

RESEARCH ARTICLE

Cytogenetic characterization of *Eurysternus caribaeus* (Coleoptera: Scarabaeidae): evidence of sex-autosome fusion and diploid number reduction prior to species dispersion

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Abstract

Mitotic and meiotic chromosomes of several populations of *Eurysternus caribaeus* (Coleoptera: Scarabaeidae) were analysed through conventional staining, C-banding, base-specific fluorochromes, silver nitrate staining and fluorescent *in situ* hybridization (FISH). All specimens showed $2n = 8$ in their karyotypes, with a neo-XY sex system (Y is a submetacentric and X a metacentric) and three pairs of submetacentric autosomes. The analysis of constitutive heterochromatin (CH) revealed small blocks located in the centromeric region of all chromosomes which do not present positive staining under the fluorochromes CMA₃ and DAPI. Silver nitrate staining revealed that the nucleolar organizer region (NORs) is associated with the sex chromosomes. The FISH technique revealed that rDNA sites in the X and Y are different in size. Data from different populations indicate that the diploid number reduction ($2n = 8$) observed in *E. caribaeus* is established and presumably has preceded the dispersion of this species. Moreover, this reduction occasioned the translocation of rDNA sites to the sex chromosomes, X and Y, an uncommon pattern in Scarabaeidae that was observed for the first time by the FISH in this work.

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Introduction

The cosmopolitan beetle family Scarabaeidae comprises approximately 2000 genera and 25,000 species. In Neotropical region, about 4706 species have been recorded, and 1777 species were identified in Brazil (Costa 2000). Some of their representatives show important functions as pollinators of plants, organic matter recyclers and biologic controllers of agricultural plagues, acting moreover as indicators for the analysis of biodiversity in tropical forest (Halffter and Favila 1993).

Despite the large number of species, there are few studies about the chromosomal diversity of Scarabaeidae

representatives and approximately, only 390 species (1.57%) have been analysed, predominantly using conventional staining. This family shows conserved karyotypes with more than 50% of the species presenting the diploid number $2n = 20$, Xy_p sex mechanism and banded chromosomes. This condition has been considered primitive to this group and also to the whole order Coleoptera (Smith and Virkki 1978; Yadav *et al.* 1979; Martins 1994; Moura *et al.* 2003; Bione *et al.* 2005a,b; Cabral-de-Mello *et al.* 2008). However, variations of diploid number, sex mechanism and, to a lesser extent, chromosomal morphology have also been recorded. The species *Eurysternus caribaeus* has the lowest ($2n = 8$), while *Autoserica assamensis* the highest diploid number for the family ($2n = 30$) (Yadav *et al.* 1979; Cabral-de-Mello *et al.* 2007). The family presents seven sex mechanisms

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(XY, Xy, XY_p, Xy_p, Xy_r, XO and neo-XY) with variations in the chromosomal morphology in some species (Yadav et al. 1979; Moura et al. 2003; Bione et al. 2005a,b; Cabral-de-Mello et al. 2007; Dutrillaux et al. 2007).

In some Coleopteran families, wide karyotypic structure variation has been reported, as described for Buprestidae ($2n = 12$ to $2n = 46$) and Elateridae ($2n = 4$ to $2n = 23$), whereas karyotypic conservation has been observed in others, such as Lampyridae ($2n = 19$, XO), and Cantharidae ($2n = 13$, XO) (Smith and Virkki 1978; Machado et al. 2001; Karagyan et al. 2004; Dias et al. 2007; Schneider et al. 2007a). In the insect class, as a whole, there are groups that present modal and conserved diploid number, i.e. acridid grasshopper (Orthoptera), Libellulidae (Odonata), and groups less conserved showing variation in number of autosomes and/or sex chromosomes, for example in Phaneropterinae (Tettigoniidae, Orthoptera) and Reduviidae (Heteroptera) (Hewitt 1979; Mola et al. 1999; Ferreira and Mesa 2007; Poggio et al. 2007).

About 70 Scarabaeidae species have been studied using differential or molecular cytogenetic techniques, such as C-banding, base-specific fluorochromes, silver nitrate staining or fluorescence *in situ* hybridization (FISH) (Moura et al. 2003; Wilson and Angus 2004, 2005, 2006; Bione et al. 2005a,b; Angus et al. 2007; Dutrillaux et al. 2007). The constitutive heterochromatin (CH) in this family is predominantly located in the pericentric region of the chromosomes and this genomic component shows wide heterogeneity regarding AT-richness and GC-richness. Moreover, species with telomeric, interstitial CH and diphasic chromosomes have been described in this family (Colomba et al. 1996, 2000, 2006; Moura et al. 2003; Bione et al. 2005a; Angus et al. 2007; Macaisne et al. 2006). The nucleolar organizer regions (NORs) are predominantly located in a single autosomal pair or in the X chromosome. However, some species show more than one rDNA site clustered in different chromosome pairs (Moura et al. 2003; Bione et al. 2005a,b; Macaisne et al. 2006).

Previous cytogenetic studies have been carried out in one of the populations of *E. caribaeus* and showed the presence of the smallest diploid number observed in the superfamily Scarabaeoidea (Cabral-de-Mello et al. 2007). In the present study, conventional staining, C-banding, silver nitrate staining, base-specific fluorochromes CMA₃ and DAPI, and FISH were used to characterize the karyotype of several populations of *E. caribaeus*. The results obtained indicate that the chromosome structure has remained conserved during the dispersion of the species and fusion of autosomal and sexual elements had occurred in the generation of the karyotype of *E. caribaeus*.

Materials and methods

Mitotic and meiotic chromosomes of 22 male specimens of *E. caribaeus* were analysed. The animals were col-

lected from forest areas of Pernambuco state (Brazil), Paudalho (11 specimens) ($7^{\circ}57'56''S$ and $35^{\circ}00'07''W$), Igarassu (three specimens) ($7^{\circ}48'37''S$ and $34^{\circ}27'25''W$), and Caruaru (five specimens) ($8^{\circ}42'S$ and $35^{\circ}15'W$). Further, three specimens from botanical garden of Santa Cruz de la Sierra city ($17^{\circ}47'21''S$ and $63^{\circ}11'48''W$), Bolivia, were analysed.

The insects were anesthetized with ether, and their testicular follicles were dissected and fixed in Carnoy (3:1, ethanol:acetic acid). Chromosome preparations were obtained by the classic testicular follicles crushing technique. For conventional staining 2% lacto-acetic orcein was used. The C-banding was performed according to Sumner (1972), with modifications. The material was incubated with 0.2 N HCl, followed by treatment with 5% barium hydroxide and $2\times$ SSC at $60^{\circ}C$. Triple combination of the fluorochromes CMA₃/DA/DAPI and silver staining were performed using the protocols of Schweizer (1976) and Rufas et al. (1987) respectively, and FISH followed the procedure of Moscone et al. (1996), using a probe of 45S rDNA isolated from *Arabidopsis thaliana*. The images were captured in Leica DM LB microscope (Leica Microsystems, Wetzlar, Germany) and the figures were organized using the Corel Photo-Paint 12 software (Corel corporation 2003, Ottawa, Canada).

Results

Conventional staining

The 22 analysed individuals of *E. caribaeus* from the different populations showed diploid number $2n = 8$ and a neo-XY sex chromosome mechanism (meioformule 3II+neo-XY). The karyotype of this species is symmetric with gradual reduction in size. The autosomes and the Y showed submetacentric morphology and the X is a metacentric element (figure 1, a&b)

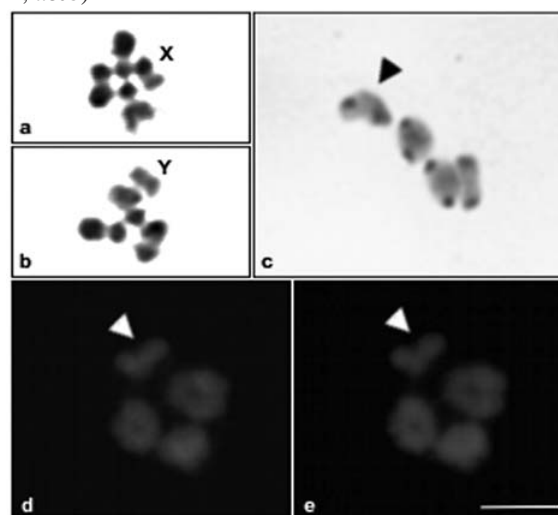


Figure 1. Meiotic chromosomes of *E. caribaeus*. (a&b) Conventional staining of metaphases II, (c) C-banding, (d) CMA₃, and (e) DAPI fluorochrome staining of metaphase I chromosomes. Arrowheads indicate the sex bivalent. Bar = $5\ \mu m$.

C-banding and fluorochrome staining

The analysis using C-banding technique allowed the identification of small constitutive heterochromatic blocks located in the centromeric region of all chromosomes (figure 1,c). The base-specific (CMA₃ and DAPI) fluorochromes staining did not reveal positive blocks indicating that the CH in this species is not enriched with AT or GC base pairs (figure 1, d&e).

Silver nitrate staining and FISH

The silver nitrate staining detected an amorphous region corresponding to nucleolar remnants in the sex bivalent neo-XY (figure 2,a), and marked the heterochromatic regions of chromosomes. However, it did not stain the sex chromosomes in advanced meiotic stages; indicating the absence of argiophilic proteins in the configuration of the sexual bivalent (figure 2,b). The physical chromosomal localization of rRNA genes detected the presence of rDNA sites on the X and Y chromosomes. The site of rDNA in the X chromosome is larger than that observed in the Y (figure 2,c).

Discussion

The diploid number of *E. caribaeus* analysed specimens corroborates the previous description by Cabral-de-Mello *et al.* (2007). Although belonging to different geographically isolated populations, and despite the significant diploid number reduction to $2n = 8$, all specimens showed the same karyotype through the conventional staining. The genus *Eurysternus* is strictly neotropical, with 28 described species so far, and shows as dispersion centre the Brazilian forests and the Guiana region, extending up from Mexico to north Argentina, without record in the fauna of Chile and Uruguay (Halffter and Halffter 1976; Jessop 1985; Martinez 1988;

Vaz-de-Mello 2000). The absence of chromosome polymorphisms in *E. caribaeus* indicates that presumably its diploid number reduction occurred in one ancestral population, before the dispersion process of this species. The reduced chromosome number of *E. caribaeus* is probably the resultant of pericentric inversions followed by fusions (Cabral-de-Mello *et al.* 2007), as observed in other species belonging to Scarabaeidae, for instance, *Bubas bubalus* $2n = 18$, Xy (Angus *et al.* 2007), *Dichotomius geminatus* $2n = 18$, Xy_p, (Cabral-de-Mello *et al.* 2008), *Isocoprissus inhiata* $2n = 18$, Xy_p (Bione *et al.* 2005a), and *Macraspis festiva* $2n = 18$, Xy_p (Bione *et al.* 2005b). In some Scarabaeidae species, reduction in the diploid number caused by fusion between autosomes and autosome-X has been observed. Fusions that involve the sex chromosomes are responsible for diploid number reduction and the origin of neo-XY sex mechanism. These rearrangements, in addition to fissions, chromosome losses and increase in the size of Y, are responsible for karyotype differentiation in Scarabaeidae during their evolution (Yadav and Pillai 1979; Yadav *et al.* 1979; Bione *et al.* 2005b; Macaisne *et al.* 2006; Cabral-de-Mello *et al.* 2007; Cabral-de-Mello *et al.* 2008; Angus *et al.* 2007; Dutrillaux *et al.* 2007).

Chromosome fusions have been observed in other groups of Coleoptera, insects and arthropods as a whole, and they are related to diploid number reduction and generation of neo-XY sex mechanism, as also observed in *E. caribaeus*. In Coleoptera, examples of diploid number reduction and/or neo-XY origin were observed in Elateridae, *Conoderus stigmatus* ($2n = 16$, neo-XY), Lampyridae, *Bicellonycha lividipennis* ($2n = 18$, neo-XY) and other families (Schneider *et al.* 2006; Dias *et al.* 2007). This pattern of chromosomal evolution in insects has also been seen in other orders, for e.g., Heteroptera (Bressa *et al.* 1999), Odonata (Mola and Papeschi 1994), and to arthropods in

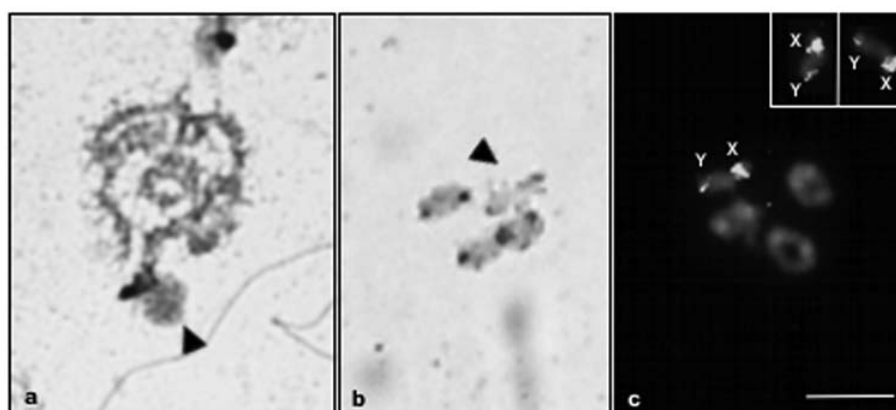


Figure 2. Meiotic chromosomes of *E. caribaeus* subjected to silver nitrate staining and fluorescence *in situ* hybridization (FISH). Silver nitrate staining of (a) zygotene, showing the NOR associated with the sex bivalent, (b) metaphase I, showing the affinity of the silver to the CH blocks, and (c) mapping of rRNA genes to metaphase I chromosomes, indicating the presence of 45S rDNA sites on the X and Y chromosomes (note the size difference of rDNA sites in the X and Y chromosomes (insert)). Arrowheads indicate the sex bivalent. Bar = 5 μm.

general in spiders (Rezác *et al.* 2006) and pseudoscorpions (Stahlavsky and Kral 2004).

The C-banding pattern observed in *E. caribaeus* is quite common among Scarabaeidae representatives and also to Coleoptera as a whole (Moura *et al.* 2003; Colomba *et al.* 2004, 2006; Rozek *et al.* 2004; Wilson and Angus 2004, 2005, 2006; Bione *et al.* 2005b; Schneider *et al.* 2006). Meanwhile, particular cases of CH distribution have been reported in some Scarabaeidae; *Cetonia aurata* and *Bubas bison* have CH blocks located in the terminal region of eight autosomal bivalents, in addition to the pericentromeric blocks (Colomba *et al.* 1996, 2006; Dutrillaux *et al.* 2006). In some species of Scarabaeidae, such as *Diabroctis mimas* and *Isocoprís inhiata*, the occurrence of diphasic chromosomes that present one heterochromatic and another euchromatic arm has been described (Bione *et al.* 2005a). In addition to these patterns, there are still some species with almost total heterochromatic chromosomes or with CH absence. In *Lygirus ebenus*, *Geniates borelli* and *Pelidnota pallidipennis*, the X chromosome is almost totally heterochromatic, while the Y do not show CH blocks (Bione *et al.* 2005b).

The CH neutrality observed using base-specific fluorochromes CMA₃ and DAPI in *E. caribaeus* is presumably related to the presence of AT and GC base pairs intercalated repetitions or to the minute size of CH blocks, which disables the visualization of positive marks (Colomba *et al.* 1996, 2000, 2006; Moura *et al.* 2003; Bione *et al.* 2005a,b). The presence of neutral blocks has been observed in two other species, *Phyllophaga capillata*, with eight bivalents having neutral blocks, and *Strategus surinamensis hirtus*, that has one small autosomal pair that shows absence of fluorochrome marks (Moura *et al.* 2003; Bione *et al.* 2005b). However, Scarabaeidae has shown heterogeneity regarding CH base pair composition. In addition to species with neutral CH blocks, species with AT-rich blocks have also been recorded, such as *Bubas bison* and *Pelidnota pallidipennis*, as well as species with GC-rich blocks, such as *Geniates borelli* and *Gymnopleurus sturmi*, and also species with AT-rich and GC-rich blocks as observed in *Phyllophaga (Phytalus) vestita* (Colomba *et al.* 1996, 2000, 2006; Moura *et al.* 2003; Bione *et al.* 2005b). The patterns of distribution and quality of CH indicate that the repetitive DNA in this group has distinct patterns of differentiation by accumulation of mutation over the evolutionary diversification of this lineage. This mechanism is common in some groups of insects and results from gene conversion, unequal crossing over, slippage replication and transposition (Charlesworth *et al.* 1994; Dover 2002; Palomeque and Lorite 2008).

In *E. caribaeus*, the nucleolar organizer region is located in the sex bivalent, differing from the most common pattern detected in Coleoptera, that is the location of NORs in one autosomal pair (Moura *et al.* 2003; Almeida *et al.* 2006; Schneider *et al.* 2007b). The observation of rDNA sites on the X and Y chromosomes of *E. caribaeus* using the FISH technique suggests the occurrence of fusion be-

tween an autosomal pair, carrying rDNA sequences, and the sex chromosomes, with the fusion also being responsible for the diploid number reduction in this species. Using conventional staining, Cabral-de-Mello *et al.* (2007) proposed only the occurrence of autosome fusions, but in this work the differential cytogenetic techniques permitted the observation of autosome-X fusions. The presence of NORs in both sex chromosomes is uncommon to Scarabaeidae and Coleoptera as a whole. In Scarabaeidae only the species *Jumnos ruckeri* had NORs detected by silver nitrate in the X and Y chromosomes (Macaisne *et al.* 2006). Some other analysed species of Scarabaeidae presented NORs associated with the sex bivalent, but the rDNA sites are located only in the X chromosome, as observed in *Phyllophaga (Phytalus) vestita*, *Lyogenys fuscus* and *Megasoma actaeon* (Moura *et al.* 2003; Dutrillaux *et al.* 2007). Moreover, Scarabaeidae species with rDNA sites in a single autosomal bivalent have also been described, as *Phyllophaga capillata* and *Isocoprís inhiata* (Moura *et al.* 2003; Bione *et al.* 2005a). There are yet other species with different patterns of rDNA cistrons location. In *Bubas bison*, these sites are present in eight chromosomes, while in *Diabroctis mimas* two autosomic pairs and the X chromosome have rDNA sites (Bione *et al.* 2005a; Colomba *et al.* 2006). This variability in the number and location of rDNA sites is probably caused by the transposition process of this DNA in the genome that is favoured by the chromocenter formation during the meiosis in this group. The differences in size of rDNA sites on the X and Y chromosomes in *E. caribaeus* is possibly the result of differential amplification of this sequence in the sex chromosomes, caused by several mechanisms of evolution of the repetitive DNA.

The absence of silver nitrate staining in the sex bivalent in metaphases I of *E. caribaeus* corroborates the presence of a sex mechanism distinct from the parachute, that remains strongly marked by the silver nitrate in some representatives of Scarabaeidae during different phases of meiosis, independent of whether or not the NORs are located in this bivalent (Virkki *et al.* 1991; Moura *et al.* 2003; Bione *et al.* 2005b). This phenomenon is related with the presence of argiophilic proteins in the lumen of the Xy_p. According to Virkki *et al.* (1990, 1991), these proteins are responsible for the 'adhesiveness' of these chromosomes acting in the maintenance of the parachute configuration and in the correct disjunction of this bivalent during cell divisions. The CH staining by silver nitrate observed in this work is probably related to the presence of argiophilic proteins in the molecular organization of the chromatin. This phenomenon has been frequently seen in Scarabaeidae species, as described in *Lyogenys fuscus*, *Geniates borelli*, *Isocoprís inhiata* and *Glyphoderus sterquilinus* (Colomba *et al.* 1996; Moura *et al.* 2003; Bione *et al.* 2005a,b).

Although *E. caribaeus* has undergone diverse chromosome rearrangements that generated a significant diploid number reduction, the four populations studied here did not

show polymorphisms for diploid number, sex mechanism and chromosome morphology. The fusion events are probably the main rearrangement responsible for the chromosome differentiation in this species, acting in diploid number reduction and in the translocation of rDNA sites to the sex chromosomes. This is the first time that a rDNA site is observed in the Y chromosome in Scarabaeidae family using FISH, a more reliable technique than silver nitrate staining that show affinity to the heterochromatic blocks and mark only the active NORs. Other species of *Eurysternus* need to be examined using differential cytogenetic techniques for the realization of a comparative analysis, which will elucidate the evolutionary mechanisms involved in the chromosome differentiation of this genus and family.

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