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ORIGINAL ARTICLE

Chromosome mapping of 28S ribosomal genes in 11 species of Cassidinae (Coleoptera: Chrysomelidae)

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Abstract. In this study, we examined for the first time the distribution of the 28S ribosomal genes in beetles of the subfamily Cassidinae. More than 55% of the species in this subfamily have a similar karyotype, 2n = 16 + Xy_p. For this work, we selected species belonging to the tribes Cassidini and Mesomphaliini, which have, respectively, the most conserved and diversified karyotype characteristics within the Cassidinae. An analysis of 11 species revealed that rDNA sites on one pair of autosomes is the most frequent pattern, occurring in 10 species. This condition occurs in the seven genera examined and in species of both of the tribes, Cassidini and Mesomphaliini. Nevertheless, the differences in the locations of 28S rDNA were more pronounced in the tribe Cassidini and among species with similar karyotype characteristics. On the other hand, in Mesomphaliini, the increase in the diploid number was not accompanied by an increase in the number of ribosomal sites. Moreover, the comparison of the number and localization of major rDNA sites with the distribution of constitutive heterochromatin indicates that there is no direct correlation between the dispersion of constitutive heterochromatin and 28S rDNA genes in Cassidinae.

INTRODUCTION

The repetitive DNA sequences constitute a large fraction of the eukaryote genomes and include satellite, microsatellite and minisatellite regions, multigene families and transposable elements (Charlesworth et al., 1994). The major ribosomal gene is one of these multigene families and is formed by a transcription unit that encodes the 18S, 5.8S and 28S rRNA genes. In the genome, these genes occur as multiple copies organized in tandem, which are located in one or more nucleolar organizer regions (NORs) (Long & Dawid, 1980; Cabrero & Camacho, 2008; Nguyen et al., 2010). The rDNA is considered to be a useful chromosomal marker in cytogenetic studies because it can reveal synapomorphies and provide information about the mechanisms of chromosomal evolution in related groups of species (Bombarová et al., 2007; Cabrero & Camacho, 2008; Nguyen et al., 2010).

The location of the NORs is widely used in cytogenetic studies, which are mainly revealed by silver impregnation (Ag-NOR) (Howell & Black, 1980), because it is a simple and low cost technique (Rufas et al., 1982). However, the Ag-NOR technique stains only active rDNA sites. On the other hand, the fluorescent in situ hybridization (FISH) allows the precise location of the rDNA genes, independent of their activity, and it may also reveal variations in the size of the sites on different chromosomes (Datson & Murray, 2006; Nguyen et al., 2010).

The order Coleoptera has approximately 360,000 taxonomically described species (Costa, 2003), but only 4,852 of them are cytogenetically analyzed (Blackmon & Demuth, 2015). Among these species, 372 included in the suborders Adephaga (Carabidae) and Polyphaga (Hydrophilidae, Geotrupidae, Lucanidae, Passalidae, Melyridae, Scarabaeidae, Buprestidae, Elateridae, Coccinelidae, Meloidae, Tenebrionidae, Chrysomelidae and Curculionidae) are investigated in terms of the location of NORs or rDNA genes. Of these species, 158 were analyzed using Ag-NOR, 126 by FISH with an rDNA probe and 88 species were studied using both techniques. In the latter case, 16 species exhibit different locations for Ag-NOR and rDNA when analyzed using silver impregnation and FISH (Schneider et al., 2007; Dutrillaux et al., 2008; Holecová et al., 2008, 2013; Lachowska et al., 2008; Moura et al., 2008; Arcanjo et al., 2009, 2013; Dutrillaux & Dutrillaux, 2009, 2012;

Silva et al., 2009; Almeida et al., 2010; Cabral de Mello et al., 2010, 2011a, b; Mendes-Neto et al., 2010; Oliveira et al., 2010, 2012a, b; Giannoulis et al., 2011; Proença et al., 2011; Lira-Neto et al., 2012; Karagyan et al., 2012; Goll et al., 2013, 2015).

Most species of Coleoptera have two rDNA clusters located on one autosomal pair. This pattern occurs in approximately 80% and 65% of the species of Adephaga and Polyphaga, respectively, including those with very divergent diploid numbers and/or sex chromosome systems, for example, 2n = 56 + X0, 2n = 18 + $X_1X_2X_3Y$, 2n = 18 + Xy_p, 2n = 14 + neoXY and 2n = 10 + XY (see revision in Schneider et al., 2007). Among the Polyphaga, most of the data on the distribution of rDNA is for the family Scarabaeidae, with more than 120 species characterized to date. The occurrence of two autosomal NORs is also widespread within this family, but closely related species do differ in the presence of a ribosomal cluster on more than one autosomal pair and/or on sex chromosomes (Colomba et al., 2006; Silva et al., 2009; Cabral-Mello et al., 2010, 2011a, b; Oliveira et al., 2010, 2012b).

In Chrysomelidae, the distribution of NOR is known for only 23 species in the subfamilies Alticinae, Cassidinae and Chrysomelinae. Among the 17 species for which the number of NORs is determined, 14 have one site located on one autosomal pair, two species have two pairs of autosomes with NORs, and one species has only one NOR located on the neoX chromosome (Petitpierre, 1970, 1976, 1996; Virkki, 1983; Virkki & Denton, 1987; Postiglioni et al., 1990, 1991; Yadav et al., 1992; Schneider et al., 2002; Gómez-Zurita et al., 2004; Almeida et al., 2006, 2010).

The subfamily Cassidinae s.l. comprises approximately 6,000 species in 43 tribes (Chaboo, 2007). Cytogenetically, only 124 species, grouped in 14 tribes, have been studied, of which 52% of the species have the conserved karyotype 2n = 16 + Xy_p, with biarmed chromosomes (De Julio et al., 2010; Lopes et al., 2016). Among the 14 tribes of Cassidinae cytogenetically characterized, the Cassidini and Mesomphaliini are the most homogenous and variable in terms of karyotypes, respectively. In this latter tribe, the karyotype variability probably reflects its non-monophyletic nature (Chaboo, 2007; Lopes et al., 2016).

Within Cassidinae, only two species of Mesomphaliini are investigated regarding the location of Ag-NOR. In *Chelymorpha varians* (Blanchard, 1851) (2n = 20 + Xy_p), the NOR is coincident with the secondary constriction on the short arm of the 5th pair of autosomes. In *Botanochara angulata* (Germar, 1824) (2n = 51 = 48 + $X_pneoXneoY_p$), NORs can be seen on some autosomal chromosomes, which are not identified (Yadav & Pillai, 1975; Postiglioni et al., 1990). In this study, we mapped the 28S rDNA gene in 11 species of the tribes Cassidini and Mesomphaliini, with the aim of understanding the role of the variation in the number and location of this gene in the evolution of chromosomes in these groups.

Table 1. The species of Cassidinae analyzed in the present study, including the number of individuals and the locality where collected in the state of São Paulo, Brazil.

Species	No. of male specimens	Collecting locality
Cassidini		
Agroiconota inedita (Boheman, 1855)	1	Saltinho
	1	São Pedro
Charidotella immaculata (Oliver, 1790)	3	Saltinho
	3	São Pedro
Charidotella sexpunctata (Fabricius, 1781)	2	Saltinho
	1	São Pedro
Deloyala cruciata (Linnaeus, 1758)	3	Saltinho
Microctenochira gnata (Spaeth, 1926)	1	São Pedro
Microctenochira optata (Boheman, 1855)	1	Saltinho
Microctenochira stigmatica (Boheman, 1855)	1	Saltinho
Mesomphaliini		
Chelymorpha cribraria (Fabricius, 1775)	1	Saltinho
	4	São Pedro
Chelymorpha inflata (Boheman, 1854)	1	Jundiaí
	1	São Pedro
Cyrtonota cyanea (Linnaeus, 1758)	2	São Pedro
Paraselenis flava (Linnaeus, 1758)	3	Saltinho
	1	São Pedro

Geographical coordinates: Jundiaí (23°8´S, 46°53´W); Saltinho (22°50´S, 47°40´W); São Pedro (22°3´S, 47°57´W).

MATERIALS AND METHODS

The species analyzed in this work and their respective collection localities are listed in Table 1. The voucher specimens were deposited in the entomological collection of the Museu Paraense Emilio Goeldi (MPEG, curator O.T. Silveira), Belém, state of Pará, Brazil.

The chromosomal preparations were obtained from testes of adult individuals, following the method of Lopes et al. (2016). Total genomic DNA of *Charidotella immaculata* (Olivier, 1790) was extracted from the abdomen using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The amplifications of the 28S rDNA gene were done using the primers 28S-F 5'-GACCCGTCTTGAAACACGGA and 28S-R 5'-TCGGAAGGAACCAGCTACTA designed by Nunn (1992). The PCR was carried out in a total volume of 25 µL, using 1 µL DNA template (20 ng/µL), 1 µL of each primer (10 pmol/µL), 2.5 µL10 × reaction buffer, 5 µL dNTP mix (2 mM), 1 µL $MgCl_2$ (50 mM) and 0.3 µL *Taq* DNA polymerase (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA). The reaction was performed in an Applied Biosystem thermal cycler (Life Technologies) following the program: 3 min initial denaturation at 94°C; 30 s denaturation at 94°C, 30 s annealing at 50°C, 1 min extension at 72°C (35 cycles); 10 min final extension at 72°C.

The 28S rDNA probes were labelled with digoxigenin-11-dUTP with DIG-Nick Translation Mix (Roche Diagnostics GmbH, Mannheim, Germany). The FISH was performed following the technique of Pinkel et al. (1986) with modifications described by Almeida et al. (2010). However, before the FISH procedure, the chromosome preparations were incubated overnight in 70% acetic acid solution. Hybridization signals were detected with anti-digoxigenin-rhodamine (Roche Diagnostics). The chromosomes were counterstained with 4',6-diamidino-2-fenylindol (DAPI) and mounted in an anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA, USA). The images of the chromosomes were taken using a Zeiss Imager A2 equipped with an Axio Cam digital camera, using Axion Vision software (Carl Zeiss AG, Jena, Germany).

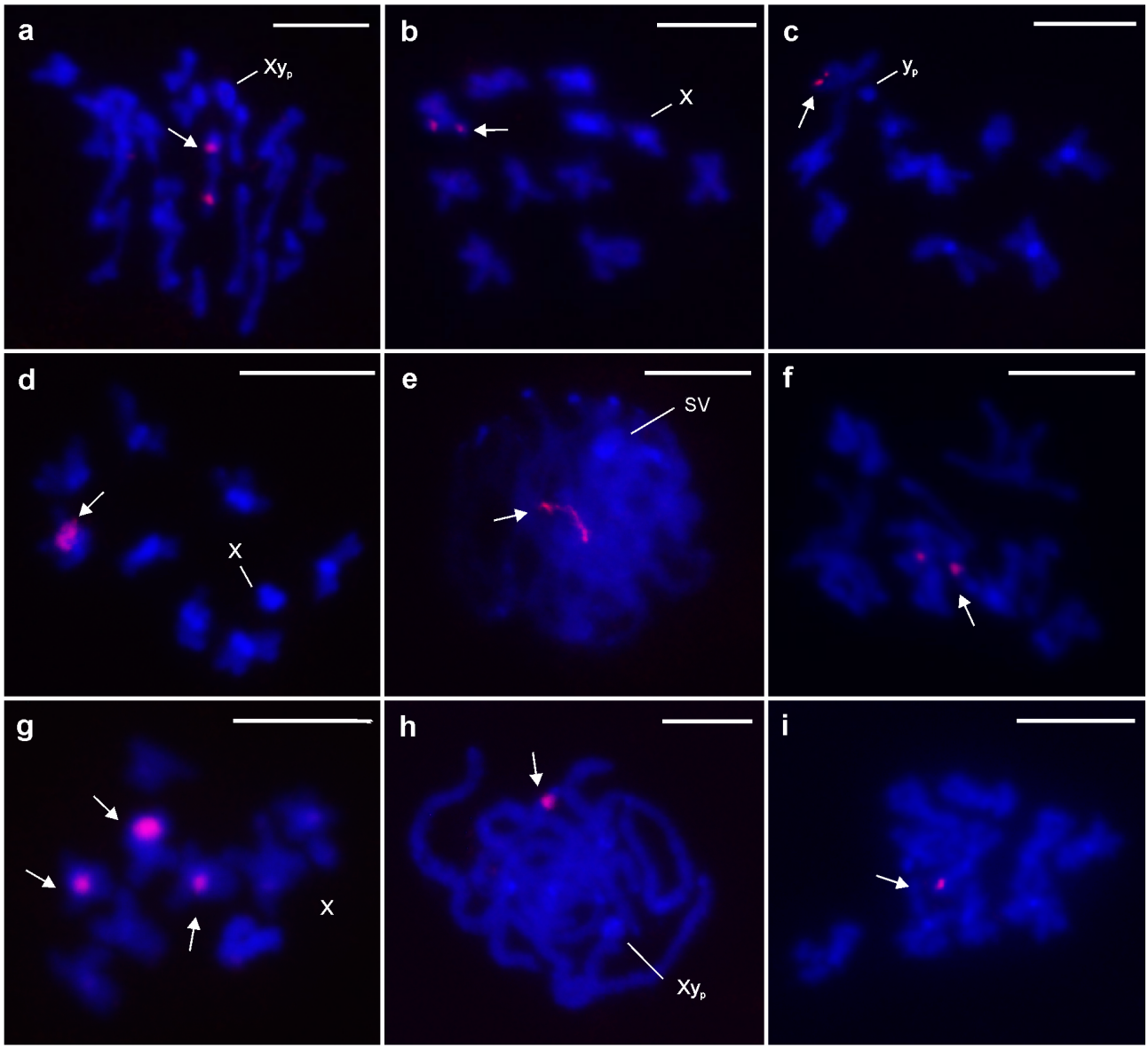

Fig. 1. Localization of the 28S ribosomal genes in male meiotic cells of species of Cassidini using FISH with a 28S rDNA probe. a – *Agroiconota inedita*, diplotene – 2n = 20II + Xy$_p$; b – *Charidotella immaculata,* metaphase II – n = 10 + X; c – *Charidotella sexpuctata*, metaphase II, n = 10 + y$_p$; d – *Deloyala cruciata*, metaphase II – n = 8 + X; e–f – *Microctenochira gnata*, pachytene and metaphase II (n = 9), respectively; g – *Microctenochira optata*, metaphase II – n = 8 + X; h–i – *Microctenochira stigmatica*, pachytene and metaphase II (n = 11), respectively. SV – sex vesicle. The red regions on the chromosomes (arrow) correspond to major rDNA sites. Scale bar = 10 μm.

RESULTS

The cytogenetic analysis of five Cassidini species revealed the following diploid numbers and sex chromosome systems: 2n = 38 + Xy$_p$ in *Agroiconota inedita*, 2n = 20 + Xy$_p$ in *Cha. immaculata* and *Cha. sexpunctata*, and 2n = 16 + Xy$_p$ in *Deloyala cruciata* and *Microctenochira optata*. In the Mesomphaliini, it revealed 2n = 20 + Xy$_p$ in *Che. cribraria* and 2n = 40 + Xy$_p$ in *Paraselenis flava*. In addition, we examined for the first time, the karyotypes of two species of Cassidini: *M. gnata* with 2n = 18 and *M. stigmatica* with 2n = 20 + Xy$_p$, and two species of Mesomphaliini: *Cyr. inflata* with 2n = 20 + Xy$_p$ and *Cyr. cyanea* with 2n = 38 + Xy$_p$. All these species have biarmed chromosomes. Due to the absence of diplotene cells, it was not possible to determine the sex chromosome system of *M. gnata*.

In the species of Cassidini, three patterns in the distribution of the ribosomal clusters were revealed by FISH (Fig. 1): terminal/subterminal region of one autosomal pair in *A. inedita*, *Cha. immaculata*, *Cha. sexpuctata* and *M. stigmatica* (Fig. 1a–c, h–i); interstitial region of one autosomal pair in *D. cruciata* and *M. gnata* (Fig. 1d–f); terminal region of two autosomal pairs and interstitial region of one pair in *M. optata* (Fig. 1g). In *M. gnata*, the hybridization signals in early prophase I cells indicate an out of sex vesicle, confirming the presence of autosomal rDNA.

The hybridization signals in species with one terminal rDNA clusters are located in chromosomes of different size, i.e., in *A. inedita*, *Cha. immaculata* and *M. stigmatica*, the 28S rDNA is located in medium-sized metacentric chromosomes, while in *Cha. sexpuctata* this gene occurs

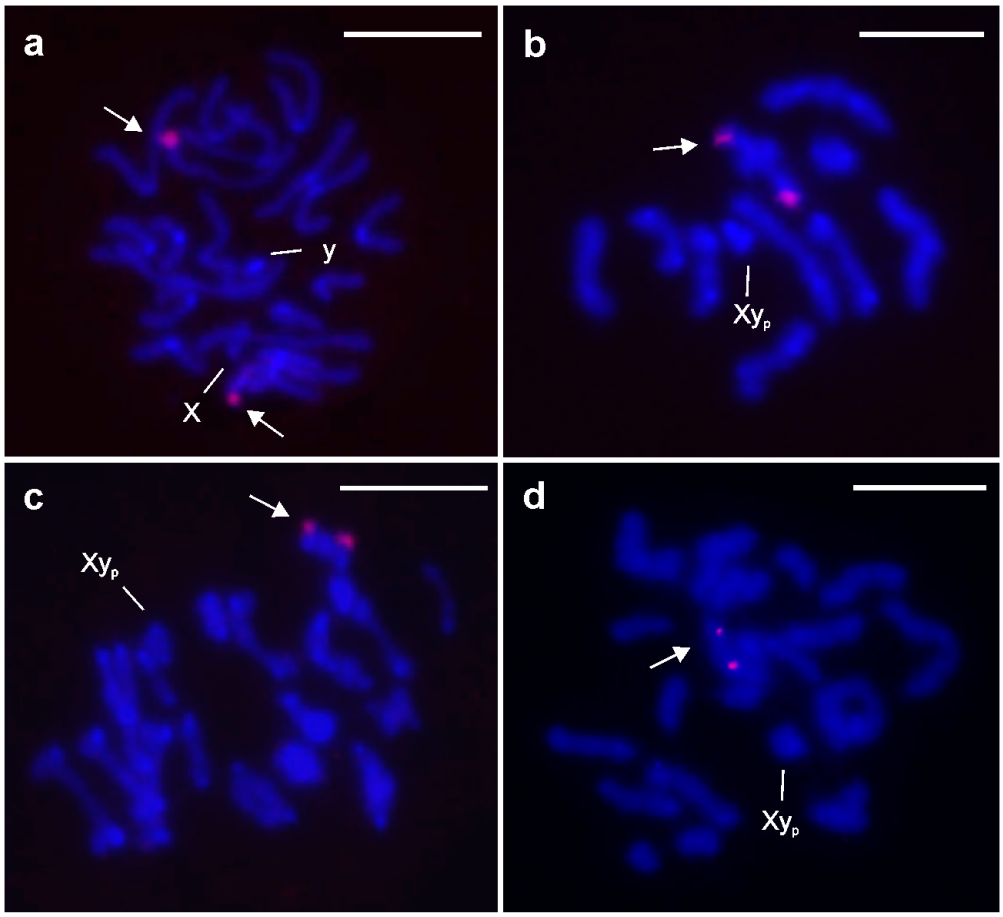

Fig. 2. Localization of the 28S ribosomal genes in species of Mesomphaliini revealed using FISH with a 28S rDNA probe. a – *Chelymorpha cribraria*, mitotic metaphase – 2n = 20 + Xy_p; b – *Chelymorpha inflata*, diakinesis – 2n = 10II + Xy_p; c – *Cyrtonota cyanea*, diakinesis – 2n = 19II + Xy_p; d – *Paraselenis flava*, diplotene – 2n = 20II + Xy_p. The red regions on the chromosomes (arrow) correspond to major rDNA sites. Scale bar = 10 μm.

in a large metacentric pair. In *D. cruciata* and *M. gnata*, rDNA is hybridized in one metacentric pair of medium size. The three rDNA clusters in *M. optata* are located in medium-sized metacentric chromosomes.

In all species in the tribe Mesomphaliini, rDNA-FISH revealed 28S sites located in the terminal/subterminal region of only one autosomal pair. However, in *Che. cribraria* and *Che. inflata*, the rDNA clusters occur in submetacentric chromosomes of large and medium size, respectively (Fig. 2a–b); in *Cyr. cyanea* and *P. flava*, the chromosomes with hybridization signals have a metacentric morphology and are small (Fig. 2c–d).

DISCUSSION

The analyses of male mitotic and meiotic cells in five species of Cassidini: *A. inedita, Cha. immaculata, Cha. sexpunctata, D. cruciata* and *M. optata*, and two Mesomphaliini species: *Che. cribraria* and *P. flava*, confirmed the diploid number and the sex chromosome system previously described for these species (Lopes et al., 2016). For the two species of Cassidini, characterized here for the first time, only *M. stigmatica* (2n = 20 + Xy_p) had a diploid number greater than those recorded for the other species in this genus: *M. aciculata*, *M. gnata*, *M. optata* and *M. quadrata* (Lopes et al., 2016; this work). This increase in the diploid number may correspond to a derived condition within the genus *Microctenochira* and even in the tribe Cassidini, taking into account that approximately 60% of the species in this tribe, belonging to 11 distinct genera, have a diploid number 2n = 18 (De Julio et al., 2010; Lopes et al., 2016).

In the tribe Mesomphiliini, the diploid number is 2n = 22 and an Xy_p sex chromosome system, verified here for *Che. inflata,* are similar to those recorded for *Che. cassidea, Che. cribraria, Che. indigesta, Che. nigricolis* and *Che. varians* (Stevens, 1906; De Vaio & Postiglioni, 1974; Vidal, 1984; Postiglioni et al., 1990, 1991; Virkki et al., 1992; Lopes et al., 2016). On the other hand, *Cyr. cyanea* has the karyotype 2n = 38 + Xy_p. Among the species of Mesomphaliini, diploid numbers similar to or greater than 2n = 40, occur predominantly in the genera *Botanochara*, whose species also invariably have multiple sex chromosome systems (for review see De Julio et al., 2010). In this tribe, the only exception is *P. flava* with 2n = 40 + Xy_p. Thus, only a phylogenetic analysis of the Mesomphaliini can clarify if the large diploid number is a shared characteristic of this tribe or appeared independently in some genera.

Physical mapping of the 28S rDNA gene in Cassidinae revealed that 10 of the 11 species studied have a conserved pattern regarding the number of ribosomal sites. In addi-

tion, it revealed the presence of rDNA on one autosomal pair in seven distinct genera and in species of both the tribes Cassidini and Mesomphaliini. All these species have the Xy_p sex chromosome system type and the constitutive heterochromatin is predominantly located in the pericentromeric region, but they differ in terms of their diploid number, chromosome sizes and number of chromosomal pairs with constitutive heterochromatin (for details see Lopes et al., 2016). These results corroborate the data obtained for the Coleoptera as a whole, in which the presence of rDNA sites on a pair of autosomes seems to be the most stable evolutionary pattern and may be the ancestral condition in this order. The occurrence of one pair of autosomal rDNA clusters in beetles remains unchanged in species with a conserved diploid number and same sex chromosome system as well as in those with derived karyotypes (Schneider et al., 2007).

In Chrysomelidae, the presence of one pair of NORs or rDNA sites is reported in more than 80% of the species, e.g. *Chelymorpha variabilis* ($2n = 20 + Xy_p$), *Chrysolina americana* ($2n = 22 + Xy_p$), *Chr. bankii* ($2n = 22 + X0$), *Diabrotica speciosa* ($2n = 20 + X0$), *O. octoguttata*, *O. personata* ($2n = 20 + X + Y$), *Paranaita opima* ($2n = 20 + XY$), *Timarcha espanoli* ($2n = 24 + Xy_p$), *T. fallax, T. lugens, T. marginicollis, T. perezi* ($2n = 18 + Xy_p$), *T. granadensis* ($2n = 20 + Xy_p$) and *T. punctella* ($2n = 26 + Xy_p$) (Petitpierre, 1970, 1976, 1996; Virkki, 1983; Postiglioni & Brum-Zorrilla, 1988; Postiglioni et al., 1990, 1991; Schneider et al., 2002; Gómez-Zurita et al., 2004; Almeida et al., 2006, 2010).Variations in this pattern occurs only in four species, in which the rDNA genes are present on two autosomal pairs, as in *Zygogramma bicolorata* ($2n = 22 + Xy_p$) and *Omophoita magniguttis* ($2n = 20 + Xy$), just on the sex chromosome, as on the neoX of *Timarcha aurichalcea* ($2n = 16 + neoXY$), or on autosomes and the Y chromosome, as in *Alagoasa januaria* ($2n = 20 + X + Y$) (Virkki, 1983; Yadav et al., 1992; Gómez-Zurita et al., 2004; Almeida et al., 2010).

Among the 11 species examined in this study, only in *Cha. immaculata* the ribosomal cistrons are co-localized with constitutive heterochromatin on the terminal region of the chromosomes. The co-localization of these two chromosomal markers is reported in other coleopteran families, such as Curculionidae, Elateridae, Geotrupidae, Lucanidae, Scarabaeidae and Tenebrionidae (Virkki & Sepúlveda, 1990; Virkki et al., 1990; Colomba et al., 1996, 2000a, b, 2004; Vitturi et al., 1999, 2003; Moura et al., 2003; Bione et al., 2005; Schneider et al., 2006; Dutrillaux et al., 2007; Cabral-Mello et al., 2010; Lira-Neto et al., 2012). Cabral-de Mello et al. (2011a), in their comparison of the number and localization of major rDNA sites with the distribution of constitutive heterochromatin in 22 beetles in the subfamily Scarabaeinae, verified two patterns: (1) a stable number of rDNA sites (one autosomal pair) in species with constitutive heterochromatin predominantly located in the centromeric/pericentromeric region and (2) an increased number of rDNA cistrons in species with constitutive heterochromatin dispersed in the chromosomes. Consequently, these authors suggest that the same mechanism of chromosomal alteration (ectopic recombination) could be associated with the dispersion of these two chromosomal regions in the genome. Among the species studied by us, *Cha. immaculata* differs from other species of Cassidini in having rDNA genes located only in the terminal region of one autosomal pair, but constitutive heterochromatin dispersed among pericentromeric, telomeric and interstitial regions on autosomes (Lopes et al., 2016). In contrast, *D. cruciata* and *M. optata* has a different number and/or location of the ribosomal sites, compared to other species of Cassidini, *D. cruciata* with two interstitial rDNA sites and *M. optata* with three sites of rDNA, two terminal and one interstitial, but constitutive heterochromatin located only in the pericentromeric region of the chromosomes. These results seem to indicate that there is no direct correlation between the dispersion of constitutive heterochromatin and the 28S rDNA gene in Cassidini, and other mechanisms, such as inversion or transposition, may have been involved in the movement of the rDNA sites in these species. However, it is now necessary to use additional techniques to confirm the presence and identify the heterochromatin in species of Cassidinae.

In all the species studied here, the 28S rDNA is located in terminal/subterminal region of the chromosomes, with the exception of *D. cruciata*, *M. gnata* and *M. optata,* in which there are signals of hybridization in the interstitial regions. It is important to emphasize that these three species have diploid numbers and sex chromosome systems similar to most Cassidini, i.e., $2n = 16 + Xy_p$. In Coleoptera, the location of the major rDNA genes (or NOR) is only established in around 30% of the species investigated, that is 22% of the species with terminal rDNA, 1.6% with interstitial and 6.4% with centromeric rDNA. The species with interstitial and/or centromeric rDNA cistrons are mainly members of the families Scarabaeidae and Chrysomelidae (subfamily Alticinae). As recorded in the present study, the changes in the location of the sites of rDNA are described in species with similar karyotype characteristics (Virkki, 1983; Yadav et al., 1992; Bione et al., 2005; Almeida et al., 2006, 2010; Arcanjo et al., 2009, 2013; Silva et al., 2009; Cabral de Mello et al., 2010, 2011b; Oliveira et al., 2010, 2012b). The differences in the location of rDNA in closely related species with similar chromosomal characteristics may be a result of small chromosomal rearrangements, which change the position of the ribosomal site without modifying the metacentric chromosomal morphology.

In summary, the results obtained in this study reveal that the changes involving the 28S rDNA are pronounced in the tribe Cassidini, which has the most uniform karyotype within the subfamily Cassidinae. In the tribe Mesomphalini, the increase in the diploid number is not accompanied by an increase in the number of ribosomal genes. Finally, we show that the changes in the number and location of the ribosomal genes occur between species with similar karyotypes, indicating that the rearrangements in these specific genes may have resulted in the chromosomal evolution in this group.

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