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Cytogenetic analysis of *Epicauta atomaria* (Meloidae) and *Palembus dermestoides* (Tenebrionidae) with Xy_p sex determination system using standard staining, C-bands, NOR and synaptonemal complex microspreading techniques

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The mitotic and meiotic chromosomes of the beetles *Epicauta atomaria* (Meloidae) and *Palembus dermestoides* (Tenebrionidae) were analysed using standard staining, C-banding and silver impregnation techniques. We determine the diploid and haploid chromosome numbers, the sex determination system and describe the chromosomal morphology, the C-banding pattern and the chromosome(s) bearing NORs (nucleolar organizer regions). Both species shown $2n = 20$ chromosomes, the chromosomal meioformula $9 + Xy_p$, and regular chromosome segregation during anaphases I and II. The chromosomes of *E. atomaria* are basically metacentric or submetacentric and *P. dermestoides* chromosomes are submetacentric or subtelocentric. In both beetles the constitutive heterochromatin is located in the pericentromeric region in all autosomes and in the X_p chromosome; additional C-bands were observed in telomeric region of the short arm in some autosomes in *P. dermestoides*. The y_p chromosome did not show typical C-bands in these species. As for the synaptonemal complex, the nucleolar material is associated to the 7th bivalent in *E. atomaria* and 3rd and 7th bivalents in *P. dermestoides*. Strong silver impregnated material was observed in association with Xy_p in light and electron microscopy preparations in these species and this material was interpreted to be related to nucleolar material. *Key words*: *karyotype, chromosome, heterochromatin, pachytene, meiosis, nucleolus, polyphaga, coleoptera.*

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The order Coleoptera has approximately 350,000 known species; the total number may be ten times higher. The families Meloidae and Tenebrionidae comprise, respectively, about 1% and 6% of this order (COSTA 1999). According to SMITH and VIRKKI (1978) and JUAN and PETITPIERRE (1989), 24 species of Meloidae and 200 of Tenebrionidae have well characterized karyotypes, i.e. the diploid number, the chromosome morphology and the type of the sex chromosome determination system are known.

As postulated by SMITH and VIRKKI (1978), the great majority of the cytogenetically described coleopteran species possess the basic chromosome meioformula $9 + Xy_p$, which is considered primitive for this group. The "p" represents an special meiotic configuration of the associated sex chromosomes which resembles a parachute during metaphase I. According to VIRKKI (1984), the sex determination system of the type Xy_p can not represent a primitive condition, but it could have an adaptive advantage that could explain its widespread presence in a great number of coleopterans.

During meiosis, the association between the X and y sex chromosomes may vary depending on the species and on the chromosome differentiation during past evolution. This association can be established by nucleolar material and/or by achiasmatic synapsis (SMITH and VIRKKI 1978; JUAN et al. 1993; PETITPIERRE 1996).

Until the sixties, all cytogenetic analyses on Coleoptera were made using sectioning or "squash" techniques. These techniques showed great karyotype uniformity in the majority of the studied species. However, at the end of the sixties, new techniques arose shedding light to longitudinal differentiation of the chromosomes establishing the banding techniques. The presence and the identification of specific regions on mitotic and meiotic Coleoptera chromosomes, such as constitutive heterochromatin (C-banding pattern) and nucleolar organizer regions (NORs)

allowed a better characterization of each beetle karyotype. They shed information on the level of structural differentiation of the chromosomes and facilitated the comparison of karyotype of related species. It was shown that during meiosis the association of sex chromosomes could be established by heterochromatic regions or by NORs.

The study of heterochromatin and NORs in the chromosomes in a few number of species of Coleoptera was reported giving information mainly about karyotype differentiation among species that possess the basic chromosome meioformula for the group ($9 + Xy_p$). As examples we can cite Coccinellidae (MAFFEI et al. 2000), Curculionidae (VIRKKI et al. 1991), Carabidae (Rozek and Maryánska-Nadachowska, 1991), Chrysomelidae (PETITPIERRE 1996), Hydrophilidae (ANGUS 1983), Tenebrionidae (JUAN et al. 1993; PETITPIERRE et al. 1995) and Cicindelidae (GALIÁN et al. 1995).

In general, the constitutive heterochromatin in the chromosomes of these beetles is found in the pericentromeric region. Some species may show additional heterochromatic regions (additional C bands) in the interstitial and/or telomeric regions. In the sex chromosomes, the constitutive heterochromatin has a variable occurrence and can be located in the pericentromeric region and/or distributed along the chromosome according to the level of chromosome differentiation. The pattern of NORs is not well established in beetle families since it can be located in the autosomic pairs and/or sex chromosomes. When not evident in the autosomes, the visualization of the NORs is restricted to the meiotic cycle and located between the chromosome X and y (JOHN and LEWIS 1960).

Here we attempt to characterise the karyotype of two coleopterans species, *Epicauta atomaria* (Meloidae) and *Palembus dermestoides* (Tenebrionidae). We analyse the mitotic and meiotic chromosomes preparations to describe the diploid number, the chromosome morphology, the type of sex chromosome determination system, the C-banding pattern, and the chromosomes bearing NORs.

MATERIAL AND METHODS

We used 77 *E. atomaria* and 65 *P. dermestoides* adult males. The *E. atomaria* beetles were collected at the Instituto de Biociências Experimental Garden, UNESP (Rio Claro, SP, Brazil) and at the Horto Florestal Navarro de Andrade (Rio Claro, SP, Brazil), and the *P. dermestoides* specimens were obtained from domestic strains from Limeira (SP, Brazil).

The chromosome preparations were obtained from adult male testes. The material was immersed in hypotonic solution (tap water, for 3 min) and fixed in Carnoy I (methanol: glacial acetic acid, 3:1 parts by volume, for at least 30 min). Each fixed testis was transferred into a drop of acetic acid solution (45%), that was placed on the surface of the slide and then the material was macerated to form a cellular suspension. The slides were dried at 35–40°C (heating metal plate). Standard staining was accomplished using Giemsa (3% of Merck commercial solution in phosphate buffer at pH 6.8, for 12–15 min), rinsed briefly with distilled water, and air-dried. C-banding and NOR silver impregnation were performed according to the methodologies described by SUMNER (1990) and HOWELL and BLACK (1980), respectively. Light microscopy (LM) for routine cytological analyses and photomicrography were carried out using a Zeiss photomicroscopy. For photomicrography it was employed an Agfa-Gevaert Copex Pan A.H.U. film.

Synaptonemal complex (SC) microspreading analyses were accomplished according to the methodology described by LOIDL and JONES (1986). The analyses of silver impregnated (HOWELL and BLACK 1980) SC preparations were performed using a Zeiss EM9-S2 transmission electron microscopy (TEM) and the electron micrography were made on Kodak 4489 film plates.

RESULTS

Standard staining

The spermatogonial metaphase of *E. atomaria* and *P. dermestoides* cells showed a diploid number of $2n = 20$ chromosomes (Fig. 1). The chromosome meioformula $9 + Xy_p$ was observed in all cells in diakinesis and metaphase I (Fig. 2a–b, Fig. 2e–f). In all metaphases II observed, the haploid complement $n = 9 + X_p$ or $n = 9 + y_p$ was evidenced, indicating that chromosomes segregate normally during anaphase I (Fig. 2c–d, Fig. 2g–h). The karyotype of *E. atomaria* spermatogonial metaphases shows that the diploid complement is formed by metacentric and submetacentric chromosomes while the *P. dermestoides* diploid complement is formed by submetacentric and subtelocentric chromosomes. The X_p chromosome of both species is metacentric of medium size and usually is difficult to be identified due its similarity with autosomal chromosomes of the same size. The y_p is metacentric and is characterized as the smallest chromosome of the karyotype in both species (Fig. 1).

In both beetles it was observed in the mitotic metaphase and metaphase II, that negative heteropycnosis appears in the pericentromeric region, in the majority of the chromosomes, and in the short arms of some chromosomes (Fig. 1, Fig. 2c–d, Fig. 2g–h).

Additionally, it was observed that in some of these cells the y_p chromosome possesses negative heteropycnosis.

In diakinesis cells it was noted the occurrence of one chiasma per autosomal bivalent and that the X_p and y_p chromosomes are associated by the parachute configuration (Fig. 2a, Fig. 2e). Furthermore, the y_p chromosome showed negative heteropycnosis in all analysed diakinesis and metaphase I cells (Fig. 2a–b, Fig. 2e–f).

C-banding

The *E. atomaria* and *P. dermestoides* spermatogonial metaphases showed that the constitutive heterochromatin of the autosomes and X_p chromosome is located in the pericentromeric region (Fig. 3). The *E. atomaria* y_p shows a tenuous C-band probably in the pericentromeric region (Fig. 3b) and the *P. dermestoides* y_p does not exhibit any C-band (Fig. 3d). In both species, the pericentromeric C-band extends towards to the short arm. In *P. dermestoides* additional heterochromatin was also detected in the telomeric region of the short arm of some chromosomes (Fig. 3d).

In the metaphase I cells of both species, the pericentromeric C-bands extend along the short arm in almost all the autosomes and in the X_p sex chromosome (Fig. 4). In the metaphase I cells of *E. atomaria* the y_p chromosome shows a tenuous C-band (Fig. 4a–b) while no distinguishable band was observed in the *P. dermestoides* y_p chromosome at the same meiotic phase (Fig. 4c–d). Analyses of metaphase II have evidenced similar C-banding pattern to that one noted in the metaphase I cells (Fig. 5).

Silver nitrate impregnation

LM of testicular chromosome preparations of *E. atomaria* and *P. dermestoides*, which were previously analysed with conventional staining and then submitted to silver impregnation, did not show the typical NOR silver impregnation in the spermatogonial metaphase chromosomes.

On the other hand, TEM analysis of *E. atomaria* pachytenic cells showed that the nucleolar material is

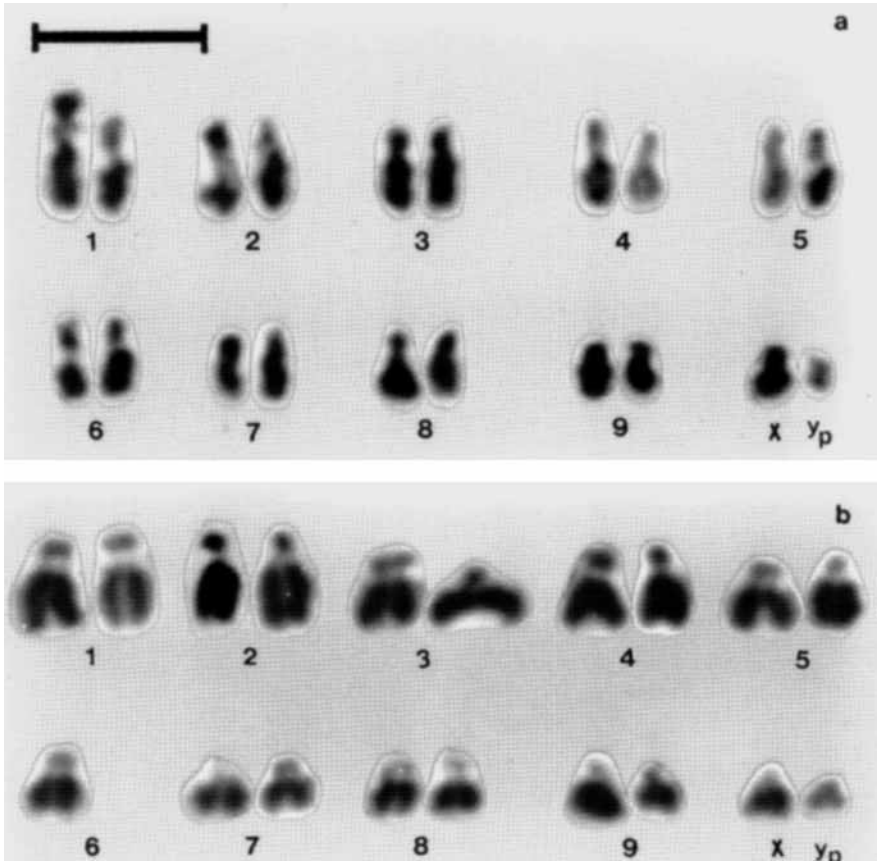

Fig. 1a and b. Karyotypes of *Epicauta atomaria* **a** and *Palembus dermestoides* **b** spermatogonial metaphases ($2n = 18 + Xy_p$) standard staining with Giemsa. The chromosomes are metacentric, submetacentric, and subtelocentric. Bar = 5 μm.

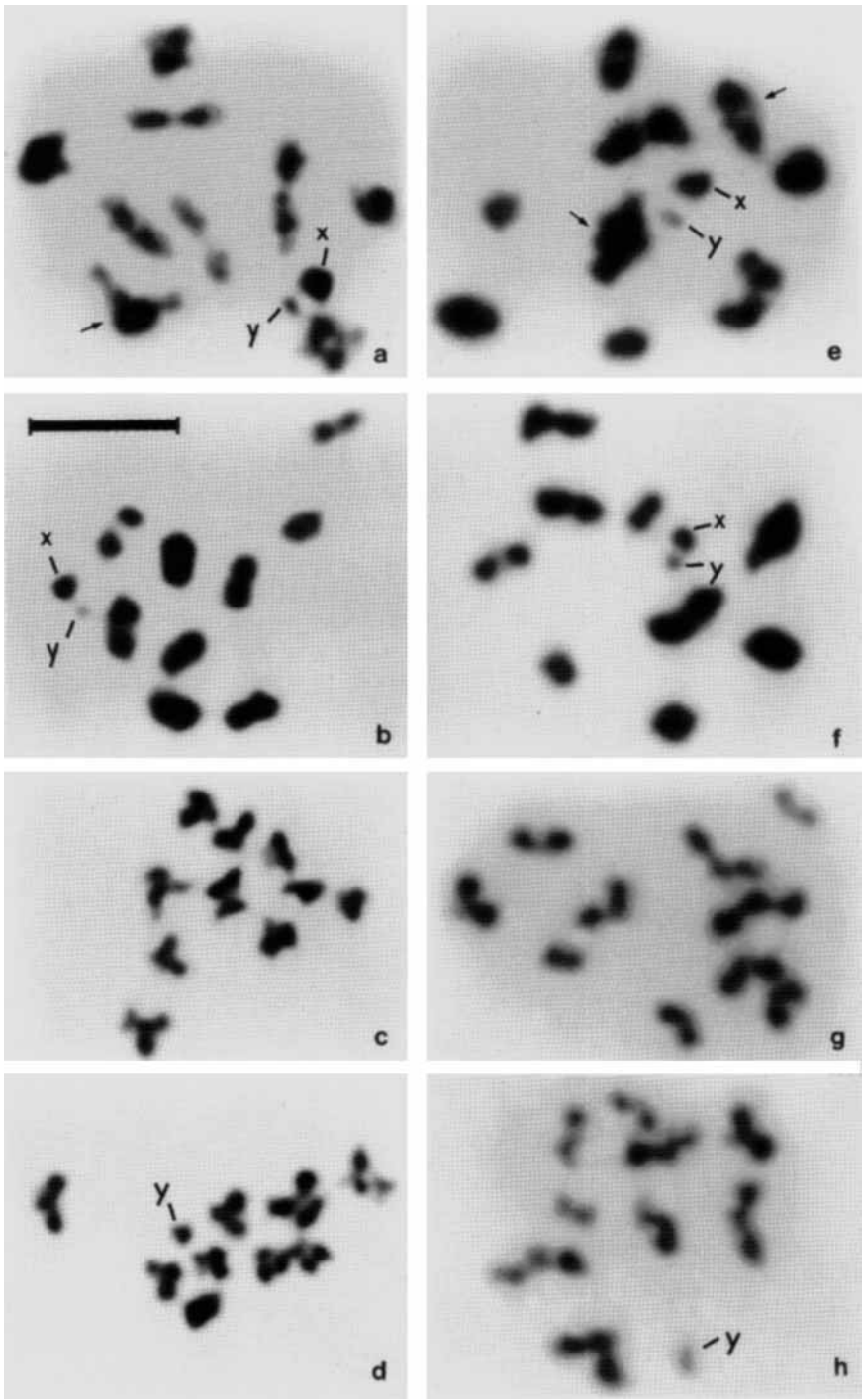

Fig. 2a–h. *Epicauta atomaria* **a–d** and *Palembus dermestoides* **e–h** male meiotic chromosomes analysed by standard staining. Diakinesis **a** and metaphase I **b** showing the meioformula $9 + Xy_p$ and the negative heteropycnotic y_p chromosome; metaphases II with $n = 9 + X_p$ **c** and $n = 9 + y_p$ **d**. Diakinesis **e** and metaphase I **f** evidencing the meioformula $9 + Xy_p$ and also the negative heteropycnotic y_p chromosome; metaphases II with $n = 9 + X_p$ **g** and $n = 9 + y_p$ **h**. Arrows indicate chiasmata. Bar = 5 μm.

associated to the 7th autosomal bivalent (Fig. 6a). In *P. dermestoides*, the nucleolar material is associated to 3rd and 7th autosomal bivalents (Fig. 6b). Additionally, in both species strong silver impregnation

was detected in association to Xy_p sexual bivalent and it was interpreted as being related to nucleolar material (Fig. 6).

Light microscopy showed strong silver impregnation of nucleolar material only in the Xy_p sexual bivalent in all cells in diakinesis and metaphase I (Fig. 7), in both species. LM analysis of metaphase II microspreading *E. atomaria* cells showed nucleolar material associated to only one chromosome of the haploid complement (Fig. 8).

DISCUSSION

Recently, some authors have employed more refined techniques to analyse, to characterise, and to better differentiate the chromosomes of the beetles that have the basic chromosome meioformula $9 + Xy_p$ (ANGUS 1983; POSTIGLIONI et al. 1991; VIRKKI et al. 1991; UGARKOVIC et al. 1994; PETITPIERRE 1996).

The standard staining analysis of the karyotypes and the behaviour of the *E. atomaria* and *P. dermestoides* meiotic bivalents show the general coleopteran basic cytogenetic characteristics. The chromosomal morphology differs between these species, except in that of the y_p sex chromosome. The morphological variability observed in the *E. atomaria* and *P. dermestoides* chromosomes seems to show that there have been structural rearrangements such as pericentric inversions or alterations in the constitutive heterochromatin content.

Some karyotypic characteristics observed in *E. atomaria*, such as the diploid chromosome number and the sex chromosome system agree with the ones described for others species of the same genera, for example, *Epicauta anthracina*, *Epicauta cinerea*, *Epicauta isthmica*, *Epicauta murina*, *Epicauta pennsylvanica*, *Epicauta ruffipedes* and *Epicauta* n.sp (SMITH and VIRKKI 1978; FERREIRA and MESA 1977). Chromosome morphology has been described only for *E. anthracina*, in which all chromosomes were classified as metacentric (FERREIRA and MESA 1977).

Positive heteropycnosis in the sex chromosomes during the meiosis is observed in the majority of beetles; this heteropycnosis seems, however, to vary among related species (FERREIRA and MESA 1977; YADAV et al. 1985; JUAN et al. 1993). Positive heteropycnosis was also observed in X_p and y_p sex chromosomes of Meloidae (SMITH and VIRKKI 1978) and Tenebrionidae (BISOI and PATNAIK 1988; JUAN et al. 1993).

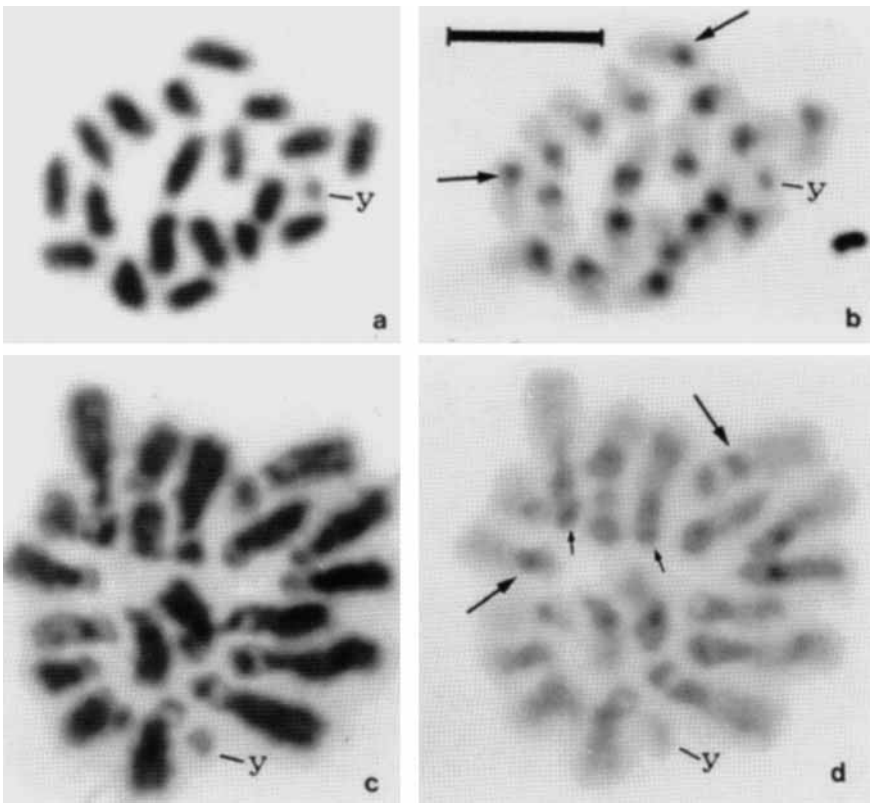

Fig. 3a–d. *Epicauta atomaria* **a–b** and *Palembus dermestoides* **c–d** spermatogonial metaphases submitted to both standard staining **a and c** and C-banding **b and d**. The large and the small arrows indicate pericentromeric and telomeric C band, respectively. Bar = 5 μm.

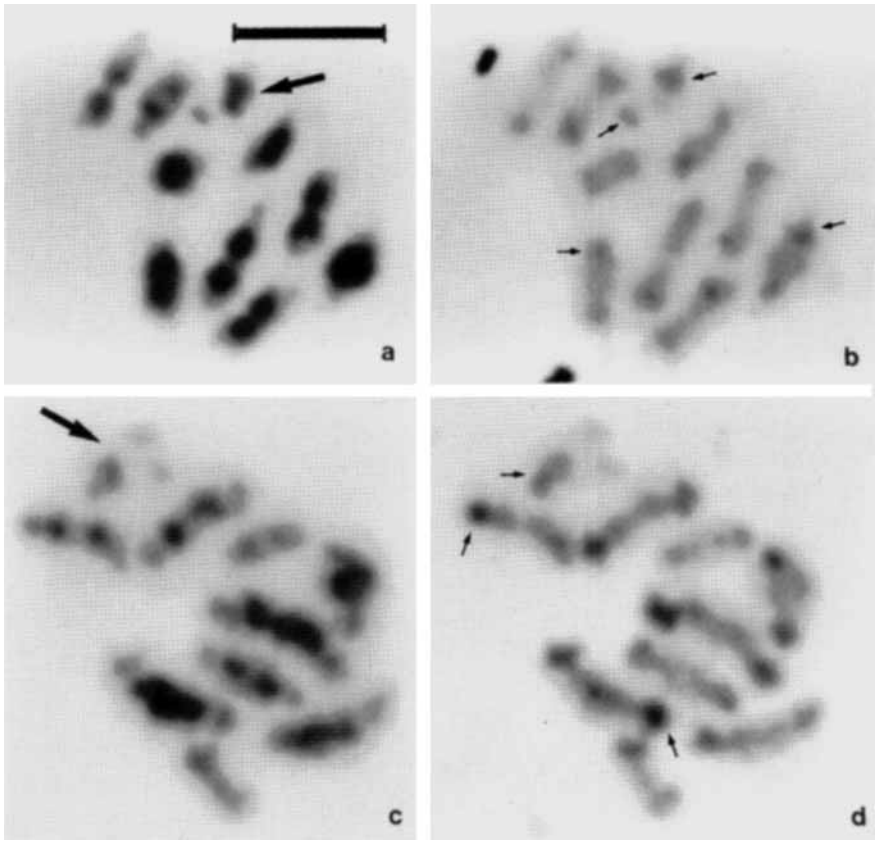

Fig. 4a–d. *Epicauta atomaria* **a–b** and *Palembus dermestoides* **c–d** metaphases I submitted to both standard staining **a and c** and C-banding **b and d**. Small and large arrows indicate respectively C band and Xy_p bivalent. Bar = 5 μm.

The heteropycnotic pattern observed in the *E. atomaria* and *P. dermestoides* X_p and y_p chromosomes, i.e. X_p allocyclic and y_p negative heteropycnotic, differs of the positive heteropycnotic pattern found in these chromosomes of other Meloidae species (*E. rufipedes*, *E. isthmica*, *E. grammica*, *Mylabris thunbergi*, *Panyculolytta* and *Tetraonyx frontalis*) (SMITH and VIRKKI 1978; BISOI and PATNAIK 1988) and Tenebrionidae (*Hoplobrachium dentipes*, *Pachycera?coromandelensis* and *Misolampus goudoti*) (BISOI and PATNAIK 1988; JUAN et al. 1993). The difference involving heteropycnosis can be due to the chromosome condensation and/or the presence of special type of chromatin. Besides, the difference in size of the X and y sex chromosomes among species of the same family or of the same genera is very common in Coleoptera, due to the occurrence of independent process of differentiation. The Meloids *Epicauta* n.sp. and *Pyrota decorata* have extremely large X and y chromosomes when compared to the ones found in *E. isthmica* and *Paniculolytta sanguineoguttata* (SMITH and VIRKKI 1978). Difference in size between sex chromosomes also occur in Tenebrionidae, for example, *Gonocephalum patruele*, in which the X_p is very large, and *G. rusticum*, in which the X_p is very small (JUAN and PETITPIERRE 1990).

The pericentromeric C-banding pattern noted in the *E. atomaria* and *P. dermestoides* mitotic and meiotic chromosomes agrees with that described for most Coleoptera species (ANGUS 1983; JUAN et al. 1993; UGARKOVIC et al. 1994). Additional C bands were seen in the telomeric region of the short arm in some chromosomes of *P. dermestoides*. Telomeric C-bands were also detected in chromosomes of *Misolampus goudoti* (Tenebrionidae) by JUAN et al. (1993). These additional C-bands have probably arisen by means of small tandem duplications as proposed by KING and JOHN (1980) when they analysed the C-banding pattern of some species of Orthoptera.

In both beetles, the y_p chromosome does not present a particular heterochromatic marking. The C-banding technique does not show all types of the heterochromatin (SUMNER 1990), so that we can not exclude the possibility of the y_p chromosome being heterochromatic.

The employment of more refined techniques, such as in situ hybridization using satellite DNA probes,

will certainly provide information about the nature of the chromatin in the y_p chomosome. JUAN et al. (1993) have confirmed the presence of repetitive DNA in the y_p chromosome of coleopterans species using fluorescent in situ hybridization and fluorochrome staining.

Even through the two beetles that we have studied share the same basic karyotype, they are different at the C-band level.

In the spermatogonial metaphases, diakinesis and the metaphases I and II, negative heteropycnosis appears in the pericentromeric region and along the short arms in almost all autosomal chromosomes, in the X_p and, also, along the y_p chromosome of the two unrelated beetles. In these phases, the C-band is totally or partially coincident with negative heteropycnosis in the majority of the chromosomes, but not in the y_p, showing that this chromosome may contain a different type of chromatin.

The analysis of the C-banding pattern obtained for the Xy_p bivalent in the metaphase I of both species allowed to indicate that the parachute configuration is not assembled by a constitutive heterochromatic association.

Although the C-banding technique does not evidence all the particular types of chromatin, the em-

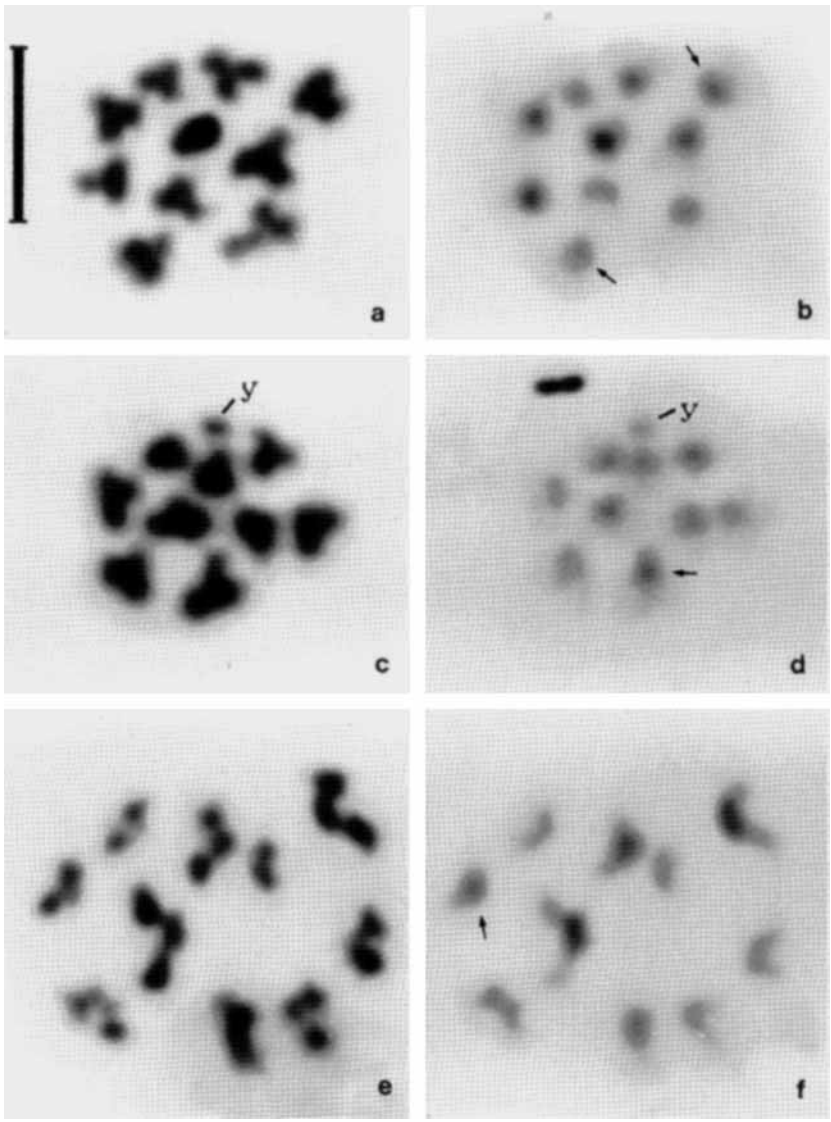

Fig. 5a–f. *Epicauta atomaria* **a–d** and *Palembus dermestoides* **e–f** metaphases II with X_p **a, b, e and f)** and y_p chromosomes **c–d. a and c** Standard staining. **b and d** The same cells as in **a** and **c** submitted to C-banding. **e** Standard staining. **f** The same cell as in **e** subjected to C-banding. Arrows indicate pericentromeric C band. Bar = 5 μm.

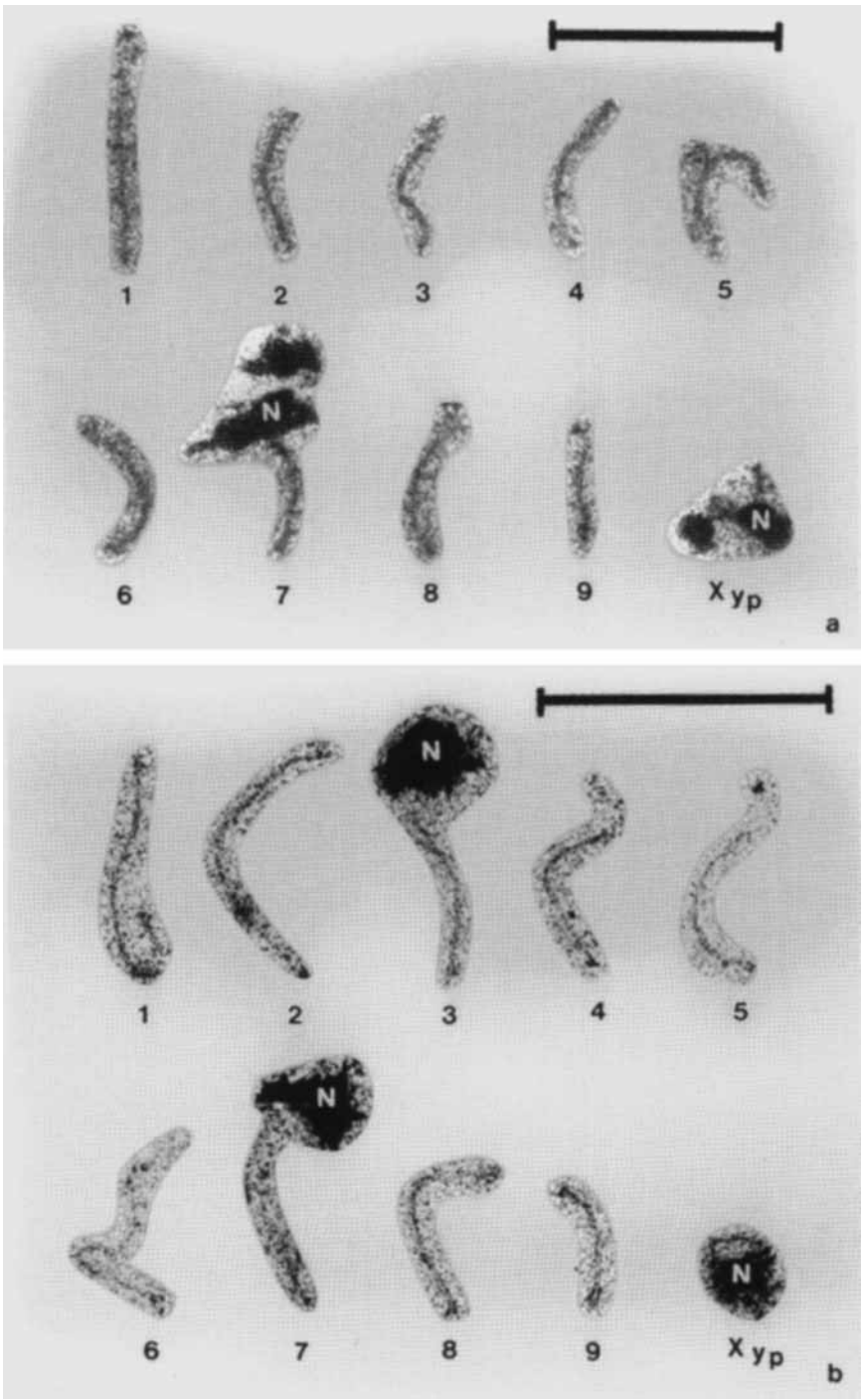

Fig. 6 a and b. *Epicauta atomaria* **a** and *Palembus dermestoides* **b** ultra-structural karyotypes of pachytenic nucleus, meioformula $9 + Xy_p$, analysed by silver nitrate impregnation. N = nucleolar material. Bar = 10 μm.

ployment of this technique has shown that the X_p and y_p chromosomes are totally differentiated in some Coccinelinae and Tenebrionidae species, being associated by heterochromatic regions (DRETS et al. 1983; JUAN et al. 1993; UGARKOVIC et al. 1994).

Silver nitrate impregnation of *E. atomaria* and *P. dermestoides* spermatogonial metaphases did not show NORs. As postulated by GOODPASTURE and BLOOM (1975) the absence of NOR(s) in mitotic chromosomes can be due to a circumstance that ribosome cistrons are disposed in small groups in the

chromosomes and, consequently, the proteins responsible to silver nitrate precipitation could be small so that NOR(s) are not seen. Nevertheless, nucleolar material was observed associated to the *E. atomaria* 7th and Xy_p bivalents and *P. dermestoides* 3rd, 7th and Xy_p bivalents, which probably remains associated to the bivalents in the meiotic division for longer than that one associated to the chromosomes in the mitotic division.

The presence of nucleolar material in the Xy_p bivalents of *E. atomaria* and *P. dermestoides* does not mean that ribosome cistrons are located in this bivalent, but the nucleolar material could be transferred to Xy_p bivalent during the early prophase contributing for regular association and segregation during meiosis. The occurrence of ribosome cistrons in the Xy_p bivalent could be confirmed only using rDNA probes as reported by JUAN et al. (1993).

The occurrence of nucleolar material associated to Xy_p sex bivalent and, in some cases, also associated to autosomal bivalents, was observed in Scarabaeidae (SMITH and VIRKKI 1978), in Dermestidae (JOHN and SHAW 1967), and in Tenebrionidae (JUAN et al. 1993). In Cicindelidae (Adephaga), which have the male XXXy type of sex determining system, the presence of a NOR associated to only one X chromosome and to y chromosome was evidenced. In two other species of the same family, which show the X0 sex determining system, it was observed the occurrence of NOR associated to an autosomal bivalent and not to the X chromosome (GALIÁN et al. 1995).

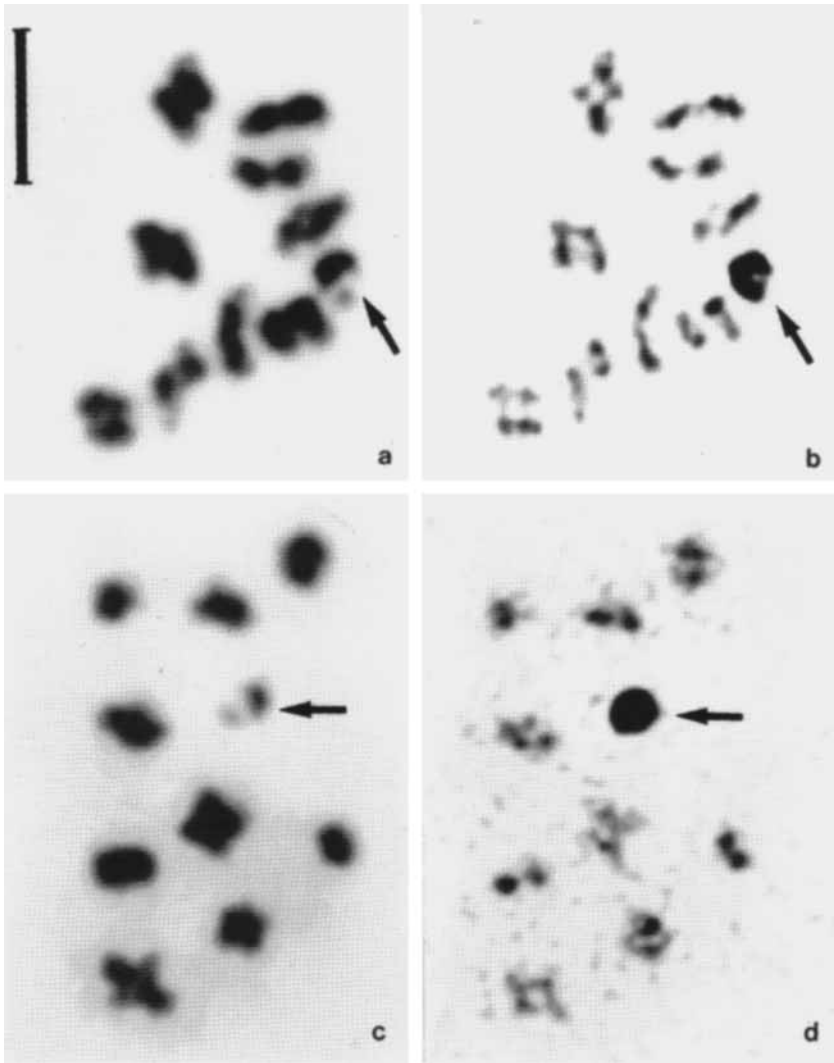

Fig. 7 a–d. *Epicauta atomaria* **a–b** and *Palembus dermestoides* **c–d** diakinesis, meioformula $9 + Xy_p$, submitted to both standard staining **a and c** and silver nitrate impregnation **b and d**. The Xy_p is deeply impregnated by silver nitrate (arrows). Bar = 5 μm.

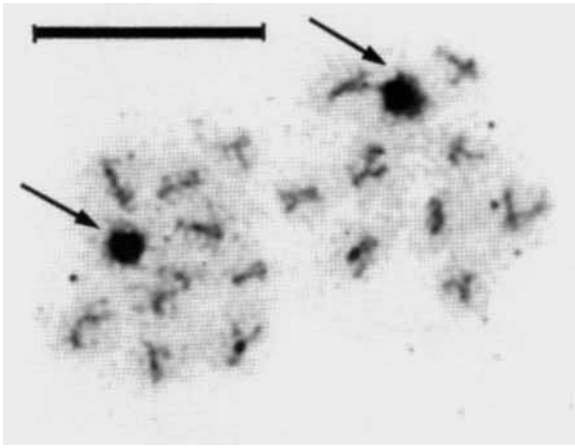

Fig. 8. *Epicauta atomaria* metaphases II (n = 10 in each cell) submitted to silver nitrate impregnation showing silver precipitation (arrow) in one chromosome of the haploid complement. Bar = 5 μm.

The presence of a NOR only in the autosomic chromosomes was also verified in Coccinellidae (DRETS et al. 1983), in Chrysomelidae (PETITPIERRE 1996) and in Curculionidae (VIRKKI et al. 1991), all having Xy_p sex determining system.

JUAN et al. (1993) and GALIÁN et al. (1995) explain the absence of NOR region in the sex chromosomes of some coleopterans through a translocation to autosomes. It is not certain if the coleopteran NOR is originally located in the sex chromosome(s).

The determination of the pattern of distribution of the NOR(s) in more coleopterans, in particular ones considered to be less derived from taxonomic point of view, shed light on the karyotypic evolution within this order. The presence of NOR labelling in the sex chromosomes since pachytene until late phases of the meiosis, such as *E. atomaria* metaphases II and *P. dermestoides* metaphases I, indicates that the nucleolar material drives the association of the sex chromosomes during meiosis I and in this way can cooperate with regular segregation of these chromosomes during anaphase I. Similar conclusions were reported by JOHN and LEWIS (1960) and JUAN et al. (1993), which have studied beetle chromosomes bearing NORs with histochemical and FISH methods, respectively.

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