



Chromosomal diversification of diploid number, heterochromatin and rDNAs in two species of *Phanaeus* beetles (Scarabaeidae, Scarabaeinae)

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Abstract

The genus *Phanaeus* is included in the tribe Phanaeini, one of the most diverse tribes within the subfamily Scarabaeinae in terms of chromosomal characteristics. However, so far the species of this genus were not studied with differential cytogenetic techniques, limiting any inference of the probable mechanisms responsible for this diversity. In this work, several techniques were applied with the aim of cytogenetically characterizing two *Phanaeus* species. The karyotype found for *Phanaeus (Notiophanaeus) chalconelas* was $2n = 12$, neo-XY, and that of *P. (N.) splendidulus* was $2n = 20$, Xy_p , considered primitive for the family Scarabaeidae. The chromosomes of both species showed a high amount of constitutive heterochromatin (CH), with blocks rich in base pairs GC (CMA₃⁺). Moreover, in *P. (N.) chalconelas* the marks revealed by C-banding and fluorochrome staining were different in size, showing CH variability. Sites of 18S ribosomal DNA (rDNA) were identified in one autosomal pair of *P. (N.) chalconelas* and in five autosomal pairs of *P. (N.) splendidulus*. On the other hand, only one autosomal pair exhibited 5S rDNA sequences in these species. The results suggest that the karyotype differentiation of the *Phanaeus* species studied here involved pericentric inversions and centric fusions, as well as mechanisms related to amplification and dispersion of CH and rDNA sequences.

Keywords: karyotype, chromosome evolution, repetitive DNA, FISH.

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Introduction

The subfamily Scarabaeinae (Coleoptera: Scarabaeidae) comprises a group of insects with widespread geographic distribution and high diversity, consisting of approximately 6,000 species distributed in 11 tribes (Hanski and Cambefort, 1991). The tribe Phanaeini encompasses 12 genera and 150 species restricted to Neotropical and Nearctic regions. *Phanaeus* is considered one of the most derived genera in this tribe and comprises two subgenera: *Notiophanaeus*, the more primitive, with 19 species divided into five groups, and *Phanaeus* s.str., with 35 species divided into eight groups. Among the *Notiophanaeus*

groups, *chalconelas* is considered an annectant taxon between the two subgenera (Edmonds, 1994; Philips *et al.*, 2004; Edmonds and Zidek, 2012).

Even though the number of cytogenetically analyzed Phanaeini species is small, the results have shown a great variability, defining it as one of the most diverse tribes in terms of chromosomal characteristics within the subfamily Scarabaeinae. Among the 20 species studied, three different diploid numbers ($2n = 12$, 19 and 20) and six sex chromosome mechanisms (Xy_p , XY_p , Xy , XY , $X0$ and neo-XY) were observed, contrasting with the majority of Scarabaeinae species that display the karyotype $2n = 20$, Xy_p , considered modal and primitive for the group (Cabral-de-Mello *et al.*, 2008; Oliveira *et al.*, 2010, 2012). So far, six representatives of genus *Phanaeus* (corresponding to 14.29% of all described species) were cytogenetically studied, four belonging to the subgenus *Phanaeus* s.str., and

two to the subgenus *Notiophanaeus*. The karyotypes observed in these species differed greatly from $2n = 20, Xy_p$, showing diploid number reduction to $2n = 12$ and the occurrence of two distinct sex chromosome mechanisms in the genus, XY and neo-XY (Cabral-de-Mello *et al.*, 2008, 2011a).

Differential cytogenetic techniques, such as C-banding, base-specific fluorochrome staining, silver nitrate (AgNO₃) staining and fluorescent *in situ* hybridization (FISH) using major ribosomal DNA (45S rDNA) as probe, were applied only in nine Phanaeini representatives (Bione *et al.*, 2005a; Oliveira *et al.*, 2010, 2012; Cabral-de-Mello *et al.*, 2011a). The use of these techniques has enabled a more detailed chromosomal analysis of different Scarabaeinae species, helping to identify major events involved in the karyotype evolution of the group (Bione *et al.*, 2005a,b; Silva *et al.*, 2009; Cabral-de-Mello *et al.*, 2010a,b, 2011a).

In order to contribute to a better understanding of the trends in the chromosomal diversification of *Phanaeus* representatives, the species *Phanaeus (Notiophanaeus) chalconelas* (Perty, 1830) (*chalconelas* group) and *P. (N.) splendidulus* (Fabricius, 1781) (*splendidulus* group) were analyzed here by conventional and differential staining, and mapping of rDNA genes by FISH. The data obtained point to pericentric inversions and centric fusions as the main mechanisms responsible for the chromosomal differentiation in these species. Furthermore, the amplification and dispersion of constitutive heterochromatin and rDNA sequences may also be involved in their karyotype diversification.

Materials and Methods

Mitotic and meiotic chromosomes from six adult males of the species *Phanaeus (Notiophanaeus) chalconelas* and 50 of *P. (N.) splendidulus* were analyzed. The six *P. (N.) chalconelas* specimens were collected in the Botanical Garden (Jardín Botánico) of Santa Cruz de La Sierra (17°47'21" S; 63°11'48" W), Bolivia, and the 50 *P. (N.) splendidulus* specimens in the Ecologic Park (Parque Ecológico) João Vasconcelos Sobrinho of Caruaru (08°18'30" S; 36°00'10" W), Pernambuco, Brazil. The individuals analyzed were deposited in the Scientific Collection of Scarabaeidae, at the Instituto de Ciências Biológicas, Universidade de Pernambuco, Brazil.

The insects were anesthetized with ethyl ether and then their testes were dissected. Subsequently, the materials were fixed in 3:1 Carnoy (ethanol:acetic acid) and stored at -20 °C. The cytological preparations were obtained by the classical method of testicular follicles squashing, followed by conventional staining with 2% lacto-acetic orcein. C-banding was performed according to Sumner (1972), with modifications related to the exposure time of chromosomes to the different solutions, and the triple stain-

ing with CMA₃/DA/DAPI followed the method established by Schweizer (1976). Silver nitrate (AgNO₃) staining was done according to Howell and Black (1980), and FISH according to Cabral-de-Mello *et al.* (2010c). The probes used for FISH consisted of cloned partial sequences of 5S and 18S rDNAs, obtained by PCR from the genomic DNA of the scarab beetle *Dichotomius semisquamosus* (GenBank accession numbers GQ443313 for the 18S rRNA gene, and GQ443312 for the 5S rRNA gene). The probes were labeled with biotin-14-dATP (Invitrogen, San Diego, CA, USA) and detected with an avidin-FITC conjugate (Sigma, St Louis, MO, USA). The preparations were counterstained with propidium iodide (50 µg/mL) and mounted with Vectashield mounting medium (Vector Laboratories Ltd, Cambridgeshire, UK). For each species, approximately 50 metaphase cells were analyzed using conventional staining and about 30 metaphases were processed by differential techniques.

The images were captured in an Olympus BX61 microscope connected to an Olympus DP71 digital camera, and organized using Corel Photo-Paint X3 software®. The morphological classification of chromosomes followed the criteria established by Levan *et al.* (1964).

Results

Karyotypes

The species *Phanaeus (N.) chalconelas* displayed $2n = 12$ and neo-XY sex chromosomes (Figure 1a). The morphology of its chromosomes could not be precisely determined, but they may be meta-submetacentric. *Phanaeus (N.) splendidulus* exhibited the modal karyotype for the family Scarabaeidae, $2n = 20, Xy_p$ (Figure 1c), with submetacentric pairs 1 and 9 and X chromosome, a metacentric pair 7, acrocentric pairs 2-6 and 8, and a punctiform Y chromosome (Figure 1d). The karyotypes of both species showed chromosomes with gradual reduction in size.

Constitutive heterochromatin

The C-banding technique showed chromosomes rich in constitutive heterochromatin in both species. In *Phanaeus (N.) chalconelas* there were large C-banded blocks in all chromosomes, with the pericentromeric heterochromatin extending along the chromosome arms (Figure 1b). In *P. (N.) splendidulus*, pericentromeric C-banded blocks were observed in all autosomes. Moreover, pairs 3-6 and 8 showed additional large C-banded blocks in the terminal region of the long arms. Pair 9 was diphasic, with an entirely heterochromatic short arm and an euchromatic long arm. C-banded blocks were observed in the pericentromeric region of the X chromosome and in part of its short arm. A small C-banded block was detected on the Y element, but its small size and the low amount of heterochromatin did not allow a precise determination of the

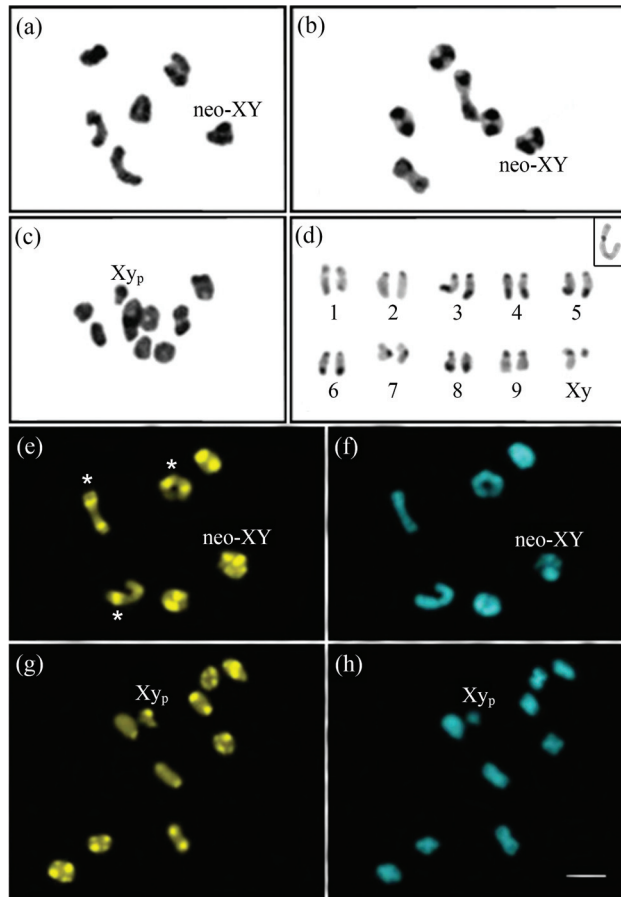


Figure 1 - Conventional staining, C-banding and triple staining with CMA₃/DA/DAPI in the two species studied: Conventional staining in metaphases I of *Phanaeus (Notiophanaeus) chalconelas* (a) and *P. (N.) splendidulus* (c); C-banding in metaphase I of *P. (N.) chalconelas* (b) and in the karyotype of *P. (N.) splendidulus* (d); CMA₃⁺ blocks in metaphase I chromosomes of *P. (N.) chalconelas* (e) and *P. (N.) splendidulus* (g) and the corresponding DAPI negative blocks (f and h). The insert in (d) indicates the bivalent 1 in pachytene showing the pericentromeric C-banded block; the asterisks in (e) indicate the bivalents with CMA₃⁺ blocks restricted to the pericentromeric region. Scale bar = 5 µm.

location or extent of the heterochromatic regions (Figure 1d).

Triple fluorochrome staining with CMA₃/DA/DAPI allowed the identification of CMA₃-positive (rich in G+C base pairs) heterochromatic blocks in all chromosomes of *P. (N.) chalconelas* and *P. (N.) splendidulus* (Figures 1e and g). These blocks were DAPI-negative (Figures 1f and h). In *P. (N.) chalconelas*, three bivalents showed CMA₃⁺ blocks restricted to the pericentromeric region, while the others displayed the same pattern revealed by C-banding. In *P. (N.) splendidulus*, the CMA₃⁺ blocks corresponded to the C-banded blocks.

Nucleolus organizer regions and ribosomal DNA sites

The chromosomal mapping of major rDNA clusters revealed that they were located in autosomes. *Phanaeus*

(*N.*) *chalconelas* presented one autosomal pair carrying 18S rDNA sequences (Figure 2a), while in *P. (N.) splendidulus* five pairs showed these sequences (Figure 2c). FISH with a 5S rDNA probe revealed the presence of one autosomal pair carrying these genes in both species analyzed (Figures 2b and d). In *P. (N.) chalconelas*, the 18S and 5S rDNA sites were located in different chromosomes: the 18S rDNA was located in a pair relatively larger than the bivalent carrying the 5S rDNA sequences (Figures 2a and b). In *P. (N.) splendidulus*, it was not possible to carry out this analysis, due to the similar size of the chromosomes.

Silver nitrate (AgNO₃) staining showed that three to five major rDNA sites could be active as nucleolus organizer regions (NORs) in *P. (N.) splendidulus* (Figures 2e-g). In *P. (N.) chalconelas*, it was not possible to obtain cells in appropriate stages to identify active NORs by this technique.

Discussion

The two species analyzed in this study, although belonging to the same subgenus, showed remarkable differences in their macro-chromosomal structure, and exhibited divergences with regard to the karyotype considered primitive and modal for the subfamily Scarabaeinae (2n = 20, Xy_p with meta-submetacentric chromosomes), reinforcing the high karyotype variability described in Phanaeini (Bione *et al.*, 2005a; Cabral-de-Mello *et al.*, 2008; Oliveira *et al.*, 2010).

The karyotype 2n = 20, Xy_p observed in *Phanaeus (N.) splendidulus* is similar to that considered ancestral for Scarabaeinae, however, in this species a prevalence of acrocentric chromosomes (six pairs) was observed. This modification in chromosome morphology can be attributed to pericentric inversions in ancestral meta-submetacentric chromosomes. Pericentric inversions have been described in other species of this subfamily, *e. g.*, *Bubas bison*, *Paracopris ramosiceps* and *Diabroctis mimas* (Bione *et al.*, 2005a; Angus *et al.*, 2007), and comprise, along with autosomal fusions and fissions, X-autosome fusions and Y chromosome loss, the main rearrangements responsible for the karyotype variability observed in Scarabaeinae (Cabral-de-Mello *et al.*, 2008).

The reduced diploid number (2n = 12) and the derivative sex chromosome mechanism (neo-XY) found in *P. (N.) chalconelas* were also observed in four other species of the subgenus *Phanaeus* s.str. (Smith and Virkki, 1978). Edmonds (1994) reported other similarities between the species belonging to the chalconelas group and *Phanaeus* s.str., considering this group as an annectant between the two subgenera. Diploid number reductions have been reported in some beetles, and probably result from fusions involving only autosomes, or autosomes and sex chromosomes, as observed in Scarabaeinae species such as *Eurysternus caribaeus*, *Deltotilum calcaratum* and

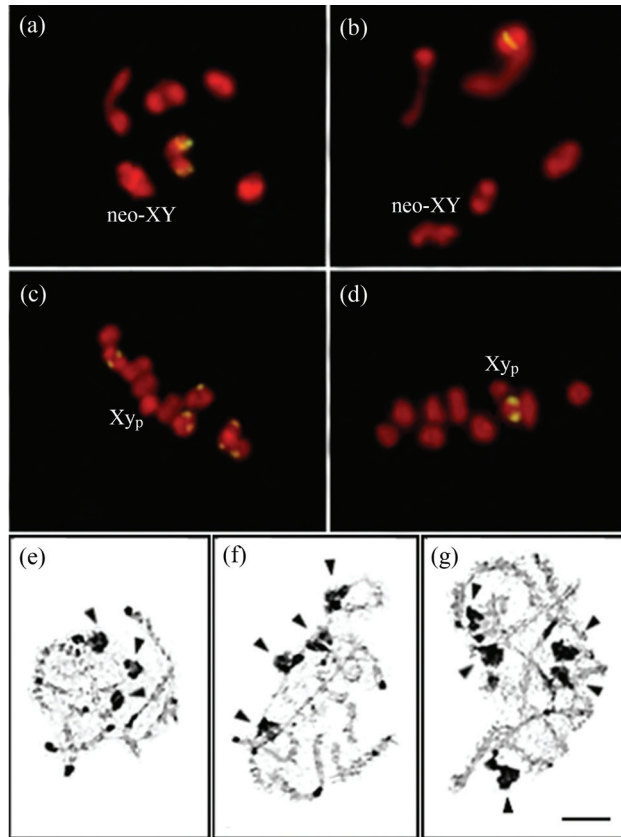


Figure 2 - Fluorescent *in situ* hybridization and silver nitrate staining in the two *Phanaeus* species: FISH using 18S rDNA as probe in metaphases I of *Phanaeus (Notiophanaeus) chalconelas* (a) and *P. (N.) splendidulus* (c); Metaphases I hybridized with 5S rDNA as probe in *P. (N.) chalconelas* (b) and *P. (N.) splendidulus* (d); AgNO₃ staining in pachytene of *P. (N.) splendidulus*, showing three, four and five pairs with active NORs, respectively (e-g). Arrowheads indicate active NORs. Scale bar = 5 μm.

Deltochilum aff. *morbillosum* (Smith and Virkki, 1978; Arcanjo *et al.*, 2009; Cabral-de-Mello *et al.*, 2010b). Apparently, fusions represent the main mechanism of chromosomal diversification in *Phanaeus*, the only genus of Phanaeini in which this event was described so far. The results presented here are in agreement with the phylogenetic analysis made by Price (2009), based on combined molecular and morphological data. According to this phylogeny, *P. (N.) splendidulus* is located in a more basal group that is a sister of a clade including all other *Phanaeus* species displaying the chromosomal reduction. The cytogenetic data for this genus indicate that more derived species have reduced diploid numbers.

The great amount of constitutive heterochromatin (CH) observed in the karyotypes of the species analyzed here was also described in other Scarabaeinae representatives, such as *Deltochilum calcaratum*, *D. aff. morbillosum* (*Deltochilini*) and some Phanaeini species, such as *Coprophanaeus (Coprophanaeus) cyanescens*, *C. (Megaphanaeus) ensifer* and *Diabroctis mimas* (Bione *et al.*, 2005a; Cabral-de-Mello *et al.*, 2010b; Oliveira *et al.*,

2010). However, the subfamily Scarabaeinae shows different patterns of CH quantity and distribution, including species that conserve the pattern that is probably the ancestral condition of the family Scarabaeidae, with a predominance of centromeric/pericentromeric heterochromatin blocks (Moura *et al.*, 2003; Wilson and Angus, 2004, 2005; Bione *et al.*, 2005b). Although belonging to distinct genera, the karyotypes of all the Phanaeini representatives studied display high amounts of CH, indicating that this condition may be characteristic to the tribe. This high amount of heterochromatin found both in *Phanaeus* representatives and in other Scarabaeinae suggests amplification/dispersion of this DNA fraction in the genomes of these species. Different mechanisms have been appointed to play major roles in the expansion and spreading of DNA sequences in the genomes, such as unequal crossing over, occurrence of extrachromosomal circular DNAs, slippage replication, rolling circle replication, and conversion-like mechanisms (Charlesworth *et al.*, 1994; Ugarkovic and Plohl, 2002; Palomeque and Lorite, 2008).

The heterochromatic blocks of the *Phanaeus* species analyzed in this study presented a similar composition with regard to base pairs. However, in *P. (N.) chalconelas*, CMA₃ staining revealed a pattern different from that observed by C-banding, indicating that part of the heterochromatin of this species has a distinct base pair content, which could be related to the presence of different repetitive DNA families in the heterochromatic regions. These differences in the base pair content of C-banded blocks were already reported for some Scarabaeinae species, *e. g.*, *Coprophanaeus ensifer*, *Deltochilum* aff. *morbillosum*, *Dichotomius bos*, *D. laevicollis*, among others (Cabral-de-Mello *et al.*, 2010b, 2011b; Oliveira *et al.*, 2010).

As a general trend, both in *P. (N.) chalconelas* and in *P. (N.) splendidulus*, the heterochromatin diversification involved an increase in the amount and variability of its base pair content. Ugarkovic and Plohl (2002) suggested that these variation mechanisms could occur concomitantly, promoting an increase in the number of satellite DNA copies (frequently associated to heterochromatin) and modification in nucleotide sequences. According to the “Library model”, proposed by Fry and Salser (1977), closely related species share a set of repetitive sequences which can originate new variants by the accumulation of mutations. These sequences undergo dispersion by different mechanisms, such as unequal crossing-over and gene conversion. These processes could also explain the variability in the quantity and base pair composition observed in the heterochromatin of Scarabaeinae species.

The neo-XY sex bivalent of *P. (N.) chalconelas* contains a higher amount of heterochromatin than the ancestral Xy_p mechanism present in species *P. (N.) splendidulus*. These data corroborate the hypothesis raised by Dutrillaux *et al.* (2007) that the accumulation of heterochromatin contributes to the maintenance of the coleopteran neo-XY

mechanism, promoting the isolation between ancestral gonosomal and autosomal segments. In some groups, such as rodents, bats and *Drosophila*, the accumulation of heterochromatin seems to allow the permanence of X-autosome translocations in the karyotype, probably avoiding the deregulation of autosomal genes associated with X components (Parish *et al.*, 2002; Dobigny *et al.*, 2004; Dutrillaux *et al.*, 2007). In contrast, neo-XY sex chromosome systems with heterochromatin restricted to the centromere were observed in *Eurysternus caribaeus*, Scarabaeinae (Arcanjo *et al.*, 2009), indicating that the origin and differentiation of the neo-XY may occur by distinct mechanisms of accumulation/non-accumulation of heterochromatin, even in related species.

The occurrence of major rDNA sites on autosomes in both species studied was similar to the pattern that is most frequent in the order Coleoptera, in which the majority of analyses were made using silver nitrate staining (reviewed by Schneider *et al.*, 2007). In *P. (N.) splendidulus*, the presence of these sites was observed in some autosomal pairs, indicating dispersion of this gene cluster, including a polymorphic condition compared to the pattern described by Cabral-de-Mello *et al.* (2011a) in which four autosomal pairs displayed 18S rDNA sites. The presence of multiple rDNA sites seems to be common in Phanaeini and has been described in *Diabroctis mimas*, *Coprophanaeus (C.) cyanescens* and *C. (M.) ensifer*, which presents the highest number of 18S rDNA sites in Coleoptera (Bione *et al.*, 2005a; Oliveira *et al.*, 2010).

The dispersion of rDNA sites in Phanaeini could have occurred from only one pair (one autosomal bivalent), which is apparently the ancestral characteristic of Coleoptera (revealed mostly by silver nitrate staining), present in about 60% of the species analyzed (Schneider *et al.*, 2007). This hypothesis is reinforced by the occurrence of two species in the tribe with 18S rDNA sites in one bivalent, *Phanaeus (N.) chalconelas*, analyzed here, and *Coprophanaeus (Metallophanaeus) pertyi* (Oliveira *et al.*, 2012). As observed for instance in the *Coprophanaeus* species (Oliveira *et al.*, 2010, 2012) and *P. (N.) splendidulus*, the differences in number and location of major rDNA sites appear to be related to processes such as amplification/dispersion rather than to macro-chromosomal rearrangements like translocations. In contrast to the variability in number of major rDNA sites observed in Phanaeini, both *Phanaeus* species showed only one autosomal pair carrying 5S rRNA genes, which was also observed in most of the Scarabaeinae studied until now (Cabral-de-Mello *et al.*, 2011a,c). The data obtained suggest that, in the genus *Phanaeus*, the distinct rDNA multigene families are controlled by distinct evolutionary forces for spreading/non-spreading, as described for other Scarabaeinae groups (Cabral-de-Mello *et al.*, 2011a).

Among the main chromosomal evolutionary mechanisms which might explain the great karyotype divergence

between the species studied in this work, the relevant ones are apparently: (1) fusions between autosomes and at least one fusion between autosomes and sex chromosomes, generating the reduced diploid number and neo-XY mechanism in *P. (N.) chalconelas*; (2) pericentric inversions in chromosomes of *P. (N.) splendidulus*; (3) amplification and dispersion of constitutive heterochromatin and 18S rDNA sites. In addition, the data obtained demonstrate the intense dynamic of heterochromatin and major rDNA sites in the tribe Phanaeini.

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