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Karyotypic characterization of representatives from Melolonthinae (Coleoptera: Scarabaeidae): karyotypic analysis, banding and fluorescent in situ hybridization (FISH)

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Meiotic chromosomes of *Phyllophaga* (*Phytalus*) *vestita*, *Phyllophaga* (*Phyllophaga*) *aff capillata* and *Lyogenys fuscus* (Melolonthinae) were analyzed by conventional staining, C-banding, fluorochromes, silver nitrate and FISH. The three species had a diploid number of 2n = 20 and a sex mechanism of the (Xy_p; XY_p) parachute type. *P.* (*Phytalus*) *vestita*, *P.* (*Phyllophaga*) *aff capillata* and *Lyogenys fuscus* showed pericentromeric constitutive heterochromatin (CH) in all autosomal bivalents and on X chromosomes. Staining with CMA_3 and DAPI fluorochromes showed that the CH of *P.* (*Phytalus*) *vestita* is not specifically rich in AT and GC-base pairs, whereas in *P.* (*Phyllophaga*) *aff capillata* the sex bivalent and one autosomal pair were found to be enriched in GC base pairs with CMA_3, and in *Lyogenys fuscus* CH was positive for DAPI. Silver nitrate staining revealed nucleolar remnants in all three species. However, FISH obtained a precise identification of nucleolar organizing regions with an rDNA 18S and 25S probe. A signal of hybridization was seen in each species, being detected in the X chromosome of *P.* (*Phytalus*) *vestita* and *Lyogenys fuscus*, and in a small autosomal bivalent of *P.* (*Phyllophaga*) *aff capillata*.

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The superfamily Scarabaeoidea is represented by 13 families the world over, among them, the Scarabaeidae family is particularly important by having undergone wide adaptive spreading, with a fauna comprising approximately 2000 genera and 25000 species. A total of 362 genera and 4706 species have been recorded in the Neotropical region, and about 1777 species belonging to 204 genera have been recorded in Brasil (COSTA 2000).

Although Scarabaeidae represents a rich and diversified fauna, chromosomal studies of this family are still scarce. About 323 species have been studied karyotypically thus far, corresponding to 1.29 % of the species described (SMITH and VIRKKI 1978; YADAV and PILLAI 1979; YADAV et al. 1979; VIDAL and NOCERA 1984; COLOMBA et al. 1996, 2000a; BIONE 1999). A considerable predominance of a diploid number of 2n = 20, biarmed chromosomes and a sex mechanism of the Xy_p type has been widely detected.

The use of C-banding, silver nitrate staining and base-specific fluorochromes is limited to few scarabeoid species, such as *Enema pan* (VIDAL and GIACOMOZZI 1978), *Glyphoderus sterquilinus*, *Eucranium arachnoids*, *Anomiopsoides heteroclyta* (VIDAL and NOCERA 1984), *G. sterquilinus*, *Bubas bison* (COLOMBA et al. 1996), *Macraspis festiva*, *Pelidnota pallidipennis*, *Lygyrus ebenus*, *Geniates borelli* (BIONE 1999), and *Gymnopleurus sturmi* (COLOMBA et al. 2000a). On the other hand, fluorescent in situ hybridization (FISH) with rDNA probes has been used in a few beetles species (GÁLIAN et al. 1995; DE LA RÚA et al. 1996; PETITPIERRE 1996; COLOMBA et al. 2000a).

The aim of this study is to investigate the meiotic chromosomes of males of the species *Phyllophaga* (*Phytalus*) *vestita*, *Phyllophaga* (*Phyllophaga*) *aff capillata* and *Lyogenys fuscus* using conventional staining, differential chromosome banding techniques and fluorescent in situ hybridization (FISH). The use of these techniques permitted a comparative analysis of the three Melolonthinae species studied.

MATERIAL AND METHODS

The number and sources of the specimens studied were as follows. *Phyllophaga* (*Phytalus*) *vestita* (10 specimens), *Phyllophaga* (*Phyllophaga*) *aff capillata* (12) and *Lyogenys fuscus* (15) were collected in differ-

ent areas of the Atlantic Forest in the State of Pernambuco, northeastern Brazil.

Male gonads from adult beetles were fixed in Carnoy (ethanol and acetic acid, 3:1) and used to obtain meiotic chromosomes. Cytological preparations were obtained by the classical squashing method and the chromosomes were stained with 2% lactoacetic orcein. C-banding was performed following SUMNER (1972), with some slides being stained with Giemsa (CBG) and other with DAPI (CB/DAPI). Triple staining with CMA_3/DA/DAPI was performed according to SCHWEIZER et al. (1983) and double staining with CMA_3/DA and DAPI/DA was also applied in the three species. Silver nitrate staining ($AgNO_3$) was performed by the method of RUFAS et al. (1987). In the fluorescent in situ hybridization (FISH) we used 18S and 25S probes (rDNA) of *Arabidopsis thaliana* (UNFRIED et al. 1989; UNFRIED and GRUNDLER 1990) and the technique of MOSCONE et al. (1996). The probes were labeled with bio-11-dUTP by nick translation (Life Technologies) and detected by rat antibiotin antibodies (Dakopatts M0743, DAKO) and anti-rat antibodies (Dakopatts R0270, DAKO) produced with a rabbit TRITC (tetramethyl-rhodamine isothiocyanate) conjugate. The preparations were counterstained with DAPI (2 µg/ml) and mounted with Vectashield H-1000 (Vector).

Slides were examined with a Leitz Orthoplan photomicroscope. Conventional staining, fluorescence and FISH were photographed with Kodak Imagelink ASA 25 film, T-MAX 400 ASA film and Kodacolor 400 ASA film (Kodak), respectively.

RESULTS

Conventional staining

Phyllophaga (*Phytalus*) *vestita*, *Lyogenys fuscus* and *Phyllophaga* (*Phyllophaga*) *aff capillata* presented a diploid number of $2n = 20$, meioformula $9II + Xy_p$, in the former ones, being $9II + XY_p$ in the *P.* (*Phyllophaga*) *aff capillata*, sex-determining mechanisms of the parachute type and an achiasmatic association between the sex chromosomes. All the three species have symmetrical karyotypes and a predominance of apparently biarmed chromosomes, as observed in metaphases I (Fig. 1a–c). The male sex chromosomes are represented by a heteromorphic pair, which form a typical parachute in *P.* (*Phytalus*) *vestita* (Fig. 1a) and *L. fuscus* (Fig. 1c), with a very small X chromosome and a punctiform Y chromosome (Xy_p). In *P.* (*Phyllophaga*) *aff capillata* the sex chromosomes (XY_p) are small and present identical size (Fig. 1b).

C-banding and fluorochrome staining

The localization of constitutive heterochromatin (CH) blocks was defined by C-banding only in *P.* (*Phytalus*) *vestita*, which presented pericentromeric blocks in all autosomes. This pattern permitted the identification of the occurrence of biarmed chromosomes in this species. The sex bivalent was almost

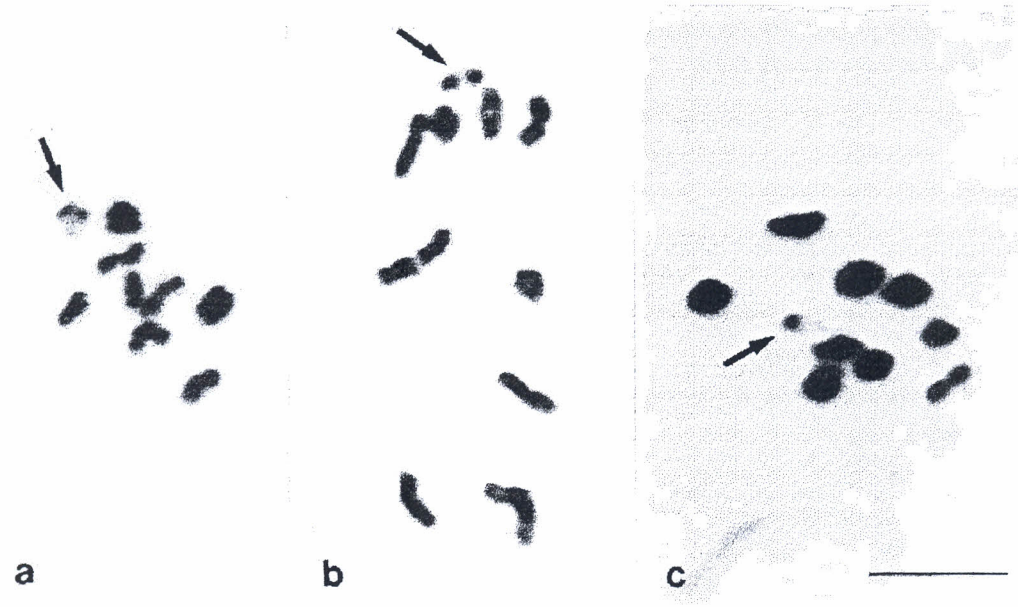

Fig. 1a–c. Conventional staining in the three Melolonthinae species. Metaphases I in *Phyllophaga* (*Phytalus*) *vestita* (**a**), *P.* (*Phyllophaga*) *aff capillata* (**b**) and *Lyogenys fuscus* (**c**). Note the parachute configuration of the sexual bivalent Xy_p and XY_p (arrows). Bar = 10 µm.

fully heterochromatic (Fig. 2a–b). In contrast, for *P. (Phyllophaga) aff capillata* (Fig. 2e) slides pretreated by C-banding and stained with DAPI (CB-DAPI) showed the presence of apparently smaller CH blocks compared to *P. (Phytalus) vestita*. Triple staining with CMA_3/DA/DAPI in *P. (Phytalus) vestita* did not reveal any differential amount of CH and showed that all blocks were labeled both by CMA_3^+ and $DAPI^+$ (Fig. 2c–d). However, DAPI labeling was more intense than CMA_3. In *P. (Phyllophaga) aff capillata* double staining with CMA_3/DA showed the presence of CMA_3^+ blocks only in the sex bivalent and in a small autosomal bivalent (Fig. 2f). No $DAPI^+$ blocks were detected in this species (result not shown). On the other hand, the CH of the entire chromosome complement in *L. fuscus* showed homogeneous CMA_3 staining (Fig. 2g) and strongly positive DAPI staining (Fig. 2h), clearly showing that the CH is AT-rich.

$AgNO_3$ staining and fluorescent in situ hybridization (FISH)

Silver nitrate staining showed the presence of nucleolar remnants in only one chromosome pair of each species. In *P. (Phyllophaga) aff capillata* the active NOR was observed in an autosome (Fig. 3c), in contrast to *P. (Phytalus) vestita* (Fig. 3a) and *L. fuscus* (Fig. 3e) in which the NORs were located in the sex bivalents. The sex bivalent was strongly stained by silver and continued to be labeled during the different phases of meiosis, as can be seen in *P. (Phyllophaga) aff capillata* and *L. fuscus* (Fig. 3g–h). Silver nitrate also labeled the CH of the autosomal bivalents (Fig. 3g–h). In meiotic bivalents FISH with the 18S and 25S ribosomal probe (rDNA) only showed one signal of hybridization in each species (Fig. 3b,d,f), confirming the $AgNO_3$ staining. Nevertheless, in *P. (Phyllophaga) aff capillata* (Fig. 3d), labeling occurred in a small-sized autosome, whereas in *P. (Phytalus) vestita* (Fig. 3b) and *L. fuscus* (Fig. 3f) the hybridization signals were present in the X chromosome.

DISCUSSION

The karyotypic constitution of $2n = 20,Xy_p$ in males (meioformula $9II + Xy_p$) in *Phyllophaga (Phytalus) vestita*, *Phyllophaga (Phlyllophaga) aff capillata* and *Lyogenys fuscus* corresponds to the karyotype most frequently encountered in the family Scarabaeidae. This karyotype has also been detected in more than 50 % of the Melolonthinae species analyzed thus far (SMITH and VIRKKI 1978; YADAV and PILLAI 1976 1979). Although this subfamily is considered to be chromosomally conserved, generally presenting biarmed autosomes and a sex mechanism of the parachute type (Xy_p), some chromosome rearrangements have led to changes in chromosome morphology, size and diploid numbers. Some examples are *Apogonia* sp with $2n = 21,XO$ (SAHA 1973), *Apogonia nigricans* and *Apogonia* sp with $2n = 19,XO$ (MANNA and LAHIRI 1972; YADAV and PILLAI 1974) and *Ophthalmoserica karafutoensis* with $2n = 18$ (KUDOH et al. 1973).

Although C-bands are localized in the pericentromeric region of most of the Scarabaeidae species analyzed (BIONE 1999; COLOMBA et al. 2000a), as also observed here in *P. (Phytalus) vestita* and *L. fuscus*, other scarabeoids such as *Enema pan* (VIDAL and GIACOMOZZI 1978), *Glynoderus sterquilinus* and *Eucranium arachnoides* (VIDAL and NOCERA 1984) show interstitial and telomeric C-bands. This has also been reported for representatives of other Coleoptera families (JUAN et al. 1991; ROZEC 1992; ROZEC and RUDEK 1992). On the other hand, extra heterochromatic segments have also been detected in *Bubas bison* (COLOMBA et al. 1996).

Intra- and interspecific CH heterogeneity were observed after using fluorochrome staining in all the species. In *P. (Phytalus) vestita* the positive labeling in response to the CMA_3 and DAPI indicates that the heterochromatin does not present a specific riches in terms of AT or GC base composition (CH with no specific abundance). Among grasshoppers there are also reports of species that respond positively to stains with different specificity, such as *Arcyptera fusca* and *A. tornosi*, whose chromosomes are labeled with antagonist stains when they have been pretreated for C-banding (BELLA and GOSÁLVES 1991). According to these authors, this seems to occur due to protein removal during pretreatment. However, in *P. (Phytalus) vestita* the fluorescence pattern was obtained in chromosomes that had not been pretreated for C-banding, supporting the suggestion that the CH is heterogeneous. In contrast, *P. (Phyllophaga) aff capillata* presented a predominantly neutral CH for the fluorochromes used, only a small autosome and the sex bivalent showed CMA_3^+ labeling (GC-rich). This was also observed in *Macraspis festiva*, in which only a small CMA_3^+ labeling was detected in an sexual pair (BIONE 1999). On the other hand, the abundance of AT base pairs in CH observed in all chromosomes of *L. fuscus* was also detected in most of the chromosomes of the scarabeoid *Pelidnota pallidipennis* (BIONE 1999). Despite the reduced number of Scarabaeoidea species analyzed with fluorochromes thus far, there is evidence that the CH of this group is quite heterogeneous, presenting both quantitative and qualitative variations (BIONE 1999; VITTURI et al. 1999; COLOMBA et al. 2000a). On the contrary, to the

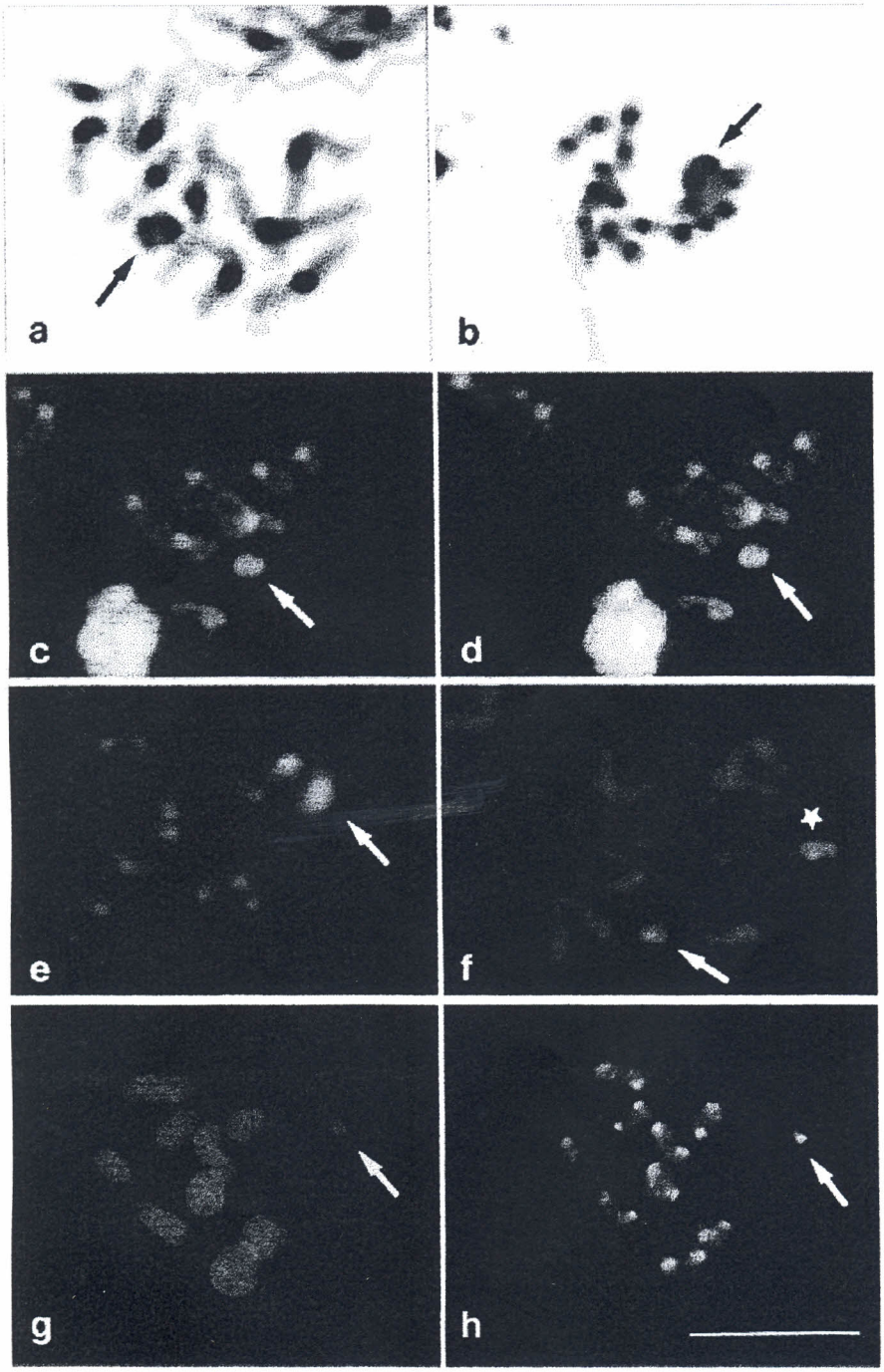

Fig. 2a–f. Giemsa C-banding in *Phyllophaga* (*Phytalus*) *vestita* (**a, b**) and CB/DAPI in *P.* (*Phyllophaga*) *aff capillata* (**e**). Triple staining (CMA_3/DA/DAPI) in *P.* (*Phytalus*) *vestita* (**c, d**) and *Lyogenys fuscus* (**g, h**) and double staining (CMA_3/DA) in *P.* (*Phyllophaga*) *aff capillata* (**f**). Pachytene (**a**), metaphase I (**b**) and diplotenes (**c, d**) in *P.* (*Phytalus*) *vestita*. Note in (**a–b**) an almost completely heterochromatic X chromosome. In (**c–d**) the CH blocks positively labeled with CMA_3 and DAPI. Zygotene (**e**) and pachytene (**f**) in *P.* (*Phyllophaga*) *aff capillata*. Note in **f** the CMA_3^+ blocks in an autosomal bivalent and in the XY_p. Metaphases I (**g, h**) in *L. fuscus*, with homogeneous CMA_3 staining and positive DAPI staining. The arrows indicate the sex bivalents. Bar = 10 μm.

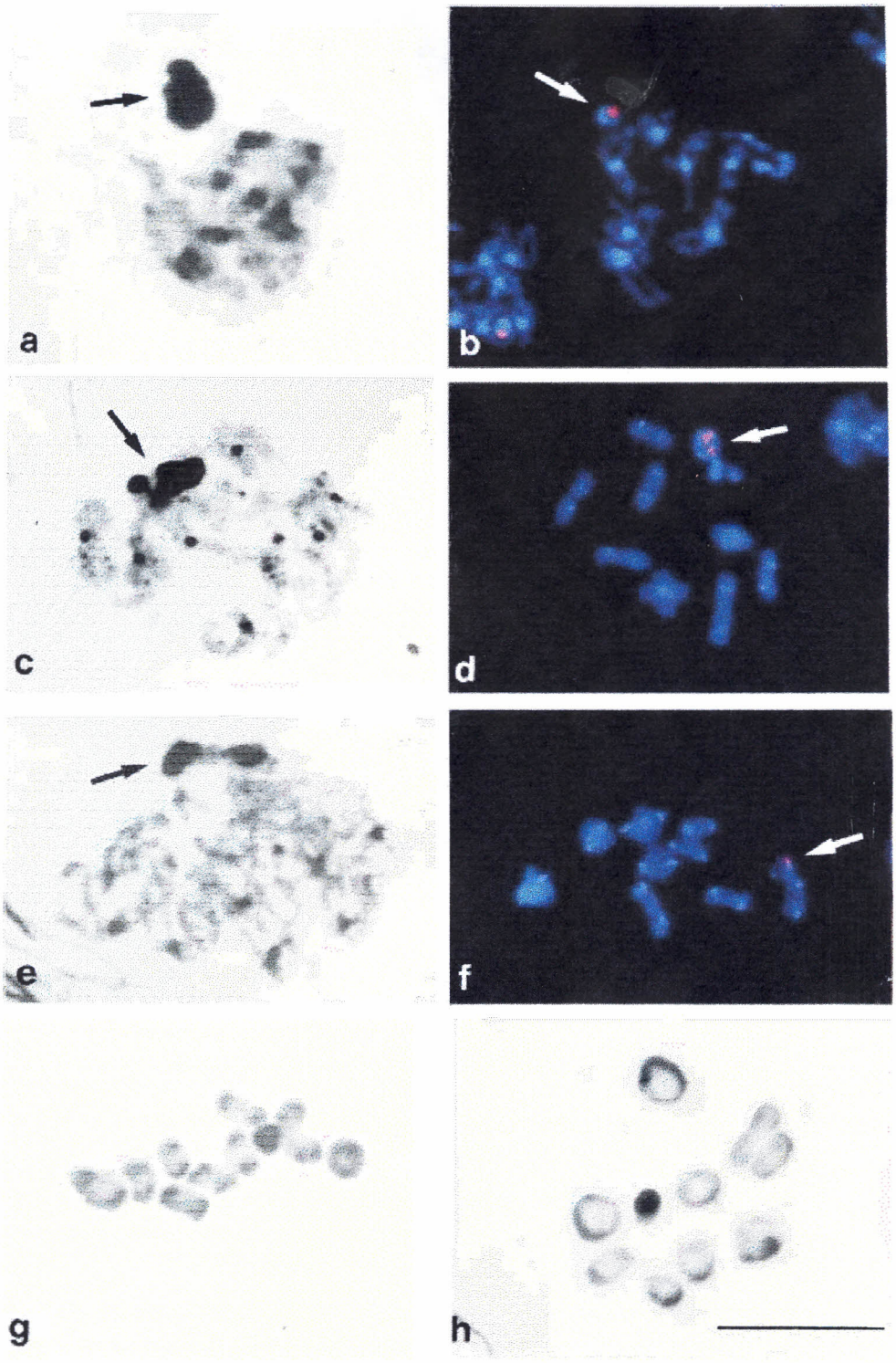

Fig. 3a–h. Silver staining and FISH in two *Phyllophaga* species and in *Lyogenys fuscus*. Zygotene (**a**) and diplotene (**b**) in *P.* (*Phytalus*) *vestita*. Pachytene (**c**) and metaphase I (**d**) in *P.* (*Phyllophaga*) *aff capillata*. Pachytene (**e**) and metaphase I (**f**) in *L. fuscus*. Note the NORs in **a**, **c** and **e** (arrow) and the rDNA sites in **b**, **d** and **f** (arrowhead). The detail in **g** and **h** shows the labeling of pericentromeric CH with silver nitrate in *P.* (*Phyllophaga*) *aff capillata* and *L. fuscus*, respectively. Observe the argyrophilic behavior of the sex bivalent in metaphase I (**g**) and in diakinesis (**h**). Bar = 10 μm.

family Tenebrionidae which has a large amount of CH and whose base composition is predominantly AT-rich (JUAN et al. 1991; PLOHL et al. 1993).

According to some investigators (VIRKKI 1983; VIRKKI et al. 1984), in Coleoptera the NORs are widely distributed in one autosome pair, as observed in *P.* (*Phyllophaga*) *aff capillata* and three other Scarabaeoidea species (BIONE 1999; VITTURI et al. 1999: COLOMBA et al. 2000a). On the other hand, in most scarabeoids the NOR is present in the sex bivalent (BIONE 1999), as also observed here for *P.* (*Phytalus*) *vestita* and *L. fuscus*. A sex bivalent deeply stained with silver has been found in various Scarabaeoidea species during different phases of meiosis. This labeling is independent of the presence of NORs in this chromosome pair (BIONE 1999; VITTURI et al. 1999; COLOMBA et al. 2000a). This pattern was also detected in all the species and may result from the presence of non-nucleolar argyrophilic substances in the lumen of the Xy_p bivalent. These substances may facilitate the parachute configuration of the sex chromosomes and probably play an important role in the segregation of these chromosomes (VIRKKI et al. 1990, 1991).

Silver nitrate staining showed a preliminary labeling of the NORs in the three species analyzed. However, only the FISH permitted a precise identification of the NORs in these species. The present FISH study unequivocally assigned the 25S and 18S (major) rDNA loci on a single autosome pair in *P.* (*Phyllophaga*) *aff capillata* and X chromosome in *P.* (*Phytalus*) *vestita* and *L. fuscus*. On the other hand, little is known about the chromosomal location of rDNA genes in representatives of the superfamily Scarabaeoidea (VITTURI et al. 1999; COLOMBA et al. 2000a,b) in comparison with other Coleoptera groups (PETITPIERRE 1996; GÁLIAN et al. 1995; SANCHEZ-GÉA et al. 2000) in the which data have been published.

The heterochromatic regions of the three species studied were deeply stained with silver. This argyrophilic behavior of CH has been observed in representatives of Scarabaeoidea and does not depend on the base composition of CH, also occurring in species such as *Bubas bison* and *Pelidnota pallidipennis*, whose CH is AT-rich (COLOMBA et al. 1996; BIONE 1999), *Thorectes intermedius*, *Gymnopleurus sturmi* and *Geniates borelli*, whose CH is in GC-rich (BIONE 1999; VITTURI et al. 1999; COLOMBA et al. 2000a), and in *Macraspis festiva*, whose CH is neutral (BIONE 1999).

Although the species studied in the present work had a $2n = 20,Xy_p$ karyotype, considered to be more frequent for Melolonthinae, the methods used permitted us to characterize karyotypic differences between the two *Phyllophaga* species and *Lyogenys fuscus* in terms of chromosome Y, NOR localization, distribution of rDNA sites and base composition of CH, suggesting that the genomes of the two genera evolved in a different manner.

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