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1 **Evolutionary insights into the genomic organization of major ribosomal**

2 **DNA in ant chromosomes**

3

4 Gisele Amaro Teixeira^{1,2}, Hilton Jeferson Alves Cardoso de Aguiar^{3*}, Frédéric

5 Petitclerc⁴, Jérôme Orivel⁴, Denilce Meneses Lopes², Luísa Antônia Campos Barros³

6

7 1 Programa de Pós-graduação em Biologia Celular e Estrutural, Universidade Federal
8 de Viçosa, Viçosa, MG 36570-000, Brazil.

9 2 Laboratório de Citogenética de Insetos, Departamento de Biologia Geral,
10 Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil.

11 3-Universidade Federal do Amapá, Campus Binacional – Oiapoque, n°3051, Bairro
12 Universidade, 68980-000, Oiapoque, Amapá, Brazil.

13 4 CNRS, UMR EcoFoG, AgroParisTech, CIRAD, INRA, Université de Guyane,
14 Université des Antilles, Campus Agronomique, BP 316, 97379 Kourou Cedex, France.

15

16 **Short running title: Evolution of rDNA genes in ant chromosomes**

17 * Corresponding author

18 E-mail: hilton.aguiar@hotmail.com

1 **Abstract**

2 The major rDNA genes are composed of tandem repeats and are part of the nucleolus
3 organizing regions (NORs). They are highly conserved and therefore useful in
4 understanding the evolutionary patterns of chromosomal locations. The evolutionary
5 dynamics of the karyotype may affect the organization of rDNA genes within
6 chromosomes. In this study, we physically mapped 18S rDNA genes in 13 Neotropical
7 ant species from four subfamilies using fluorescence *in situ* hybridization. Furthermore,
8 a survey of published rDNA cytogenetic data for 50 additional species was performed,
9 which allowed us to detect the evolutionary patterns of these genes in ant chromosomes.
10 Species from the Neotropical, Palearctic, and Australian regions, comprising a total of 63
11 species from 19 genera within six subfamilies, were analyzed. Most of the species (48 out
12 of 63) had rDNA genes restricted to a single chromosome pair in their intrachromosomal
13 regions. The position of rDNA genes within the chromosomes appears to hinder their
14 dispersal throughout the genome, as translocations and ectopic recombination are
15 uncommon in intrachromosomal regions because they can generate meiotic
16 abnormalities. Therefore, rDNA genes restricted to a single chromosome pair seem to be
17 a plesiomorphic feature in ants, while multiple rDNA sites, observed in distinct
18 subfamilies, may have independent origins in different genera.

19

20 **Keywords:** Evolution - ribosomal DNA - gene dispersion - Formicidae - chromosome
21 rearrangements - physical mapping

1 **Introduction**

2 Eukaryotic genomes have repetitive tandem sequences such as in the major
3 ribosomal RNA genes (45S = 18S + 5.8S + 28S), herein denominated rDNA, which
4 contain highly conserved genic sequences and are therefore useful as molecular genetic
5 markers, allowing comparisons across distant taxa. However, intergenic spacers vary in
6 both sequence and length (Long and Dawid, 1980; Sumner, 2003; Symonová, 2019). The
7 45S ribosomal genes are part of the nucleolus organizing regions (NORs) and are located
8 in portions of the DNA that, after their condensation, usually appear as secondary
9 constrictions on metaphase chromosomes (Sumner, 2003).

10 The silver nitrate impregnation technique (Ag-NOR banding) shows only
11 transcriptionally active NORs by staining nuclear acid proteins involved in transcription
12 (Howell and Black, 1980; reviewed by Sumner, 2003). Species with multiple rDNA
13 clusters do not usually exhibit silver-staining in all clusters (reviewed by Sumner, 2003;
14 Vicari *et al.*, 2008). However, it must be assumed that if a species has only a single NOR
15 (or single 45S rDNA gene site), then it will be transcriptionally active, as evidenced for
16 different organisms (Dobigny *et al.*, 2002; Barros *et al.*, 2015; Falcione *et al.*, 2018;
17 Cholak *et al.*, 2020; Malimpensa *et al.*, 2020).

18 Intercellular and interindividual variations are frequently detected by the Ag-NOR
19 banding method (Zurita *et al.*, 1997; Cross *et al.*, 2003; Walker *et al.*, 2014; Schmid *et*
20 *al.*, 2017). However, the use of this technique in ant species has not produced reliable
21 results owing to low repeatability, difficulty in obtaining good-quality markings, and the
22 appearance of unspecified marks on heterochromatic regions of many chromosomes,
23 which makes using this procedure unsatisfactory and inconclusive (Imai *et al.*, 1992;
24 Hirai *et al.*, 1994; Lorite *et al.*, 1997; Barros *et al.*, 2009, 2015; Aguiar *et al.*, 2017).

1 Since the 1980s, molecular cytogenetic tools have been used to study karyotypes.
2 For example, fluorescence *in situ* hybridization (FISH) has proven to be an effective and
3 precise tool for physically mapping specific DNA sequences within chromosomes
4 (reviewed by Levsky and Singer, 2003; Liehr, 2017). The rDNA genes can be located in
5 single or multiple chromosome pairs (Sochorová *et al.*, 2018). In several organisms,
6 studies of these genes have pointed to chromosomal differences within species complexes
7 (Mantovani *et al.*, 2005; Barbosa *et al.*, 2017; Dutra *et al.*, 2020) and between species
8 with similar karyotypes (Panzera *et al.*, 2012; Golub *et al.*, 2015; Gokhman *et al.*, 2016).
9 As a consequence, inferences can be made based on chromosomal rearrangements that
10 shape the chromosomal evolution of a species (Roy *et al.*, 2005; Nguyen *et al.*, 2010;
11 Britton-Davidian *et al.*, 2011; Cabral-de-Mello *et al.*, 2011; Dutrillaux and Dutrillaux,
12 2012; Roa and Guerra, 2012; Menezes *et al.*, 2019; Degrandi *et al.*, 2020).

13 In ants, the physical mapping of rDNA genes using the FISH technique was first
14 described in Australian ants by Hirai *et al.* (1994, 1996). Since then, the number of studies
15 mapping these genes has increased (Mariano *et al.*, 2008; Santos *et al.*, 2016; Micolino
16 *et al.*, 2019a; Teixeira *et al.*, 2020) and other repetitive sequences, such as telomeres
17 (Meyne *et al.*, 1995; Pereira *et al.*, 2018; Micolino *et al.*, 2020; Castro *et al.*, 2020),
18 satellite DNA (Lorite *et al.*, 2004; Huang *et al.*, 2016), 5S ribosomal genes (Aguiar *et al.*,
19 2017), and microsatellites (Barros *et al.*, 2018; Micolino *et al.*, 2019b), have been mapped
20 in the chromosomes using the FISH technique. To date, molecular cytogenetic studies on
21 rDNA genes in ants have improved understanding of chromosomal evolution and
22 phylogeny, and provided taxonomic resolutions for different ant groups (Hirai *et al.*,
23 1994, 1996; Santos *et al.*, 2010, 2016).

24 In this study, we physically mapped 18S rDNA clusters using the FISH technique
25 and verified if they were GC-rich in 13 Neotropical ant species from four subfamilies

1 (Ectatomminae, Formicinae, Myrmicinae, and Ponerinae). In addition, we reviewed
2 previous molecular cytogenetic data related to rDNA gene clusters (45S, 18S, or 28S) in
3 ants. Using these data, we investigated whether the number and location of the ribosomal
4 gene clusters followed a specific pattern or were randomly distributed in order to
5 understand the genomic organization and evolutionary dynamics of these genes in ants.

6

7 **Results**

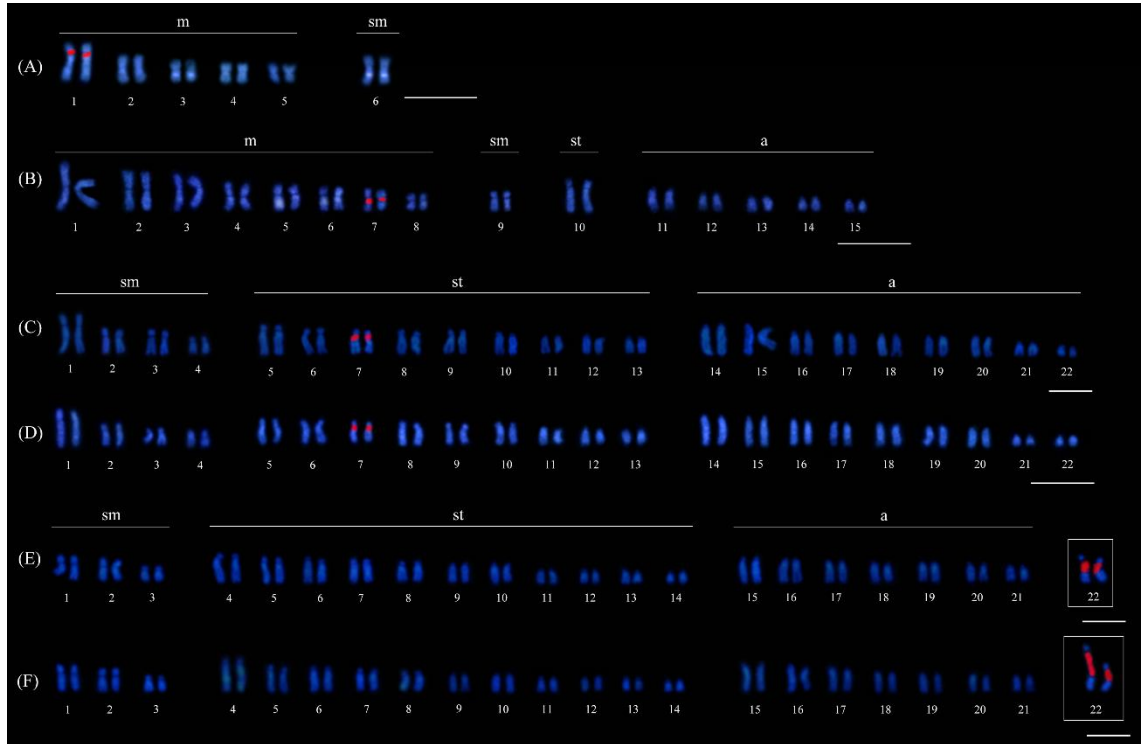
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9 **1 - Chromosome mapping of 18S rDNA clusters in 13 Neotropical ant species**

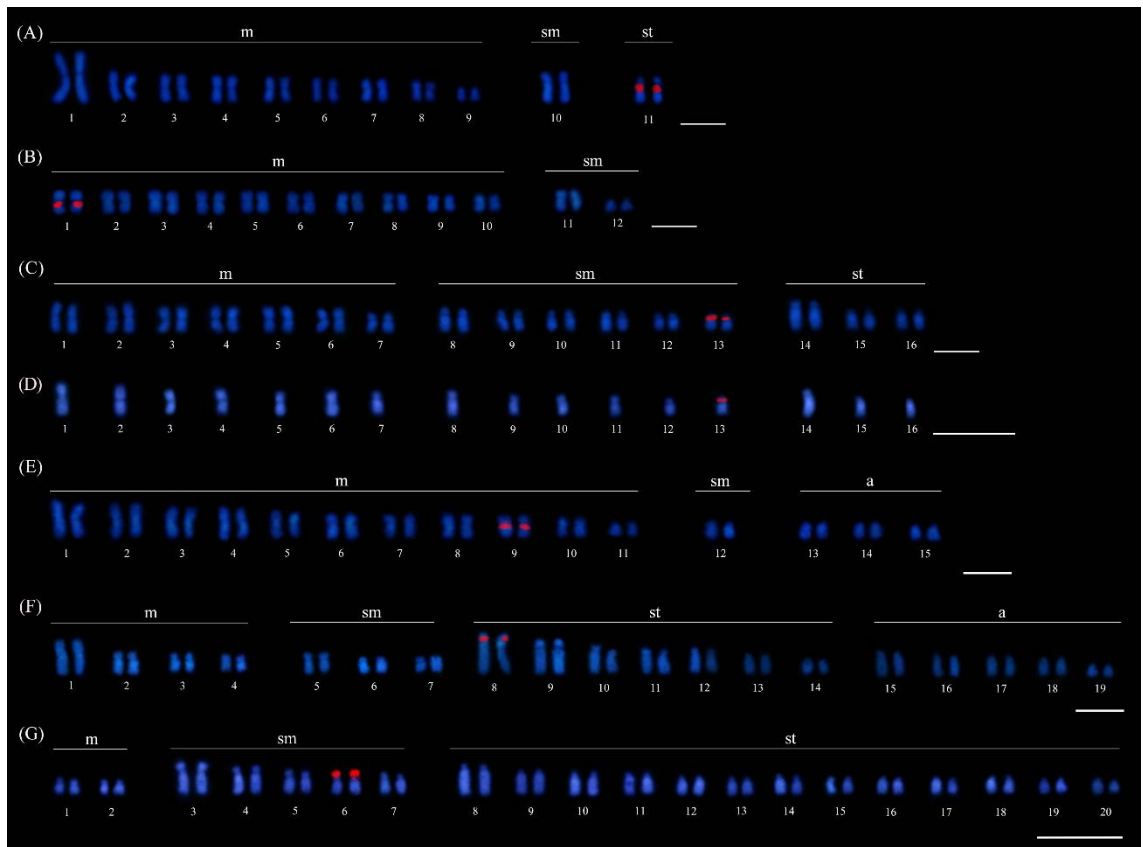
10

11 All of the studied ant species presented only a single 18S rDNA site that was co-
12 localized with GC-rich regions (CMA₃⁺) (Table 2), while AT-rich regions (DAPI⁺) were
13 not detected in any species. Most of the species presented GC-rich rDNA genes in the
14 intrachromosomal regions (pericentromeric or interstitial), including *Pseudoponera*
15 *gilberti* (Kempf, 1960) (Fig. 1A, S1A), *Anochetus targionii* Emery, 1894 (Fig. 1B, S1B),
16 *Odontomachus haematodus* (Linnaeus, 1758) (Fig. 1C, D, S1C), *Odontomachus bauri*
17 Emery, 1892 (Fig. 1E, S1D), *Pheidole germani* Emery, 1896 (Fig. 2A, S2A),
18 *Crematogaster longispina* Emery, 1890 (Fig. 2B, S2B), *Solenopsis geminata* (Fabricius,
19 1804) (Fig. 2C, D, S2C), *Myrmicocrypta* sp. (Fig. 2E, S2D), and *Acromyrmex echinator*
20 (Forel, 1899) (Fig. 2F). Additional GC-rich bands only occurred in *P. gilberti* in the
21 pericentromeric region of the 3rd and 4th metacentric chromosome pairs (Fig. S1B).
22 Differences in rDNA cluster sizes between homologous chromosomes were observed in
23 heterozygous *O. bauri* individuals. One of the homologous chromosomes showed clusters
24 approximately twice the size of those in the other chromosome (Fig. 1F, S1E). A male

- 1 individual bearing a chromosome with minor GC-rich bands was analyzed (Fig. S1F).
- 2 Homozygous individuals with duplicated clusters were not observed.
- 3



4
 5 **Figure 1.** Fluorescence *in situ* hybridization with 18S rDNA probe (red blocks) of
 6 Ponerinae ants: (A) *Pseudoponera gilberti* ($2n=12$), (B) *Anochetus targionii* ($2n=30$), (C,
 7 D) *Odontomachus haematodus* ($2n=44$, Brazil and French Guiana, respectively), (E, F)
 8 *Odontomachus bauri* ($2n=44$). In the last species, the 18S rDNA site was located on the
 9 long arm of the polymorphic chromosomal pair. The boxes show a polymorphic
 10 subtelocentric pair in (E) in the homozygous state, and (F) in the heterozygous state. Bars
 11 = 5 μm .



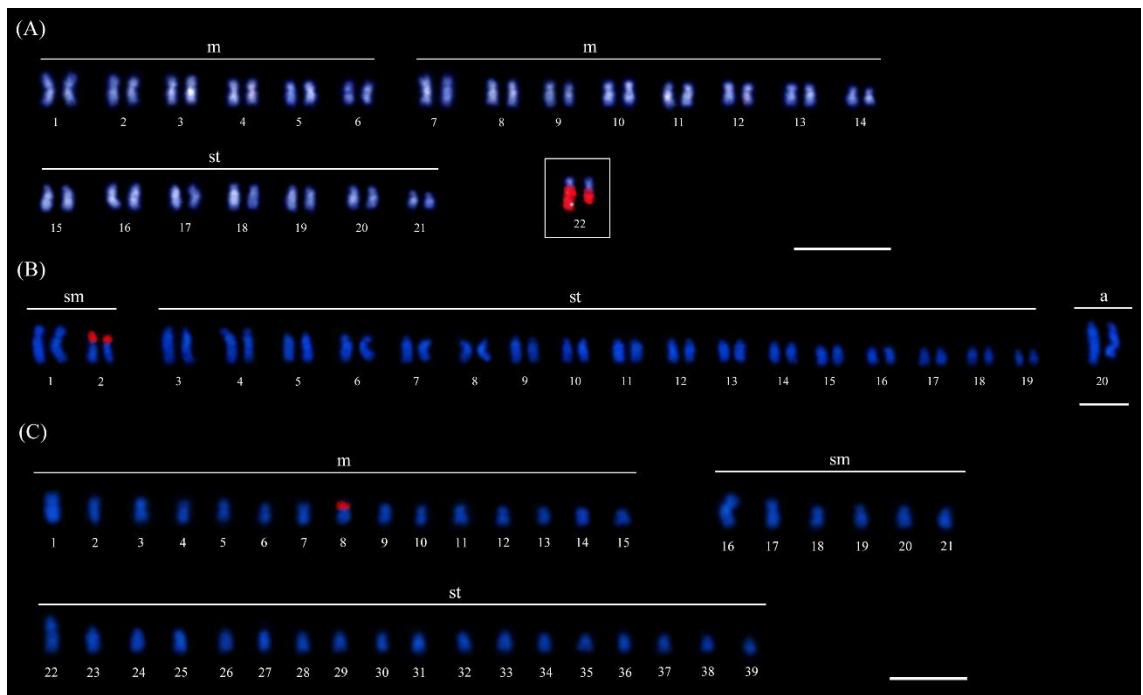
1

2 **Figure 2.** Fluorescence *in situ* hybridization with 18S rDNA probe (red blocks) of
 3 Myrmicinae ants: (A) *Pheidole germani* ($2n=22$), (B) *Crematogaster longispina*
 4 ($2n=24$), (C) *Solenopsis geminata* ($2n=32$) and (D) *Solenopsis geminata* ($n=16$), (E)
 5 *Myrmicocrypta* sp. ($2n=30$), (F) *Acromyrmex echinator* ($2n=38$), (G) *Strumigenys*
 6 *diabolus* ($2n=40$). Bars = 5 μ m.

7

8 The remaining species showed GC-rich 18S rDNA clusters across the entire
 9 chromosome arm, occupying either the long arm, as in *Gnamptogenys tortuolosa* (Smith,
 10 1858) (Fig. 3A, S3A), or residing in the short arm, as in *Strumigenys diabolus* Bolton,
 11 2000 (Fig. 2G, S2E), *Camponotus atriceps* (Smith, 1858) (Fig. 3B, S3B), and *Gigantiops*
 12 *destructor* (Fabricius, 1804) (Fig. 3C, S3C, D). Heteromorphism of 18S rDNA clusters
 13 was detected in all of the analyzed *C. atriceps* and *G. tortuolosa* individuals. In the latter
 14 species, the heteromorphism of the NOR resulted in differences in total size between

1 homologous chromosomes, which changed their morphology such that one was
2 submetacentric while the other was subtelocentric. In *G. destructor*, additional GC-rich
3 bands were located in the interstitial region of the long arm of the largest subtelocentric
4 chromosome pair (Fig. S3C, D).
5



6
7 **Figure 3.** Fluorescence *in situ* hybridization with 18S rDNA probe (red blocks) of an
8 Ectatomminae ant (A) *Gnamptogenys tortuolosa* (2n=44), and Formicinae ants (B)
9 *Camponotus atriceps* (2n=40) and (C) *Gigantiops destructor* (n=39). In *G. tortuolosa*,
10 the box shows a remarkable size heteromorphism of 18S rDNA clusters between
11 homologs. Bars: 5 μ m.

12

13 2 - Chromosome mapping review of rDNA clusters in ants

14

15 Cytogenetic data available in the literature related to the rDNA genes of 50 ant
16 species from 12 genera and six subfamilies were reviewed (Table 2; Fig. 4). Most data
17 were concentrated on Neotropical ants, with information on 33 species, while the Palearctic

1 and Australian regions had data on only one and 16 species, respectively. A single rDNA
2 site localized in the intrachromosomal region was observed in most species (Fig. 4A, B).
3 However, *Camponotus renggeri* Emery, 1894, *Dinoponera gigantea* (Perty, 1833), and
4 most of the studied *Myrmecia* species presented multiple rDNA sites over the entire short
5 chromosome arm. The subfamily Myrmicinae possessed most of the rDNA data, and the
6 Myrmeciinae subfamily showed a different pattern in relation to other subfamilies with
7 multiple rDNA sites observed in the majority of species (Fig. 4C). *Dolichoderus*
8 *voraginosus* Mackay, 1993 did not show any co-localization of 18S rDNA clusters and
9 GC-rich bands. Figure 5 summarizes the available data on the number and position of
10 NORs in ant species and the phylogenetic relationships among these species, and is based
11 on the published molecular phylogenies.

12

13 **Discussion**

14

15 **1 - Patterns of rDNA clusters in the karyotypes of specific ant groups**

16

17 Specific rDNA patterns can be observed in the karyotypes of some ant groups
18 where several species have been studied. In species of the genus *Dolichoderus*, the
19 chromosome numbers range from $2n=10$ to 58, and a single rDNA site in the interstitial
20 region has been observed in the majority of the investigated species. However, there are
21 two exceptions: *D. voraginosus* and *Dolichoderus attelaboides* (Fabricius, 1775), where
22 the rDNA clusters are located in the terminal region of the long arm and in the short arm,
23 respectively (Santos *et al.*, 2016).

24 Despite the chromosomal variation observed in *Gnamptogenys* spp. ($2n=24$ to 44),
25 they all have a single rDNA site in the intrachromosomal region (Teixeira *et al.*, 2020).

1 Even *Gnamptogenys moelleri* (Forel, 1912), a species with differing chromosome
2 numbers between two populations ($2n=34$ and 44), has a single intrachromosomal rDNA
3 site. *Gnamptogenys tortuolosa* is an exception, with rDNA clusters occurring over the
4 entire long arm (this study). In the genus *Anochetus*, the chromosome number ranges
5 from $2n=30$ to 46 , and a single pericentromeric rDNA site has been observed (Santos *et*
6 *al.*, 2010).

7 All analyzed fungus-farming ants (*Attina*) had a single rDNA site located in the
8 intrachromosomal region (Table 2). In the genus *Mycetophylax*, although chromosome
9 number differs among species ($2n=26$ to 36), a single rDNA site is located in the
10 pericentromeric or terminal region in all the species of the genus (Micolino *et al.*, 2019a).
11 The leaf-cutting ants are considered the most derived among the *Attina* species (Schultz
12 and Brady, 2008). *Atta* spp. ($2n=22$) have a single rDNA site in the interstitial region and
13 *Acromyrmex* spp. ($2n=38$) have a single rDNA site in the terminal region (Barros *et al.*,
14 2015, 2016; Teixeira *et al.*, 2017). *Acromyrmex striatus* (Roger, 1863), a sister group of
15 the leaf-cutting ants, has $2n=22$ and pericentromeric rDNA clusters that are not located
16 on the same chromosome pair relative to that in *Atta* spp. (Cristiano *et al.*, 2013; Teixeira
17 *et al.*, 2017). In *A. echinator* ($2n=38$), rDNA clusters are located in the interstitial region
18 of the same pair as observed in other *Acromyrmex* spp. (this study).

19 The chromosome number of the Australian bulldog ants is highly variable,
20 ranging from $2n=2$ to 76 (Imai *et al.*, 1994). In addition, *Myrmecia* spp. present
21 remarkable patterns of multiple 28S rDNA clusters that are highly dispersed throughout
22 their genomes. The number of rDNA sites increases with the chromosome number of the
23 species. This suggests several ribosomal gene amplification events have occurred in the
24 different species of *Myrmecia* and that they have accumulated in karyotypes throughout
25 the evolution of the genus. Only four out of 16 species from this monophyletic genus

1 have the entire arm or intrachromosomal rDNA clusters restricted to a single pair of
2 chromosomes (Hirai *et al.*, 1994, 1996; Hirai, 2020). This pattern is observed in species
3 with small chromosome numbers, which suggests that a single NOR is plesiomorphic
4 among *Myrmecia* (Hirai, 2020).

5 Carpenter ants (*Camponotus*) from the subgenus *Myrmothrix* have $2n=40$
6 chromosomes, with its studied species having a single rDNA site in the terminal region.
7 *Camponotus renggeri* is the only exception, having an additional rDNA cluster at the
8 terminal region of a medium-sized subtelocentric pair (Aguiar *et al.*, 2017).

9 In the giant ants of the genus *Dinoponera*, two contrasting patterns have been
10 observed: *D. gigantea* ($2n=82$) has multiple rDNA sites located on its short chromosome
11 arms (Aguiar *et al.*, 2011), whereas *Dinoponera lucida* Emery, 1901 has a higher
12 chromosome number ($2n=120$), but only a single rDNA site restricted to the
13 intrachromosomal region of its largest chromosome pair (Mariano *et al.*, 2008).

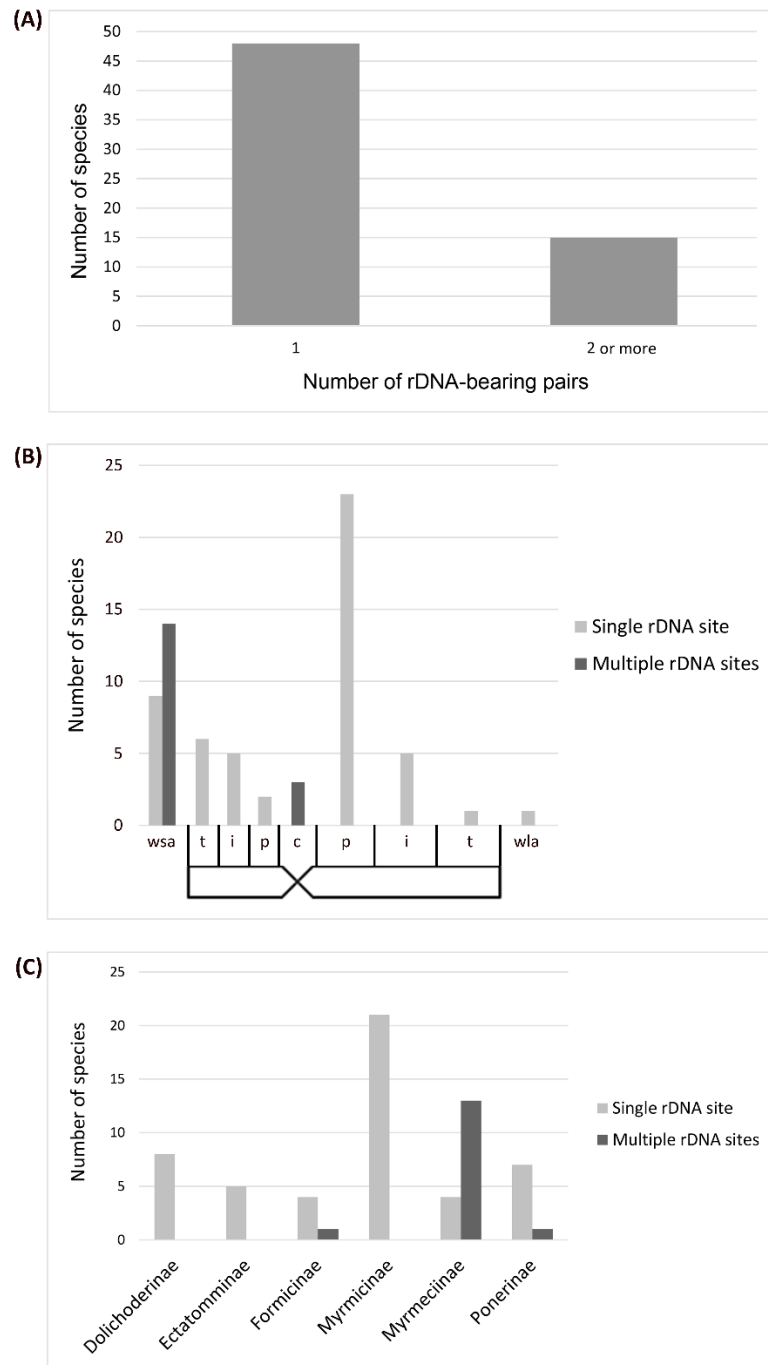
14 Homeology patterns among chromosomal pairs bearing ribosomal genes can be
15 detected in a few ant genera, such as *Gnamptogenys* (*striatula* group), *Camponotus*
16 (*Myrmothrix*), *Atta*, and *Acromyrmex* (Table 2). However, it is speculative to infer such
17 homeology patterns for the entire Formicidae family. Ants constitute an ultra-diverse
18 monophyletic group with more than 13,800 described species (Bolton, 2020). They show
19 a wide range of karyotype variation both in number ($2n=2$ to $2n=120$) and chromosomal
20 morphology (reviewed by Lorite and Palomeque, 2010; Mariano *et al.*, 2019).

21

22 **2 - Insights concerning the organizational patterns of ribosomal gene clusters in the** 23 **ant genome**

24

1 The mapping of the ribosomal gene clusters of 63 species distributed in 19 genera
2 and six subfamilies, together with information on their phylogenetic relationships,
3 demonstrated that a single pair of chromosomes bearing the GC-rich rDNA clusters is the
4 most frequent trait among the studied species, regardless of the chromosome number
5 (Table 1, Fig. 4A). Genomes carrying rDNA clusters in more than a single chromosome
6 pair have been observed in non-related taxa, such as *D. gigantea* (Aguiar *et al.*, 2011), *C.*
7 *renggeri* (Aguiar *et al.*, 2017), and *Myrmecia* spp. (Hirai *et al.*, 1994, 1996). We
8 hypothesize that having a single rDNA site should be considered a plesiomorphic trait
9 because multiple rDNA sites were observed in different non-related lineages that do not
10 share exclusive common ancestry and appear *de novo* throughout the Formicidae family.



1

2 **Figure 4.** Summary of available molecular cytogenetic data (this study and in the
 3 literature) concerning rDNA genes in ant species: (A) number of species with single and
 4 multiple rDNA sites in a karyotype, (B) location of rDNA genes in chromosomes of ant
 5 species, considering the following chromosomal regions: c, centromeric region; p,
 6 pericentromeric region; i, interstitial region; t, terminal region; wsa, whole short arm; wla,

1 whole long arm, and (C) number of species with single and multiple rDNA sites by
2 subfamilies of Formicidae.

3 In eukaryotes, it is common to observe variations in the number of rDNA clusters
4 and the location of these genes in the chromosomes within genera (Sánchez-Gea *et al.*,
5 2000; Gross *et al.*, 2010; Cabral-de-Mello *et al.*, 2011; Gokhman *et al.*, 2014; Mazzoleni
6 *et al.*, 2018), among populations (Panzera *et al.*, 2014; Ferreti *et al.*, 2019; Menezes *et*
7 *al.*, 2019), and sexes of the same species (Nakayama *et al.*, 2001; Štáhlavský *et al.*, 2018).
8 The possession of terminal rDNA clusters seems to be a common trait among mammals,
9 fish, and mollusks, but less so in arthropods (Sochorová *et al.*, 2018). Within the ultra-
10 diverse insect group, the location of rDNA clusters may follow distinct patterns in its two
11 largest orders; terminal rDNA sites are more abundant in Coleoptera, whereas
12 pericentromeric rDNA clusters are more frequent in Orthoptera (Sochorová *et al.*, 2018).
13 In Formicidae, terminal rDNA sites are a less common feature and all species with
14 multiple rDNA clusters show these genes in the entire short chromosome arms including
15 terminal/subterminal regions, such as *C. renggeri*, *D. gigantea*, and *Myrmecia* spp.
16 (Aguiar *et al.*, 2011, 2017; Hirai *et al.*, 1994, 1996).

17 Different hypotheses have been proposed to explain the cytogenetic pattern
18 (conservative or variable) of these rDNA clusters in the genome of several organisms.
19 Such hypotheses are linked to the specific locations of these rDNA clusters on the
20 chromosomes. Rearrangements, such as translocations, unequal exchange, and ectopic
21 recombination mechanisms (i.e., between non-homologous chromosomes), which can
22 lead to gene dispersion or increases in number in the genome, are more likely in the
23 terminal/subterminal regions of chromosomes and are uncommon in the
24 intrachromosomal regions (Martins and Wasko, 2004; Mantovani *et al.*, 2005; Nguyen *et*
25 *al.*, 2010; Roy and Guerra, 2012; Hirai, 2020).

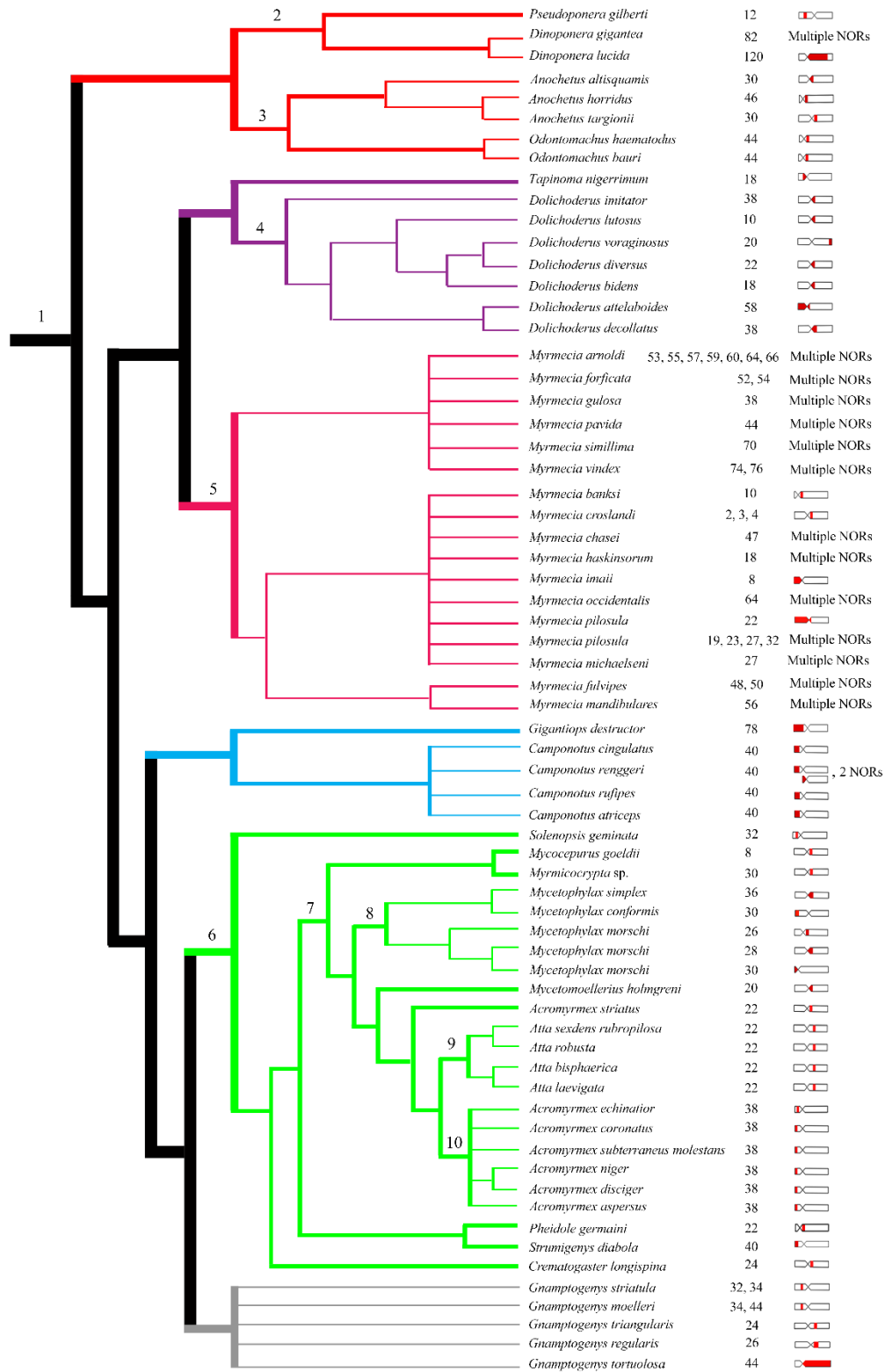
1 Based on rDNA mapping data in fish karyotypes, Martins and Wasko (2004)
2 proposed that translocations are more likely to occur in telomeric regions because of their
3 proximity within the interphase nucleus, which originates from the ordering of
4 chromosomes according to Rabl's model. Effects due to the location of ribosomal genes
5 in relation to their dispersion in the karyotype were also observed in Coleoptera
6 (Dutrillaux *et al.*, 2016) and primates (Gerbault-Seureau *et al.*, 2017). These authors
7 argued that translocations in the interstitial position could result in abnormal meiosis and,
8 therefore, unbalanced gametes. In contrast, translocations in terminal positions may
9 increase the number of rDNA genes in the genome. This would lead to fewer meiotic
10 abnormalities and highlights the selection for interstitial rDNA site stability (Dutrillaux
11 *et al.*, 2016; Gerbault-Seureau *et al.*, 2017).

12 Ectopic recombination is another mechanism suggested to explain the rDNA
13 patterning in moths and butterflies (Nguyen *et al.*, 2010) and plants (Roa and Guerra,
14 2012). It is also included in the recent model proposed by Hirai (2020). In this model,
15 two mechanisms are important: the "site effect" and the "molecular effect." The former
16 allows terminal region associations due to the proximity of these regions in a meiotic
17 bouquet. The "site effect" is a precondition for the "molecular effect," which refers to
18 systems of affinity/non-affinity due to the similarity between rDNA sequences with other
19 repetitive sequences. Thus, rDNA clusters in the terminal regions tend to associate with
20 other repetitive sequences of non-homologous chromosomes more easily, facilitating the
21 occurrence of ectopic recombination and dispersion of these genes in the genome (Hirai,
22 2020).

23 There are reports of species with multiple rDNA clusters associated with the
24 centromeres of acrocentric chromosomes (Cazaux *et al.*, 2011). In the recent model
25 proposed by Hirai (2020), the centromeric region of acrocentric chromosomes

1 (chromosomes with a short and heterochromatic arm) that have rDNA genes associated
2 with the centromere may behave as subterminal regions. Therefore, such an arrangement
3 would also facilitate eventual associations of rDNA genes with other repetitive sequences
4 and the occurrence of ectopic recombination, which leads to their dispersal in the genome
5 (for details, see Hirai, 2020).

6 The ant rDNA chromosome evolution seems to be in accordance with the above-
7 mentioned hypothesis about dispersal and NOR location because the single rDNA
8 clusters of most studied species are interstitial or pericentromeric (Fig. 4B). In ant species,
9 terminal rDNA clusters are prone to rearrangements that lead to their dispersal.
10 *Camponotus renggeri* (Aguiar *et al.*, 2017), *D. gigantea* (Aguiar *et al.*, 2011), and
11 *Myrmecia* spp. present multiple NORs in the entire short chromosome arms including
12 terminal/subterminal regions, which facilitates the association of these genes with the
13 heterochromatic sequences of other non-homologous acrocentric chromosomes during
14 meiosis and the subsequent occurrence of ectopic recombination (Hirai, 2020). This
15 pattern may be applicable to different ant groups. In addition, inversions have been shown
16 to change the position of rDNA genes in *A. echinator* (this study), *Dolichoderus* spp.
17 (Santos *et al.*, 2016), and *Myrmecia* spp. (Hirai *et al.*, 1996).



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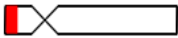
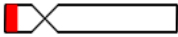
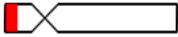
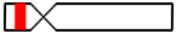
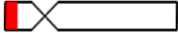

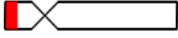




2 **Figure 5.** Summary of available data (this study and literature) concerning the number
 3 and position of rDNA genes in ant species, along with their degree of relatedness and
 4 diploid chromosome numbers. Ideograms of rDNA-bearing chromosomes show number

1 and location (terminal, interstitial, pericentromeric or centromeric) of rDNA clusters (red)
2 in the haploid complement. Phylogenetic relationships are based on 1- Moreau and Bell
3 (2013), 2 - Schmidt (2013), 3 - Larabee *et al.* (2016), 4 - Santos *et al.* (2016), 5 - Hasegawa
4 and Crozier (2006), 6 - Ward *et al.* (2015), 7 - Solomon *et al.* (2019), 8 - Micolino *et al.*
5 (2019a), 9 - Bacci *et al.* (2009), 10 - Queiroz (2015). Colours of phylogenetic branches
6 indicate the following subfamilies: red, Ponerinae; purple, Dolichoderinae; pink,
7 Myrmeciinae; blue, Formicinae; green, Myrmicinae; gray, Ectatomminae.

8 A single rDNA site located in the terminal region or entire chromosomal arm was
9 observed in some ant species. The repetitive sequences in the subterminal/terminal
10 chromosome regions probably do not form affinity systems with ribosomal genes (the so-
11 called molecular effect; for details, see Hirai, 2020) in these species. Therefore, rDNA
12 clusters are restricted to a single chromosomal pair. Future studies focusing on the
13 characterization of repetitive sequences that make up the heterochromatin of these species
14 will help clarify this hypothesis.


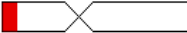


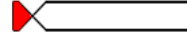



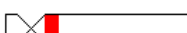

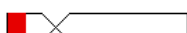


15 Size heteromorphisms are frequent in karyotypes where the rDNA clusters have
16 terminal positions in the chromosomes, as reported in this study (*G. tortuolosa* and *C.*
17 *atriceps*) as well as in other ants (Aguiar *et al.*, 2017) and insects in general (Cabral-de-
18 Mello *et al.*, 2011; Maryńska-Nadachowska *et al.*, 2016; Andrade-Souza *et al.*, 2018).
19 Subtle variations in the size of the rDNA clusters between homologous chromosomes can
20 be observed as a result of late condensation during cell division (Sumner, 2003).
21 However, large variations, such as those mentioned above, at the terminal region on the
22 chromosome are usually related to duplications/deletions as a result of unequal exchange
23 (Schubert and Lysack, 2011). It is believed that exchanges are less common in
24 intrachromosomal regions (Hirai, 2020).

1 **Table 1.** Summary of the available molecular cytogenetic data concerning major ribosomal genes (45S, 28S or 18S) detected by FISH in ants

Ant species	2n/(n)	Location of rDNA genes in karyotype	Ideogram	Co-localization CMA ₃ /rDNA	Reference CMA ₃ /rDNA
Neotropical ants					
Subfamily Myrmicinae					
<i>Acromyrmex aspersus</i>	38	Largest subtelocentric pair		Yes	Teixeira et al. (2017)
<i>Acromyrmex coronatus</i>	38	Largest subtelocentric pair		Yes	Barros et al. (2016)
<i>Acromyrmex disciger</i>	38	Largest subtelocentric pair		Yes	Barros et al. (2016)
<i>Acromyrmex echinator</i>	38	Largest subtelocentric pair		Yes	Barros et al. (2016)/ Present study
<i>Acromyrmex niger</i>	38	Largest subtelocentric pair		Yes ^a	Barros et al. (2016)
<i>Acromyrmex striatus</i>	22	2nd metacentric pair		Yes ^a	Cristiano et al. (2013)/ Teixeira et al. (2017)
<i>Acromyrmex subterraneus molestans</i>	38	Largest subtelocentric pair		Yes	Teixeira et al. (2017)
<i>Atta bisphaerica</i>	22	4th metacentric pair		Yes	Barros et al. (2014)/ Teixeira et al. (2017)
<i>Atta laevigata</i>	22	4th metacentric pair		Yes	Barros et al. (2014)/ Teixeira et al. (2017)
<i>Atta robusta</i>	22	4th metacentric pair		Yes	Barros et al. (2015)
<i>Atta sexdens rubropilosa</i>	22	4th metacentric pair		Yes	Barros et al. (2014)/ Teixeira et al. (2017)


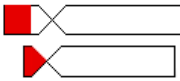
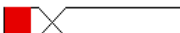
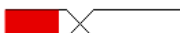




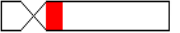

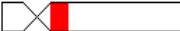

2

1 **Table 1. Cont.**

Ant species	2n/(n)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA₃/rDNA	Reference CMA₃/rDNA
<i>Crematogaster longispina</i>	24	Largest metacentric pair		Yes	Present study
<i>Mycetophylax conformis</i>	30	11th metacentric pair		Yes	Cardoso et al. (2014)/ Micolino et al. (2019a)
<i>Mycetophylax morschi</i>	26	2nd submetacentric pair		–	Micolino et al. (2019a)
<i>Mycetophylax morschi</i>	28	7th metacentric pair		–	Micolino et al. (2019a)
<i>Mycetophylax morschi</i>	30	Acrocentric pair		–	Micolino et al. (2019a)
<i>Mycetophylax simplex</i>	36	Smallest metacentric pair		Yes	Cardoso et al. (2014)/ Micolino et al. (2019a)
<i>Mycocepurus goeldii</i>	8	2nd metacentric pair		Yes ^a	Barros et al. (2010)/ Barros et al. (2012)
<i>Myrmicocrypta</i> sp.	30	9th metacentric pair		Yes	Present study
<i>Pheidole germani</i>	22	Subtelocentric pair		Yes	Present study
<i>Solenopsis geminata</i>	32	Smallest submetacentric pair		Yes	Present study
<i>Strumigenys diabola</i>	40	4th submetacentric pair		Yes	Present study
<i>Trachymyrmex holmgreni</i>	20	4th metacentric pair		Yes	Barros et al. (2018)
Subfamily Formicinae					
<i>Camponotus atriceps</i> ^c	40	2nd submetacentric pair		Yes	Present study

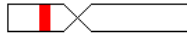
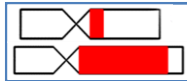
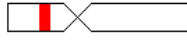
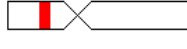






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1 **Table 1. Cont.**

Ant species	2n/(n)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA ₃ /rDNA	Reference CMA ₃ /rDNA
<i>Camponotus cingulatus</i> ^c	40	2nd submetacentric pair		Yes	Aguiar et al. (2017)
<i>Camponotus renggeri</i>	40	2nd submetacentric pair and medium-sized subtelocentric pair		Yes	Aguiar et al. (2017)
<i>Camponotus rufipes</i> ^c	40	2nd submetacentric pair		Yes	Aguiar et al. (2017)
<i>Gigantiops destructor</i>	(39)	8th metacentric pair		Yes ^a	Present study
Subfamily Ponerinae					
<i>Anochetus altisquamis</i> ^c	30	3rd submetacentric pair		–	Santos et al. (2010)
<i>Anochetus horridus</i> ^c	46	4th telocentric pair		–	Santos et al. (2010)
<i>Anochetus targionii</i>	30	7th metacentric pair		Yes	Present study
<i>Dinoponera gigantea</i>	82	Multiple pairs	-	–	Aguiar et al. (2011)
<i>Dinoponera lucida</i>	(59)	Largest pair (A ^{Mt})		Yes	Mariano et al. (2008)
<i>Odontomachus bauri</i> ^d	44/(22)	2nd subtelocentric pair	 	Yes	Present study
<i>Odontomachus haematodus</i>	44	3rd subtelocentric pair		Yes	Present study
<i>Pseudoponera gilberti</i>	22	Largest metacentric pair		Yes ^a	Present study



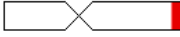



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1 **Table 1. Cont.**

Ant species	2n/(n)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA₃/rDNA	Reference CMA₃/rDNA
Subfamily Ectatomminae					
<i>Gnamptogenys moelleri</i>	34, 44	4th metacentric pair		Yes	Teixeira et al. (2020)
<i>Gnamptongeys regularis</i> ^d	26	Metacentric and submetacentric homologous		Yes	Teixeira et al. (2020)
<i>Gnamptogenys striatula</i>	32	5th metacentric pair		Yes	Teixeira et al. (2020)
<i>Gnamptogenys striatula</i>	34	4th metacentric pair		Yes	Teixeira et al. (2020)
<i>Gnamptogenys triangularis</i>	24	Largest metacentric pair		Yes	Teixeira et al. (2020)
<i>Gnamptogenys tortuolosa</i> ^c	44	Subtelocentric and submetacentric homologous		Yes	Present study
Subfamily Dolichoderinae					
<i>Dolichoderus attelaboides</i>	58	Largest submetacentric pair		Yes	Santos et al. (2016)
<i>Dolichoderus bidens</i>	18	Largest metacentric pair		Yes ^a	Santos et al. (2016)
<i>Dolichoderus decollatus</i>	38	2nd metacentric pair		Yes	Santos et al. (2016)
<i>Dolichoderus diversus</i>	22	Largest metacentric pair		Yes ^a	Santos et al. (2016)

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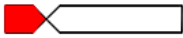

1 **Table 1. Cont.**

Ant species	2n/(n)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA ₃ /rDNA	Reference CMA ₃ /rDNA
<i>Dolichoderus imitator</i>	38	Largest metacentric pair		Yes	Santos et al. (2016)
<i>Dolichoderus lutosus</i>	10	2nd metacentric pair		Yes ^a	Santos et al. (2016)
<i>Dolichoderus voraginosus</i>	20	Largest metacentric pair		No ^a	Santos et al. (2016)
Australian ants					
Subfamily Myrmeciinae					
<i>Myrmecia banksi</i> ^c	10	Smallest acrocentric pair		–	Hirai et al. (1994), (1996)
<i>Myrmecia chasei</i>	47	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia croslandi</i> ^d	2, 3, 4 ^b	Acrocentric pair	 , 	–	Hirai et al. (1994), (1996)
<i>Myrmecia forficata</i>	52 ^b , 54	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia fulvipes</i>	48, 50	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia gulosa</i>	38 ^b	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia haskinsorum</i>	18	Multiple pairs	-	–	Hirai et al. (1994), (1996)

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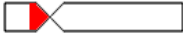
1 **Table 1.** Cont.

2

Ant species	2n/(n)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA ₃ /rDNA	Reference CMA ₃ /rDNA
<i>Myrmecia imaii</i> ^c	8	Largest acrocentric pair		–	Hirai et al. (1994), (1996)
<i>Myrmecia mandibularis</i>	56 ^b	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia michaelseni</i>	27	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia occidentalis</i>	64 ^b	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia pavidata</i>	44	Multiple pairs	-	–	Hirai et al. 1996
<i>Myrmecia pilosula</i>	22 ^b	Acrocentric pair		–	Hirai et al. (1996)
<i>Myrmecia pilosula</i>	19, 23 ^b , 27, 32	Multiple pairs	-	–	Hirai et al. (1994), (1996)
<i>Myrmecia simillima</i>	70 ^b	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia arnoldi</i>	53, 55, 57, 59, 60 ^b , 64, 66	Multiple pairs	-	–	Hirai et al. (1996)
<i>Myrmecia vindex</i>	74, 76	Multiple pairs	-	–	Hirai et al. (1996)

Palearctic ant

Subfamily Dolichoderinae

<i>Tapinoma nigerrimum</i>	18	Largest submetacentric pair		Yes	Lorite et al. (1997)
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3 ^a: Additional markers CMA₃⁺. ^b: Data showed by Hirai et al. (1996). ^c: Heteromorphism of rDNA clusters between the homologous.4 ^d: Polymorphism of rDNA clusters between the homologous. – unavailable data.

1 **Table 2.** The relationship between Neotropical ant species studied using 18S rDNA FISH and their chromosome number
 2

Ant species	Subfamily	Locality	2n	3
<i>Acromyrmex echinator</i> (Forel, 1899)	Myrmicinae	Barro Colorado – Panamá	38	4
<i>Anochetus targionii</i> Emery 1894	Ponerinae	Campus Agronomique, Kourou – FG	30	5
<i>Camponotus atriceps</i> (Smith, 1858)	Formicinae	Viçosa – Minas Gerais – BR	40	6
<i>Crematogaster longispina</i> Emery, 1890	Myrmicinae	La Montagne des Singes – FG	24	7
<i>Gigantiops destructor</i> (Fabricius, 1804)	Formicinae	Sinnamary – FG	78	8
<i>Gnamptogenys tortuolosa</i> (Smith, 1858)	Ectatominae	Sinnamary – FG	44	9
<i>Myrmicocrypta</i> sp.	Myrmicinae	Sinnamary – FG	30	10
<i>Odontomachus bauri</i> Emery, 1892	Ponerinae	Açailândia – Maranhão – BR	44	11
<i>Odontomachus haematodus</i> (Linnaeus, 1758)	Ponerinae	Campus Agronomique, Kourou – FG	44	12
		Ubá – Minas Gerais – BR	44	13
<i>Pheidole germaini</i> Emery, 1896	Myrmicinae	Viçosa – Minas Gerais – BR	22	14
<i>Pseudoponera gilberti</i> (Kempf, 1960)	Ponerinae	Sinnamary – FG	12	15
<i>Solenopsis geminata</i> (Fabricius, 1804)	Myrmicinae	Sinnamary – FG	32	16
<i>Strumigenys diabolus</i> Bolton 2000	Myrmicinae	Sinnamary – FG	40	17
			40	18
				19
				20

21 FG: French Guiana, BR: Brazil.

1 Size variations in the rDNA clusters can be observed when these genes are located
2 in the interstitial/pericentromeric region of the chromosomes, as seen in *Gnamptogenys*
3 *regularis* Mayr, 1870 (Teixeira *et al.*, 2020) and in *O. bauri* (this study). A different path
4 seems to be involved in the evolution of these karyotypes compared to the rearrangements
5 involved in terminal rDNA heteromorphisms. In these cases, the mechanism may be
6 associated with the formation of extrachromosomal circular DNA (eccDNA), which is
7 likely to form tandem repetitive sequences similar to the rDNA genes (Cohen and Segal,
8 2009). These eccDNAs may be lost, leading to deletions in the original rDNA sequences,
9 or they may be replicated via a rolling circle mechanism and reintegrated into the original
10 chromosome, producing duplications of these repetitive sequences (Cohen and Segal,
11 2009).

12 In ants as well as in other eukaryotes, rDNA clusters are located in GC-rich
13 regions (Symonová, 2019) and, therefore, usually coincide with CMA₃⁺ bands, possibly
14 as a result of GC-biased gene conversion (gBGC) over the course of evolutionary time
15 (Escobar *et al.*, 2011). This co-localization of GC-rich regions and rDNA was observed
16 for all the ants studied to date (Table 2), with the exception of *D. voraginosus* (Santos *et*
17 *al.*, 2016). However, GC-rich chromatin is not always an indication of ribosomal genes,
18 as seen here in *G. destructor*, *P. gilberti*, and in some *Dolichoderus* spp. and fungus-
19 farming ants (Table 2).

20 In insects, a compilation of previous data concerning rDNA genes has been
21 conducted, for example, in moths and butterflies (Lepidoptera; Nguyen *et al.*, 2010),
22 beetles (Coleoptera; Dutrillaux and Dutrillaux, 2012), and kissing bugs (Heteroptera;
23 Panzera *et al.*, 2012). However, this is the first survey of Hymenoptera species. We have
24 compiled available information and new data on 13 Neotropical ant species. In different
25 organisms, including ants, the number and location of chromosomes bearing rDNA

1 clusters within the genome follow general patterns that govern the modes of evolution for
2 these genes (Martins and Wasko, 2004; Nguyen *et al.*, 2010; Dutrillaux *et al.*, 2016;
3 Gerbault-Seureau *et al.*, 2017; Hirai, 2020; this study). We can conclude that having only
4 a single pair of chromosomes bearing rDNA clusters is more common in the ant genome
5 because of the pericentromeric/interstitial location of these genes on the chromosomes.
6 Intrachromosomal regions are sites with low frequencies of rearrangements, such as non-
7 Robertsonian translocations and ectopic recombination, and are therefore less prone to
8 meiotic abnormalities. It should be assumed that the chromosomal location of rDNA
9 clusters influences the dispersion of these genes within the karyotype.

10 Future studies will allow the mapping of rDNA genes in more ant taxa, including
11 the other remaining subfamilies. Other repetitive sequences, such as 5S rDNA and histone
12 genes, may also be mapped in ant species as a tool to investigate further patterns that
13 reflect the relationship between chromosomal location and dispersion in the genome.
14 Finally, a solid understanding of the evolutionary patterns of ribosomal gene dispersal in
15 ant chromosomes may provide a comparative model for other insects.

16

17 **Experimental Procedures**

18

19 **1 - Obtaining samples for analysis**

20

21 Field surveys to collect ant colonies were performed in French Guiana, Brazil, and
22 Panama (Table 1) from the following locations: *La Montagne des Singes*, Kourou
23 (5.07225, -52.69407), Campus Agronomique, Kourou (5.17312, -52.65480), and
24 Sinnamary (5.28482, -52.91403), all in French Guiana; Viçosa (-20.757041, -42.873516)

1 and Ubá (-21.128880, -42.937646), both in Minas Gerais, Brazil, and Açailândia (-
2 4.84200, -47.29667) in Maranhão, Brazil; and Barro Colorado Island (9.150000, -
3 79.83333) in Panama. Sampling permit in Brazil was provided by the Instituto Chico
4 Mendes de Conservação da Biodiversidade (ICMBio) (SISBIO accession numbers 32459
5 and 62598). Adult specimens were identified by Dr. Jacques H. C. Delabie and deposited
6 in the ant collection at the Laboratório de Mirmecologia do Centro de Pesquisas do Cacau
7 (CPDC/Brazil), in Bahia, Brazil, under records #5802, #5803, and #5804.

8 In this study, previous molecular cytogenetic data related to rDNA genes (45S,
9 18S, or 28S) mapped by FISH from 50 ant species were used. For comparative analysis,
10 the following traits were considered for each species: chromosome number, number of
11 rDNA-bearing chromosomes, location of rDNA clusters in the karyotype, and co-
12 localization of CMA₃ fluorochrome and rDNA clusters. Ag-NOR data were disregarded
13 due to the highly variable patterns and unreliable results (for details, see Introduction).

14

15 **2 – Chromosome preparation**

16

17 Mitotic metaphase chromosomes were obtained from the cerebral ganglia of
18 larvae after meconium elimination, using colchicine hypotonic solution (0,005%) and
19 fixatives according to the methods described by Imai *et al.* (1988).

20

21 **3 - Staining with fluorochromes chromomycin A₃ (CMA₃) and 4'6- diamidino-2- 22 phenylindole (DAPI)**

23

24 Metaphase chromosomes of all the species, except *Ac. echinator*, were stained
25 with the fluorochromes CMA₃ and DAPI for the detection of GC and AT-rich regions,

1 respectively, based on the technique proposed by Schweizer (1980). The *Ac. echinator*
2 samples studied here correspond to the same colonies studied by Barros *et al.* (2016) and
3 the CMA₃/DAPI staining in this species was performed by these authors.

4 **4 - Fluorescent *in situ* hybridization with rDNA 18S probe**

5
6
7 The 18S rDNA probes were obtained by amplification via polymerase chain
8 reaction (PCR) using *Melipona quiquefasciata* Lepeletier, 1836, rDNA primers 18SF1
9 (5'-GTC ATA GCT TTG TCT CAA AGA-3') and 18SR1.1 (5'-CGC AAA TGA AAC
10 TTT AAT CT-3') (Pereira, 2006) in the genomic DNA from the ant *Camponotus rufipes*
11 (Fabricius, 1775). Gene amplification was performed following Pereira (2006). The
12 probes were labeled by an indirect method using digoxigenin-11-dUTP (Roche Applied
13 Science, Mannheim, Germany), and the FISH signals were detected with anti-
14 digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

15 The rDNA 18S genes were mapped by FISH, following the protocol of Pinkel *et*
16 *al.* (1986). The slides were treated with RNase A (100 µg/mL) and kept in a moist
17 chamber at 37°C for 1h. After that, they were washed in 2×SSC for 5 min, incubated in
18 5 µg/ml pepsin in 0.01 N HCl for 10 min, washed in 1×PBS for 5 min and dehydrated in
19 50%, 70% and 100% alcohol series for 2 min each. After this pretreatment, metaphase
20 chromosomes were denatured in 70% formamide/2×SSC at 75°C for 3 min, and 20 µL of
21 hybridization mix including 200 ng of labeled probe, 2×SSC, 50% formamide, and 10%
22 dextran sulfate was denatured for 10 min at 85°C and added on preparations. The slides
23 were kept in a moist chamber at 37°C overnight. Then, the slides were washed in 2×SSC
24 for 5 min; the detection solution including anti-digoxigenin-rhodamine was added on
25 slides that were kept in a moist chamber at 37°C for 1h. The slides were washed in

1 4×SSC/Tween, and dehydrated in an alcohol series. Finally, counterstaining with DAPI
2 (DAPI Fluoroshield, Sigma Aldrich) was performed.

3

4 **5 - Chromosomal analysis**

5

6 Chromosomes were arranged in order of decreasing size and based on the ratio of
7 the chromosomes arm lengths ($r = \text{long arm/short arm}$), according to the classification
8 proposed by Levan *et al.* (1964). The chromosomes were classified as m = metacentric ($r =$
9 $= 1-1.7$), sm = submetacentric ($r = 1.7-3$), st = subtelocentric ($r = 3-7$), and a =
10 acrocentric ($r > 7$); they were organized using Adobe Photoshop® 21.1.1 and measured
11 using Image Pro Plus®. Ideograms of the NOR-bearing chromosome/chromosomes (i.e.,
12 graphical representation of the chromosomes concerning the rDNA clusters) of the ant
13 species were then designed with the Easy Idio software (Diniz and Xavier, 2006).

14 For the fluorochrome staining and FISH 18S rDNA technique, 30 metaphases
15 from at least three individuals of each species were analyzed. In the case of *O. bauri*,
16 which presented a chromosomal polymorphism involving rDNA clusters, seven
17 individuals were analyzed (six females and one male). The metaphases were analyzed
18 and photographed using a fluorescence microscope, Olympus BX60, attached to an image
19 system, QColor Olympus®, with the filters WB (450–480 nm), WU (330-385 nm), and
20 WG (510-550 nm) for the fluorochromes CMA₃, DAPI, and rhodamine, respectively.

21

22 **6 - Phylogenetic relationships**

23

24 The phylogenetic relationship among ant species was determined by associating
25 with previously published molecular phylogenies. The resultant cladogram topology at

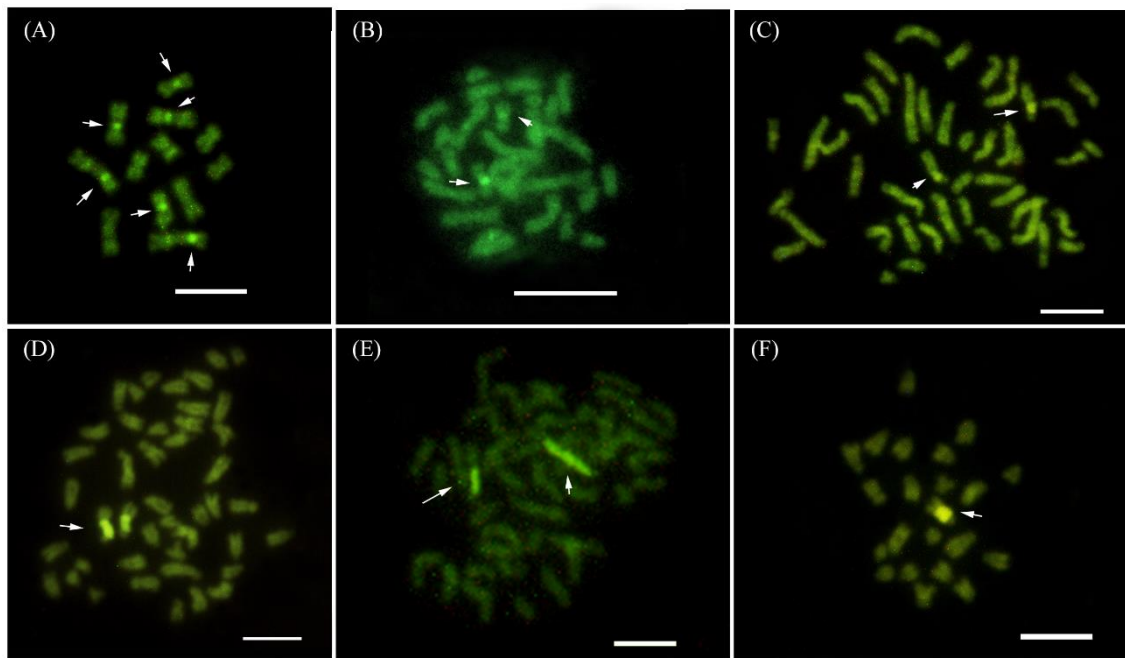
1 the subfamily level was determined following Moreau and Bell (2013). The Poneroid
2 clade topology was determined according to Schmidt (2013) and Larabee *et al.* (2016);
3 the clade topology for the subfamily Dolichoderinae was determined according to Santos
4 *et al.* (2016) and that for the subfamily Myrmicinae was determined according to Bacci
5 *et al.* (2009), Ward *et al.* (2015), Queiroz (2015), Solomon *et al.* (2019), and Micolino *et*
6 *al.* (2019a). The topology of the species groups within the subfamily Myrmeciinae was
7 determined according to Hasegawa and Crozier (2006).

8

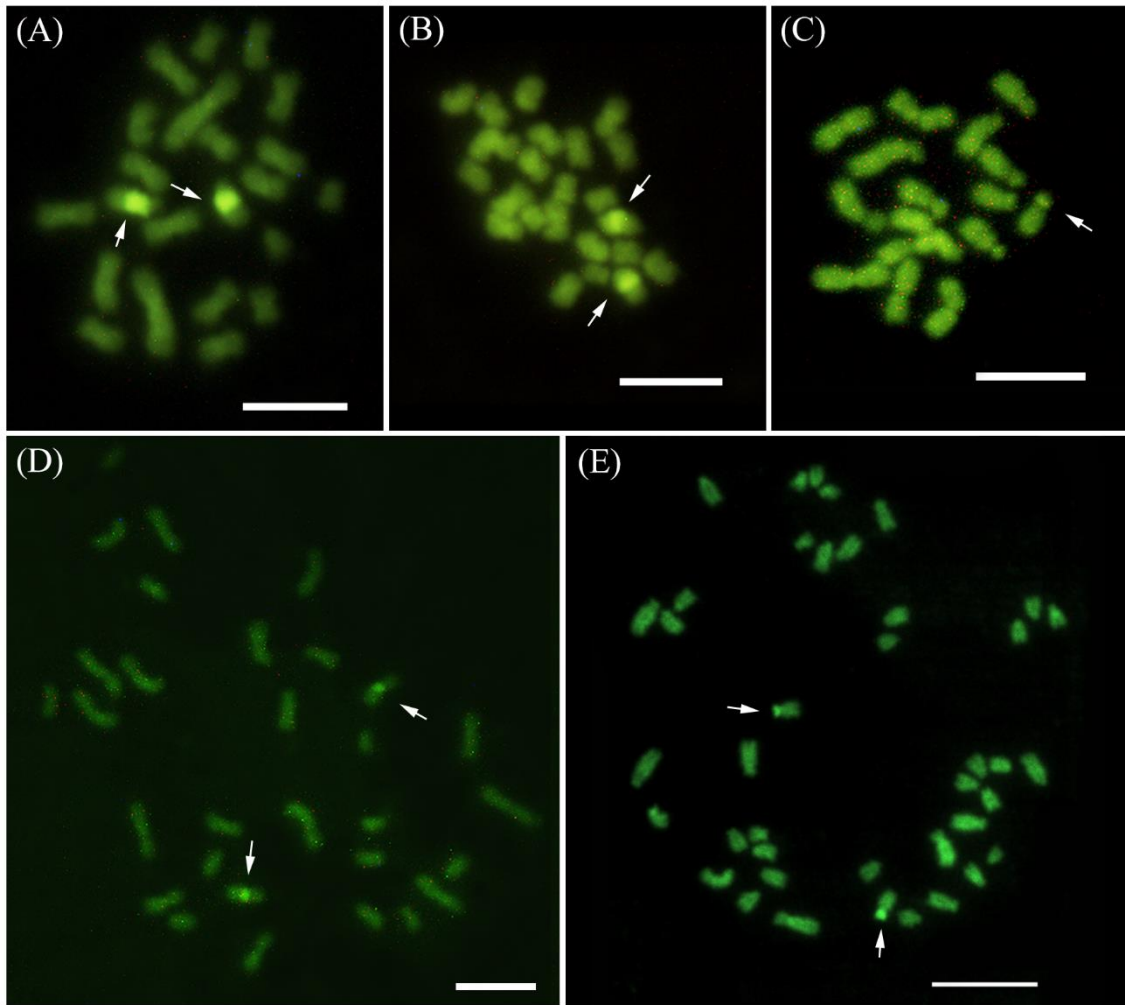
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10

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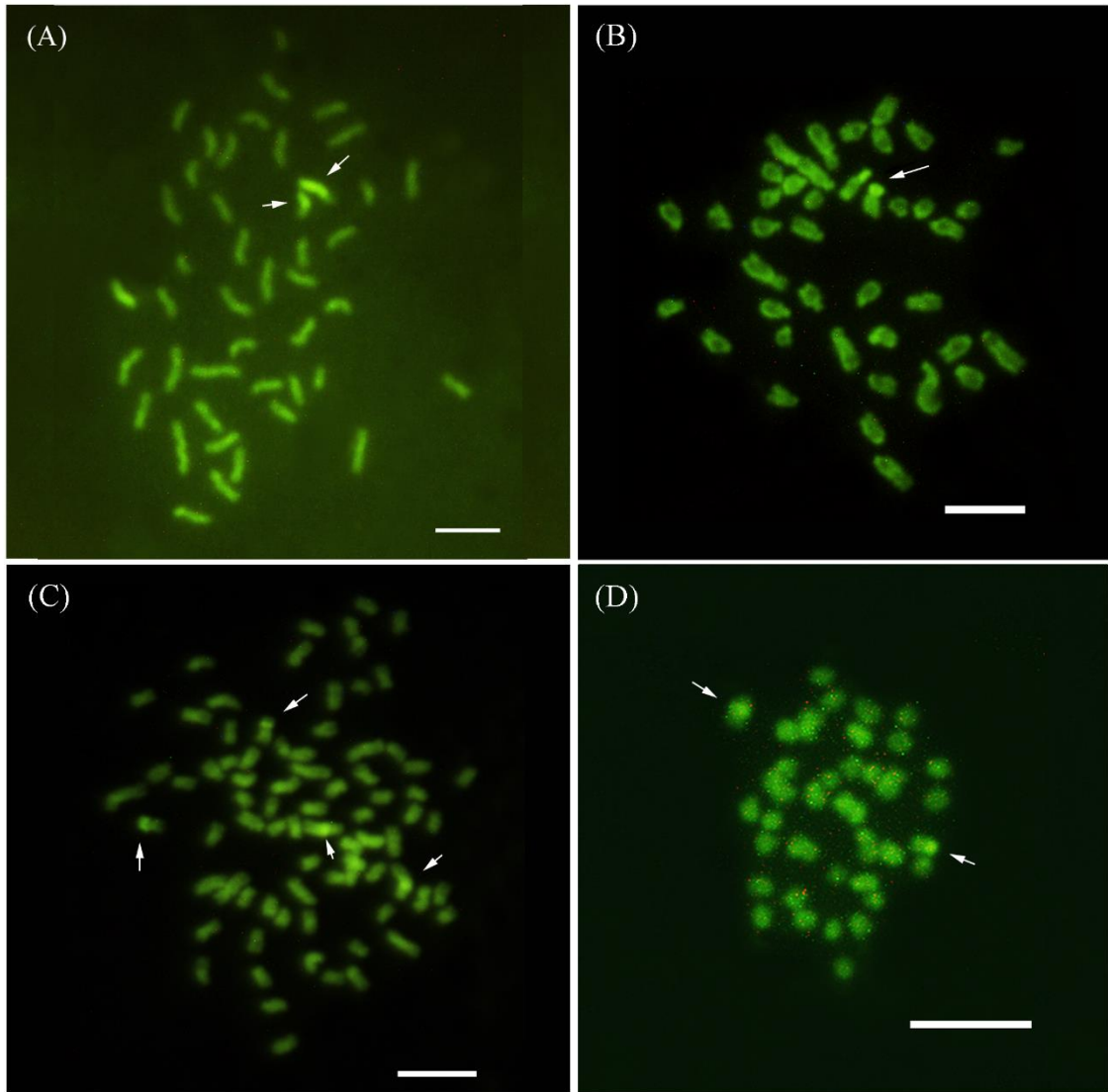


1
 2 **Figure S1.** Staining with chromomycin A₃ showing GC-rich regions (arrows) of
 3 Ponerinae ants: (A) *Pseudoponera gilberti* (2n=12), (B) *Anochetus targionii* (2n=30), (C)
 4 *Odontomachus haematodus* (2n=44, French Guiana), (D, E) *Odontomachus bauri*
 5 showing the homozygous and heterozygous states, respectively (2n=44), and (F) male of
 6 *Odontomachus bauri* with the smaller size regions rich in GC base pairs. Bars = 5 μm.



1

2 **Figure S2.** Staining with chromomycin A₃ showing GC-rich regions (arrows) of
3 Myrmicinae ants: (A) *Pheidole germani* (2n=22), (B) *Crematogaster longispina*
4 (2n=24), (C) *Solenopsis geminata* (n=16), (D) *Myrmicocrypta* sp. (2n=30), (E)
5 *Strumigenys diabolus* (2n=40). Bars = 5 μ m.



1

2 **Figure S3** Staining with chromomycin A₃ showing GC-rich regions (arrows) of an
 3 Ectatomminae ant (A) *Gnamptogenys tortuolosa* (2n=44), and Formicinae ants (B)
 4 *Camponotus atriceps* (2n=40) and (C, D) *Gigantiops destructor* (2n=78, n=39). Bars = 5
 5 μm.

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