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Cytogenetic characterization of the ant *Trachymyrmex fuscus* Emery, 1934 (Formicidae: Myrmicinae: Attini) with the description of a chromosomal polymorphism

Luísa Antônia Campos Barros[a,b], Hilton Jeferson Alves Cardoso de Aguiar[a,b,f], Cléa dos Santos Ferreira Mariano[c,d], Jacques Hubert Charles Delabie[c,e]* & Silvia das Graças Pompolo[a]

[a]Departamento de Biologia Geral; Laboratório de Citogenética de Insetos, Universidade Federal de Viçosa; Viçosa-MG, 36570–000, Brazil; [b]Programa de Pós-graduação em Genética e Melhoramento. Universidade Federal de Viçosa – UFV – MG, Brazil; [c]Laboratório de Mirmecologia, CEPEC/CEPLAC; Itabuna-BA, CP 7, Brazil; [d]Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz; 45650–000 Ilhéus-BA, Brazil; [e]Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz; 45650–000 Ilhéus-BA, Brazil; [f]Universidade Federal do Amapá; Oiapoque – AP, Brazil

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Summary. The genus *Trachymyrmex* is a key group in the tribe Attini because of its close phylogenetic relationship to leaf-cutter ants, *Acromyrmex* and *Atta*. Cytogenetic data are only available for five taxa of *Trachymyrmex*, with chromosome numbers of 2n = 12, 18, 20 and 22, and morphology with predominantly metacentric chromosomes. The aim of the present study was to characterize the karyotype of the ant *Trachymyrmex fuscus* Emery, 1934, by means of the number and morphology of its chromosomes, heterochromatin pattern, CMA_3 and DAPI fluorochromes in the population of two nests collected at Paraopeba, state of Minas Gerais, Brazil. Nineteen females presented 2n = 18 chromosomes (16m + 2sm) and a single male presented n = 9 (8m + 1sm). A size chromosomal polymorphism involving the short arm of the submetacentric pair was confirmed by statistical analysis, with three character conditions: heterozygous SB (with a difference in size between the short arms), standard SS (smaller short arms) and homozygote BB (bigger short arms). In the first nest, both SB and SS workers were observed. The other nest contained heterozygous (SB), homozygous (BB), and a male carrying the B chromosome (larger size). The presence of heterochromatin on all centromeric and pericentromeric chromosomes of *T. fuscus* suggests that the size difference observed in the submetacentric pair in the SB and BB workers is not related to the heterochromatin but to a duplication of euchromatic regions through intra- or inter- chromosomal rearrangements. The fluorochrome CMA_3 matched the C-banding markings, indicating that the heterochromatin is rich in GC base pairs. As far as we know, this is the first chromosomal polymorphism reported in the tribe Attini.

Résumé. Caractérisation cytogénétique de la fourmi *Trachymyrmex fuscus* Emery 1934 (Formicidae : Myrmicinae : Attini) avec la description d'un polymorphisme chromosomique. Le genre *Trachymyrmex* est un groupe clé dans la tribu Attini à cause de sa proximité phylogénétique avec les fourmis coupeuses de feuilles des genres *Acromyrmex* and *Atta*. Des données cytogénétiques sont disponibles seulement pour cinq taxons de *Trachymyrmex*, avec le nombre chromosomal variant de 2n = 12, 18, 20, 22 et une morphologie où predominent les chromosomes métacentriques. Notre objectif est d'étudier le caryotype de la fourmi *Trachymyrmex fuscus* Emery, 1934, en considérant aussi le nombre et la morphologie des chromosomes, la distribution de l'hétérochromatine et des fluorochromes CMA_3 et DAPI dans la population de deux colonies récoltées à Paraopeba, état de Minas Gerais, Brésil. Dix-neuf femelles présentaient 2n = 18 chromosomes (16m + 2sm) alors qu'un unique male possédait n = 9 (8m + 1sm). Un polymorphisme chromosomique de taille impliquant le bras court de la paire submétacentrique a été confirmé par l'analyse statistique, avec trois états: hétérozygote SB (bras courts avec une différence de taille), homozygote "standard" SS (petits bras courts) et homozygote BB (bras courts plus allongés). Dans la première colonie, aussi bien des ouvrières SB que des ouvrières SS workers ont été observées. La seconde colonie renfermait des femelles hétérozygotes (SB), homozygotes (BB), et un male porteur du chromosome B (bras court allongé). La présence d'hétérochromatine sur tous les chromosomes centromériques et péricentromériques de *T. fuscus* suggère que la différence de taille observée dans la paire submétacentrique chez les ouvrières SB et BB n'est pas liée à l'hétérochromatine, mais à une duplication des régions euchromatiques par l'intermédiaire de réarrangements intra- ou inter-chromosomiques. Le marquage par fluorochrome CMA_3 coincide avec celui de bande C, ce qui indique que l'hétérochromatine est riche en paires de bases GC. Autant que nous sachions, il s'agit ici du premier cas connu de polymorphisme chromosomique signalé pour la tribu Attini.

Keywords: karyotype; polymorphism; chromosome banding; evolution; tribe Attini

The tribe Attini is a monophyletic group (Chapela et al. 1994; Schultz & Meier 1995; Mueller et al. 1998; Schultz & Brady 2008; Mehdiabadi & Schultz 2010) and includes 15 genera with 297 species (Schultz & Brady 2008; Brandão et al. 2011). The genus *Trachymyrmex* Forel, 1893 is suspected to be a paraphyletic group (Schultz &

*Corresponding author. Email: jacques.delabie@gmail.com

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Brady 2008; Mehdiabadi & Schultz 2010) and is considered key for understanding the phylogenetics of the "higher" Attini because it is suspected to be the sister group of the leaf-cutter ants *Acromyrmex* Mayr, 1865 and *Atta* Fabricius, 1804 (Wetterer et al. 1998; Brandão & Mayhé-Nunes 2007; Schultz & Brady 2008; Mehdiabadi & Schultz 2010; Cristiano et al. 2013). Furthermore, there are reports of leaf-cutting habits in the *Septentrionalis* species group of *Trachymyrmex* (Schultz & Meier 1995; Schultz & Brady 2008).

Cytogenetic data are available for more than 750 ant taxa (Lorite & Palomeque 2010), with great variation in the chromosome numbers, ranging from 2n = 2 chromosomes in *Myrmecia croslandi* Taylor, 1991 (Crosland & Crozier 1986) to 2n = 120 in *Dinoponera lucida* Emery, 1901 (Mariano et al. 2008). Cytogenetic data are available for five taxa of the genus *Trachymyrmex*, with basic information of the chromosome numbers and morphology only (Murakami et al. 1998; Barros et al. 2013). In this genus, the diploid number ranges from 2n = 12 to 2n = 22, with three taxa studied in the Barro Colorado island, Panama, by Murakami et al. (1998): *Trachymyrmex* sp.1 (2n = 12m), *Trachymyrmex* sp.2 (2n = 18m) and *Trachymyrmex septentrionalis* (McCook, 1881) (2n = 20m), and two taxa in Brazil: *T. relictus* Borgmeier, 1934 (2n = 20m) and *Trachymyrmex* sp. (2n = 22m) (Barros et al. 2013). The difficulties obtaining cytogenetic data are mainly due to the necessity of getting live larvae and deep attine nests are the main cause of the present reduced amount of data available for evolutive discussions.

Banding technique data are available for two *Trachymyrmex* taxa through the detection of heterochromatic regions by C-banding technique. *Trachymyrmex* sp.1 (2n = 12) presented centromeric markings in all chromosomes and interstitial marking in the fourth pair (Murakami et al. 1998) and *T. relictus* showed centromeric markings in all chromosomes (Barros et al. 2013).

The minimum interaction theory (Imai et al. 1994) proposes that chromosomal fission plays a more important role than fusion in ant karyotype evolution, resulting in karyotypes with higher fitness. Although chromosome fissions play an important role in ant karyotype evolution, rearrangements have been reported, including the presence of polymorphisms (reviewed in Lorite & Palomeque 2010).

Sumner (1990) used the expression chromosomal polymorphism to refer only to those cases originating by inversions and other structural rearrangements. Under this definition, only two forms are possible (normal and rearranged) and these are clearly discontinuous. According to White (1973) chromosomal polymorphisms can be found in almost all natural populations.

Cytogenetic studies of the tribe Attini are important for increasing the available data necessary for further comparative studies. Moreover, detailed comparisons between species will only be possible by the application of chromosomal banding techniques. The aim of the present study was to characterize the karyotype of *T. fuscus* by means of its chromosome number, morphology, heterochromatic pattern and base-specific fluorochrome staining.

Materials and methods

Cytogenetic studies were carried out in two nests of *T. fuscus* from Paraopeba, state of Minas Gerais, Brazil (19°17′ S, 44°29′ W) in October 2009. The nests were kept in a 25°C incubation chamber (Biochemical Oxygen Demand, Fanem, Model 347 CD) for larval production at the Laboratório de Citogenética de Insetos, Universidade Federal de Viçosa, MG, Brazil. Mitotic metaphases were obtained from cerebral ganglia or testis by dissecting female or male pharate pupae (soon after meconium rejection) according to Imai et al. (1988). Two colonies (the first with 16 and the second with three workers and a male) with at least 10 metaphases per slide were analyzed. Some slides were analyzed using conventional coloration (Giemsa 4%) and others were submitted to the C-banding technique or fluorochrome staining. Metaphases were observed and photographed using an Olympus® BX 60 microscope attached to a Q Color 3 Olympus® image capture system (Quantitative Imaging Corp., Burnaby, British Columbia, Canada). The karyotypes were assembled by pairing the chromosomes (in females) in order by decreasing size following Levan et al. (1964) using the ratio of the chromosome arms (r). The chromosomes were arranged in karyotypes with Corel Photopaint X3® (Corel Corporation, Ottawa, Canada) and Image Pro Plus® (version 4.5, Image House, Copenhagen, Dinamarca) software. C-banding was done according Sumner (1972) with modifications from Barros et al. (2013). The GC and AT base-specific regions were detected using the fluorochromes Chromomicin A_3 (CMA_3) and 4',6-diamidino-2-phenylindole (DAPI), respectively (Schweizer 1980).

The *t*-test was applied to test any possible size difference between the arms of the only submetacentric chromosome pair of *T. fuscus*. Nineteen workers were used for contrasting the mean length of the short arms between the homologous chromosomes and the same was done for long arms to identify differences in the chromosome. The workers that did not present size difference between short arms of the submetacentric pair (including a male) were analyzed using the F test for variance analysis using the mean ratio of the submetacentric pair (long arm/short arm) of eight workers and a male; the chromosome means were compared by the Tukey test if ANOVA was significant. The significance value in the statistic tests adopted in the present study was $p \leq 0.05$ and the software used was SigmaStat® 3.0 (Systat Software, San Jose, CA, USA).

The standard submetacentric chromosome of *T. fuscus* was identified by the letter S (small) and the chromosome that presented size differences compared with the homologous standard was denominated as B (big). Therefore, workers could be heterozygous SB (with a difference in size between the short arms), standard SS (smaller short arms) or homozygous BB (bigger short arms).

Imagoes of both colonies were deposited in the ant collection at the Laboratório de Mirmecologia do Centro de Pesquisas do Cacau (CPDC/Brazil), under the record #5570.

Results

Trachymyrmex fuscus females presented 2n = 18 chromosomes (16m + 2sm) (Figure 1A–C, E, F) and males n = 9 chromosomes (8m + 1 sm) (Figure 1D). All chromosomes presented markings for C-banding (Figure 1E) and

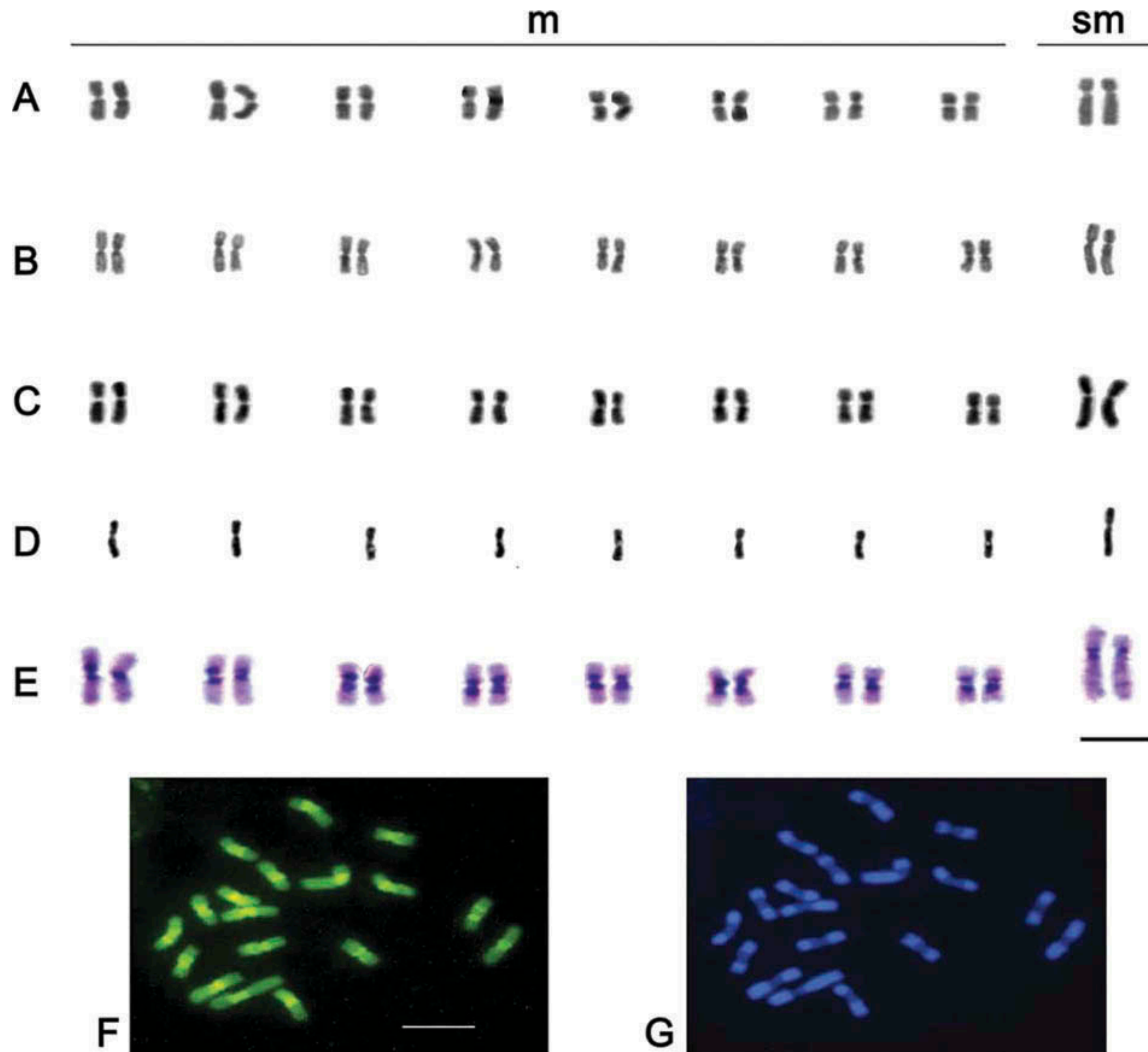

Figure 1. Karyotype of *Trachymyrmex fuscus* ($2n$ = 18, n = 9): **A,** female with the standard submetacentric pair SS (smaller short arms); **B,** heterozygous female SB for the polymorphism in the submetacentric pair (with a difference in size between the short arms); **C,** homozygous female BB (bigger short arms); **D,** male with increased size in the submetacentric chromosome (B); **E,** C-banding in heterozygous female for the polymorphism, indicating centromeric and pericentromeric markings; **F,** CMA_3, and **G,** DAPI staining in heterozygous female for the polymorphism, indicating centromeric and pericentromeric markings for CMA_3 and complementary negative markings in the same regions for DAPI. Scale bar = 5 µm.

fluorochrome CMA_3 (Figure 1F) at the centromeric and pericentromeric regions. The fluorochrome DAPI presented negative markings for the same regions stained with the CMA_3 and specific markings for DAPI were not observed in the chromosomes of *T. fuscus* (Figure 1G).

A statistical size difference was observed in the short arm between the homologous of submetacentric pair in *T. fuscus* (Table 1) confirming cytological observations (Figure 1). It was also possible to differentiate three groups of individuals from the two colonies (Table 2): standard SS (smaller short arms) (Figure 1A), heterozygous SB (with size difference between the short arms: a small and a big short arms) (Figure 1B, E, F) and homozygous BB (bigger short arms) (Figure 1C, D). It was possible to differentiate individuals from each colony, as those from colony 1 presented nine SB workers and seven SS workers, whereas colony 2 had two SB workers, one BB worker and a B male.

Discussion

All chromosomes of *T. fuscus* females (2n = 18) presented metacentric morphology, with the exception of the biggest chromosome pair being submetacentric. Similarly the five

Table 1. Chromosome arm measurements: comparison of the short and long arms mean length (in micrometers) of the ant *Trachymyrmex fuscus* submetacentric chromosome pair (sm) here denominated chromosome sm1 and sm2. A total of 19 workers from two colonies (16 of Colony 1 and three of Colony 2) was submitted to the *t*-test. SB = with a difference in size between the short arms; B = big chromosome, S = small chromosome

		Short arm (mean)[1]				Long arm (mean)[1]			
Worker	Colony	Chromosome sm1	Chromosome sm2	*p*-value	Cytological correspondence	Chromosome sm1	Chromosome sm2	*p*-value	Cytological correspondence
1	1	2.126	1.393	0.006*	Heterozygous worker (SB)	3.534	3.419	0.833	No difference
2		1.581	1.194	0.010*	Heterozygous worker (SB)	2.598	2.605	0.987	No difference
3		1.813	1.189	0.005*	Heterozygous worker (SB)	2.779	2.895	0.787	No difference
4		2.217	1.552	0.026*	Heterozygous worker (SB)	3.469	3.430	0.943	No difference
6		1.186	1.111	0.373	No difference	2.781	2.656	0.446	No difference
7		1.767	1.252	0.049*	Heterozygous worker (SB)	2.802	2.833	0.952	No difference
8		1.416	1.327	0.555	No difference	3.066	3.121	0.905	No difference
9		0.996	0.925	0.439	No difference	2.240	2.245	0.983	No difference
10		1.310	1.224	0.562	No difference	2.846	2.838	0.985	No difference
11		1.718	1.284	< 0.001*	Heterozygous worker (SB)	2.949	2.793	0.343	No difference
12		1.391	1.109	0.002*	Heterozygous worker (SB)	2.587	2.582	0.977	No difference
13		1.434	1.115	0.006*	Heterozygous worker (SB)	2.417	2.386	0.916	No difference
14		1.332	1.257	0.619	No difference	3.039	3.069	0.920	No difference
15		1.273	1.017	0.036*	Heterozygous worker (SB)	2.188	2.179	0.983	No difference
16		1.343	1.241	0.408	No difference	2.962	2.918	0.876	No difference
17		1.255	1.176	0.616	No difference	2.758	2.611	0.729	No difference
1	2	1.620	1.255	0.030*	Heterozygous worker (SB)	2.759	2.820	0.842	No difference
3		1.852	1.343	0.043*	Heterozygous worker (SB)	2.902	3.062	0.705	No difference
5		1.474	1.354	0.285	No difference	2.573	2.466	0.600	No difference

*Significant by *t*-test, at the 5% probability level;
[1]Obtained from 10 metaphases.

Table 2. Mean ratios between long/short arms of the submetacentric chromosome pair of *Trachymyrmex fuscus* using the measurements of 10 metaphases of the workers that did not present significant differences between the short arm sizes of the submetacentric pair. The measurement of a male that belongs to the second colony is also included. B = big chromosome; S = small chromosome.

Individual	Colony	Mean ratio	Cytological correspondence
6	1	2.360 a	Standard worker (SS)
14	1	2.304 a	Standard worker (SS)
9	1	2.257 a	Standard worker (SS)
16	1	2.194 a	Standard worker (SS)
17	1	2.174 a	Standard worker (SS)
10	1	2.147 a	Standard worker (SS)
8	1	2.122 a	Standard worker (SS)
5	2	1.753 b	Homozygous worker (BB)
male	2	1.627 b	Hemizygous male (B)

Note: Means followed by the same letter are not different at 5% probability, using Tukey test.

taxa of the genus already studied for cytogenetics also presented metacentric chromosomes. *Trachymyrmex fuscus* presented the same chromosome number of *Trachymyrmex* sp.2 as studied by Murakami et al. (1998).

The size difference between the short arms of the submetacentric pair in *T. fuscus* through statistical analysis confirmed the presence of a chromosomal polymorphism. In colony 1, nine workers were SB and seven SS; therefore, about half of them (56.2%) presented the heterozygous pair. Our interpretation is that the queen founder of colony 1 was heterozygous (SB) while her haploid mate (the male reproducer) presented the smaller chromosome (S), since only standard (SS) and heterozygous (SB) individuals were observed in her offspring. The probability of finding this phenotype in the offspring of a parental generation with this genotype is theoretically 50%, if the polymorphism does not decrease the fitness of the heterozygous individuals. In colony 2 both heterozygous (SB) and homozygous (BB) pairs were observed, in addition to one male bearing the B chromosome. Our interpretation is that the founder was heterozygous (SB) while her haploid mate presented the bigger submetacentric chromosome (B). These results confirm this species as monogynous and monoandrous (Villesen et al. 2002; Mehdiabadi & Schultz 2010).

Tandem duplication due to unequal crossing-over between homologous chromosomes (Schubert & Lysak 2011) may have originated this kind of polymorphism (Figure 1). Another possibility that cannot be disregarded is the polymorphism arising by non-homologous chromosome translocations, which in practice results in the observed polymorphism (Imai et al. 1977). The different situations may originate a chromosome bearer of tandem duplication for one or more loci and another with a deficiency, beyond the standard chromosome initially present. The translocations are more common in the family Formicidae in species with low chromosome numbers ($n \leq 12$) (Imai et al. 1977), such as *T. fuscus* which presents $n = 9$. The existence of pericentromeric inversion could be discarded because there is no difference by the length of the long arms (Table 1).

Duplications can become fixed in the populations and also may not be neutral since duplicated genes can produce higher quantities of the genic product (Imai et al. 1977; Camacho et al. 1986; Futuyma 2003). The grasshopper *Oedipoda fuscocincta* Lucas, 1849 (Orthoptera, Acrididae) presented higher activity of the nucleolus organizer regions (NORs) due the duplication of these genes, because of a translocation between non-homologous chromosomes (Camacho et al. 1986).

Moreover, genic loci that are present in duplicate can accumulate independent mutations and acquire new functions, by becoming paralogous, without disturbing the adaptability of the organism, since the unmodified alleles could perform the original function of the locus (Bridges 1935; Stebbins 1970; Koszul & Fischer 2009), eventually contributing to speciation events (Bridges 1935; Palomeque et al. 1993).

Structural chromosomal alterations are not always easy to observe by means of light microscopy, especially if they involve small chromosomal regions (White 1973). However, in mitotic or meiotic chromosomes, duplicated segments have already been observed in different organisms such as grasshoppers (Camacho et al. 1984, 1986, Camacho & Cabrero 1987; Rufas et al. 1988) and fishes (Maistro et al. 2001).

Chromosomal duplications have been identified in different ant subfamilies such as Dolichoderinae, Formicinae and Myrmicinae (Imai et al. 1977; Palomeque et al. 1993). This kind of polymorphism is probably a frequent means of chromosomal variation in ants, and studying the characteristics and behavior of polymorphisms in these organisms will throw some light on the origin and evolution of the chromosome modifications. The species studied here, *T. fuscus*, has the first reported chromosomal polymorphism in the tribe Attini.

Imai et al. (1977) reported the occurrence of a chromosomal polymorphism involving chromosomal duplication in *Monomorium* sp.1, *Crematogaster* sp.1 and *Monomorium rothsteini* Forel, 1902. In the latter species, two colonies were observed without chromosomal duplication; a third colony was with heterozygous and homozygous females, and a fourth had females homozygous for the duplication only.

It is improbable that the inverse process (e.g. the deletion of part of the short arm) occurred in *T. fuscus*, because deletions of euchromatic parts of the chromosome are generally deleterious and have less significance for the evolution of the organism (Stebbins 1970; Imai et al. 1977; Futuyma 2003, Schubert & Lysak 2011).

The C-banding technique indicated the presence of heterochromatic blocks restricted to the centromeric and pericentromeric regions of all chromosomes of *T. fuscus*. Consequently, the size differences observed in the submetacentric pair in the SB and BB individuals did not correspond to the differential heterochromatic growth, but to the differences in the euchromatic region of the short arm of one or both homologous chromosomes, indicating the duplication of a euchromatic region. The fluorochrome CMA_3 presented centromeric and pericentromeric markings (Figure 1E) coinciding with the same pattern observed with the C-banding technique. The GC-rich heterochromatin was also found in other two attines of different genera: *Mycocepurus goeldii* (Barros et al. 2010) and *Acromyrmex striatus* (Cristiano et al. 2013). The existence of GC-rich heterochromatin (CMA^+) is unusual, since commonly insect heterochromatin is AT rich (Palomeque & Lorite 2008). As ongoing cytogenetic investigation in attine ants is on course (Barros et al., in preparation), so far it seems that the GC richness of the constitutive heterochromatin is not as rare as once it was thought to throughout the tribe Attini.

Extra chromosome segments are usually heterochromatic; however there are reports of species with duplications of euchromatic chromosomal segments (Camacho et al. 1984; Palomeque et al. 1993), such as those we saw in *T. fuscus*, which presented a duplication in a euchromatic region.

The presence of heterochromatin in *T. fuscus* was observed in both centromeric and pericentromeric regions. This differs from *Trachymyrmex relictus* (Barros et al. 2013) which only presents centromeric markings in all chromosomes, and *Trachymyrmex* sp. 1 (2n = 12) which also presents centromeric heterochromatin in all the chromosomes with interstitial markings in four of them (Murakami et al. 1998).

Chromosomal rearrangements represents an important key to better understanding the evolution of the genus *Trachymyrmex*, therefore mapping the location and composition of heterochromatin in future studies in other species of *Trachymyrmex* will provide more information on the chromosomal rearrangements involved in the evolution of the tribe Attini. Moreover, the microdissection and the construction of a probe for the short arm of the submetacentric chromosome of *T. fuscus* and hybridization in different individuals (SS, SB and BB) would be informative and contribute to understanding how this polymorphism arises.

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