and small Ag-granules in all bivalents at metaphase-I. A second reason is that, besides in *C. vulgatum*, in other molluscan species such as two Prosobranchia (VITTURI and CATALANO 1990, VITTURI et al. 1993) and two Pulmonata (VITTURI et al. 1991a; VITTURI 1992) a diffuse silver stainability of metaphase-I chromosomes was observed. The same feature was also described in some other taxonomically widely separated invertebrate organisms namely, *Clavelina lepadiformis* (Ascidiacea, Aplousobranchiata) (VITTURI et al. 1991b), *Pelagia noctiluca* (Coelenterata, Scyphomedusae) (VITTURI et al. 1994) and *Kalothermes flavicollis* (Isoptera, Kalothermitidae) (GOLDONI and FONTANA 1991).

Here we demonstrated that rDNA FISH to metaphase-I chromosomes of the species under study mapped ribosomal sites on one bivalent per spread (Fig. 7c). This allows us to conclude that silver stainability of metaphase-I bivalents – probably in correspondence of the centromeric region – is due to the presence of argentophilic proteins, which are not directly related to the presence of rDNA. On the other hand, it is known that NOR proteins which are multifunctional components of the nucleolus, can migrate from the nucleolus to the chromosomes (HERNANDEZ-VERDUN et al. 1993).

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 *C. vulgatum* chromosome complement. The results demonstrated that the hexanucleotide occurred at the ends of all pachytene chromosomes. This finding brings the number of known gastropod species with telomeric $(TTAGGG)_n$ repeats to a total of six. The others are the prosobranchs *Patella coerulea* L. (pers. unpubl. data), *Melarhaphe neri-*

toides (Linnaeus, 1758) (COLOMBA et al. 2002) and *Fasciolaria lignaria* (VITTURI et al. 2000a), the opisthobranch *Bulla striata* Bruguière 1792 and the Pulmonata *Cantareus aspersus* (Miller 1774) (pers. unpubl. data). Since these species represent the three high taxa (i.e. sub-classes) of Gastropoda, we conclude that the hexa-telomere repeat is commonly widespread within this class.

Unlike gastropods, other invertebrates such as Polychaeta (shown in VITTURI et al. 2002), several arthropods (SAHARA et al. 1999) and ciliates (LIU and FREDGA 1999) have not conserved this telomeric motif.

In the genome of *C. vulgatum*, no evident label were revealed by the $(GATA)_n$ FISH, except for a minute hybridization site. This result reinforces a previous assumption (COLOMBA et al. 2002) according to which a heterogeneous distribution of the microsatellite $(GATA)_n$ occurs among gastropods. In fact, this sequence which was found to be very abundant in the genome of the tulip shell *F. lignaria* (VITTURI et al. 2000a) and of the slug *Milax nigricans* (COLOMBA et al. 2002), was absent or, if present, occurred in a very low amount in the periwinkle *Melarhaphe neritoides* (COLOMBA et al. 2002) and in *C. vulgatum* (this paper).

In conclusion, even if FISH using repeated nucleotide sequences has rarely been employed in Gastropoda, however, fom these studies it has emerged that repeated DNAs are very useful to examine the genomic constitution at the molecular level and to investigate on the reconstruction of chromosomal changes during evolution. For instance, a 18S rDNA probe has been employed to examine Robertsonian chromosome rearrangements in the Atlantic dogwhelk *Nucella lapillus* (PASCOE et al. 1996), whereas a telomeric $(TTAGGG)_n$ probe has been employed to demonstrate the chromosome fusion point of a Robertsonian translocation in the freshwater snail *Biwamelania habei* (NOMOTO et al. 2001). Moreover, the use of the microsatellite $(GATA)_n$, which was found to have a sex-linked arrangement in certain eukaryotic species (NANDA et al. 1992), has revealed that this sequence did not occur in the Y chromosome of *F. lignaria* (VITTURI et al. 2000a).

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