ELSEVIER

Micron 36 (2005) 351–357

micron

www.elsevier.com/locate/micron

Cytogenetics of the land snails *Cantareus aspersus* and *C. mazzullii* (Mollusca: Gastropoda: Pulmonata)

Roberto Vitturi[a], Angelo Libertini[b], Luca Sineo[a], Ignazio Sparacio[a], Antonella Lannino[a], Armando Gregorini[c], Mariastella Colomba[d,*]

[a]*Dipartimento di Biologia Animale, Università di Palermo, Via Archirafi 18, 90123 Palermo, Italy*
[b]*Istituto di Scienze Marine, CNR, Riva 7 Martiri 1364/a, 30122 Venezia, Italy*
[c]*Istituto di Psicologia, Università di Urbino "Carlo Bo", Via Zeppi, 61029 Urbino (PU), Italy*
[d]*Istituto di Ecologia e Biologia Ambientale, Università di Urbino "Carlo Bo", Via Sasso 75, 61029 Urbino (PU), Italy*

Received 30 September 2004; revised 1 November 2004; accepted 2 December 2004

Abstract

A cytogenetic study was carried out on the chromosomes and nuclear DNA contents of the land snails *Cantareus aspersus* and *C. mazzullii* (Gastropoda: Pulmonata). Chromosomes were studied using Giemsa staining, banding methods and fluorescent in situ hybridization (FISH) with three repetitive DNA probes [18S rDNA, $(GATA)_n$ and $(TTAGGG)_n$]. Results were very similar in the two species both showing (1) 54 bi-armed chromosomes [submetacentrics (SM) + metacentrics (M) + subtelocentrics (ST)]; (2) 10 terminal NORs after sequential application of rDNA FISH and silver staining; (3) uniform DNA fluorescence with CMA_3 and DAPI staining and (4) genomic composition considerably enriched both in highly- and moderately-repeated DNAs. The telomeric $(TTAGGG)_n$ sequence hybridized with the termini of all of the chromosomes in the two species. In spite of their apparent karyological uniformity, flow cytometry DNA assays showed that *C. aspersus* and *C. mazzullii* are characterized by different nuclear DNA content (*C* values are 3.58 and 3.08 pg, respectively) and slightly different base composition in their genomes. Present data on GS and AT% in *C. mazzullii* and *C. aspersus* confirm the trend toward high GS values and GC percentages among land snails.

1. Introduction

It is well known that banding techniques such as silver impregnation (Ag-NOR banding), C-banding and fluorochrome staining have been successfully used to obtain knowledge on genomic DNA complexity and organization in animals and plants (Sumner, 1990). An important contribution in information has been provided by results obtained by means of fluorescent in situ hybridization (FISH) that enables a clear visualization of nucleic acid probes on target chromosomes. For instance, the use of homologous or heterologous ribosomal sequences (rDNA FISH) proved to be essential for conclusively mapping major (18S–28S) ribosomal clusters, otherwise unidentifiable after silver and/or chromomycin A_3 staining, in the karyotype of several vertebrate and invertebrate species (see for example: Sánchez et al., 1995; Pendás et al., 1993; Lorite et al., 1997; Colomba et al., 2000; Libertini et al., 2000). Furthermore, the human telomeric $(TTAGGG)_n$ probe was used to investigate the mechanisms underlying various types of Robertsonian translocation (Slijepcevic, 1998), a chromosome rearrangement universally considered one of the most frequent in karyotype evolution.

Land snails, freshwater snails and slugs (Mollusca: Gastropoda: Pulmonata), include some 30,000–35,000 species (Solem, 1984). Evolutionary inter-relationships within Pulmonates remain largely unresolved as proved by several rearrangements of their systematics (Shileyko, 1979; Tillier, 1989; Nordsieck, 1993; Wade et al., 2001). Cytogenetic studies may contribute additional characters

Abbreviations: NOR, nucleolar organizer region; CMA_3, chromomycin A_3; DAPI, 4,6-diamidino-2-phenylindole; C, DNA content of the haploid genome; GS, genome size; AT, adenine and thymine; GC, guanine and cytosine.

* Corresponding author. Tel.: +39 722 303 438; fax: +39 722 303 436.
E-mail address: m.colomba@uniurb.it (M. Colomba).

0968-4328/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micron.2004.12.010

but unfortunately, up-to-date karyological data are scarce and include: (1) the chromosome number (haploid and/or diploid) of 71 species; (2) silver staining of 12 species; (3) C-banding of one species; and (4) genome size of 17 species (Thiriot-Quiévreux, 2003; Gregory, 2004 for reviews). Two studies dealt with the physical mapping of repeated DNA chromosomal sequences. In particular, these studies included cloning, sequencing and mapping of the telomere $(TTAGGG)_n$ repeat in the freshwater snail *Biwamelania habei* (Nomoto et al., 2001) and the use of single- and double colour FISH methods to specify the relative position of three multigene families in the slug *Milax nigricans* (Vitturi et al., 2004).

Helix aspersa (Müller, 1774) and *Cantareus mazzullii* (De Cristofori and Jan, 1832) (Pulmonata: Stylommatophora: Helicidae) were for long time included into two different genera. On the basis of the result of an accurate anatomical comparison, Giusti et al. (1995) recently placed both species in the same genus *Cantareus*. However, the two snail species show pronounced differences in geographical distribution and ecology. *C. aspersus* has a West European-holomediterranean distribution and occurs from coastal regions up to an altitude of about 1000 m in many different biotopes including woods, rocky ground and antropized sites. *C. mazzullii* appears to be endemic of Sicily in the restricted sense, never having been reported outside this island, and is mainly found in stone screes and in rock cavities.

Chromosome analysis in the two land snails was carried out by conventional staining, banding and molecular methods. Moreover, genome sizes and AT-DNA nuclear content were also investigated.

2. Material and methods

Analyses were carried out on the following material: 40 individuals of *Cantareus (Helix) aspersus* from the meadows near Palermo (north-western Sicily, Italy) and 30 individuals of *C. mazzullii* from the Monte Pellegrino promontory (Palermo district). Collections were made during the years 2001 and 2002, but sexually mature specimens only occurred from October 2001 to March 2002.

In order to increase the yield in mitotic metaphases, specimens were immersed for 2 h in a freshwater colchicine solution (0.01%) at room temperature. The male portion of the gonad was dissected and treated following the air-drying technique for molluscan species described elsewhere (Vitturi, 1992).

Slides were processed for direct Giemsa staining, C-banding (Sumner, 1990) and silver staining of NORs (Howell and Black, 1980). For fluorescent pattern analysis, chromosomes on different slides were stained separately with the AT-specific dye DAPI or the GC-specific one CMA_3 (Sumner, 1990).

Chromosome morphology was described according to the criteria proposed by Levan et al. (1964). Sub-telocentric chromosomes were considered as bi-armed.

FISH was performed on fixed chromosomes from the testis as described by Vitturi et al. (2000a) using three different probes: a sea urchin (*Paracentrotus lividus*) 18S rDNA probe; a $(TTAGGG)_n$ telomeric hexanucleotide, and a $(GATA)_n$ sequence. The latter two probes were obtained by PCR in the absence of template (Ijdo et al., 1991) using as primers $(TTAGGG)_5$ along with $(CCCTAA)_5$, and $(GATA)_7$ along with $(TATC)_7$, respectively. The PCR products were DIG-labeled following the random priming protocol (Roche). Nick translation labeling with digoxigenin of the 18S rDNA was performed according to manufacturer's instructions (Roche). Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and a Leica I3 filter set (BP 450-490, LP 515) allowed the simultaneous visualization of fluorescein-labeled hybrid (yellow) and chromosomal DNA (red).

Chromosomes were observed with a Leica microscope and photographed with a 800 ASA film. GS and AT base-pair DNA nuclear content were evaluated through flow cytometric assay performed on cell suspensions of *C. aspersus* (10 specimens) and *C. mazzullii* (20 specimens) obtained from the foot muscle, by means of a BRITE-HS cytometer (Bio-Rad Laboratories Inc.) equipped with a xenon-mercury lamp. Peripheral blood erythrocytes from chicken (GS (2C) $=2.54$ pg, AT-DNA (2C) $=1.39$ pg; Ronchetti et al., 1995) were added to sample cell suspensions as internal standard. Nuclei were stained with propidium iodide and DAPI for GS and AT-DNA evaluation, respectively. For each sample at least 2500 cells were examined, and the DNA index (mean channel number of the G1/G0 peak of the land-snail cells over the mean channel number of the G1/G0 peak of the chicken cells) was evaluated after elaboration of the fluorescence data by means of Modfit software (Verity Software House). The average DNA indices among the analyzed samples, multiplied by half of the DNA content of the standard, gave the *C*-values assigned to the investigated species (data are reported as mean $\pm$ standard deviation).

3. Results

3.1. Nuclear DNA content

The mean *C* values of GS and AT-DNA of *C. mazzullii* were determined as 3.08 ± 0.06 pg and 1.62 ± 0.02, respectively; calculated AT percentage was 52.68% of the whole genome weight. In *C. aspersus* GS was 3.58 ± 0.09 pg, AT-DNA 1.82 ± 0.03 pg, and AT percentage 50.78%.

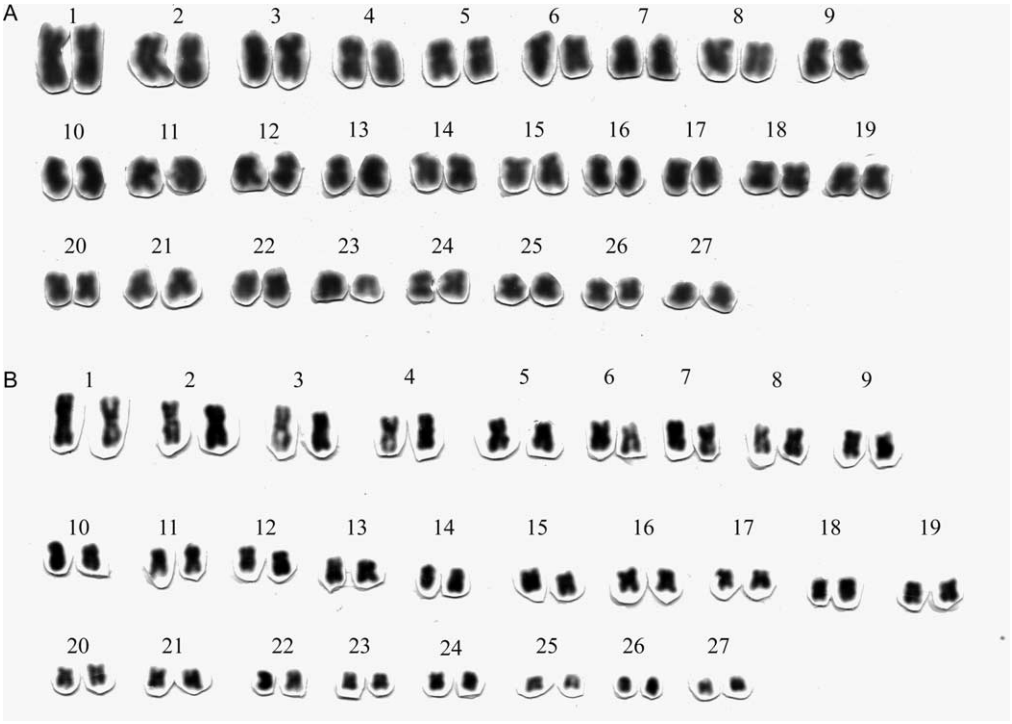

Fig. 1. Giemsa karyotype obtained from stained spermatogonial metaphase chromosomes of *C. aspersus* (A) and *C. mazzullii* (B).

3.2. Chromosome analyses

With respect to all chromosome techniques here employed, individuals of the two species show very similar results. For this reason, the two species are not further distinguished in this section. Chromosome images referring to *C. aspersus* and *C. mazzullii* are designated as (A) and (B), respectively.

3.2.1. Untreated chromosomes and karyotyping

Karyotype consisted of $2n = 54$ bi-armed chromosomes (SM + M + ST) (Fig. 1(A) and (B)). Chromosome pairs could not be divided into size classes, since their lengths gradually varied.

3.2.2. Fluorochrome staining

CMA_3 (Fig. 2(A) and (B)) and DAPI (Fig. 3(A) and (B)) staining showed that all chromosomes fluoresced with uniform intensity. Therefore, the two fluorochromes were uninformative for detecting any repeated and/or compartmentalized DNA sequence, including the nucleolus organizer regions.

3.2.3. rDNA FISH and silver staining

18S rDNA probe labeled the terminal region of 10 chromosomes (Fig. 4(A) and (B)). Silver impregnation carried out on the same metaphases produced NOR-banding patterns always corresponding to the hybridization ones (Fig. 5(A) and (B)).

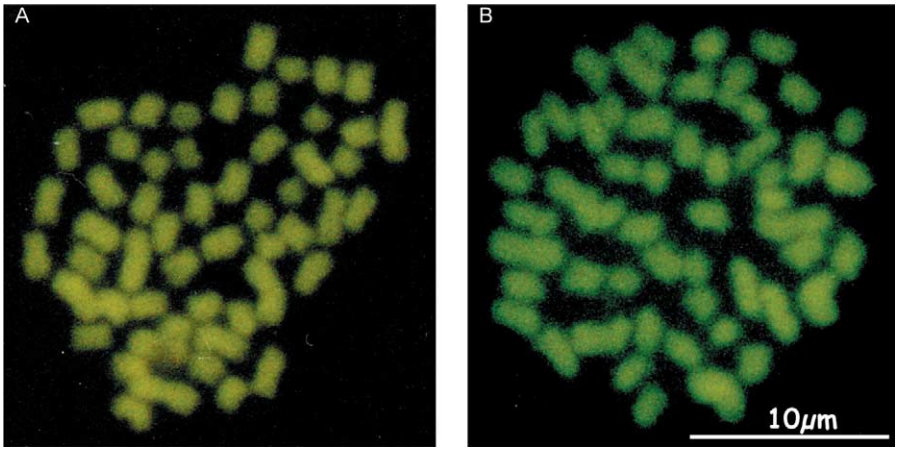

Fig. 2. CMA_3 stained spermatogonial chromosomes of *C. aspersus* (A) and *C. mazzullii* (B).

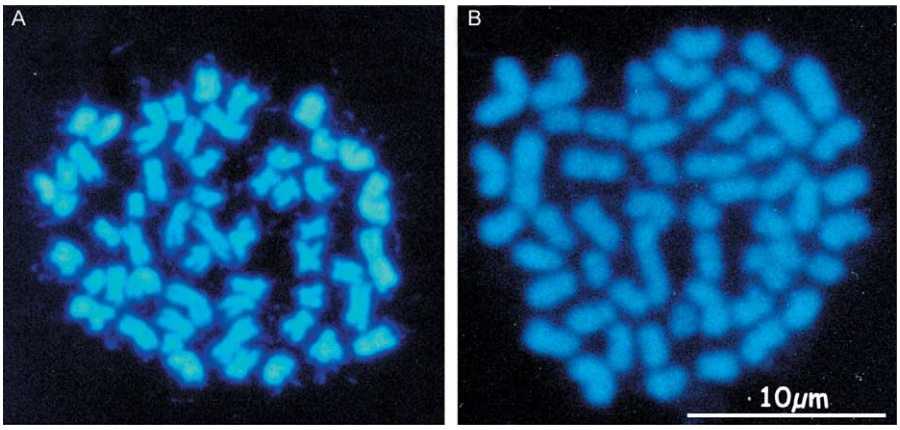

Fig. 3. DAPI stained spermatogonial chromosomes of *C. aspersus* (A) and *C. mazzullii* (B).

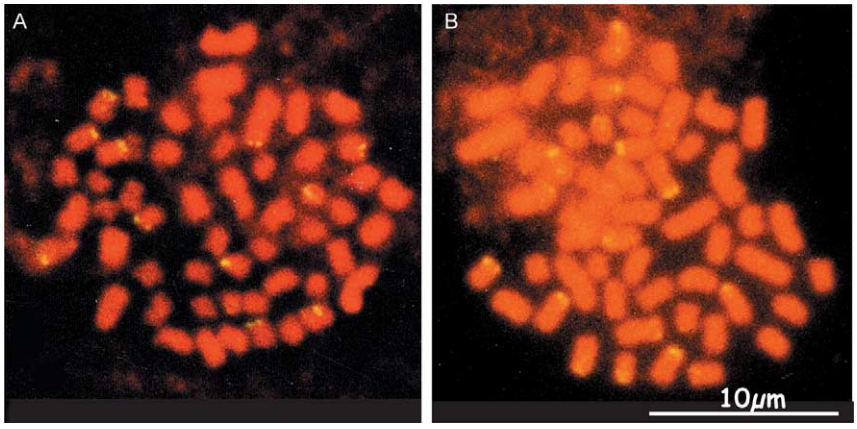

Fig. 4. Spermatogonial chromosomes of *C. aspersus* after FISH with 18S rDNA probe (A); spermatogonial chromosomes of *C. mazzullii* after FISH with 18S rDNA probe (B).

3.2.4. CBG-banding and GATA-FISH

Pachytene bivalents were C-positive and all displayed heterochromatic bands in the median/submedian centromeric region. Moreover, several bivalents showed additional bands which were terminally and/or interstitially located (Fig. 6(A) and (B)). The GATA probe hybridized with small regions dispersed along the chromosomal body (Fig. 7(A) and (B)). No preferential concentration of GATA labeling signals was observed.

3.2.5. $(TTAGGG)_n$ FISH

Images obtained by standard FISH showed typical hybridization signals with the $(TTAGGG)_n$ probe at the chromosomal ends (Fig. 8(A) and (B)).

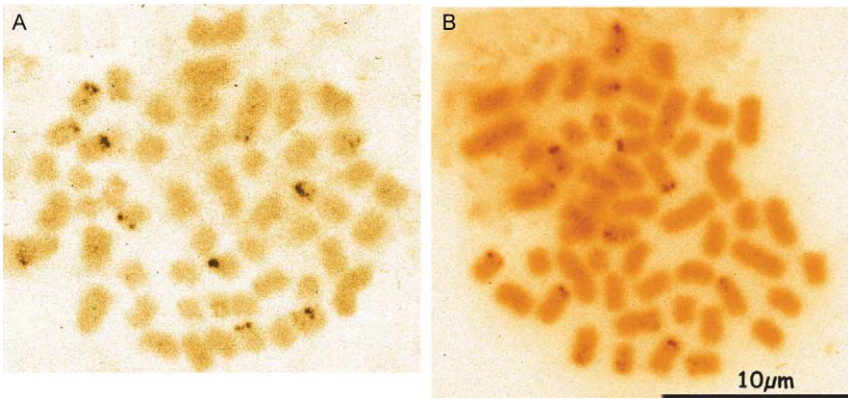

Fig. 5. Silver staining of spermatogonial chromosomes of *C. aspersus* (A) (same plate as in Fig. 4A); silver staining of spermatogonial chromosomes of *C. mazzullii* (B) (same plate as in Fig. 4B).

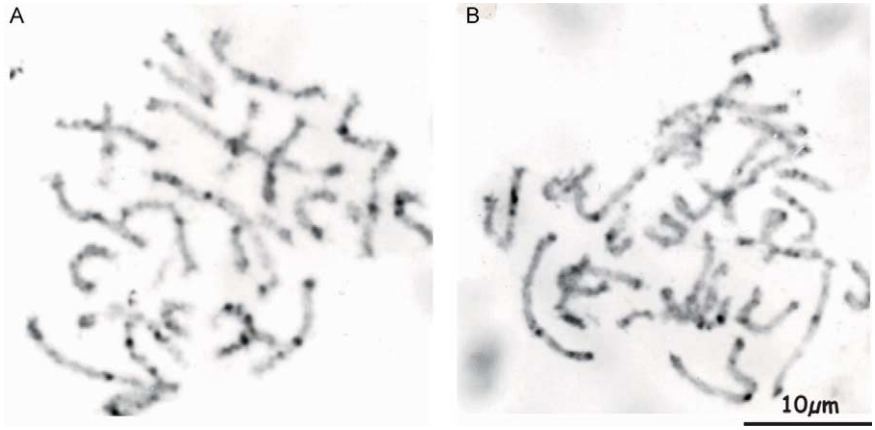

Fig. 6. C-banded pachytene chromosomes of *C. aspersus* (A) and *C. mazzullii* (B).

4. Discussion

Despite their pronounced geographical and ecological diversity, *C. aspersus* and *C. mazzullii* displayed a high similarity in karyotype gross morphology. In fact, the two species exhibit a complement of 54 bi-armed (SM+M+ST) chromosomes. Moreover, the same level of karyotypical uniformity was observed in rDNA location (NORs) and in fluorescent GC- or AT-specific staining pattern. Sequential application of silver staining and 18S rDNA FISH invariably localized NORs on the terminal region of 10 chromosomes per cell, whereas CMA_3 and DAPI dyes both yielded uniform fluorescence of chromatin. Taking into account these observations the following general remarks may be advanced: (1) the complete matching between silver impregnation and 18S rDNA FISH indicates that all ribosomal genes are transcriptionally active; (2) the homogeneous DAPI- and CMA_3-staining of genomic DNA suggests that in the two *Cantareus* species chromatin is not compartmentalized, but rather AT and GC base pairs are equally interspersed in both eu- and heterochromatin; (3) the finding that NORs are not enhanced by CMA_3 demonstrates that these regions do not contain GC-rich DNA and, at the same time, provides further evidence for the inadequacy of CMA_3 staining for a proper identification of NORs in Gastropoda (Vitturi et al., 1997, 2002).

Ten NOR-bearing chromosomes were invariably found in *C. aspersus* and *C. mazzullii* karyotypes. A similar value has never before been observed in molluscs. In gastropods, most of the species showed two NORs, whereas the dogwhelk *Nucella lapillus* (Pascoe et al., 1996) and the Mediterranean tulip shell *Fasciolaria lignaria* (Vitturi et al., 2000a) had a maximum number of four and eight NOR sites, respectively. In bivalves from two to six NOR-bearing chromosomes for karyotype were found (Thiriot-Quievreux, 2002 and references therein). Following the assumption suggested by several authors that a single pair of NORs per cell is primitive (plesiomorphic) in most vertebrates (Schmid, 1978) and invertebrates including molluscs (Pascoe et al., 1996; Thiriot-Quievreux, 2002), an increase in the number of the NOR sites could have occurred in a common ancestor of the two *Cantareus* species. Concessing the mechanism generating such a numerical change, the literature suggests different possibilities, including the translocation of ribosomal

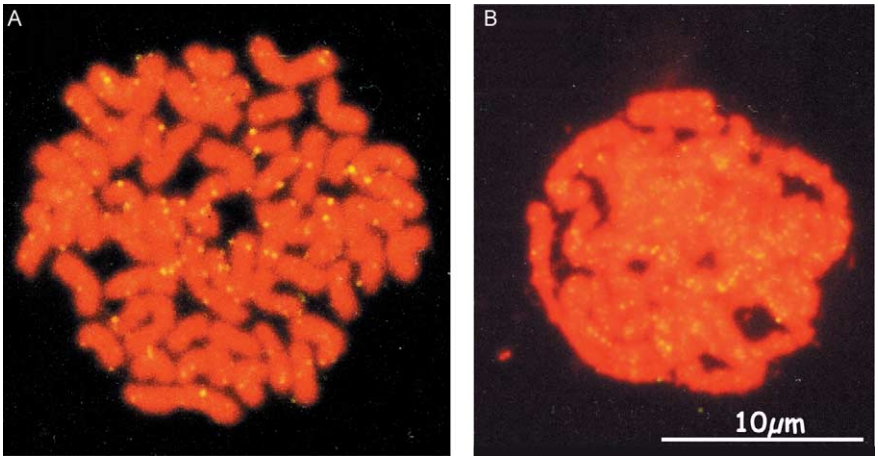

Fig. 7. Spermatogonial chromosomes of *C. aspersus* (A) and pachytene bivalents of *C. mazzullii* (B) after FISH with $(GATA)_n$ probe.

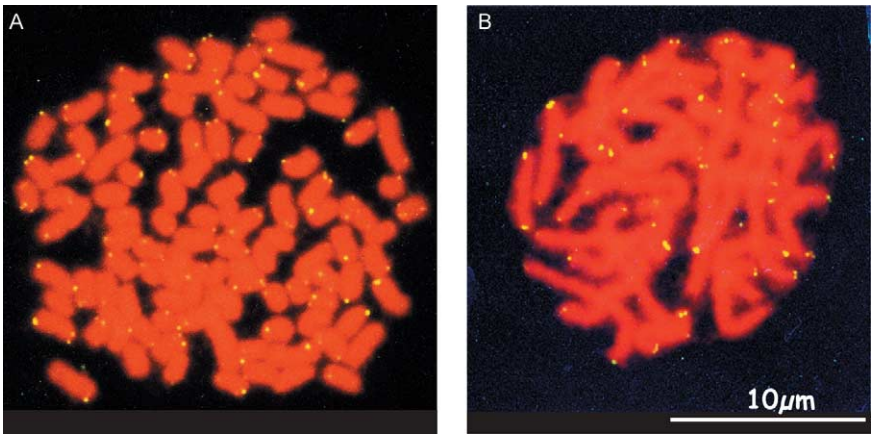

Fig. 8. Spermatogonial chromosomes of *C. aspersus* (A) and pachytene bivalents of *C. mazzullii* (B) after FISH with the telomeric probe $(TTAGGG)_n$.

cistrons by unequal crossing over. As in other species with multiple NORs (Vitturi et al., 1996), in both *C. aspersus* and *C. mazzullii*, the terminal chromosome position of the 18S rDNA clusters suggests the possibility of involvement of telomeric sequences in favouring these rearrangements of NORs.

C. aspersus and *C. mazzullii* were also screened with FISH for the presence of the human telomere repeat $(TTAGGG)_n$. Its occurrence in both species brings the number of pulmonates with this type of telomeric sequence to a total of seven, one freshwater snail (Nomoto et al., 2001), four slugs (Vitturi et al., 2004; personal unpublished data) and two land snails (present paper). However, to authors' knowledge, the same sequence is absent in two other gastropod species, the opisthobranch *Oxynoe olivacea* (Vitturi et al., 2000b) and the caenogastropod *Crepidula unguiformis* (personal data), thus demonstrating that, although common, it is not the only telomere 'motif' in this class.

The GS values found in *C. aspersus* (3.58 pg) and *C. mazzullii* (3.08 pg) differ from one another and, although among the highest in the subclass Pulmonata (range 0.95–4.00 pg in Gregory, 2004), they are intermediate with those ones already determined in other two species of Helicidae: *Helix vulgaris* (4.00 pg) (Vinogradov, 2000) and *Arianta arbustorum* (2.84 pg) (Vinogradov, 1998). Since the land snails of this study showed a similar $2n = 54$ chromosomes karyotype, most or all of the genome size changes ought to have occurred within chromosomes. Surprisingly, in the two species very similar patterns were obtained after C-banding and GATA FISH, therefore these techniques did not provide any direct indication of the qualitative nature of the DNA involved in GS variation. However, it is likely that GS variation may reflect changes of repeated DNA, since the experiments on C-heterochromatin and the microsatellite $(GATA)_n$ showed that the genome in the two land snails is considerably enriched in both highly- and moderately-repeated DNAs. This assumption is consistent with the results reported for congeneric species of other animals such as the North American minnows (Cyprinidae) (Gold and Price, 1985) and the grasshoppers of the genus *Pamphagus* (Vitturi et al., 1993).

A question arises about whether repeated DNA sequences in the two land snails were accumulated or lost during their karyotypical evolution. However, since available data on GS and genome base composition in stylommatophoran pulmonates are not enough, no hypothesis can be advanced.

In a previous study on the DNA content in pulmonate gastropods (Vinogradov, 2000), significantly higher GS and GC percentages (more than 40%, i.e. corresponding to an AT percentage less than 60%) were found in terrestrial species as compared to the aquatic ones. The enrichment of GC percentage in the genome was argued to increase the DNA physical stability, which would be secured by the triple H-bonds in a GC pair. This condition, therefore, was considered as an adaptive trait for terrestrial life. Present data on GS and AT% in *C. mazzullii* and *C. aspersus* confirm the trend toward high GS values and GC percentages among land snails.

Acknowledgements

This work was supported by a MURST grant (ex 60%) to M. Colomba.

References

Colomba, M., Vitturi, R., Zunino, M., 2000. Chromosome analysis and rDNA FISH in the stag beetle *Dorcus parallelipipedus* L. (Coleoptera: Scarabaeoidea: Lucanidae). Hereditas 133, 249–253.

Giusti, F., Manganelli, G., Scembri, P.J., 1995. The non-marine molluscs of the Maltese islands. Museo Regionale di Scienze Naturali Torino. Monografie 15. Torino.

Gold, J.R., Price, H.J., 1985. Genome size variation among north American minnows (Cyprinidae). I. Distribution of the variation of five species. Heredity 54, 297–305.

Gregory, T.R., 2004. Animal genome size database. Molluscs. URL http://www.genomesize.com/molluscs.htm.

Howell, W.M., Black, D.A., 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36, 1014–1015.

Ijdo, J.W., Wells, R.A., Baldini, A., Reeders, S.T., 1991. Improved telomere detection using a telomere repeat probe $(TTAGGG)_n$ generated by PCR. Nucleic Acid Res 19, 4780.

Levan, A.R., Fredga, K., Sandberg, A.A., 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52, 201–220.

Libertini, A., Colomba, M.S., Vitturi, R., 2000. Cytogenetics of the amphipod *Jassa marmorata* Holmes, 1903 (Corophioidea: Ischyroceridae): karyotype morphology, chromosome banding, fluorescent in situ hybridization and nuclear DNA content. J. Crust. Biol. 20, 350–356.

Lorite, P., Aranega, A.E., Luque, F., Palomeque, T., 1997. Analysis of nucleolar organizing regions in the ant *Tapinoma nigerrimum* (Hymenoptera Formicidae). Heredity 78, 578–582.

Nomoto, Y., Hirai, M., Ueshima, R., 2001. Cloning of molluscan telomere DNA with $(TTAGGG)_n$ repeat and its chromosomal location in the freshwater snail *Biwamelania habei*. Zoolog. Sci. 18, 417–422.

Nordsieck, H., 1993. Phylogeny and system of the Pulmonata. Arch. Moll. 121, 31–52.

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

Pendás, A.M., Morán, P., Freije, J.P., García-Vázquez, E., 1993. Multichromosomal location of ribosomal RNA genes and heterochromatin association in brown trout. Chromosome Res. 1, 63–67.

Ronchetti, E., Salvadori, S., Deiana, A.M., 1995. Genome size and AT-content in Anguilliformes. Eur. J. Histochem. 39, 259–264.

Sánchez, A., Jimenez, R., Burgos, M., Stitou, S., Zurita, F., Delaguardia, R.D., 1995. Cytogenetic peculiarities in the Algerian hedgehog: silver stains not only NORs but also heterochromatic blocks. Heredity 75, 10–16.

Schmid, M., 1978. Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma 66, 361–388.

Shileyko, A.A., 1979. The system of the order Geophila (=Helicida) (Gastropoda: Pulmonata). Proc. Zool. Inst. Acad. Sci. USSR 80, 44–69.

Slijepcevic, P., 1998. Telomeres and mechanisms of Robertsonian fusion. Chromosoma 107, 136–140.

Solem, A., 1984. A world model of land snail diversity and abundance, in: Solem, A., van Bruggen, A.C. (Eds.), Word-wide snails: biogeographical studies on non-marine Mollusca. Leiden, Brill, pp. 6–22.

Sumner, A.T., 1990. Chromosome banding. Unwin Hyman Ltd, London.

Thiriot-Quievreux, C., 2002. Review of the literature on bivalve cytogenetics in the last ten years. Cah. Biol. Mar. 43, 17–26.

Thiriot-Quiévreux, C., 2003. Advances in chromosomal studies of gastropod molluscs. J. Moll. Stud. 69, 187–202.

Tillier, S., 1989. Comparative morphology, phylogeny and classification of land slugs and snails (Gastropoda:Pulmonata: Stylommatophora). Malacologia 30, 1–303.

Vinogradov, A.E., 1998. Variation in ligand-accessible genome size and its ecomorphological correlates in a pond snail. Hereditas 128, 59–65.

Vinogradov, A.E., 2000. Larger genomes for molluskan land pioneers. Genome 43, 211–212.

Vitturi, R., 1992. Conventionally stained chromosomes, constitutive heterochromatin and nucleolus organizer regions in *Milax nigricans* (Gastropoda: Pulmonata). Chromatin 1, 147–155.

Vitturi, R., Mansueto, C., Di Paola Ficarella, F., 1993. Heterochromatin variation in four species of the genus *Pamphagus* (Orthoptera, Pamphagidae) analysed by C-banding. Biol. Zbl. 112, 335–341.

Vitturi, R., Libertini, A., Mazzola, A., Colomba, M.S., Sarà, G., 1996. Characterization of mitotic chromosomes of four species of the genus *Diplodus*: karyotypes and chromosomal nucleolar organizer region phenotypes. J. Fish. Biol. 49, 1128–1137.

Vitturi, R., Pandolfo, A., Colomba, M.S., Damiani, F., Chemello, R., 1997. Characterization of mitotic and meiotic chromosomes of the vermetid gastropod *Dendropoma* (*Novastoa*) *petraeum* (Monterosato, 1884) (Mollusca, Caenogastropoda). Ophelia 46, 127–139.

Vitturi, R., Colomba, M.S., Gianguzza, P., Pirrone, A.M., 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.S., Jensen, K.R., Riggio, S., 2000b. Cytogenetics in the sacoglossan *Oxynoe olivacea* (Mollusca: Opisthobranchia): karyotype, chromosome banding and fluorescent in situ hybridization. Mar. Biol. 137, 577–582.

Vitturi, R., Colomba, M., Castriota, L., Beltrano, A.M., Lannino, A., Volpe, N., 2002. Chromosome analysis using different staining techniques and fluorescent in situ hybridization in *Cerithium vulgatum* (Gastropoda: Cerithiidae). Hereditas 137, 101–106.

Vitturi, R., Sineo, L., Volpe, N., Lannino, A., Colomba, M., 2004. Repetitive DNAs in the slug *Milax nigricans*: association of ribosomal (18S–28S and 5S rDNA) and $(TTAGGG)_n$ telomeric sequences in the slug *M. nigricans* (Mollusca: Gastropoda: Pulmonata). Micron 35, 255–260.

Wade, C.V.M., Mordan, P.B., Clarke, B., 2001. A phylogeny of the land snails (Gastropoda: Pulmonata). Proc. R. Soc. Lond. B 268, 413–422.