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CYTOGENETICS AS A TOOL FOR TRIATOMINE SPECIES DISTINCTION (HEMIPTERA-REDUVIIDAE)

RUBEN PEREZ; YANINA PANZERA; SANDRA SCAFIEZZO; MARIA CRISTINA MAZZELLA*; FRANCISCO PANZERA/+; JEAN PIERRE DUJARDIN** & EKATERINA SCVORTZOFF

Sección Genética Evolutiva, Facultad de Ciencias, Tristán Narvaja 1674, 11200 Montevideo, Uruguay
*Cátedra de Genética, Facultad de Agronomía, Montevideo, Uruguay
**Laboratoire de Génétique des Parasites et des Vecteurs, ORSTOM, Montpellier, France

Several cytogenetic traits were tested as species diagnostic characters on five triatomine species: Rhodnius pictipes, R. nasutus, R. robustus, Triatoma matogrossensis *and* T. pseudomaculata. *Four of them are described for the first time.*

The detailed analysis of the meiotic process and the application of C-banding allowed us to identify seven cytogenetic characters which result useful to characterize and differentiate triatomine species.

Key words: cytogenetics – *Triatoma* – *Rhodnius* – holocentric chromosomes

The Reduviid subfamily Triatominae included numerous species which are actual or potential vectors of *Trypanosoma cruzi,* causative agent of American trypanosomiasis or Chagas disease. Until recently, taxonomy of Triatominae relied almost entirely on morphological determination. Experimental crosses between species were used to understand their phylogenetic relationships (Usinger et al., 1966), or to asses their specific status in dubious cases (Corrêa & Espínola, 1964). The same approach using isoenzyme analysis was applied recently (Dujardin et al., 1988, 1991). Cytogenetics has proved to be a potential tool in species distinction (Ueshima, 1966, 1979; Schreiber et al., 1967; Vaio et al., 1985; Mello et al., 1986).

Three out of the five species we analyzed belong to the genus *Rhodnius,* where four species are closely related: *R. prolixus, R. robustus, R. neglectus* and *R. nasutus.* Morphological identification requires careful examination after genitalia dissection, and taxonomic confusion may ocurr (Dujardin et al., 1991). Similar problems could arise in the genus *Triatoma.* For example *T. pseudomaculata* (North-Eastern Brazil) and *T. maculata* (important vector in Venezuela) are very similar. As a result, there are considerable doubts about many of the distribution records of some species and consequently on their epidemiological importance.

The species described here have been found naturally infected with *T. cruzi* with the exception of *Triatoma matogrossensis* (Lent & Wygodzinsky, 1979). *Rhodnius nasutus, R. pictipes, R. robustus* and *T. pseudomaculata* are sylvatic species but sometimes they are found in domestic and/or peridomestic habitats in different countries of South America (Silveira et al., 1984; Tibayrenc & Le Pont, 1984), which may represent a transitionary phase towards domesticity and increasing vectorial importance (Schofield, 1988). Little is known of *T. matogrossensis,* which appears to be entirely sylvatic.

The present paper describes and compares the chromosome behaviour during male meiosis and the C-banding characteristics in these five species of triatomines, in order to define reliable species diagnostic characters.

MATERIALS AND METHODS

All male specimens were obtained from laboratory strains maintained in the "Labora-

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+Corresponding author.
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tório Nacional e Internacional de Referência em Taxonomia de Triatomíneos" (Instituto Oswaldo Cruz, Rio de Janeiro, Brasil). The number of male specimens studied in each species and the origin of each laboratory strain are showed in Table I.

TABLE I

Number of male specimens studied for each species and the origin of each laboratory strain

Triatoma pseudomaculata	3	Pernambuco, Brazil
Triatoma matogrossensis	4	Mato Grosso, Brazil
Rhodnius nasutus	4	Sciara & Itapagé, Brazil
Rhodnius pictipes	2	Manaus, Brazil
Rhodnius robustus	6	Manaus, Brazil

Testes were fixed in ethanol-acetic acid (3:1) and softened in a 45% aqueous solution of acetic acid. Part of the material was used in squashes stained with lacto-acetic orcein for meiotic descriptions. The remaining material was studied after C-banding technique (Sumner, 1972) to observe the distribution and behaviour of C-heterochromatin during mitosis and meiosis.

The bars in all figures correspond to 10 μm.

RESULTS

All the species studied presented the same chromosome number, 2n = 22, constituted by 20 autosomes and X-Y sex chromosomes in the male. The size differences between autosomes were small (Figs 1-2-3-5), except in *T. pseudomaculata,* where it was possible to recognize a large autosomal pair (Fig. 4).

With classical staining, the sex chromosomes appeared heteropycnotic and indistinguishable from each other during meiosis and mitosis. The application of the C-banding technique allowed the identification of autosomal C-bands and the differentiation of both sex chromosomes.

Rhodnius pictipes Stal 1872 (Figs 1, 6-13) – During early meiotic prophase, the sex chromosomes were seen associated or separated and positively heteropycnotic forming one (Fig. 6) or two (Fig. 7) conspicuous chromocenters. The autosomes did not participate in the formation of this heteropycnotic mass (Figs 6-8). A long pachytene stage was observed (Figs 6-7-8). The beginning of diplotene was characterized by a decondensation of euchromatin. It was called the diffuse stage. At this stage, nuclei enlarge and the sex chromosomes persist heteropycnotic and forming a chromocenter. At late diplotene, the association of sex chromosomes lapsed and their chromatids can be identified (Figs 9-10). Many autosomal bivalents appeared with evident chiasmata.

At metaphase I (Figs 11-12) the sex chromosomes were seen as the smallest elements of the complement. The very small Y chromosome was negatively heteropycnotic (Fig. 11) and their chromatids frequently separate to opposite poles before anaphasic segregation begins (Fig. 12). The first meiotic division was equational for the sex chromosomes.

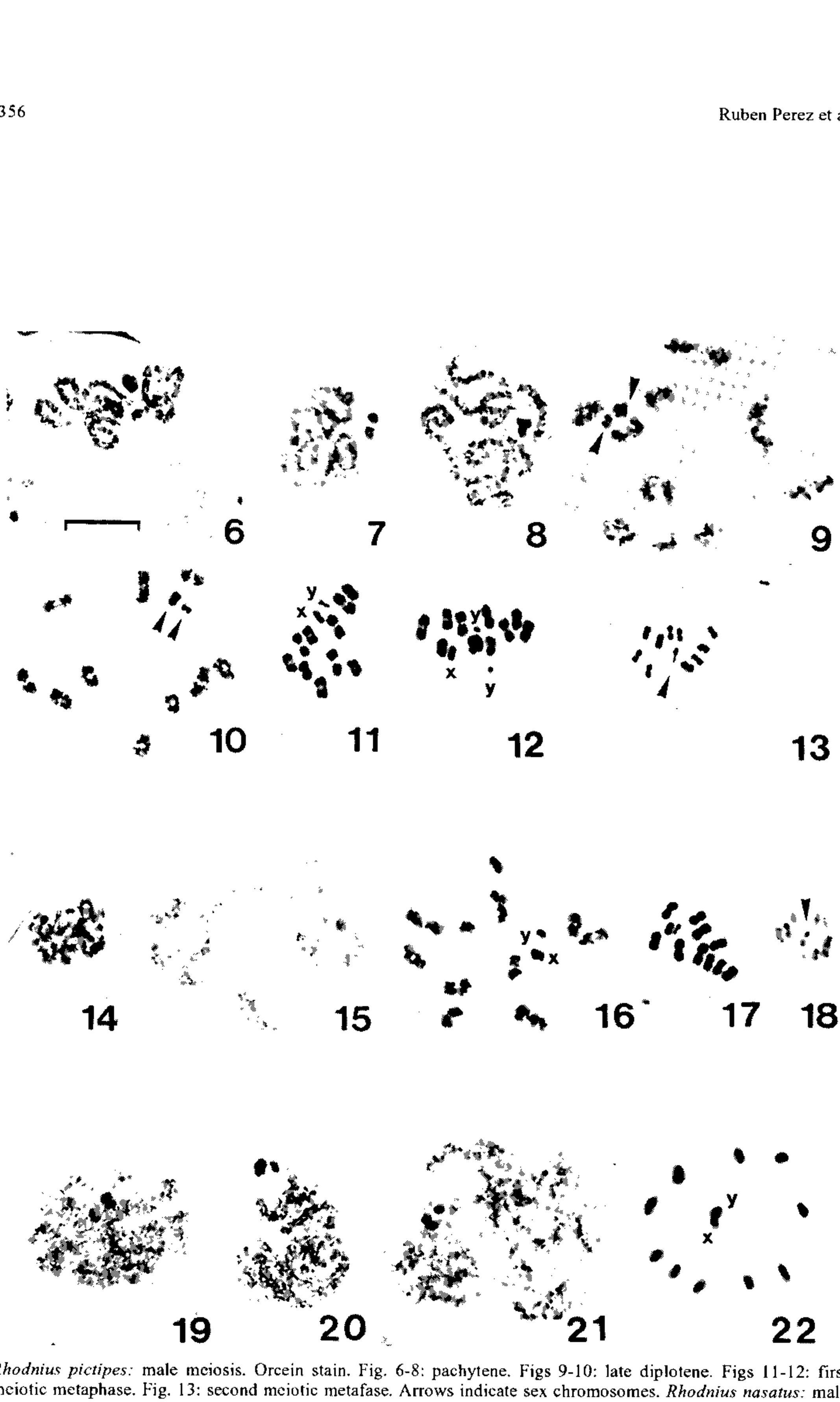
At second meiotic metaphase, the autosomes were found in the periphery of the spindle with the X and Y chromosomes forming a pseudobivalent in the center of the ring (Fig. 13 arrow). The sex chromosomes segregate to opposite poles. The second division is therefore reductional for the sex chromosomes. After C-band staining, only the smallest sex chromosome (Y) was seen heterochromatic and the autosomes did not present evident C-blocks.

Rhodnius nasutus Stal 1859 (Figs 2, 14-18) – During pachytene stage, both sex chromosomes appear forming one heteropycnotic chromocenter (Fig. 14). With C-banding, it was possible to observe small autosomal C-positive corpuscles. From diffuse stage until metaphase I, the sex chromosomes were separated (Figs 15-16-17).

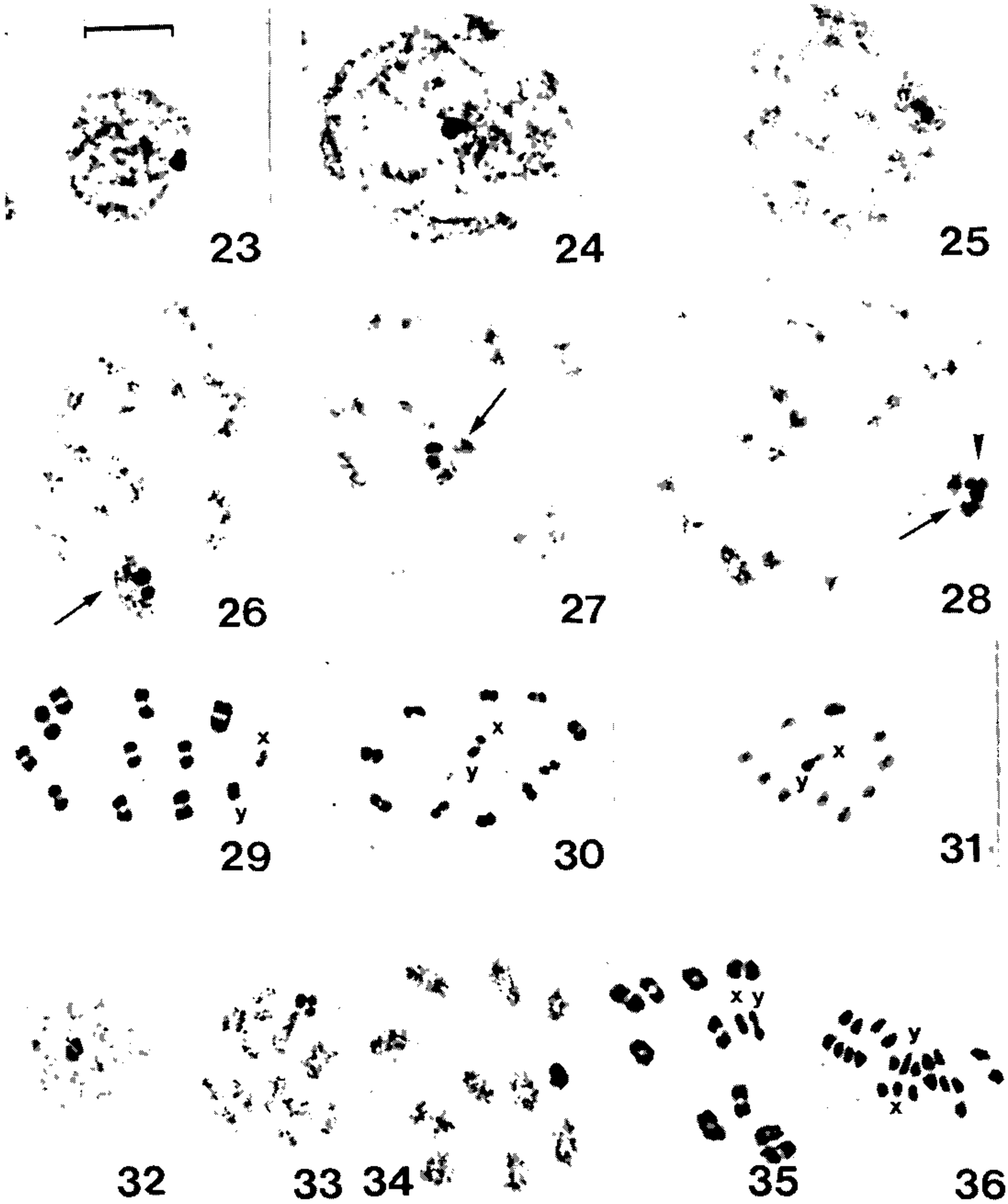
Sex chromosomes were the smallest of the complement, but one is bigger than the other (Figs 17-18). After C-banding, the small sex chromosome of *R. nasutus* was C-heterochromatic (Fig. 18 arrow), similar to that observed in *R. pictipes.* Several autosomes showed terminal C-heterochromatic dots (Fig. 2 arrows).

Rhodnius robustus Larrousse 1927 (Figs 3, 19-22) – From zygotene until the end of the diffuse stage, sex chromosomes were separated from each other and included in a particular region of the nucleus (Figs 19-20-21). The chromatids of both sex chromosomes were seen separated in zygotene (Fig. 19). At pachytene (Fig. 20), the sister chromatids presented a close association but at the diffuse stage one of the sex chromosomes was seen with its chromatids separated again (Fig. 21).

Fig. 1: *Rhodnius pictipes:* karyogram based on spermatogonial metaphase. Orcein stain. Fig. 2: *R. nasutus:* karyogram based on spermatogonial metaphase. C-banding. Arrows indicate small C-bands. Fig. 3: *R. robustus* karyogram based on spermatogonial metaphase. Orcein stain. Fig. 4: *Triatoma pseudomaculata:* karyogram based on spermatogonial metaphase. C-banding. Arrows indicate small C-bands. Fig. 5: *T. matogrossensis:* karyogram based on spermatogonial metaphase. Orcein stain.



Rhodnius pictipes: male meiosis. Orcein stain. Fig. 6-8: pachytene. Figs 9-10: late diplotene. Figs 11-12: first meiotic metaphase. Fig. 13: second meiotic metafase. Arrows indicate sex chromosomes. *Rhodnius nasatus:* male meiosis. Orcein stain. Fig. 14: pachytene. Fig. 15: middle diplotene. Fig. 16: late diplotene. Fig. 17: first meiotic metaphase. Fig. 18: second meiotic metaphase (C-banding). Arrow indicates the heterochromatic (C-positive) Y chromosome. *Rhodnius robustus:* male meiosis. Orcein stain. Fig. 19: zygotene. Fig. 20: pachytene. Fig. 21: diffuse stage. Fig. 22: metaphase II (C-banding).



Triatoma pseudomaculata: male meiosis. Orcein stain. Fig. 23: zygotene. Figs 24-27: diffuse stage. Fig. 28: late diplotene. Fig. 29: first meiotic metaphase. Fig. 30: metaphase II (Orcein stain). Fig. 31: second meiotic metaphase (C-banding). Large arrows indicate the association of the one autosomal bivalent with both sex chromosomes. Small arrow indicates the chromatids separated of one sex chromosome. *Triatoma matogrossensis:* male meiosis. Orcein stain. Fig. 32: pachytene. Fig. 33: diffuse stage. Fig. 34: diakinesis. Fig. 35: first meiotic metaphase. Fig. 36: second anaphase with reductional segregation of sex chromosomes.

TABLE II

Cytogenetic similarities and differences recorded for the five species studied here

Species	*Rhodnius*			*Triatoma*	
Cytogen. traits	*pictipes*	*nasutus*	*robustus*	*pseudomaculata*	*matogros.*
Chromosome number	22	22	22	22	22
Sex mechanism	XY	XY	XY	XY	XY
Relative size of sex chromosomes	X>Y	X>Y	X<Y	X<Y	X<Y
Sex chromosomes are separated at	Pachytene	Diffuse	Pachytene	Diffuse	Zygotene
Relative autosomal size	Small variation	Small variation	Small variation	One larger autosomal pair	Small variation
Autosomal C-bands	No	Yes	No	Yes	No
Constitution of chromocenters	only sex chroms.	only sex chroms.	only sex chroms.	sex chr. plus 1 autos. biv.	only sex chroms.

The C-heterochromatic chromosome (Y) was the largest of the sex chromosomes (Fig. 22).

Triatoma pseudomaculata Corrêa and Espínola 1964 (Figs 4, 23-31) – During early meiotic prophase, one heteropycnotic chromocenter was observed (Fig. 23). Towards the end of pachytene, nuclei abruptly increased in size and enter the diffuse stage (Fig. 24). This particular stage was quite extent and chromosomes can be followed throughout it because they were not totally decondensed. The sex chromosomes appear associated or separated forming one (Fig. 24) or two (Figs 25-26-27) dense corpuscles.

During first meiotic prophase, at least one autosomal bivalent was associated to the sex chromosomes (Figs 26-27-28 large arrows). This association lapsed towards the end of diplotene and the chromatids of the small sex chromosome were clearly identified (Fig. 28 small arrow).

At metaphase I (Fig. 29) both sex chromosomes were seen at the periphery of the spindle. At metaphase II, the sex chromosomes were situated in the centre of a ring formed by the autosomal pairs, adopting a configuration characteristic of other triatomines (Figs 30-31).

The C-banding analysis of first and second meiotic metaphases showed the largest sex chromosome totally heterochromatic and the smallest one as C-negative (Fig. 31). Some autosomal bivalents were observed having terminal C-heterochromatic bands (Fig. 4).

Triatoma matogrossensis Leite and Barbosa 1953 (Figs 5, 32-36) – During early meiotic prophase, the X and Y chromosomes appeared separated forming two heteropycnotic chromocenters (Fig. 32). During diffuse and diplotene stages, it was possible to recognize the chromatids of one sex chromosome (Fig. 33). At diakinesis, sex chromosomes were invariably seen associated and more condensed than the autosomal bivalents (Fig. 34).

At metaphase I, the sex chromosomes were the smallest of the complement, but one was slightly larger than the other (Fig. 35). At second anaphase, the sex chromosomes segregated to opposite poles (Fig. 36). The C-banding technique revealed the largest sex chromosome as being heterochromatic, similar to that observed in *T. pseudomaculata.* The autosomes did not show C-bands.

The cytogenetic similarities and differences recorded for the species studied here are summarized in Table II.

DISCUSSION AND CONCLUSIONS

Chromosome number – Chromosome numbers of about 36 species out of the 115 recognized ones are known (Ueshima, 1966; Schreiber et al., 1972; Panzera et al., 1988a,

1991, this paper). In triatomines, diploid chromosome number in the male ranged from 21 to 25. Ueshima (1966) suggested that the typical chromosome number for this subfamily is 2n = 22 with a male chromosome complement of 20A + XY. This number and complement was found in all species described here for the first time, and the same was confirmed for *T. pseudomaculata* (Schreiber et al., 1972). This finding added a total of 21 species of triatomines with these cytological characteristics.

Once more the discussion is opened towards the typical chromosome complement and its meaning in a group of insects. Some cytogeneticists considered it the ancestral karyotype (White, 1973). But others (Smith & Virkki, 1978) question this interpretation and try to explain it as the ideal cytological situation in each group, being the product of natural selection. We preferred the second explanation for the existence of a most frequent chromosome complement in an insect group.

Sex chromosomes and their relative size – The sex chromosome mechanism in triatomines ranged from XY, X1X2Y, X1X2X3Y in the male. Several authors (Schrader, 1947; White, 1973) suggested that the fragmentation was the major source of multiple sex chromosomes in Heteroptera. The ancestral XY sex mechanism is the most frequent in triatomines, and was found in the five species studied here.

Identification of each sex chromosome in triatomines is very difficult because both sex chromosomes are heteropycnotic and indistinguishable from each other with classical staining. Until now, the sex chromosomes have been named arbitrarily according to their relative size (Schreiber & Pellegrino, 1950; Ueshima, 1966). Based on the study of males and females of three triatomine species, we have proposed another way of naming the sex chromosomes according to their C-heterochromatin: the Y is the heterochromatic sex chromosome (Panzera et al., 1992). We used this nomenclature in this paper and also compared the relative size of the sex chromosomes (Table II).

In *R. pictipes* and *R. nasutus* the Y chromosome was the smallest of both sex chromosomes, while in *R. robustus* the C-heterochromatic one was the largest. Thus the nomenclature using size differences would be wrong in this case. With the same new criterion of naming the sex chromosomes, both *T. pseudomaculata* and *T. matogrossensis* had a large C-heterochromatic chromosome.

Meiotic behaviour of the sex chromosomes – In all species studied here the sex chromosomes were positively heteropycnotic during the first meiotic prophase. However their meiotic behaviour varied significantly between them. During early prophase they were tightly associated, close together or inside a vesicle. In this stage, the first separation between both sex chromosomes were quite specific for each species (Table II). The same happened with the separation of sister chromatids during the first meiotic prophase. It was worth noting that the separation of sister chromatids was not simultaneous for both sex chromosomes in all species.

Relative autosomal size – Schreiber & Pellegrino (1950) had already pointed out the small variation in the size of the autosomes. Striking variations were reported for *T. infestans* and *T. platensis* which possesed three large autosomal pairs (Schreiber & Pellegrino, 1950; Ueshima, 1966). Our observations indicated that *T. pseudomaculata* has one autosomal pair significantly larger than the rest of the karyotype (Fig. 4). In the other species, no differential size classes can be defined (Figs 1, 2-3 and 5).

Comparative analysis of metaphase I could indicate that the chromosomes are smaller in the genus *Rhodnius* than in the genus *Triatoma* (Figs 11, 17, 29 and 35). However, intergenus or interspecific comparison of chromosome size are difficult to estimate due to different stages of chromosome condensation. Neverthless, it is quite feasible to use the relative chromosome size within species as a differential character.

Another approach, though not directly related to chromosome size could be to compare DNA content between species. Schreiber et al. (1972) showed indeed that the *Rhodnius* species had a low value of DNA compared with *Triatoma* and *Panstrongylus* species. It would be of great interest to make DNA content determination in triatomine species in order to advance in the interpretation of their evolutionary relationships.

Autosomal C-bands – Autosomal C-bands may be used as interspecific as well as infraespecific analysis. Infraspecific variability was studied for *T. infestans* (Panzera et al., 1988b, 1992). For species comparisons, the character to be used would be the presence or the absence of C-bands. Until now, only two triatomine species (*T. infestans* and *T. platensis*) presented evident C-heterochromatic blocks mainly in the largest autosomes (Solari, 1979; Panzera et al., 1988a). In *T. pseudomaculata* and *R. nasutus* some autosomes had C-heterochromatic dots or small bands (Figs 2 and 4). The rest of species studied here did not present autosomal C-bands (Table II).

Constitution of chromocenters – The association (or separation) of both heteropycnotic sex chromosomes during first prophase and, in some cases, the association of heteropycnotic regions of the autosomes, were responsible of the diversity found in the configuration (shape, aspect) and number of the meiotic chromocenters of these five species (see Table II).

In four species (Table II) the chromocenters were exclusively constituted by the sex chromosomes. In *T. pseudomaculata,* one autosomal pair was associated to the sex chromosomes and contributed to the chromocenter formation. In two other species of triatomines, *T. infestans* and *T. platensis,* the mitotic and meiotic chromocenters were formed also by the association of the sex chromosomes with a varying number of C-heterochromatic autosomal bivalents (Solari, 1979; Panzera et al., 1988a, 1992).

Advantages and limitations of cytogenecit analysis – Advantages: (a) Species distinction by cytogenetics does not require to analyze a large number of individuals; (b) Low cost of consummables. Limitations: (a) In triatomines only adults and in some cases the 5th instar have the mature gonads, necessary for cytogenetic studies; (b) Time required to complete the cytogenetic identification of one species could be one or two weeks for one research. However, only a few specimens would be needed.

The seven cytogenetic markers evidenced by this study may be used together for species distinction. Cytogenetic appears to be a reliable and low cost tool for species identification in case of taxonomic confusion, and as a complement to other genetic methods when they remain inconclusive.

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