Cytogenetic Analysis of 3 Populations of *Diabrotica speciosa* (Chrysomelidae, Galerucinae): Constitutive Heterochromatin and Nucleolus Organizer Regions

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Summary The genus *Diabrotica* is characterized by having a great uniformity of karyotype in respect to the chromosome number and morphology, with the exception of *Diabrotica speciosa*. The diploid chromosome number, the morphology of chromosomes, the type of sex determination, the pattern of constitutive heterochromatin distribution and the chromosomes bearing the nucleolus organizer regions (NORs) were studied in specimens from 3 different Brazilian populations of *D. speciosa*. The analysis of mitotic cells revealed $2n=21$ in the males and $2n=22$ in the females. All the chromosomes of the complement were metacentric and the type of sex determination was XO/XX. Additionally, mitotic metaphases of 1 female from the Ponta Grossa population showed the occurrence of heteromorphic homologous chromosomes in pair 9. The male meiocytes showed 10 bivalents and a univalent X chromosomes. One or 2 chiasmata were found per bivalent in diplotene. The constitutive heterochromatin was found in the centromeric region of all the autosomes and in both arms of the X chromosome. Besides, some autosomes of 1 specimen from the Ponta Grossa population were heteromorphic for C bands. NORs were found the telomeric region of pair 9 in all the individuals of the populations.

Key words Meiosis, C-banding, NOR, Karyotype, Sex determination.

The family Chrysomelidae includes many species, which have great heterogeneity of chromosome numbers and types of sex determination. The most frequent types of sex determination are Xyp and XO in this family (Smith and Virkki 1978). The sex determination of Xyp/XX type is found in the majority of the species of Coleoptera and XO/XX type has been observed most frequently in Polyphaga, mainly in the Lycidae, Lampyridae, Cantharidae, Tenebrionidae and Passalidae families (White 1973, Smith and Virkki 1978, Yadav *et al.* 1985). The subfamily Galerucinae includes approximately 95 cytogenetically analyzed species and is characterized by having diploid chromosome numbers ranging from $2n=59$ in *Rhaphidopalpa femoralis*, to $2n=13$ in *Gynandrobrotica nigrofasciata*. In that subfamily, the sex determination of Xyp, XO, Xy, XXy, neoXY and Xyr types occur in a similar way to those described for other Chrysomelidae subfamilies.

A review of cytogenetic information on 34 species of the genus *Diabrotica* described in literatures shows that this genus possesses a uniform chromosome number $2n=18+X$ in the male, and the complement consists of meta/submetacentric chromosomes; this karyotypic homogeneity is uncommon within the genera of Chrysomelidae (Smith 1971, Ennis 1972). Little variations of karyotypes found among the species of *Diabrotica* are related to the number of supernumerary chromosomes (B chromosomes) and the chiasma frequency. Ennis (1972) postulated that the mean chiasma frequency can be used as a parameter for interspecific comparisons. Among all the species of *Diabrotica*, only karyotypic exception is *Diabrotica speciosa*, described by Ennis (1972), which shows

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$2n=20+X$ in the male and has 2 acrocentric autosomal pairs in the complement.

According to White (1973), the supernumerary chromosomes are found in several groups of animals and plants, and are encountered in some individuals of the population. In the Coleoptera, the supernumerary chromosomes occur in approximately 50 species and subspecies (White 1973, Smith and Virkki 1978, Araújo *et al.* 2000). In the genus *Diabrotica*, 11 species have supernumerary chromosomes, which vary in number from 1 to 5 per individual (Smith 1971, Smith and Virkki 1978).

Many supernumerary chromosomes are constitutive heterochromatic, in other words, genetically inert; however in some species, these chromosomes possess transcriptional activity (Camacho *et al.* 2000).

The purpose of this work is to determine the diploid chromosome number, the morphology of chromosomes, the type of sex determination, the pattern of constitutive heterochromatin distribution and the chromosomes bearing the nucleolus organizer regions (NORs) in 3 different Brazilian populations of *Diabrotica speciosa*.

Materials and methods

In this work 54 specimens of *Diabrotica speciosa* were analyzed. The specimens were caught at 3 different states of Brazil: 35 males and 2 females from Ponta Grossa, Paraná State, 9 males and 2 females from Nonoai, Rio Grande do Sul State, and 5 males and 1 female from Itapeva, São Paulo State. The gonads from these individuals were removed in a physiological salt solution for insects, placed in a hypotonic solution (tap water, for 5 min) and fixed in Carnoy I (methanol 3: acetic acid 1, for at least 30 min). For obtaining mitotic metaphases, some gonads were previously treated with 0.05% colchicine solution (in a physiological salt solution for insects) for 2 h. For the preparations of the slides, the gonads were macerated in 45% acetic acid and dried on a heating metal plate at a temperature of 35–40°C. The slides were stained with 3% Giemsa solution for 12 min. C bands were obtained by the technique of Sumner (1972), and NORs were stained by the technique of Howell and Black (1980). Light microscope photomicrography was carried out using a Zeiss photomicroscopy and an Imagelink HQ Microfilm (Kodak) was employed.

Results

Standard staining

The mitotic cells of *D. speciosa* showed $2n=21$ in the males and $2n=22$ in the females, sex determination of XO/XX type, all the chromosomes being metacentric (Fig. 1A, B). In most oögonial metaphases, one of the X chromosomes was totally positive heteropycnotic (Fig. 1B). These characteristics were common in individuals of all the analyzed populations, with the exception of 1 female from the Ponta Grossa population, in which metaphase of gonial cells showed heteromorphic chromosomes of pair 9 of different sizes (Fig. 2A).

In all populations, interphase of testicular cells showed a precocious heteropycnotic X chromosome (Fig. 3A). At pachytene 10 long filaments of autosomal bivalents, and a single positive heteropycnotic X chromosome was found (Fig. 3B). Diplotene and diakinesis of the male meiocytes showed 10 autosomal bivalents and 1 univalent of sex chromosome ($n=10+X$) with 1 or 2 chiasmata per autosomal bivalent (Fig. 3C, D). The metaphases II showed $n=10+X$ and $n=10$, indicating the regular segregation of the sex chromosome (Fig. 3E, F). In 2 individuals from the Ponta Grossa population metaphase I and II showed an extremely small supernumerary chromosome (Fig. 3G, H).

Fig. 1. Mitotic chromosomes of *Diabrotica speciosa*. A) Spermatogonial karyotype in standard staining with $2n=20+X$. B) oogonial karyotype standard staining showing $2n=20+XX$, and one positive heteropycnotic sexual chromosome (arrow). C) The same cell seen in B, submitted to the C-banding technique, evidencing the presence of centromeric C bands in all the chromosomes (small arrows) and one of the strongly stained X chromosomes (large arrow). D) Spermatogonial karyotype, with silver nitrate impregnation, showing the telomeric NORs in pair 9 (arrow). E) Chromosomes in pair 9, evidencing telomeric NORs. Bar= $5\mu\text{m}$.

C-banding

Observation of the oogonial metaphase of *D. speciosa* using the sequential standard staining/C-banding technique revealed the presence of constitutive heterochromatin blocks in the centromeric region of all the autosomes (Figs. 1C, 2B). In these cells, one X chromosome exhibited strongly marked arms (Fig. 1C), but did not show centromeric C band. The other X chromosome showed a homogeneous staining, without a marked region. This pattern of heterochromatin distribution is similar in almost all individuals of the populations. However, the oogonial mitotic metaphas-

Fig. 2. Female karyotype of *Diabrotica speciosa* of the Ponta Grossa population with $2n=20+XX$. A) Standard staining showing the heteromorphism in size between the chromosomes of pair 9. B) The same cell observed in A, submitted to C-banding, evidencing the occurrence of centromeric marks in all chromosomes of the complement. Additional C band is observed in the telomeric region of pairs 8, 9 and 10, interstitial region in one of the chromosomes of pair 9 (arrow), and in both arms of X chromosomes. Bar= $5\mu\text{m}$.

Fig. 3. Male meiotic cells of *Diabrotica speciosa* submitted to standard staining. A) Interphasic nucleus, evidencing the positive heteropycnosis of the X chromosome (arrow). B) Pachytene cell, with 10 autosomal bivalent and 1 univalent sexual chromosome (arrow). C, D) Diplotene and diakinesis nuclei with $n=10+X$, showing the chiasma (arrows) in the autosomal bivalent. E, F) Metaphases II with $n=10+X$ or $n=10$, evidencing the regular segregation of the chromosomes. G) Metaphase I with $n=10+X+S$. H) Metaphase II with $n=10+S$. S=supernumerary chromosome. Bar= $5\mu\text{m}$.

es of 1 specimen of the Ponta Grossa population showed a variation in the C-banding pattern, *i.e.*, the presence of additional C bands in the telomeric region of the short arm of autosomal pairs 8, 9 and 10, and in the interstitial region of the long arm in 1 chromosome of the autosomal pair 9 (Fig.

Fig. 4. A, C) Diplotene and metaphase II of *Diabrotica speciosa* standard stained. B, D) The same cells observed in A, C) respectively, submitted to the C-banding technique, showing the presence of constitutive heterochromatin in the centromeric region of the autosomes (arrow) and the totally heterochromatic X chromosome. Bar=5 μ m.

2B). In these cells, the sex chromosomes showed clear centromeric C band and intensely marked blocks in both chromosome arms.

At diplotene and metaphase II the cells showed the presence of constitutive heterochromatin in the centromeric region of all the chromosomes of the complement, excepting the X chromosome, which was totally heterochromatic (Fig. 4). This C-banding pattern corresponds to the pattern of positive heteropycnosis. In all individuals of all the populations, the meiocytes cells showed the C-banding pattern described above.

Silver nitrate impregnation

Spermatogonial metaphases of *D. speciosa* submitted to silver impregnation revealed the presence of NORs in the telomeric region of the short arm of pair 9 in all individuals of the populations (Fig. 1D, E).

Discussion

Some characteristics of the karyotype of *D. speciosa* observed in the present study, such as the diploid chromosome number and the type of sex determination, are in agreement with those described by Ennis (1972) who studied the same species from the São Paulo population. However, the morphology of chromosomes disclosed in the present study using specimens from the Rio Grande do Sul (Nonoai), Paraná (Ponta Grossa) and São Paulo (Itapeva) populations differs from that described by Ennis (1972). Namely, all the chromosomes in the complement of the specimens used in the present study were metacentric, whereas 2 autosomal pairs were acrocentric in Ennis (1972). This difference between the metacentric and acrocentric chromosomes in specimens from the same population can be explained by pericentric inversions or deletions of heterochromatic material.

Considering the original chromosome number $2n=18+X$ proposed by Smith (1971) for the genus *Diabrotica*, the diploid number $2n=20+X$ of *D. speciosa* could have arisen by fission of an autosomic to increase the diploid chromosome number and to give rise to acrocentric chromosomes, as found in the karyotype of the São Paulo population studied by Ennis (1972). Subsequently, pericentric inversions could have occurred to change the acrocentric chromosomes to metacentric ones, as observed in the karyotype of the populations in the present study.

The presence of positive heteropycnotic sex chromosomes during the meiosis is common in the Coleoptera families, varying among related species. The difference among species involving heteropycnosis can be due to the chromosome condensation and/or the presence of a certain type of chromatin (Virkki 1967, Ennis 1972, Ferreira and Mesa 1977, Yadav *et al.* 1985, Juan *et al.* 1993, Almeida *et al.* 2000). The positive heteropycnosis of the sexual chromosomes during the meiosis, as observed in *D. speciosa*, has been considered to indicate heterochromatinization by many authors (Smith and Virkki 1978, Drets *et al.* 1983, Juan *et al.* 1993, Plohl *et al.* 1993).

The occurrence of 1 or 2 chiasmata per bivalent in meiocytes of *D. speciosa* is in agreement with the chiasma frequency described by Ennis (1972) for other species of *Diabrotica* and within the range of variation of the chiasma frequency proposed for the genus. Only 2 specimens of the Ponta Grossa population showed the presence of 1 supernumerary chromosome. The occurrence of supernumerary chromosome in the genus *Diabrotica*, has been reported in such species as *D. lemniscata*, *D. longicornis*, *D. scutellata*, *D. tibialis* and *D. undecimpunctata* (Smith 1971, Smith and Virkki 1978).

The centromeric C-banding pattern in the autosomes of *D. speciosa* is similar to one described in most Coleoptera species. The presence of C bands in the centromeric region of all the chromosomes of the complement was observed in 3 species of Hydrophilidae (Angus 1983), and in 1 species of Coccinellidae (Maffei *et al.* 2000). The C band in the pericentromeric region of all the chromosomes of the complement was verified in *Epilachna paenulata* (Drets *et al.* 1983) and in *Tenebrio molitor* (Weith 1985). In *Palembus dermestoides* and *Epicauta atomaria*, pericentromeric C bands occur in the autosomes and in Xp (Almeida *et al.* 2000). Moreover, some autosomes of *P. dermestoides* have telomeric marks.

The C-banding pattern in the sex chromosomes of *D. speciosa*, *i.e.*, large blocks in both chromosome arms show that the XO/XX system is differentiated not only by loss of the y chromosome, but also by increasing a considerable quantity of constitutive heterochromatin. This pattern differs from one described for other species with XO/XX system, in which the constitutive heterochromatin only occurs in the centromeric region, such as in 4 *Trechus* (Carabidae) species (Rozek 1998), and in 67 species of Acridoidea (King and John 1980, Santos *et al.* 1983, Cabrero and Camacho 1986).

The heteromorphic chromosomes of pair 9 in 1 specimen of *D. speciosa* of the Ponta Grossa population can be explained by duplication or deletion of a small segment of chromosomes, producing a difference between the chromosome length. Alternatively, the heteromorphism can occur due to the differential activity of the telomeric NOR in pair 9. In addition, the interindividual variation of C-banding pattern in the telomeric region of pairs 8, 9 and 10 and the interstitial band in the long arm of the pair 9 of *D. speciosa* can be explained by small duplications of heterochromatic segments.

Among the Chrysomelidae the occurrence of NORs in the autosomes was already verified in some species, such as *Botanochara angulata*, *Chrysolina americana*, *Chrysolina bankii* and *Chelymorpha variabilis*. In the majority of these species, the NOR-bearing chromosomes were not identified, with the exception of *Chelymorpha variabilis*, in which the NOR is located in the 5th autosome pair (Postiglioni *et al.* 1990, 1991, Petitpierre 1996). This pattern of autosome NOR found in some Chrysomelidae species reinforces the possibility that in this family the NOR is not associated to sexual chromosomes. The presence of telomeric NOR in autosomes was found in *Epicauta atomaria* (Meloidae) and *Palembus dermestoides* (Tenebrionidae), when the microspreading analysis of meiotic prophase was used (Almeida *et al.* 2000). Thus, although the species of *Diabrotica* show little karyotypic diversities as far as the standard staining was conducted, it is possible that other species of this genus show karyotype variations as those observed in the *D. speciosa*, if the chromosomes of these species will be analyzed with different staining techniques.

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