

FISH mapping of 18S-28S and 5S ribosomal DNA, (GATA)_n and (TTAGGG)_n telomeric repeats in the periwinkle *Melarhappe neritoides* (Prosobranchia, Gastropoda, Caenogastropoda)

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Spermatocyte chromosomes of *Melarhappe neritoides* (Mollusca, Prosobranchia, Caenogastropoda) were studied using fluorescent *in situ* hybridization (FISH) with four repetitive DNA probes (18S rDNA, 5S rDNA, (TTAGGG)_n and (GATA)_n). Single-colour FISH consistently mapped one chromosome pair per spread using either 18S or 5S rDNA as probes. The telomeric sequence (TTAGGG)_n hybridized

with termini of all chromosomes whereas the (GATA)_n probe did not label any areas. Simultaneous 18S-5S rDNA and 18S-(TTAGGG)_n FISH demonstrated that repeated units of the three multicopy families are closely associated on the same chromosome pair.

Heredity (2002) **88**, 381–384. DOI: 10.1038/sj/hdy/6800070

Keywords: chromosomes; repetitive DNA; FISH; invertebrate; Mollusca; Caenogastropoda

Introduction

Fluorescence *in situ* hybridization (FISH) is a powerful technique enabling the visualization of nucleic acid probes on target chromosomes, nuclei, cells and tissues. To date, this method has seldom been employed in the molluscan class Gastropoda. 18S-28S rDNA has previously been used as a probe to map major ribosomal clusters to the chromosomes of two neogastropod species, the Atlantic dogwhelk *Nucella lapillus* (Pascoe *et al.*, 1996) and the Mediterranean tulip shell *Fasciolaria lignaria* (Vitturi *et al.*, 2000a), whereas nothing is known about the 5S rDNA localization among gastropods.

Only two papers deal with chromosome FISH mapping of other multigene families in these organisms. The first reports on *in situ* hybridization with the (TTAGGG)_n telomeric repeat to the chromosomes of *Oxynoe olivacea* (Opisthobranchia, Sacoglossa) (Vitturi *et al.*, 2000b) and *F. lignaria* (Vitturi *et al.*, 2000a), revealing the genomic occurrence of the hexanucleotide sequence only in the latter species. The second one shows that in *F. lignaria* (Vitturi *et al.*, 2000a), the (GATA)_n sequence is abundant and dispersed throughout the genome.

In the present paper we used single-colour FISH to spermatocyte chromosomes of the periwinkle *Melarhappe neritoides* (Prosobranchia, Gastropoda, Caenogastropoda)

to map repeated units of the two rDNA families (18S-28S rDNA and 5S rDNA) and to test the presence of (GATA)_n and (TTAGGG)_n repeats in the genome of this species. Available literature data report on the localization of major (18S-28S rDNA) and minor (5S rDNA) ribosomal clusters on different chromosome pairs as the most frequent configuration in vertebrates (see Lucchini *et al.*, 1993; Suzuki *et al.*, 1996). Since very little is known about rDNAs mapping in gastropods, we employed in addition to single colour FISH, simultaneous double-colour FISH to investigate about their physical relationship. Furthermore, a few works concerning the application of molecular DNA analysis were highly informative for the study of phylogeny and evolution in some gastropod genera (Ozawa and Okamoto, 1993; Collins *et al.*, 1996; Reid *et al.*, 1996) nevertheless, the molecular approach was never applied to chromosomes.

Mediterranean periwinkles are represented by three species, which according to the recent revision of Littorinidae (Reid, 1989), are *Littorina saxatilis*, *Nodilittorina punctata* and *M. neritoides*; the conventional chromosome analysis and the genome size of these species were previously reported (Vitturi *et al.*, 1988, 1995). In particular, the *M. neritoides* male complement was found to consist of 33 chromosomes including 16 autosomal pairs (four mono-armed (pair nos. 4, 5, 6, and 8) and 12 bi-armed (all others)) and one unpaired metacentric X chromosome (16AA+X0).

Materials and methods

A total of 225 *M. neritoides* specimens were examined. The periwinkles were sampled during November and December 2000 from two geographically distinct areas: Addaura (Palermo, NW Sicily, S Italy; 180 specimens) and the Lagoon of Venice (NE Italy, 45 specimens) and identified according to the guidelines of Parenzan (1970). Most of the collected specimens were not useful for the present study because they were females and chromosome spreads were difficult to get from them, therefore data reported herein were obtained from about eighty sexually mature males. Moreover, since adult *M. neritoides* specimens are small in size, chromosome preparations were carried out from pooled testes of 6–8 individuals at a time, after overnight *in vivo* colchicine treatment (1 ng/L in sea water).

Single-colour FISH was performed on fixed spermatocyte chromosomes as described by Vitturi *et al* (2000b) using four different probes: (a) a sea urchin (*Paracentrotus lividus*) rDNA probe consisting of sequences of the 18S rDNA; (b) a PCR-obtained 5S rDNA probe using as primers F (5'-TGCACGTAGTGTCCCAAGC) and R (5'-ACGACCATAACCAGTTGAATAC) deduced from the 5S coding sequence of insects available in Genbank, according to the protocol described by Mandrioli *et al* (2000); (c) a PCR-obtained telomeric hexanucleotide (TTAGGG)_n; and (d) a PCR-obtained (GATA)_n sequence. Both telomeric and (GATA)_n sequences were generated by PCR (PCR DIG-Probe Synthesis Kit: Roche) in the absence of a 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 manufacturer's (Boehringer Mannheim) instructions, while the remaining three probes were DIG-labelled following the manufacturer's protocol (Roche).

For multiple fluorescent *in situ* hybridization the 18S rDNA probe was labelled with biotin (Biotin-Nick Translation Mix: Roche) whereas both 5S rDNA and (TTAGGG)_n probes were the same as those employed in single-colour experiments. Chromosomes were denatured for 4 min in 70% formamide/2×SSC at 72°C. The mixed probe solution (4 ng/μl of each probe) was denatured for 5 min at 80°C. Hybridization was allowed to proceed in a moist plastic chamber at 37°C overnight. Slides were washed twice in 50% formamide/2×SSC at 37–38°C (5 min each), twice in 2×SSC at 37–38°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. Probe hybridization sites were detected using both Anti-DIG-Fluorescein, Fab Fragments and Streptavidin Texas Red conjugate according to the manufacturers' (Boehringer Mannheim, Molecular Probes) instructions. Slides were mounted in an antifade solution containing DAPI (3 μg/ml) and viewed under a Leica three-colour filter set (B/G/R) which allowed the simultaneous visualization of fluorescein- and Texas red-labelled hybridization sites (green and red, respectively) and chromosomal DNA (blue).

Chromosomes were observed with a Leica microscope and photographed with Kodak Ektacolor 1000 ASA film.

Results

With respect to all techniques here employed, specimens of both populations did not show any inter-population differences. For this reason they are not further distinguished.

18S rDNA FISH on 15 spermatogonial metaphases of three different specimens always showed two small metacentric elements bearing a hybridization signal in the short arm (Figure 1a). Homology of these chromosomes was conclusively demonstrated by the presence of a single hybridized bivalent in 25 examined spreads at metaphase I (Figure 1b).

Due to the scantiness of spermatogonial plates in chromosome preparations, FISH using 5S rDNA, (TTAGGG)_n and (GATA)_n probes was performed only on meiotic spreads. 5S rDNA FISH showed one hybridized bivalent per spread at metaphase I (Figure 2); the telomeric (TTAGGG)_n repeat hybridized to the termini of each bivalent (Figure 3); the microsatellite did not show any labelled areas on diakinetid spreads (Figure 4). During the same experiment, the (GATA)_n probe hybridized strongly to the chromosomes of *F. lignaria* (Vitturi *et al*, 2000a) chosen as a positive control, and to the slug *Milax nigricans* (Mollusca, Gastropoda, Pulmonata) (Figure 5).

Simultaneous 18S-5S rDNA FISH to the chromosomes of the same plate, allowed individuation of the two probes as partial distinct signals (red and green, respectively) because they were closely joined to each other (Figure 6).

Simultaneous *in situ* hybridization using 18S rDNA and (TTAGGG)_n as probes showed that ribosomal (red spot) and telomeric (green spot) sequences were located adjacently at the terminal region of the rDNA-bearing bivalent (Figure 7).

Discussion

Major ribosomal genes of specimens of *M. neritoides* from the Lagoon of Venice were previously localised using silver staining. They were on the short arm of a variable number of chromosomes, most frequently three, of which two were small metacentrics and the third a large subtelocentric (Vitturi *et al*, 1995). In the present study, single-colour FISH with 18S rDNA demonstrates that the silver positive area previously observed in the large subtelocentric chromosome does not contain any ribosomal clusters, since NORs were constantly mapped to one small metacentric chromosome pair in all three examined periwinkles.

In the same manner as 18S, the 5S rDNA probe hybridized to a single metaphase I bivalent. Single-colour FISH, however, was unable to determine whether the hybridized bivalents corresponded in the two experiments.

By means of simultaneous double-colour FISH, it was possible to establish that 18S-28S rDNA and 5S rDNA were co-localized and, presumably, interspersed, due to overlapping of the two hybridization signals.

18S and 5S rDNA co-localization is rare in vertebrates (Liu and Fredga, 1999). Among invertebrates, the 18S-28S and 5S rDNAs were found in the same chromosome in the nematode *Meloidogyne arenaria* (Vahidi *et al*, 1991) and in seven out of 11 species of calanoid copepods so far analyzed.

In situ hybridization using the (GATA)_n probe revealed a lack of evident labelling of the genome of *M. neritoides*,

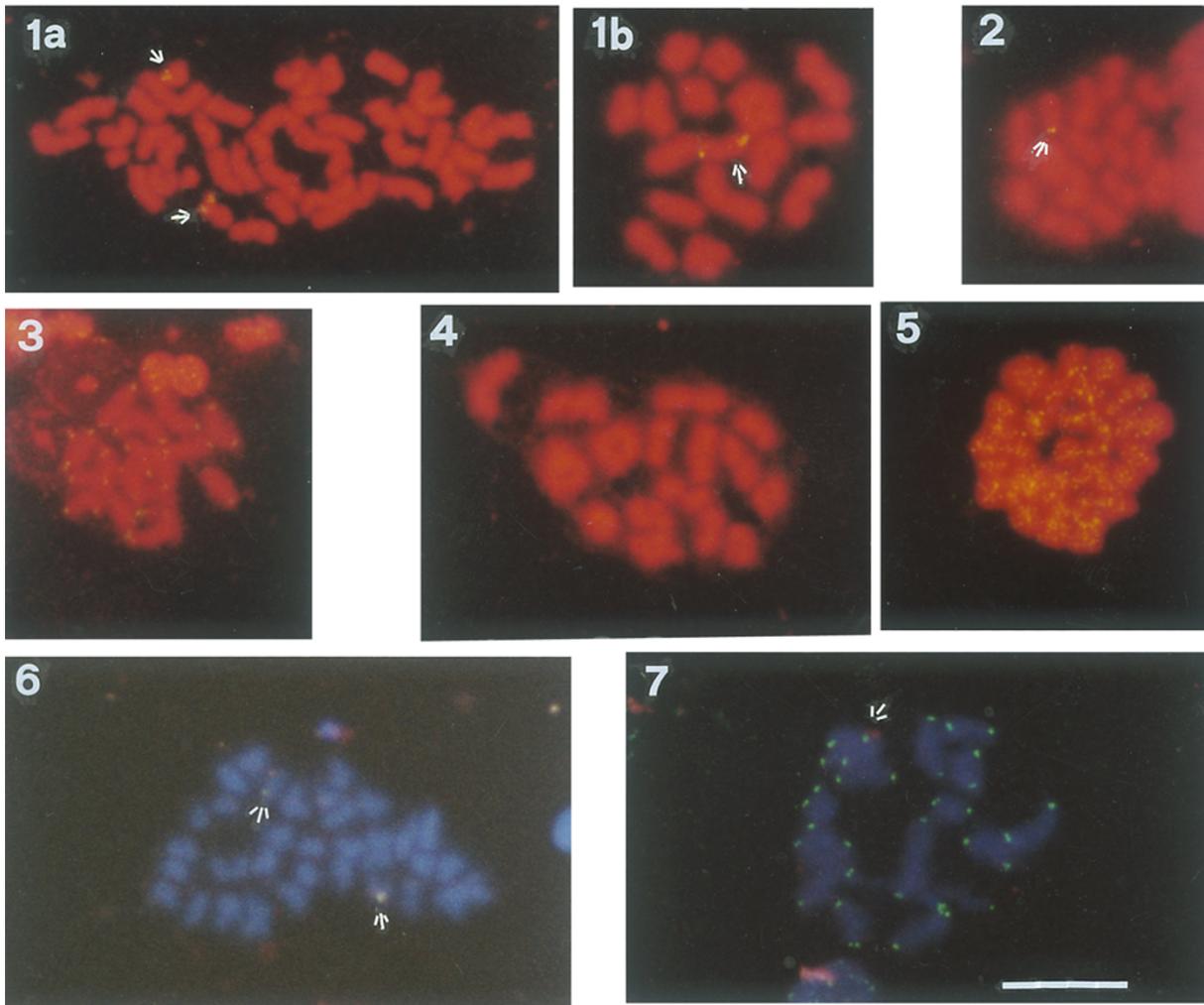


Figure 1 Spermatocyte chromosomes of *Melarhaphé neritoides* after rDNA FISH treatment with a heterologous 18S rDNA probe from the sea urchin *Paracentrotus lividus*: spermatogonial metaphase (a), diakinetive bivalents (b). (Arrows indicate hybridization signals).
Figure 2 Diakinetic bivalents after rDNA FISH treatment with a PCR obtained 5S rDNA probe. (Arrow indicates the hybridized bivalent.)
Figure 3 Pachytene chromosomes of *Melarhaphé neritoides* after FISH treatment with a PCR obtained (TTAGGG)_n sequence.
Figures 4 and 5 Diakinetic bivalents after FISH treatment with a PCR obtained (GATA)_n sequence of *Melarhaphé neritoides* (4) and *Milax nigricans* (5).
Figure 6 Spermatogonial chromosomes of *Melarhaphé neritoides* after simultaneous double-colour FISH treatment with 18S and 5S rDNAs as probes. (Arrows indicate co-localization of hybridization signals.)
Figure 7 Diakinetic bivalents of *Melarhaphé neritoides* after simultaneous double-colour FISH treatment with 18S rDNA and telomeric (TTAGGG)_n sequence as probes. (Arrow indicates the hybridized bivalent.)

suggesting that this sequence might be absent in the periwinkle or, if present, is in a very low amount. Any technical shortcoming can be ruled out about this finding because, during the same experiment, the (GATA)_n probe hybridized strongly to the chromosomes of *F. lignaria* (Vitturi *et al*, 2000a) chosen as a positive control, as well as to the chromosomes of the slug *Milax nigricans* of which FISH chromosome analysis was undertaken at the same time. These results show a heterogeneous distribution of the microsatellite (GATA)_n among gastropods.

FISH using the (TTAGGG)_n probe demonstrates that the hexanucleotide is present at the chromosomal ends of the periwinkle examined here. It is worth remarking that, among the Mollusca, this sequence has also been found in the neogastropod *F. lignaria* (Vitturi *et al*, 2000a) and in two species of the class Bivalvia (Guo and Allen, 1997; González-Tizón *et al*, 1998), whereas it is absent in

another gastropod species, the sacoglossan *Oxynoe olivacea* (Opisthobranchia) (Vitturi *et al*, 2000b). Other telomeric sequences, considered to be derivatives of this, have been found, including, among others, the pentamere (TTAGG)_n repeat in insects (Okazaki *et al*, 1993; Sahara *et al*, 1999), the (TTGGGG)_n in ciliates (Liu and Fredga, 1999) and the *Arabidopsis*-type (TTTAGGG)_n in plants (Görtner *et al*, 1998).

Double colour FISH has been performed to examine the relationship between repeated units of major rDNA and telomeric sequences. A close association between repeated units of the two multigene families could be observed. Since results of this study also demonstrated a co-localization between 18S-28S and 5S, it can be concluded that, in the periwinkle, three multicopy families are linked on the same chromosome pair.

Some authors (Dover, 1986; Liu and Fredga, 1999)

claim that co-localization or interspersions of repeated units of different multigene families may be a selective advantage. In fact, an adjacent disposition of such genes might cause unequal crossing-over with consequent heteromorphism which would play an important role in the maintenance of a conserved and multiple array.

Data from the literature indicate that this assumption may be true only for some species. In fact, among vertebrates, an adjacent disposition between NORs and telomeres is reported to be an unusual finding (see Liu and Fredga, 1999) and a co-localization of 18S-28S and 5S rDNAs is not considered the rule, but rather the exception (Sola *et al*, 2000; Mandrioli *et al*, 2000). For example, in fish, a co-localization of repeated units of ribosomal DNAs has been described only in four out of 20 species so far analyzed (Pendás *et al*, 1994; Morán *et al*, 1996; Mandrioli *et al*, 2000).

Finally, among invertebrates, a high degree of rDNA polymorphism occurs (eg, Vitturi *et al*, 1999, 2000c), and terminal NORs are often observed. This implies that major rDNA clusters are physically associated to telomeric sequences. Among these organisms next to nothing or very little is known about the physical relationship between repeated units of the 18S-28S and 5S rDNA because, at the present time, this feature has seldom been investigated.

Acknowledgements

We gratefully acknowledge Dr R Barbieri for providing the sea urchin rDNA probe and Prof AM Pirrone for supplying telomeric sequence and labelling all probes. This work was supported by a MURST grant (ex 60%) to R Vitturi.

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