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Research Article

Karyotype characterization, constitutive heterochromatin and nucleolus organizer regions of *Paranaita opima* (Coleoptera, Chrysomelidae, Alticinae)

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Abstract

Species of the subtribe Oedionychina not only have a highly uniform diploid number of 2n = 22 (20+X+y) but have the karyotypic peculiarity of possessing extremely large sex chromosomes. We analyzed *Paranaita opima* embryos and gonadal cells to determine their diploid number, chromosomal morphology, type of sex determination system, constitutive heterochromatin pattern and which chromosomes bear nucleolus organizer regions (NORs). The diploid number of *P. opima* was 2n = 22 (20+XY/XX) with all chromosomes being metacentric. Chromosome pair 6 showed an interstitial secondary constriction on the short arm. The C-banding technique revealed centromeric constitutive heterochromatin in all chromosomes, which, in pair 6, extended up to the secondary constriction of the short arm, additional C-bands also being present on the Y chromosome. Silver nitrate nucleolar organizer region (Ag-NOR) staining showed NORs on the secondary constriction of pair 6. Fluorochrome analysis with chromomycin A_3 (CMA_3), 4'-6-diamidino-2-phenylindole (DAPI) and the distamycin A (DA) counterstain showed that the short arm of chromosome pair 6 exhibited a GC-rich block extending from the proximal to the median region, including part of the secondary constriction. The same techniques also showed AT-rich blocks at the centromeric region of all chromosomes and at the terminal region of the short arm of pair 6. The basic karyotype characteristics and C band pattern of *P. opima* are similar to those described for other species in the subtribe Oedionychina. The pattern of autosomal NORs observed in *P. opima* corresponds to that registered in the majority of the Chrysomelidae species.

Key words: C bands, chromosome, embryos, fluorochromes, NOR.

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Introduction

The tribe Oedionychini (Alticinae) includes two subtribes, Disonychina and Oedionychina. Cytogenetic studies performed on 20 Disonychina species have shown that this group possesses high inter- and intraspecific variability in relation to the diploid number and type of sex determination system. An idea of the extent of such variation is given by the karyotypic variability described in the *Disonycha* species *D. spilotracheta* (2n = 29 ($13II+X_1y+X_2$), Virkki, 1988b), *D. bicarinata* (2n = 64 ($30II+X_1Y+X_2X_3$) Virkki, 1988b), *D. nigrita* (2n = 34 (16II+X+x) Virkki, 1964 and 2n = 33 ($15II+X_1y+x_2$) Virkki, 1988b) as well as *Phenrica austriaca* which has been variously described as having a karyotype of 2n = 49 (22II+Xy+3y) by Virkki (1970) and 2n = 49 ($22II+X_1y+X_2+X_3+X_4$) by Virkki (1988b). In this group, the chromosomal morphology is predominantly metacentric and the sex determination system is variable, with frequent occurrence of multiple systems, involving mainly the X chromosome (Smith and Virkki, 1978; Vidal, 1984; Virkki, 1988b).

In contrast, 74 of the 113 species of the Oedionychina subtribe which have been cytogenetically analyzed have shown high karyotype uniformity concerning the diploid number and type of sex determination system, which is 2n = 22 (20+X+y) with the sex chromosomes being asynaptic during meiosis (Smith and Virkki, 1978; Virkki, 1970, 1971, 1988a, 1989). In the Oedionychina, diploid numbers higher than 2n = 22 are due to an increase in the number of y chromosome, while numbers lower than 2n = 22 are due to alterations involving autosomes. In spite of the great number of Oedionychina species studied, little information

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exists on their chromosomal morphology, making it impossible to establish a morphological pattern for the subtribe. Of the 18 Oedionychina species whose chromosomes have been morphologically classified, about 50% have exhibited solely metacentric autosomes, 25% only acrocentric autosomes and the other 25% metacentric and acrocentric autosomes with a predominance of metacentrics (Smith and Virkki, 1978; Virkki, 1985, 1989; Virkki *et al*. 1991; Virkki and Santiago-Blay, 1993, 1996). In all these species, the X chromosome is invariably metacentric and the y chromosome is predominantly metacentric, with the exception of two species in which the y chromosome is acrocentric.

A peculiarity of all Oedionychini species is the presence of extremely large sex chromosomes, sometimes corresponding to 50% of the entire genome (Virkki, 1985).

In general, the chromosomes of the Oedionychini species have been analyzed by means of standard staining, and only 7 species have been studied with regard to their C-banding pattern and nucleolus organizer regions (NORs). In these species, the constitutive heterochromatin occurs in the pericentromeric region of all chromosomes, and additionally in the interstitial regions of the sex chromosomes. The use of silver staining to reveal the location of NORs on the chromosomes of these species have shown similar patterns to those produced by C-banding (Virkki, 1983; Virkki and Denton, 1987), which probably does not correspond to the NORs pattern. Consequently, the NORs pattern has not yet been established for this group.

Considering that little karyotype information exists in the literature on Oedionychina species (1 Disonychina and 38 Oedionychina) from the Brazilian fauna, the purpose of this work was to characterize for the first time the karyotype of *Paranaita opima* in relation to its diploid number, chromosomal morphology, type of sex determination system, constitutive heterochromatin distribution pattern, and NOR-bearing chromosomes.

Material and Methods

We analyzed 17 *Paranaita opima* (Germar, 1824) specimens (Figure 1), of which 5 were male embryos and 12 were adults (9 males and 3 females). The adults were collected at the Experimental Garden of the Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro (22°24' S, 47°33' W), São Paulo, Brazil and the embryos were obtained in the laboratory from females naturally fertilized in the field and in captivity.

The mitotic chromosomes were obtained from the embryos and also from the gonads of adult *P. opima*. The embryos and adult gonads were removed in physiological saline solution for insects and were processed according to the methodology of Webb *et al*. (1978). The testes, but not the embryos, were submitted to a hypotonic treatment (tap water) for 3 min and then testes and embryos were fixed in Carnoy I mixture (3:1 methanol: acetic acid) for 30 min and then transferred to a drop of 45% (w/v) aqueous acetic acid

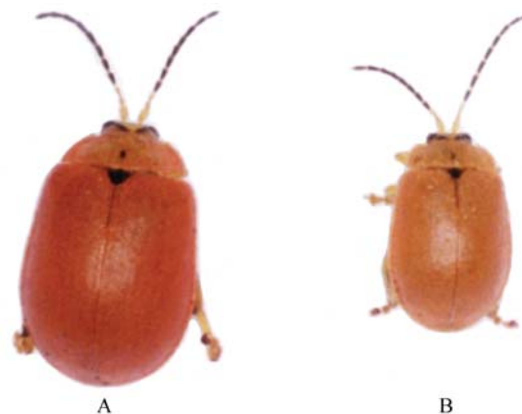

Figure 1 - Specimens of *Paranaita opima*. A. Female. B. Male. Magnification = 5.2X.

on a microscope slide and the material macerated to form a cell suspension, the slides being dried at 35-40 °C on a hot-plate, stained with 3% (w/v) Giemsa in phosphate buffer (pH 6.8) for 12 min, rinsed in distilled water and air-dried. The slides were examined by bright-field optical microscopy using a 100x oil-immersion objective fitted to a Zeiss optical photomicroscope and Kodak Imagelink HQ Microfilm.

The C-banding (Sumner, 1972) and silver nitrate nucleolar organizer region (Ag-NOR) staining (Howell and Black, 1980) were carried out on the Giemsa-stained chromosome preparations described above after removing the immersion oil with Xylene. Triple fluorescent-staining was applied using the GC-specific fluorochrome chromomycin A_3 (CMA_3) and the AT-specific fluorochrome 4'-6-diamidino-2-phenylindole (DAPI), both combined with the distamycin A (DA) counterstain using the technique described by Schweizer (1980).

Routine cytological analyses were carried out as described above and fluorochrome analyses were performed using an Olympus BX50 photomicroscope fitted with filters specific for the DAPI and CMA_3 fluorochromes, photomicrographs being made using Kodak T-Max Film. Chromosome morphology was characterized as described by Levan *et al*. (1964).

Results

Standard staining with Giemsa

The *P. opima* mitotic metaphases showed a chromosome complement of 2n = 22 (20+XY) for males and 2n = 22 (20+XX) in females. The karyotype obtained from embryos and gonial metaphases revealed that the diploid complement consists of three submetacentric chromosomes (pairs 1, 2 and 5), seven metacentric autosomes (pairs 3, 4 and 6 to 10) and extremely large metacentric X and Y sex chromosomes (Figure 2).

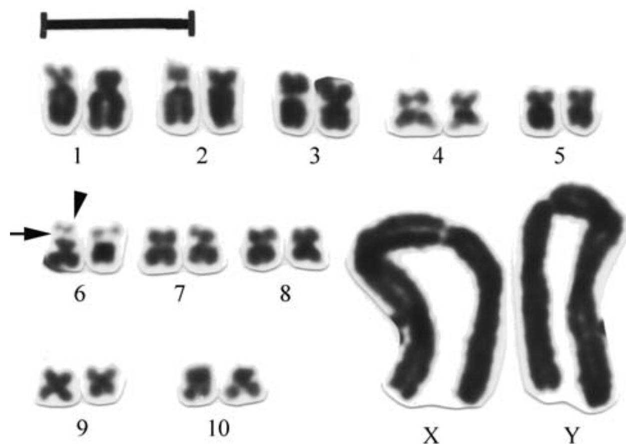

Figure 2 - Giemsa-stained *Paranaita opima* male embryo with 2n = 22 (20+XY) karyotype. Note the secondary constriction (arrow) and the negative heteropycnosis (arrowhead) on pair 6 and the extremely large sex chromosomes. Bar = 10 μm.

In most of the embryonic, spermatogonial, and oogonial mitotic metaphases, the pericentromeric region of all the chromosomes was negative heteropycnotic. Additionally, in these mitotic metaphases, a prominent secondary constriction was observed at the interstitial region of the short-arm of pair 6, and the short-arm terminal region of this pair also showed negatively heteropycnotic (Figures 2, 3A, 4A, 6A and 6F).

C-banding

In mitotic metaphases, all the chromosomes, including the sex chromosomes, showed the occurrence of strongly labeled constitutive heterochromatin in the centromeric region (Figure 3B). In pair 6, the centromeric constitutive heterochromatin extended to the short arm until the secondary constriction (Figures 3B, 6B and 6G).

In some embryonic metaphases, the chromosomes of which were more distended, the Y chromosome showed additional constitutive heterochromatin on the interstitial region of one of the chromosome arms (Figure 3B). In addition, other differential, but not well-defined, marks were visible on the arms of the sex chromosomes.

Ag-NOR staining

Some Giemsa-stained gonial metaphase cells submitted to Ag-NOR staining showed the NORs at the secondary constriction region of pair 6 (Figures 4B, 6C and 6H).

Triple fluorochrome staining

Most embryonic mitotic metaphases showed chromosomes with CMA_3-negative centromeric regions and homogeneously stained arms (Figure 5A), the exception being pair 6 which showed a CMA_3-positive chromosomal region on the short arm which extended from the proximal to the interstitial region (Figure 5A) and was partially coincident with the secondary constriction and the C-band (Figures 6A, 6B, 6D, 6F, 6G and 6I). Analysis with DAPI filter revealed the presence of fluorescent AT bands at the centromeric region of all chromosomes of the complement, including the sex chromosomes (Figure 5B). Pair 6 showed DAPI-positive fluorescence at the terminal region of the short arm (Figures 5B, 6E and 6J) in addition to that on the centromeric region and a DAPI-negative region inserted between the centromeric and telomeric DAPI-positive regions, including all positive CMA_3 region.

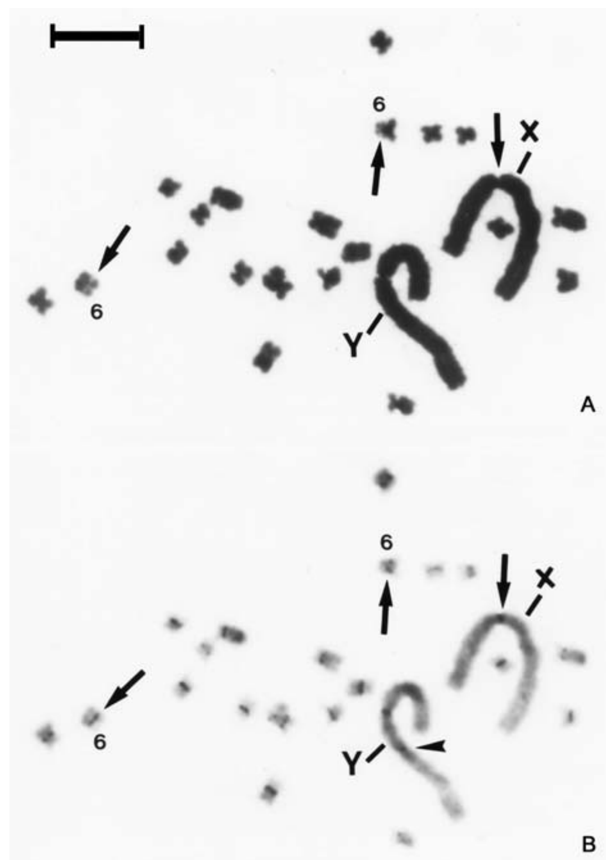

Figure 3 - Male *Paranaita opima* embryo mitotic metaphase with 2n = 22 (20+XY). A. Standard Giemsa staining. B. The same cell as shown in A submitted to Giemsa staining followed by C-banding and showing centromeric constitutive heterochromatin blocks on all chromosomes. Arrows indicate the centromeric region of some chromosomes. The arrowhead points to one of the interstitial C-bands on the sex chromosome. Bar = 10 μm.

Discussion

The basic karyotype characteristics of *P. opima*, such as diploid number, chromosomal morphology, type of sex determination system, and C-banding pattern are similar to those described for other Oedionychina species (Virkki, 1961, 1964, 1970; Smith and Virkki, 1978).

The *P. opima* chromosomal number of 2n = 22 could have been derived from 2n = 24, which has been proposed as ancestral for Chrysomelidae by Virkki (1970) and Smith and Virkki (1978), and may have resulted from fusion

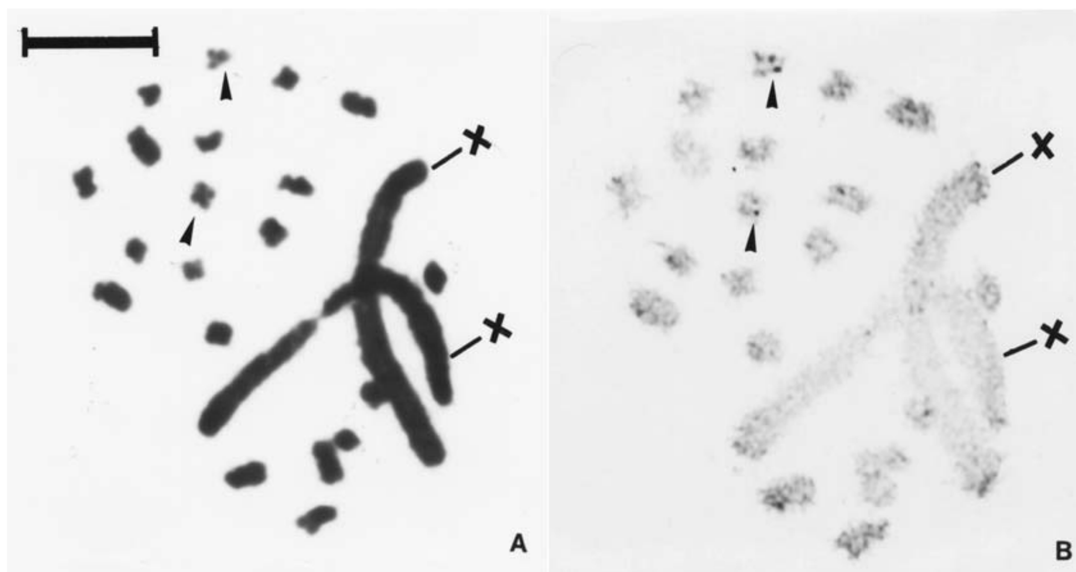

Figure 4 - Adult *Paranaita opima* oogonial metaphase with 2n = 22 (20+XX). A. Giemsa staining, showing chromosomal elements of pair 6 (arrowhead). B. The same cell as shown in A, with Giemsa and Ag-NOR sequential staining showing NORs on pair 6 (arrowhead). Bar = 10 µm.

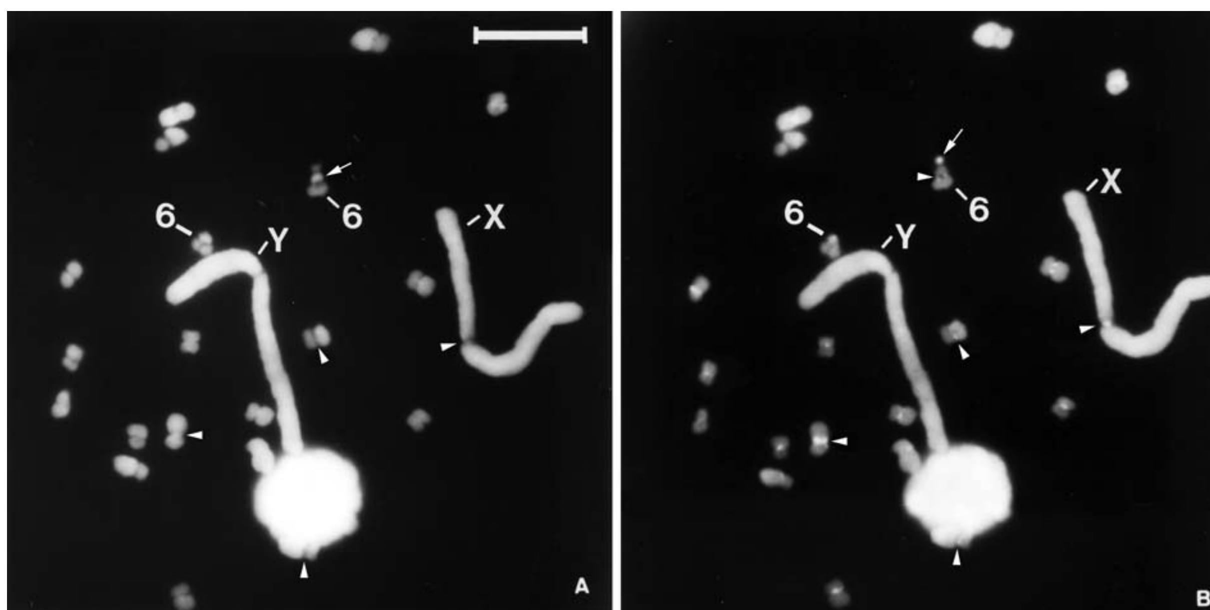

Figure 5 - Male *Paranaita opima* embryo mitotic metaphase stained with CMA_3, DA and DAPI and showing 2n = 22 (20+XY). A. Chromomycin A_3 staining, showing the CMA_3-negative centromeric region (arrowhead) of some chromosomes and the positive CMA_3 labeling at the interstitial region of the short arm of pair 6 (arrow). B. DAPI staining showing the DAPI-positive centromeric region (arrowhead) of some chromosomes, the negative DAPI interstitial region of the short arm of pair 6 and the DAPI-positive terminal region of the short arm of pair 6 (arrow). Bar = 10 µm.

events involving only autosomes or autosomes and sex chromosomes. Similar proposals have been made by Virkki (1970) and Smith and Virkki (1978) for the majority of the Oedionychina species that possesses 2n = 22 chromosomes. The presence of metacentric chromosomes in *P. opima* is in agreement with the condition that prevails in the most current Coleoptera species and that is considered by Smith and Virkki (1978) as a basic and ancestral karyotypic characteristic.

Our results show that *P. opima* has similar-sized X and Y sex chromosomes, a characteristic that has also been found in two other Oedionychina species (*Alagoasa* (under *Oedionychus*) *acutangula* and *Alagoasa extrema*), whereas the X and y chromosomes of the majority of the species of this subtribe, including *Paranaita bilimbata*, are different in size. In general, Oedionychina X and y chromosomes are asynaptic during meiosis but present regular segregation (Virkki, 1968).

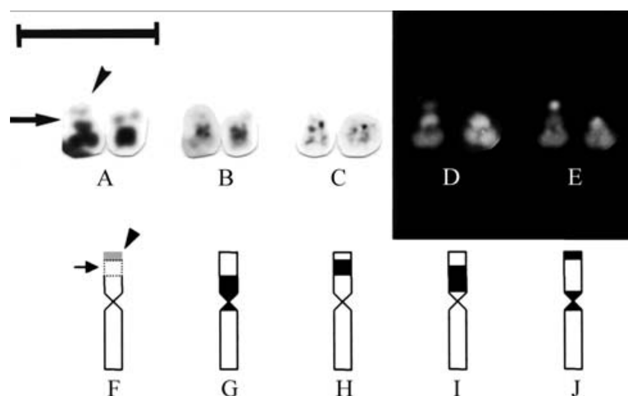

Figure 6 - *Paranaita opima* pair 6 chromosomal elements submitted to different staining techniques (A-E) and their respective schematic representation (F-J). A and F. Giemsa staining showing secondary constriction (arrow) and negative heteropycnosis (arrowhead) at the short arm terminal region. B and G. C-banding showing a large constitutive heterochromatic block at the pericentromeric region. C and H. Ag-NORs at the secondary constriction. D and I. CMA_3 and DA staining showing the GC-rich DNA at the short arm interstitial region. E and J. DA and DAPI staining showing the AT-rich DNA at the centromere and short arm terminal region.

The C-banding pattern of *P. opima* was coincident with those described by Virkki (1983) for some Oedionychina species (*Omophoita annularis*, *Omphoita personata*, *Omophoita octoguttata* and *Alagoasa januaria*) and the *P. opima* sex chromosomes also exhibit additional C-bands on the interstitial region of the chromosome arms, varying in number, position and intensity according to the degree of chromosome distention, these bands being similar to those described in *O. annularis*, *O. personata*, *O. octoguttata* and *A. januaria*.

The presence of interstitial C-bands on the large sex chromosomes of Oedionychina species could be related to the origin of this sex determination system in that these bands could represent heterochromatic material remaining from ancient autosomes incorporated into the original sex determination system of the XY type, or from ancestral y chromosomes belonging to the Xny-type sex determination system. According to Virkki (1970), the large and asynaptic sex chromosomes in Oedionychina could be derived from translocations of the X and Y chromosomes to a large pair of autosomes, such as those found in *Phyllotrupes* (Alticinae, Systenini), or from fusions among y chromosomes belonging to the Xny-type multiple sex determination system found in many Disonychina species, depending on the evolutionary relationship between the subtribes.

We found that in *P. opima* the NORs occur on pair 6, remarkably different from the pattern described for *P. bilimbata* and other Oedionychina species (*O. annularis*, *O. personata*, *O. octoguttata*, *A. januaria*, *Alagoasa bicolor*, *Omophoita cyanipennis* and *Omophoita albicollis*), which is characterized by multiple marks on the y chromosome (Virkki, 1983; Virkki and Denton, 1987). According to Virkki (1983), this pattern of multiple marks on the y chromosome could correspond to a peculiar type of chromatin and not to NORs. In other insect groups, the use of silver nitrate impregnation to detect NOR-bearing chromosomes has also shown special types of constitutive heterochromatin and other chromosome structures (Rufas *et al*., 1983; Cella and Ferreira, 1991).

In the family Chrysomelidae, NORs have been established for a few species (*Botanochara angulata*, *Calligrapha polyspila*, *Chelymorpha variabilis*, *Chrysolina americana*, *Chrysolina bankii* and *Zatrephrina meticulosa*) and are located on one autosomal pair, sometimes at the secondary constriction and sometimes not. In some of these species the NOR-bearing autosomal pair has been identified as either the largest pair or pair 5 (Postiglioni *et al*., 1990, 1991; Postiglioni and Brum-Zorrilla, 1988; Petitpierre, 1996). The distribution of NORs has only been studied in a few Coleoptera species, but in families such as the Curculionidae and Tenebrionidae the NORs occur on autosomes and on sex chromosomes, while in the Cicindelidae and Coccinellidae they occur on autosomes or on sex chromosomes (Drets *et al*., 1983; Virkki *et al*., 1991; Galián *et al*., 1995; Juan *et al*., 1993; Maffei *et al*., 2001).

Our results obtained with the CMA_3 and DAPI fluorochromes showed that the centromeric region of all *P. opima* chromosomes is AT-DNA-rich. The presence of DNA with highly repeated AT base sequences in the centromeric region has also been found in the chromosomes of some Tenebrionidae (Juan *et al*., 1991; Plohl *et al*., 1993).

In *P. opima*, the secondary constriction region on pair 6 appeared to include a GC-rich portion in addition to the NOR because one portion of this region was CMA_3 positive while the other was silver impregnated and, moreover, all this region was negative for both C-banding and DAPI. On the other hand, the short arm positive DAPI terminal region of pair 6 was CMA_3-negative, confirming that this region contains AT base pair sequences. Considering all these results, the NORs on pair 6 seems to be flanked by a special type of heterochromatin, rich in AT base sequences at the terminal side and rich in GC base sequences at the proximal side. These special types of heterochromatin flanking the NORs were not shown by the usual C-banding technique. The presence of constitutive heterochromatin flanking or inserting NORs has been found in the chromosomes of several different groups of animals (Cabrero *et al*., 1986; King *et al*., 1990; Pendás *et al*., 1993; Silva *et al*., 2000; Vicari *et al*., 2003, 2005). The presence of this NOR-associated heterochromatin can restrict genetic recombination in the adjacent region, avoiding the formation of chiasmata or inducing their formation in other chromosome regions (John and King, 1982, 1985) and also can alter the genetic expression of the NORs (Arnold and Shaw, 1985) or can represent breakpoints that facilitate the dispersion of the NORs (Moreira-Filho *et al*., 1984; Reed Phillips, 1995).

Our results offer important insights into the karyotype characteristics of *P. opima*, which may be useful in eluci-

dating relationships between species of the subtribe Oedionychina.

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