ANA PAULA ALVES SILVA

DESCRIÇÃO CITOGENÉTICA DE 13 MORFOESPÉCIES DE *Solenopsis* Westwood, 1840 (HYMENOPTERA: FORMICIDAE)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Genética e Melhoramento, para a obtenção do título de *Doctor Scientiae*.

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Luísa Antônia Campos Barros

Denilce Meneses Lopes

Carlos Roberto de Carvalho

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Silvia das Graças Pompolo
(orientadora)

“Uma vida sem desafios não vale a pena ser vivida.”

Sócrates

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SUMÁRIO

RESUMO

ALVES-SILVA, Ana Paula, D.Sc., Universidade Federal de Viçosa, novembro de 2016. **Descrição citogenética de 13 morfoespécies de *Solenopsis* Westwood, 1840 (Hymenoptera: Formicidae)**. Orientadora: Silvia das Graças Pompolo. Coorientador: Jorge Abdala Dergam.

O gênero *Solenopsis* tem aproximadamente 195 espécies que são popularmente conhecidas como formigas de fogo ou formiga lava-pé. Este gênero possui uma taxonomia difícil a nível morfológico, estando entre as mais complicadas dentre as formigas. Algumas espécies deste gênero, conhecidas como “thief ant”, são predadoras de larvas de *S. invicta* e poderiam ser usadas em seu controle, mas isso não é possível devido à dificuldade na sua identificação. O objetivo principal deste estudo foi descrever citogeneticamente o gênero *Solenopsis*. Para isso, foram analisados 30 ninhos utilizando técnicas da citogenética clássica (como coloração convencional com Giemsa, banda C e coloração sequencial com DAPI/CMA_3) e citometria de fluxo. Dentre os ninhos analisados foram encontrados 13 morfoespécies. Por meio da citogenética clássica, encontramos 7 números diploides e 11 diferentes fórmulas cariotípica. Quatro dos sete números de cromossomos são descritos pela primeira vez neste estudo para *Solenopsis* (2n = 24, 26, 28 e 42 cromossomos). Os dados de banda C mostraram que o aumento da quantidade de heterocromatina segue o aumento do número diploide, e essa diferença é mais evidente quando se compara os extremos (2n = 22 e 2n = 42 cromossomos). A identificação das regiões ricas em CG usando CMA_3 possibilitou a identificação uma possível inversão pericêntrica e de uma translocações. A citometria de fluxo foi utilizada para compreender se as células poliploides apresentadas pelas espécies do gênero *Solenopsis* são naturais e se o número de células poliploides se mantém constante durante o desenvolvimento. Para tanto, determinou-se a ploidia de células do gânglio cefálico de 4 diferentes estágios de desenvolvimento da espécie *Solenopsis saevissima* (larvas, pré-pupas jovens, pré-pupas velha e pupas) com base na medição do conteúdo de DNA nuclear. Os dados mostraram a presença de células diploides e tetraploides, e estas células apresentaram diferentes proporções dependendo da fase de

desenvolvimento analisada. As células tetraploides apresentaram uma diminuição na sua representatividade com a evolução do desenvolvimento da formiga, sugerindo que elas não são permanentes no tecido neural e, provavelmente, não estarão presentes neste tecido em formigas adultas. Esse comportamento difere do que acontece com as células diploides, que tem o seu pico de proliferação no estágio de pré-pupa jovem e em seguida apresentaram uma redução progressiva no número de células em divisão.

ABSTRACT

ALVES-SILVA, Ana Paula, D.Sc., Universidade Federal de Viçosa, November, 2016. **Cytogenetic description of 13 morphospecies of *Solenopsis* Westwood, 1840 (Hymenoptera: Formicidae)**. Advisor: Silvia das Graças Pompolo. Co-advisor: Jorge Abdala Dergam.

The genus *Solenopsis* has approximately 195 species that are popularly known as 'fire ants'. This genus has a complex taxonomy at the morphological level, figuring among the more complicated among ant species. Some species in this genus, known as 'thief ants', are predators of *S. invicta*'s larvae and could be used in the control of this pest, but this is not possible due difficulty in its identification. The main objective of this study was to describe, cytogenetically, the genus *Solenopsis*. For this, we used techniques of the classic cytogenetics (such as conventional staining with Giemsa, C band, and sequential staining with DAPI/CMA_3) and flow cytometry. We collected a total of 30 nests belonging to 13 morphospecies. By the classic cytogenetics, we found 7 diploid numbers and 11 different karyotypes formulae. Four out of seven chromosome numbers are described for the first time in this study for *Solenopsis* (2n = 24; 26; 28 e 42 chromosomes). C-banding data showed that the increase in the amount of heterochromatin follows the increase in diploid number, and this difference is more evident when comparing the extreme (2n = 22 and 2n = 42 chromosomes). The recognition of the region rich in CG using CMA3 became possible the identification one inversion pericentric, beyond possible translocations. The flow cytometry was used to understand whether the polyploid cells presented by species of the genus *Solenopsis* in the classic cytogenetic research are natural and if the number of polyploid cells keeps constant during development. For this, we determined the DNA ploidy of cephalic ganglion cells of workers in 4 different development stages (larvae, young prepupae, old prepupae and pupae) based on the measurement of nuclear-DNA content. The data showed the presence of diploid and tetraploid cells, and these cells presented different proportions depending of the development stage analysed. The tetraploid cells presented a decrease in its representativity with the advance of the ant development, suggesting that they

are not permanent in the neural tissue and, probably, will not be present in this tissue of the adult ants. This is different to what happens with diploid cells that had its proliferation peak in young prepupae stage, and then presents a significant progressive reduction in the number of cells during division.

INTRODUÇÃO GERAL

A família Formicidae (Hymenoptera) engloba aproximadamente 13 mil espécies válidas de formigas (BOLTON 2016). Esses animais são definidos como insetos sociais, devido a suas colônias possuírem castas diferenciadas em rainhas que produzem prole e operárias altruístas não reprodutivas que recolhem e processam alimentos, cuidam dos jovens, constroem ninhos e defendem as colônias, além da ocorrência de sobreposição de geração (HÖLLDOBLER & WILSON 1990).

Esses insetos apresentam um alto grau de adaptabilidade, podendo ocorrer em abundância na maioria dos ambientes terrestres, com exceção dos polos. Apesar do importante papel ecológico que desempenham, as formigas podem causar enormes prejuízos econômicos, seja no meio agrícola, como praga das mais diversas plantações, ou no meio urbano, como um perigo potencial à saúde pública (HÖLLDOBLER & WILSON 1990, FOWLER & al. 1993, DELLA LUCIA & SOUZA 2011). O seu grande poder de dispersão nos ambientes e a ausência de inimigos naturais faz com que algumas espécies de formigas sejam invasoras em potencial para certos pontos do globo (PORTER & SANVIGNANO 1990, HUMAN & GORDON 1996).

O gênero *Solenopsis*, atualmente possui 195 espécies válidas (BOLTON 2016). Duas delas, *S. saevissima* e *S. invicta,* são popularmente conhecidas como formiga de fogo, formiga-lava ou lava-pé, devido à ferroada dadas pelas operárias (PITTS & al. 2005). Essas espécies são nativas da América do Sul (ASCUNCE & al. 2011), e foram acidentalmente introduzidas no sul dos Estados Unidos no início do século XX. Elas são consideradas pragas com grande impacto nas áreas urbanas e rurais, além de acarretar grandes prejuízos à diversidade local, uma vez que leva ao desalojamento de algumas espécies nativas (WOJCIK & al. 2001). A *S. invicta* foi considerada, durante a década de 2000, uma das 100 piores espécies exóticas invasoras, tornando-se um problema global, quando invadiu ecossistemas nas ilhas do Caribe, na Oceania e na Ásia (LOWE & al. 2000, DAVIS & al. 2001, NATTRASS & VANDERWOUDE 2001, ZHANG & al. 2007).

O gênero *Solenopsis* tem uma taxonomia difícil a nível morfológico, sendo dentre as formigas uma das mais complicadas. Isto ocorre, em parte, por causa do tamanho corporal das espécies, que pode chegar a menos de 1 mm de comprimento, além da ausência de características estruturais e corporais distintivas (PACHECO & MACKAY 2013). Estas deficiências têm várias consequências, como o caso de algumas espécies no subgênero *Diplorhoptrum*, conhecido como "thief ants". Estas formigas são predadores de larvas de *S. invicta* e poderiam ser utilizadas no controle dessa praga, mas isto não é possível devido à dificuldade na identificação das espécies (PACHECO & MACKAY 2013).

Dados citogenéticos têm sido utilizados na elucidação da taxonomia de alguns grupos de animais, tais como em gêneros *Pachycondyla* e *Dolichoderus* (MARIANO & al. 2012, SANTOS & al. 2016). Apesar disso, há poucos estudos que utilizam essa ferramenta para o gênero *Solenopsis*, apenas oito espécies foram citogeneticamente estudadas. As espécies pertencentes ao subgênero *Solenopsis* (*S. invicta*, *S. saevissima*, *S. xyloni*, *S. geminata* e *S. aurea*) apresentam número diploide (2n) igual 32 cromossomos (CROZIER 1970, CROZIER 1975, GLANCEY & al. 1976, GOÑI & al. 1982, IMAI & al. 1984, TABER & COKENDOLPHER 1988). Por outro lado, aqueles que pertencem ao subgênero *Diplorhoptrum* (*S. fugaz* e *S. molesta*) mostram 2n = 22 cromossomos, e uma espécie não identificada de *Solenopsis,* coletada na Malásia*,* possui 2n = 38 (CROZIER 1970, LORITE & PALOMEQUE 2010). Estudos utilizando técnicas diferentes da coloração convencional com Giemsa não foram realizados para esse gênero.

A determinação de sexo em *Solenopsis*, assim como na maioria dos himenópteros, é dada por haplodiploidia. Nesse sistema, o sexo é determinado pelo número de conjuntos completos de cromossomos, sendo os machos, geralmente, indivíduos haploides originados de ovos não fecundados (partenogênese), enquanto as fêmeas são diploides originadas de ovos fecundados (HEIMPEL & BOER 2008). Entretanto, a ocorrência de macho diploide tem sido descrito para algumas formigas, por exemplo, *S. invicta*. O estudo realizado com um ninho dessa espécie, coletado em Hurly (Mississippi), mostrou que um terço dos indivíduos machos analisados apresentava número diploide igual a 32 cromossomos (GLANCEY & al. 1976). A presença de fêmeas triploides foi descrita em algumas populações de *S. invicta* que possuem altos índices de

machos diploides, sendo estas fêmeas originadas de ovos fecundados por espermatozoides de machos diploides (KRIEGER & al. 1999). Em outras espécies como *Aphaenogaster osimensis* (IMAI & YOSIDA 1966)*, Linepithema humile* (ARON & al. 2003)*, Crematogaster* sp.*., Camponotus ligniperda* e *Pheidole pallidula* (CROZIER 1975) foi observada a presença de células poliploides no tecido neural de larva de rainhas e/ou operárias.

O objetivo dessa tese foi estudar citogeneticamente o gênero *Solenopsis*. Para isso ela foi estruturada em dois capítulos independentes. No primeiro utilizou-se técnicas de citogenética clássica para caracterizar as morfoespécies quanto à morfologia cromossômica, padrão de distribuição e quantidade de heterocromatina, além do posicionamento e número de regiões ricas em nucleotídeos CG e AT.

No segundo capítulo, a técnica de citometria de fluxo foi empregada para confirmar a presença de células poliploides no tecido neural de larvas e pupas de operárias de *Solenopsis*. Essas células foram observadas em todas as morfoespécies estudadas no capitulo 1 dessa tese. Além de ratificar a presença de células poliploides, foi descrito o comportamento dessas células durante o desenvolvimento larval e das pupas para esse gênero.

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Capítulo I

Cytogenetic diversity of the genus *Solenopsis* Westwood, 1840 (Hymenoptera: Formicidae) in southeast Brazil.

Cytogenetic diversity of the genus *Solenopsis* Westwood, 1840 (Hymenoptera: Formicidae) in southeast Brazil.

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ABSTRACT

The genus *Solenopsis* has 195 species that are popularly known as 'fire ants'. This genus has a complex taxonomy at the morphological level, figuring among the more complicated among ant species. Some species in this genus, known as 'thief ants', are predators of *S. invicta* larvae and could be used in pest control, but this is not possible due difficulty in its identification. We used techniques of the classic cytogenetics to obtain data, which can help us to understand the karyotype evolution of the genus *Solenopsis*. We collected a total of 33 nests, belonging to 13 morphospecies, with 7 diploid numbers and 11 different karyotypes formulae. Four out of seven chromosome numbers are described for the first time in this paper for *Solenopsis* (2n = 24; 26; 28 e 42 chromosomes). C-banding data showed that the increase in the amount of heterochromatin follows the increase in diploid number, and this difference is more evident when comparing the extreme (2n = 22 and 2n = 42 chromosomes). The identification of the region rich in CG using CMA_3 became possible to identify one inversion pericentric, beyond possible translocations. *Solenopsis* presented a complex karyotype evolution, being of great importance to the understanding of karyotypic evolution in Formicidae as well as in Hymenoptera.

Keywords: fire ants; heterochromatin, DAPI/CMA_3, inversion, fission

1 – INTRODUCTION

The genus *Solenopsis* has 195 species (BOLTON 2016) that are popularly known as 'fire ants'. Within this genus, the species *Solenopsis saevissima* (Smith, 1855) and *S. invicta* Buren, 1972, native to South America (ASCUNCE & al. 2011), became pests of economic importance when they were accidently introduced in the USA, Caribbean Islands, Oceania, and Asia (DAVIS & al. 2001, NATTRASS & VANDERWOUDE 2001, ZHANG & al. 2007). They cause damages to humans, wild animals, agriculture, and electronic equipment, resulting in great expense in its control (PITTS & al. 2005, PACHECO & MACKAY 2013). On the 2000's, *S. invicta* was considered one of 100 worst exotic invasive species on a global level (LOWE & al. 2000).

The genus *Solenopsis* has a complex taxonomy at the morphological level, figuring among the more complicated among ants. This occurs, in part, because of the species body size that can be as small as 1 mm of body length, and absence of distinctive sculpture and body characteristics (PACHECO & MACKAY 2013). These impairments have several consequences, such as the case of some species in the subgenus *Diplorhoptrum,* known as 'thief ants'. These ants are predators of *S. invicta*'s larvae and could be used in pest control, but this is not possible due to the difficult identification of those species (PACHECO & MACKAY 2013).

Cytogenetic data is a very useful tool, and has been used in the taxonomic elucidation of some ant genera, such as *Pachycondyla* and *Dolichoderus* (MARIANO & al. 2012, SANTOS & al. 2016). However, there are few studies using such tools for the genus *Solenopsis*, where only eight species of two subgenera (out of the 10 described) have their cytogenetic data studied. The species belonging to subgenus *Solenopsis* (*S. invicta*, *S. saevissima*, *S. xyloni*, *S. geminata* and *S. aurea*) present diploid number (2n) equal to 32 chromosomes (CROZIER 1970, GLANCEY & al. 1976, GOÑI & al. 1982, IMAI & al. 1984, TABER & COKENDOLPHER 1988). Conversely, those belonging to subgenus *Diplorhoptrum* (*S. fugax* e *S. molesta*) show 2n = 22 chromosomes, and one unidentified species of *Solenopsis* with 2n = 38 (CROZIER 1970, LORITE & PALOMEQUE 2010). None of those species have resulted from banding or other cytogenetic techniques.

Considering the small amount of cytogenetic data on genus *Solenopsis*, we use techniques of the classic cytogenetic to obtain data about chromosome number and morphology, composition of heterochromatin, and rich regions in CG and AT. With this data, we aim to better understand the karyotypical evolution within the genus *Solenopsis*.

2 - MATERIAL AND METHODS

The cytogenetic analysis was conducted using nests collected in two localities: 1) Viçosa - Atlantic Forest secondary semideciduous (20º45'23''S, 42º52'25''W); and 2) Turmalina - Secondary fragment of Cerradão (17˚27'22.3''S, 42˚37'53.5''W). Both are in Minas Gerais, Brazil, with approximately 387 km of distance between them in a straight line (Figure 1). The National Collecting Permit was issued to Ana Paula Alves Silva (SISBIO 46427-2). The nests were transported and kept in plastic containers until we obtained larvae suitable for the protocols.

The adult specimens were assigned to morphospecies by Júlio Cézar Mário Chaul and deposited in the myrmecological collection of Laboratório de Ecologia de Comunidades, Universidade Federal de Viçosa. Morphospecies were determined by comparing a series of morphological character states presented by the workers of the karyotyped colonies. The most important characters and their states used to separate morphospecies were: the relation between the shape of the cephalic capsule; the overall shape of mesosoma; the metanotal impression; the shape of the propodeum; the degree of development of the propodeum spiracle; the shape and size of the petiole; the setae morphology; the sculpturing in the meso and metapleurae and sides of the propodeum; the body color and size were informative and used to reinforce morphospecies concepts because nest series were available and, therefore, intracolonial size differences and differential color by age of individual workers were not a source of confusion.

To obtain the metaphases, we used the cephalic ganglion of the larvae, following IMAI & al. (1988). Five individuals of each nest, with an average of 20 metaphases, were stained with Giemsa 5%. The chromosomes were classified based on centromere position, using the arm ratio (LEVAN & al. 1964). The

heterochromatic blocks were revealed by C band protocol (SUMNER 1972). Finally, the characterization of richness of CG and AT along with the chromosomes was done by 4'6-diamidino-2-phenylindole (DAPI) fluorochromes and Chromomycin A_3 (CMA_3) (SCHWEIZER 1980), respectively.

3 – RESULTS

We collected a total of 33 colonies belonging to 13 distinct morphospecies, seven diploid numbers (2n = 22, 24, 26, 28, 32, 38, and 42 chromosomes), and 11 different karyotypic formulae (Figures 2-6) (see Table 1).

All species with 22 chromosomes and *Solenopsis* sp. 1 and sp. 7 presented heterochromatic blocks in the interstitial region of the short arms in one pair of metacentric chromosomes in addition to small heterochromatic blocks in the centromere regions of some chromosomes (Figure 7a, b, and d). *Solenopsis* sp. 4 had blocks in the interstitial region of the short arms in two pairs of metacentric chromosomes (Figure 7c). All other karyotypes do not present heterochromatin in the interstitial region, but they present heterochromatic blocks in the centromeric region of all pairs of chromosomes and in the short arms of subtelocentric chromosomes (Figure 7e-i).

The sequential staining with DAPI/CMA_3 showed the absence of AT-rich regions, but it indicated the presence of CG-rich regions that varied between and within different chromosomes' numbers. The karyotypes with diploid number between 2n = 22 and 28 chromosome showed positive CMA_3 in the interstitial region in the short arm of one pair of metacentric or submetacentric chromosome (Figures 8f and 10b), or in the interstitial region of the long arm of a pair of subtelomeric chromosomes (Figures 8d, 9b, and 10d). Except *Solenopsis* sp. 6 with 26 chromosomes that presented marks in the centromeric region of some chromosomes (Figure 9d). The *Solenopsis saevissima* had positive CMA_3 in telomeric regions of two pairs of subtelocentric chromosomes (Figure 11b). *Solenopsis* sp.13, 2n = 38, showed only one chromosome with rich mark in CG in the interstitial region of a submetacentric chromosome (Figure 11d). The *Solenopsis sp.* 2, with 42 chromosomes, presented this number ranging between 4 and 5 chromosomes, all of them in telomeric regions of the short arm of the

submetacentric chromosomes (Figure 12b and d). Unfortunately, we were unable to obtain banding data from *Solenopsis* sp. 9 and *Solenopsis* sp. 12.

4 – DISCUSSION

Four out of seven chromosome numbers are described for the first time in this study for *Solenopsis* (2n = 24; 26; 28, and 42 chromosomes) (Figures 3, 4, and 6). The other diploid number (2n = 22, 32, and 38 chromosomes) was already previously described, reviewed in LORITE & PALOMEQUE (2010). When we analyzed all the diploids numbers together with their karyotypical formulae, we found that with the increase in the diploid number there is a decrease in the metacentric chromosomes' numbers, and the presence of the chromosomes with other morphologies (submetacentric and subtelocentric) (Figure 13). This behavior is predicted by the Minimum Interaction Theory, proposed by IMAI & al. (1988). This theory suggests that the chromosome evolution occurs towards the increase of the chromosome numbers by centric fission (considered as the main rearrangement in the cytogenetic evolution of eukaryotes), as a way to decrease the genetic risk of reciprocal deleterious translocations (IMAI & al. 1988, IMAI & al. 2001).

The genus *Monomorium* is a sister group of *Solenopsis* (WARD & al. 2015), and as it has a karyotypical variation that is characterized by change of chromosomal morphology with the increase in diploid number. *Monomorium dichroum* has the lowest diploid number of the genus with 2n = 16 chromosomes, being most of them metacentric. While *M. latinode* presents 2n = 70 chromosomes (the largest of the genus) and almost all are telocentric chromosomes (IMAI & al. 1984, LORITE & PALOMEQUE 2010). Based on this data, we assumed that the basal karyotype to the genus *Solenopsis* is 2n = 22 metacentric chromosomes, found in the morphospecies *Solenopsis* sp. 11 and sp. 5 (Figure 2a and b).

Although fission can be considered the main rearrangement in the chromosome evolution, it is not unique. In this study, we also identified diploid numbers associated with different karyotypic formulae, such as in 2n = 22 combined with three different karyotypic formulae. The occurrence of one or two pericentric inversions in the basal karyotype can have originated the karyotypes of the morphospecies *Solenopsis* sp. 9 (n = 10m + 1 sm; Figure 2c) and *Solenopsis*

sp. 8 (n = 9m + 2 sm; Figure 2d), respectively (Figure 13). This rearrangement can be associated with the variation found in the karyotypic formula of the morphospecies *Solenopsis* sp. 1, sp. 7 and sp. 6 (with 26 chromosomes, see Figure 3b - d) and *Solenopsis* sp. 3 and sp. 13 (with 28 chromosomes, see Figure 4).

The presence of heterochromatic blocks in interstitial regions are presented in morphospecies with smaller chromosome numbers, *Solenopsis* sp. 5, sp. 11, sp. 8 with 22 chromosomes (Figure 7a and b, respectively), *Solenopsis* sp. 4 with 24 chromosomes (Figure 7c), and *Solenopsis* sp. 1 and sp. 7 with 26 chromosomes (Figure 7d). The others, *Solenopsis* sp. 6 and the morphospecies with 28, 32, 38, and 42 chromosomes, present blocks in the short arms and centromeric regions (Figure 7e, g, h and i, respectively). It is possible to see an increase in the amount of the heterochromatin with the increase of diploid number, this difference is more evident when comparing the extremes (2n = 22 and 2n = 42 chromosomes). Those results are predicted by the MIT, because chromosomes that undergo fission are unstable and tend to grow heterochromatin on the breaking point, which stabilizes the chromosomes (IMAI & al. 2001, IMAI & al. 2002). Beside this stabilizing effect, a large heterochromatic region of C-band can be detrimental due to increase in nonspecific association and, consequently, the increase in reciprocal translocation (IMAI & al. 1977, IMAI & al. 2001, IMAI & al. 2002). To control the growth in excess of heterochromatin, there are two ways: the occurrence of centric fusion or inversion. Although the occurrence of centric fusion has been suggested to explain the karyotype evolution in some ants, such as *Myrmecia croslani* (IMAI & TAYLOR 1989)*, Strumigenys louisianae* (Formicidae: Myrmicinae) (ALVES-SILVA & al. 2014) and *Muntiacus muntjak* (Cervidae) (WURSTER & BENIRSCHKE 1970, LIN & al. 1991, LEE & al. 1993), in genus *Solenopsis*, no data suggest the occurrence of this rearrangement. However, one inversion can explain the disappearance of the heterochromatic blocks in interstitial region.

The CMA_3 staining patterns show the presence of two different groups in *Solenopsis* with down to 28 chromosomes (Figure 13). The first pattern is characterized by the presence of blocks rich in CG in the interstitial regions of the short arm in one pair of metacentric or submetacentric chromosomes (Figures 8f

and 10b), possibly associated with a heterochromatic block, and the second shows the blocks in the long arm of subtelocentric chromosomes (Figures 8d, 9b, and 10d). This suggests two different evolutionary ways to attain the same chromosome number (Figure 13). The karyotypes with 26 and 28 chromosomes that present the blocks rich in CG in the long arm of the subtelocentric chromosome, are derived from the karyotype found in the *Solenopsis* sp. 8 (Figure 13). This, in turn, was originated from the inversions pericentric in the basal karyotype. The other karyotypes with 24 and 28 chromosomes that show different patterns (8f and 10b, respectively), mark in the short arms of one pair of metacentric or submetacentric chromosome, originated directly from basal karyotype (Figure 13). *Solenopsis* sp. 1 presented a unique result (positive CMA_3 in centromeric regions), not being possible to suggest one pattern based only on the data of this work.

In ants, it is common to find one pair of the chromosomes with marks of CMA_3, as in *Dinoponera lucida* (MARIANO & al. 2008), *Strumigenys louisianae* (ALVES-SILVA & al. 2014), *Mycetophylax conformis* and *M. simplex* (CARDOSO & al. 2014). However, in this paper, the morphospecies with 32 chromosomes present two pairs of the chromosomes with CMA_3 positive that are present in the telomeric regions of the short arm of the subtelocentric chromosomes (Figure 11b). This pattern suggests the metacentric chromosomes that carry those regions in the species with 28 chromosomes may have undergone one fission along with the karyotype's formation of the 32 chromosomes. This fission possibly originated both subtelocentric chromosomes that carry the rich regions in CG. The specimens with 42 chromosomes show 4 to 5 marks (Figure 12b and d, respectively), that can have been originated by occurrence of the translocation between nonhomologous chromosomes involving repetitive DNA or even fission (FELDBERG & BERTOLLO 1985, CAMACHO & al. 1986).

In insects, the Nucleolus Organizer Regions (NORs) are generally associated with CG-rich regions, which are positive for CMA_3. This correlation was identified and ratified by FISH in many ant species (MARIANO & al. 2008, BARROS & al. 2016), therefore, being used as indirect evidence of the position and number of NORs (BRITO & al. 2003, BARROS & al. 2014). However, some ant species present multiple marks of CMA3 (CRISTIANO & al. 2013, BARROS & al. 2014) present in the

centromeric and pericentromeric regions in the chromosomes, as found in *Solenopsis* sp. 1 (Figure 9d). Whereas cytogenetic studies in ant indicated a pattern in NORs being a single pair of chromosomes, possibly, the positive CMA3 marks found in *Solenopsis* sp. 1 are not associated with NORs.

Some species of bees and ants have AT-rich regions identified by DAPI. These marks are normally present in centromeric regions, except in *W. auropunctata*, where they occur in pericentromeric regions (ROCHA & al. 2002, BRITO & al. 2003, LOPES & al. 2008, SOUZA & al. 2011). In *Solenopsis*, these regions were not identified. This absence is generally observed in ants, such as in fungus-growing ants, *Strumigenys louisianae* and *Dinoponera lucida* (MARIANO & al. 2008, BARROS & al. 2010, CRISTIANO & al. 2013, ALVES-SILVA & al. 2014, BARROS & al. 2014).

Here, *Solenopsis* presented a complex karyotype evolution, suggesting the occurrence of different rearrangements, such as fission, inversion and translocation. This result is important in understanding of karyotypic evolution in Formicidae as well as in Hymenoptera.

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Table 1: **Sample information and results about diploid number (2N) and karyotypic formulae of each morphospecies; long arm (LA); short arm (SA); centromeric regions (CR); interstitial region (IR)**

Morphospecies	Locality	Number of Nest	2N	Karyotype Formulas	CMA_3	C-band
***Solenopsis* sp. 1**	Viçosa (Horto)	3	26	20m+6sm	2sm (LA)	CR + 4sm(IR)
***Solenopsis* sp. 2**	Viçosa (Horto)	11	42	2m+12sm+28st	4-5st (SA)	CR + st (SA)
***Solenopsis* sp. 3**	Turmalina	2	28	20m+8sm	2st (SA)	CR + st (SA)
***Solenopsis* sp. 4**	Viçosa	1	24	20m+2sm+2st	2sm (SA)	4sm(IR)
***Solenopsis* sp. 5**	Viçosa	2	22	22m	2sm (SA)	CR + 4sm(IR)
***Solenopsis* sp. 6**	Turmalina	2	26	16m+2sm+8st	(CR)	CR + st (SA)
***Solenopsis* sp. 7**	Turmalina	1	26	20m+6sm	2sm(LA)	CR + 4sm(IR)
***Solenopsis* sp. 8**	Turmalina	1	22	18m+4sm	2sm (LA)	CR + 2sm(IR)
***Solenopsis* sp. 9**	Turmalina	1	22	20m+2sm	--	--
S. saevissima	Viçosa/Turmalina	3	32	18m+14sm	4st (SA)	CR + st (SA)
***Solenopsis* sp. 11**	Viçosa	1	22	22m	2sm (SA)	CR + 4sm(IR)
***Solenopsis* sp. 12**	Viçosa (Mata do Paraiso)	4	28	18m+6sm+4st	2sm (LA)	--
***Solenopsis* sp. 13**	Viçosa (Mata do Paraiso)	1	38	10m+14sm+14st	1sm (AS)	CR + st (SA)

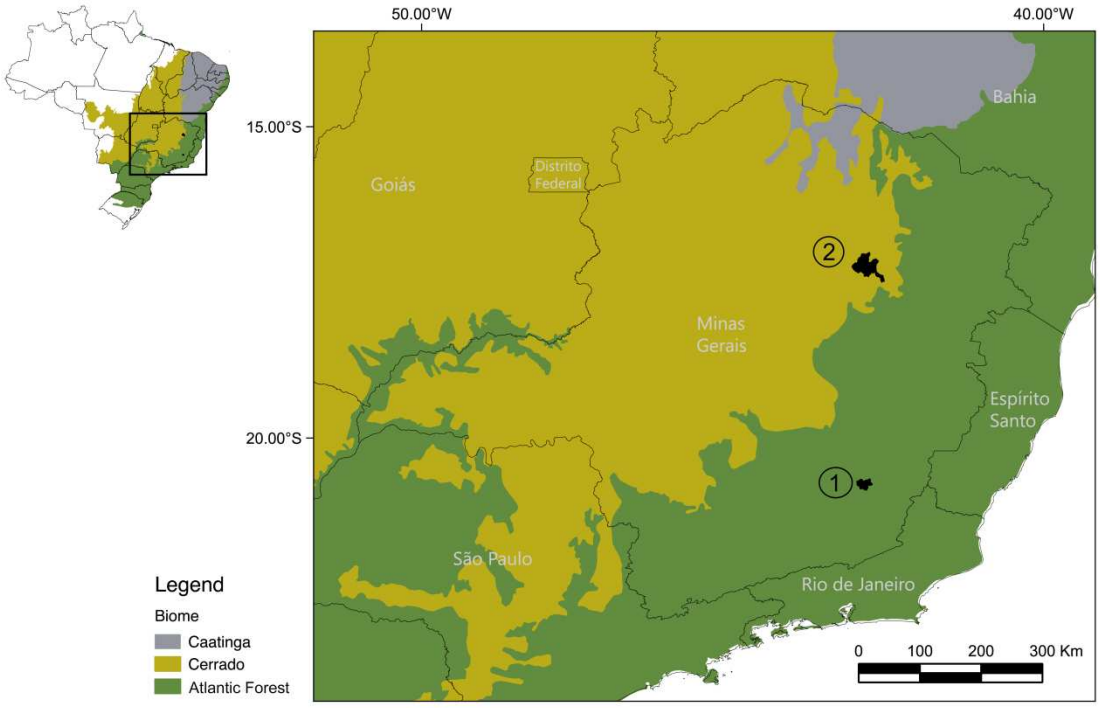

Figure 1: **Collection locales of *Solenopsis*: (1) Viçosa - Atlantic Forest secondary semideciduous (20º45'23''S, 42º52'25''W); and (2) Turmalina - Secondary fragment of Cerradão (17˚27'22.3''S, 42˚37'53.5''W);**

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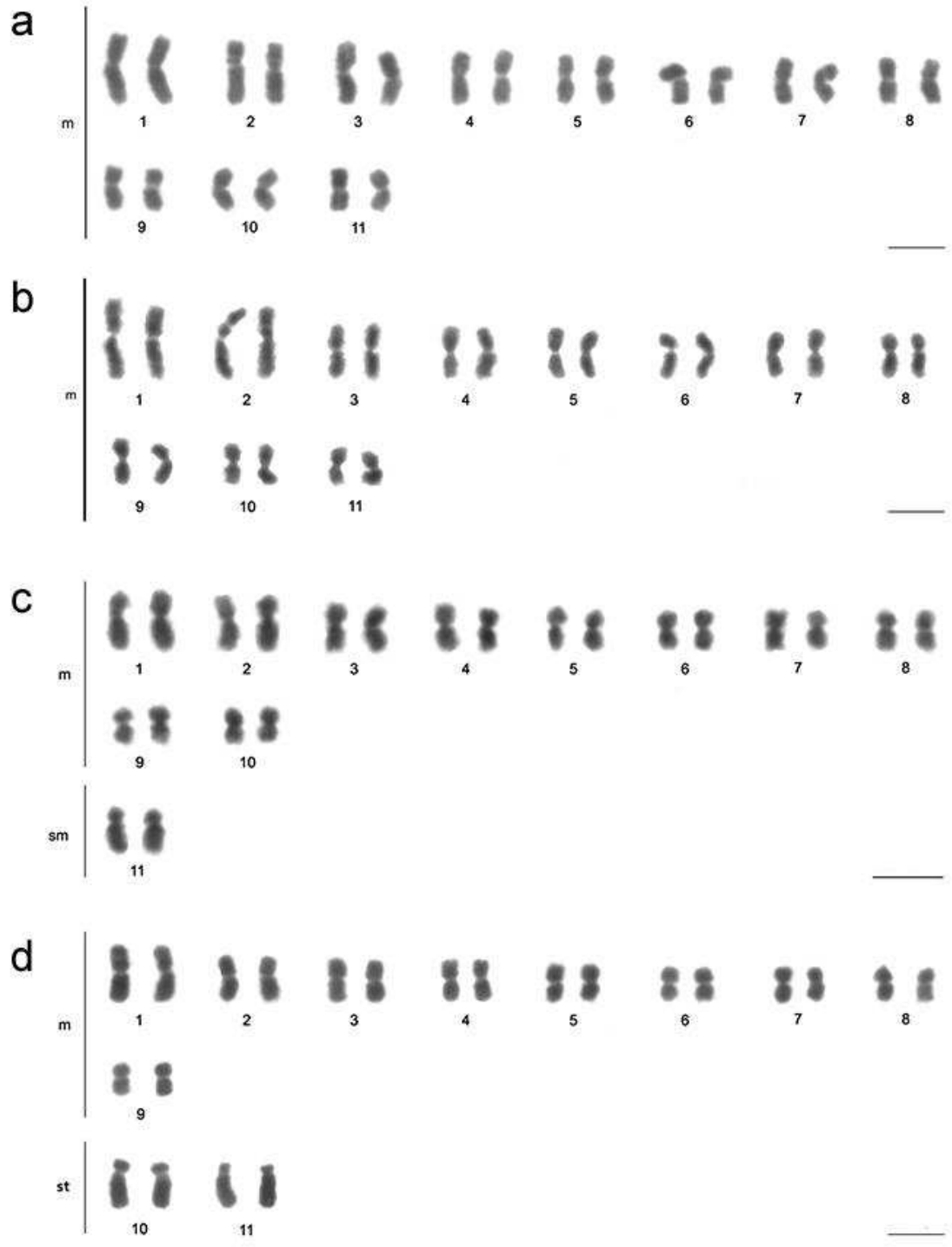

Figure 2: **Karyotypes with 2n=22 chromosomes stained with Giemsa 5% of** a) ***Solenopsis* sp. 11;** b) ***Solenopsis* sp. 5;** c) ***Solenopsis* sp. 9;** d) ***Solenopsis* sp. 8. The scale bar represents 5 µm**

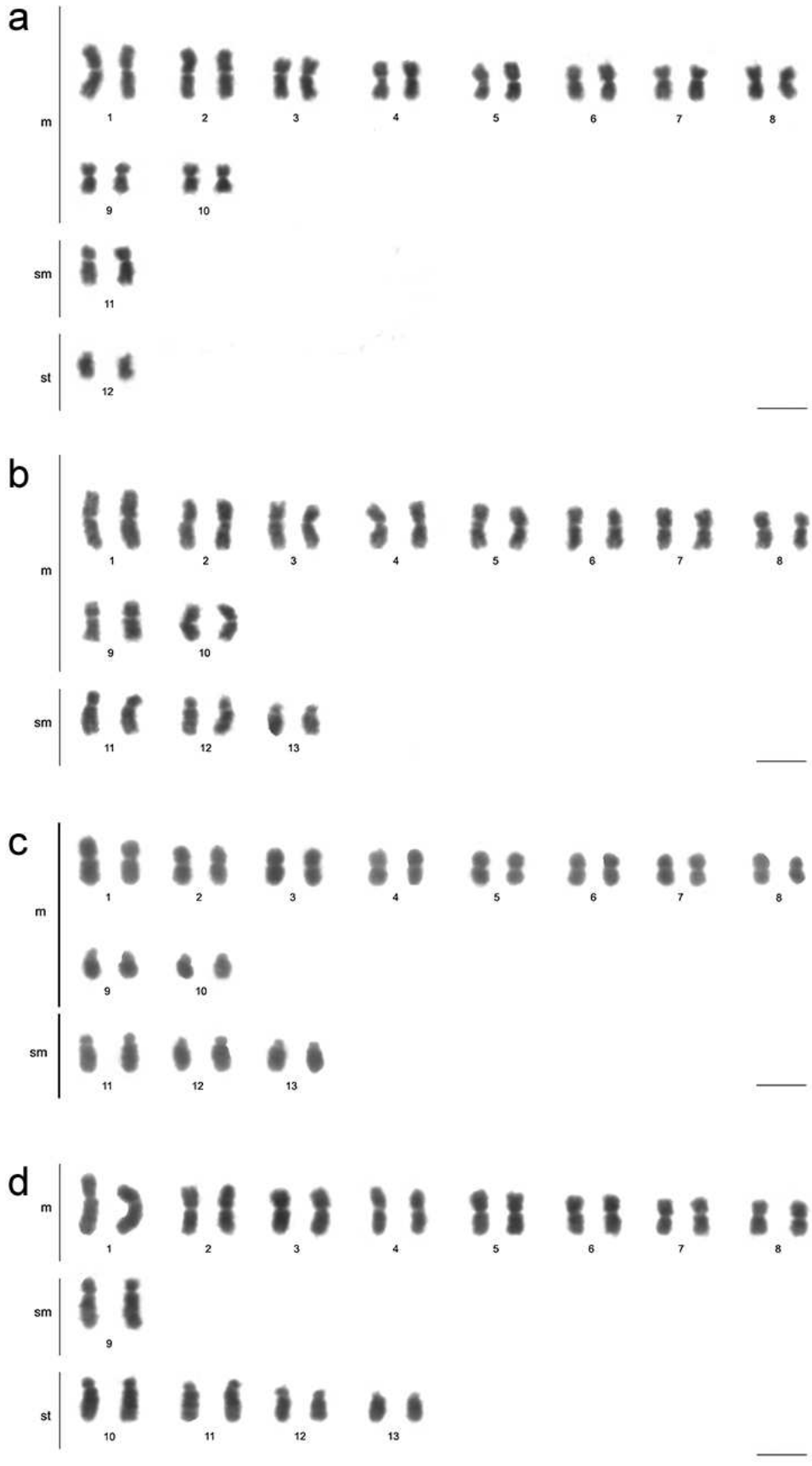
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Figure 3: **Karyotypes stained with Giemsa 5% of** a) ***Solenopsis*** **sp. 4 with 2n=24 chromosomes;** b) ***Solenopsis*** **sp. 1;** c) ***Solenopsis*** **sp. 7;** d) ***Solenopsis*** **sp. 6. The scale bar represents 5 µm**

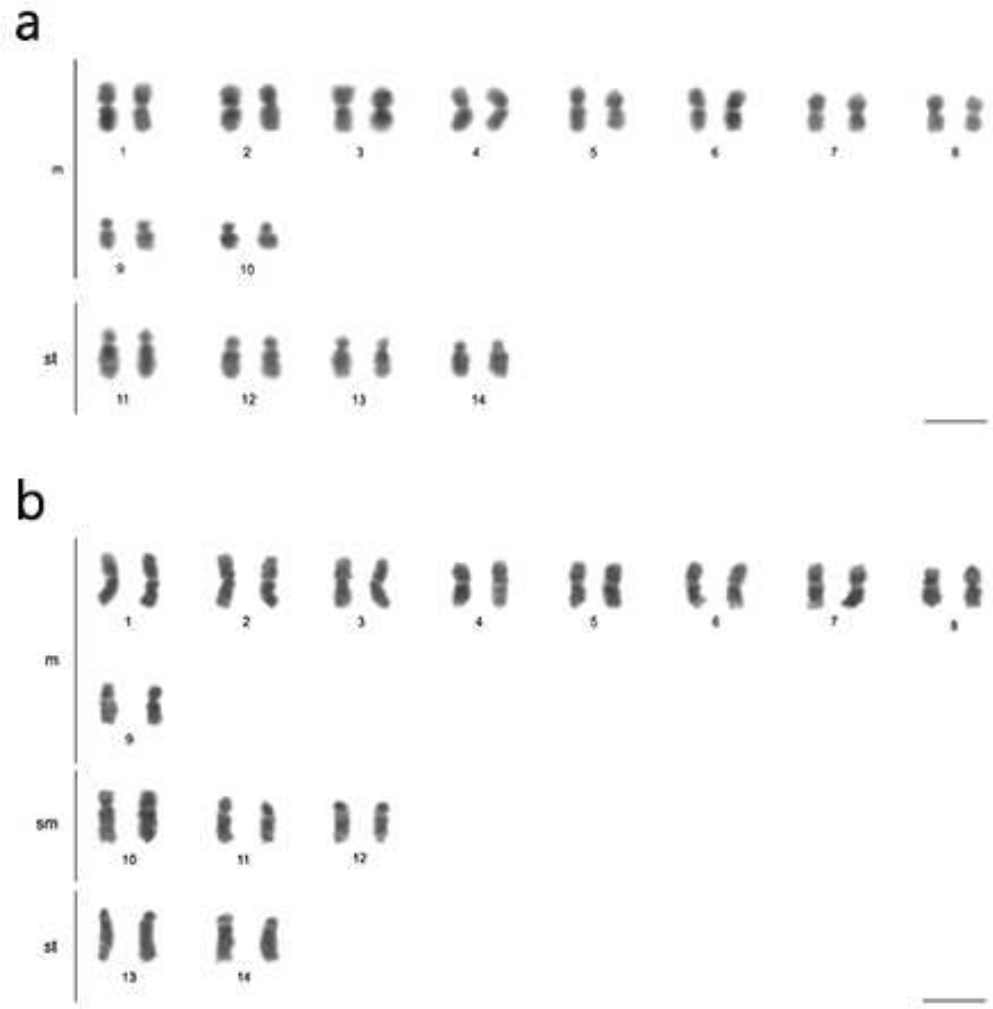

Figure 4: **Karyotypes stained with Giemsa 5% of** a) ***Solenopsis*** **sp. 3;** b) ***Solenopsis*** **sp. 12. The scale bar represents 5 µm**

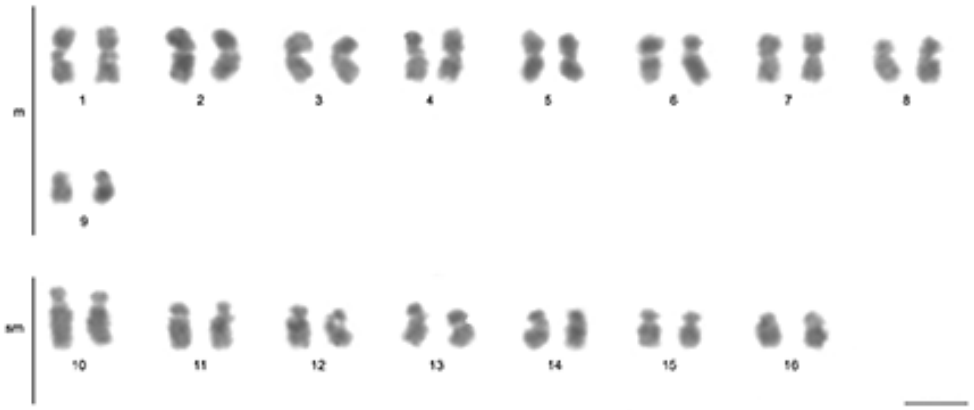

Figure 5: **Karyotype stained with Giemsa 5% of *Solenopsis saevissima*. The scale bar represents 5 µm**

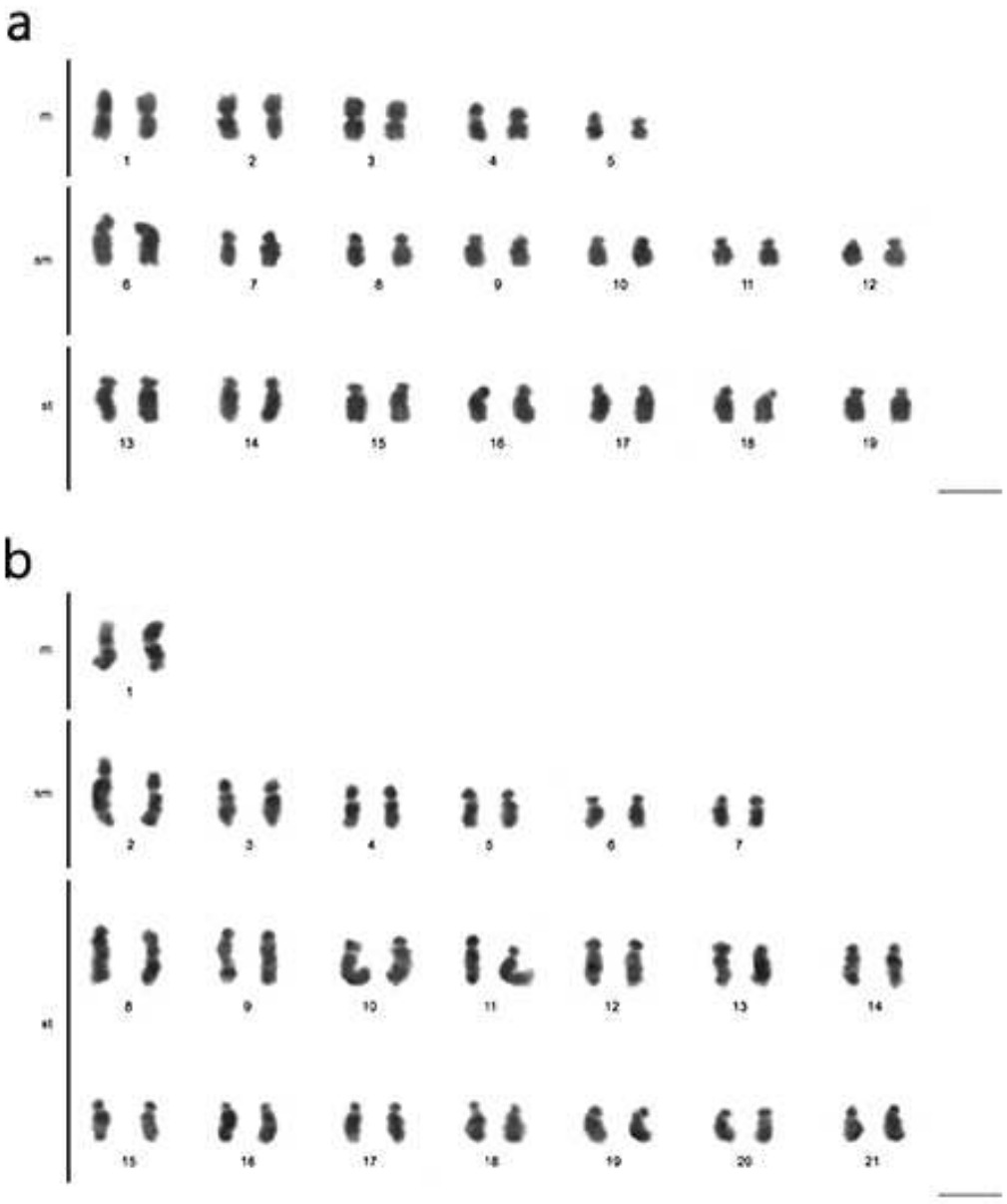

Figure 6: **Karyotypes stained with Giemsa 5% of** a) ***Solenopsis*** **sp. 13;** b) ***Solenopsis*** **sp. 2. The scale bar represents 5 µm**

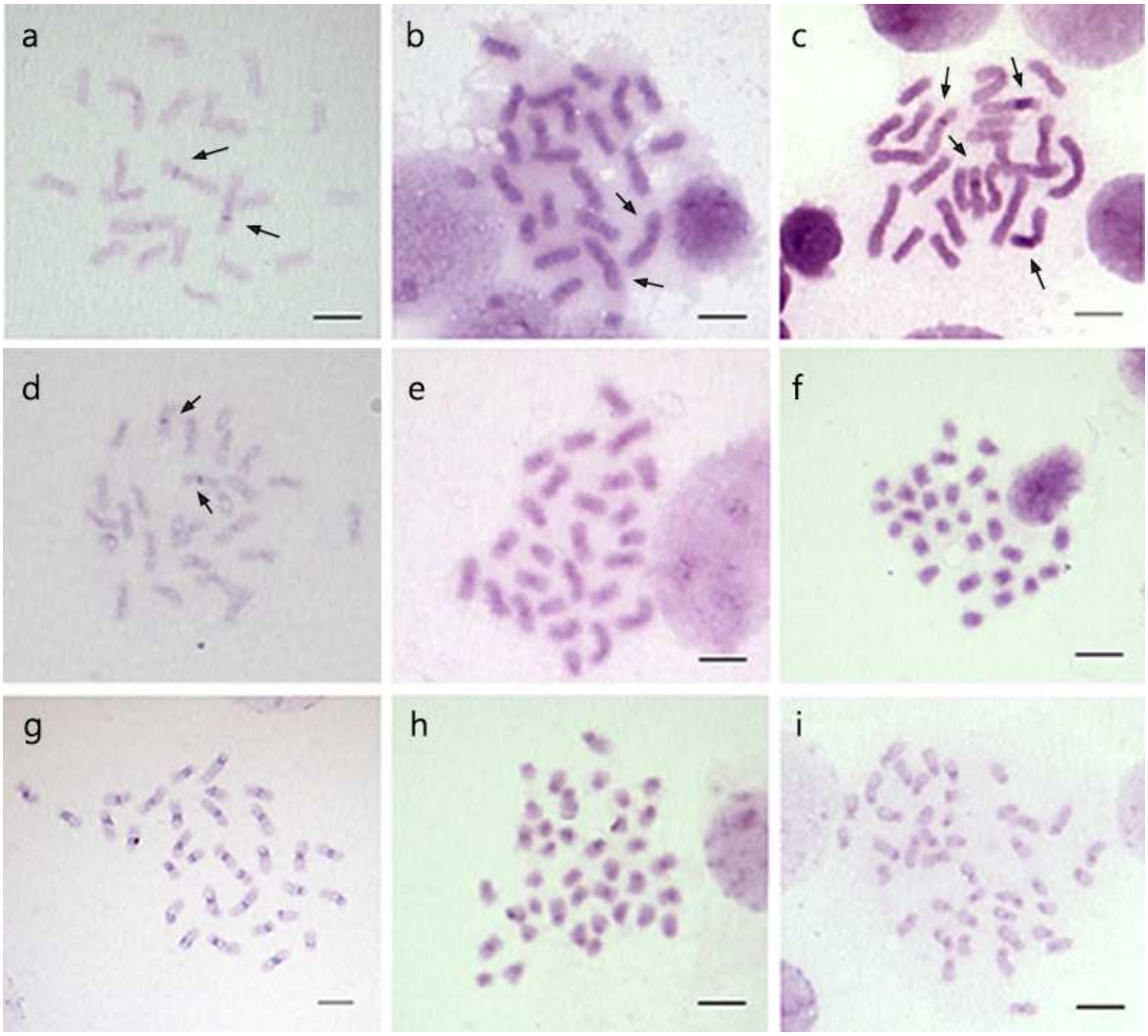

Figure 7: **C-banding pattern in: a) *Solenopsis* sp. 5 and sp. 11 (2n=22 chromosomes); b) *Solenopsis* sp. 8 (2n=22 chromosomes); c) *Solenopsis* sp. 4 (2n=24 chromosomes); d) *Solenopsis* sp. 1 and sp. 7 (2n=26 chromosomes); e) *Solenopsis* sp. 6 (2n=26 chromosomes); f) *Solenopsis* sp. 3 (2n=28 chromosomes).; g) *Solenopsis saevissima* (2n= 32chromosomes); h) *Solenopsis* sp. 13 (2n=38chromosomes); i) *Solenopsis* sp. 2 (2n=42 chromosomes). Arrows point to the interstitial heterochromatin. The scale bar represents 5 µm**

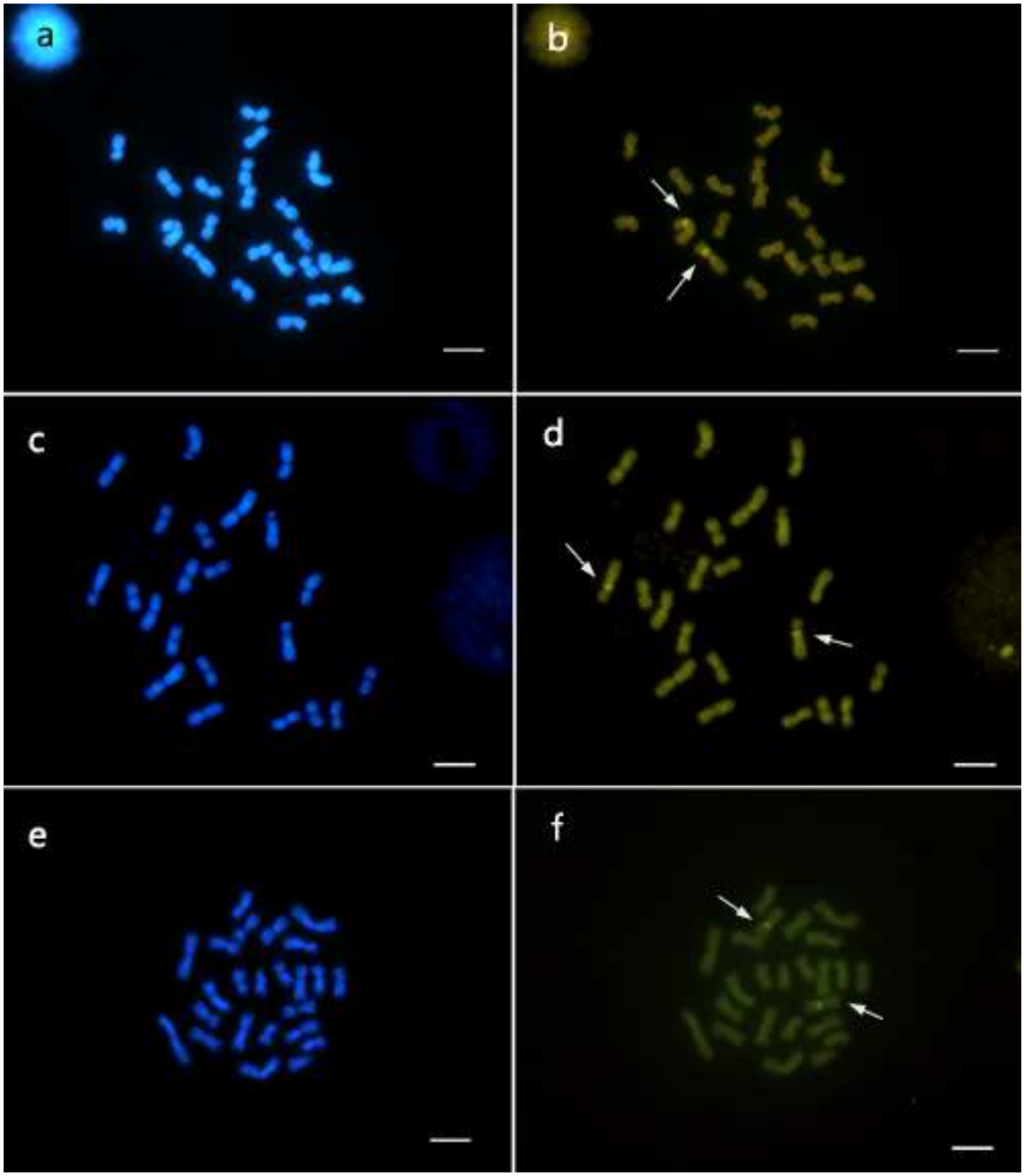

Figure 8: **Metaphases stained with the fluorochromes DAPI/CMA_3 of: a-b) *Solenopsis* sp. 5 sp. 11 (2n=22 chromosomes); c-d) *Solenopsis* sp. 8 (2n=22 chromosomes); e-f) *Solenopsis* sp. 4 (2n=24 chromosomes). Arrows point to the positive CMA_3 marks. The scale bar represents 5 µm**

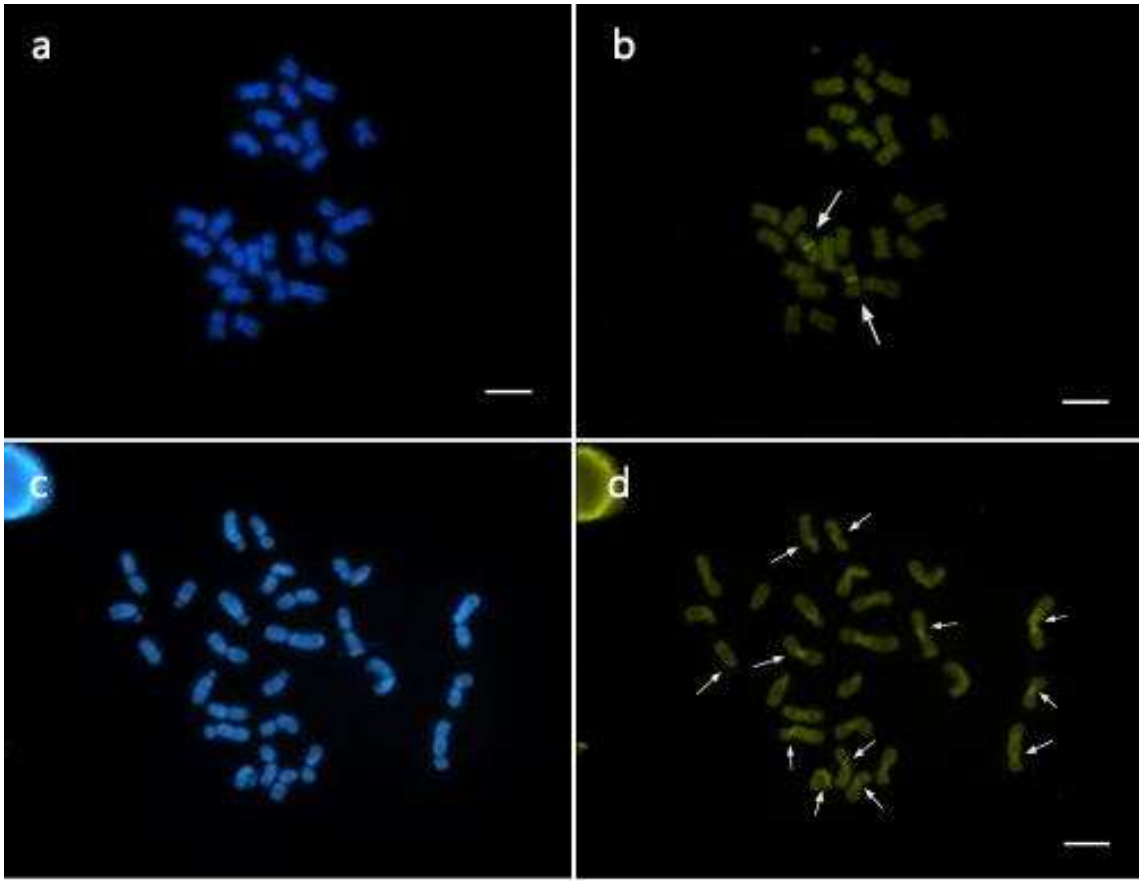

Figure 9: **Metaphases with 26 chromossomes stained with the fluorochromes DAPI/CMA_3 of: a-b) *Solenopsis* 1 and 7; c-d) *Solenopsis* sp. 6. Arrows point to the positive CMA_3 marks. The scale bar represents 5 µm**

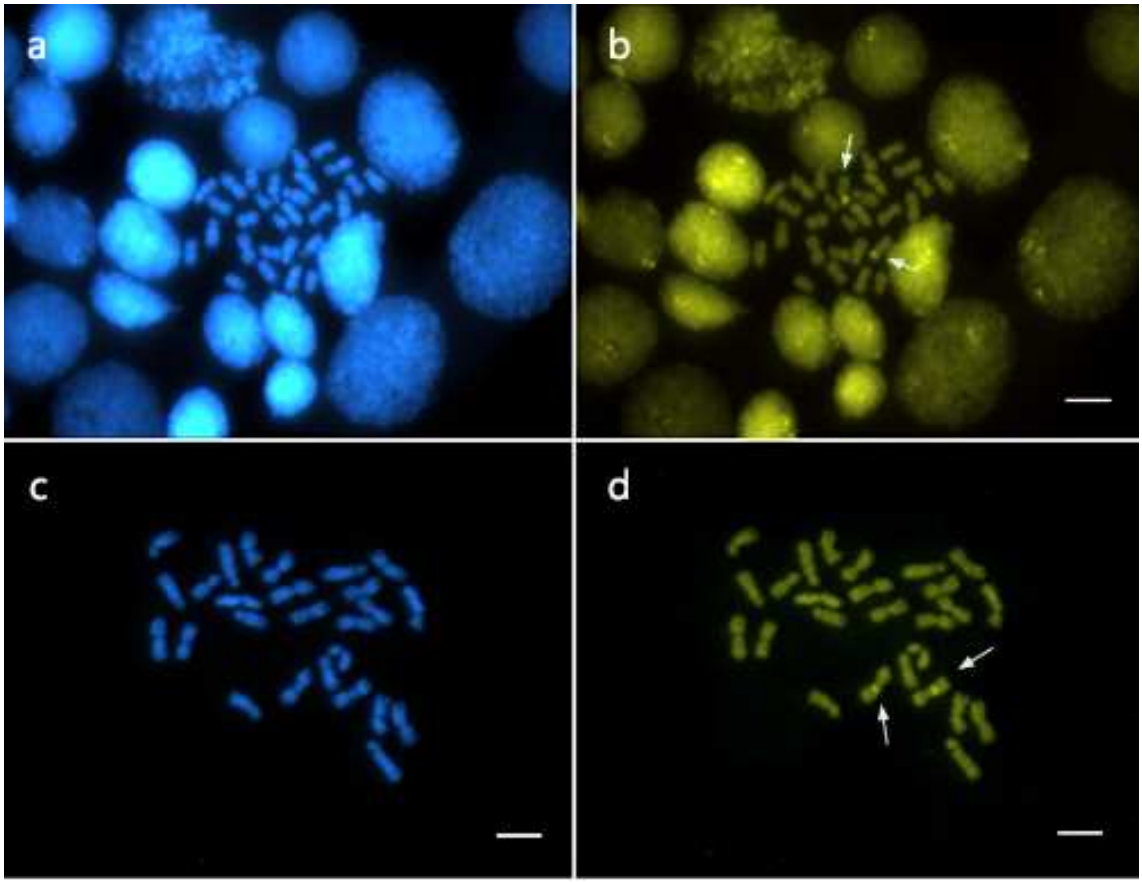

Figure 10: **Metaphases with 28 chromosomes stained with the fluorochromes DAPI/CMA_3 of: a-b) *Solenopsis* sp. 3; c-d) *Solenopsis* sp. 12. Arrows point to the positive CMA_3 marks. The scale bar represents 5 µm**

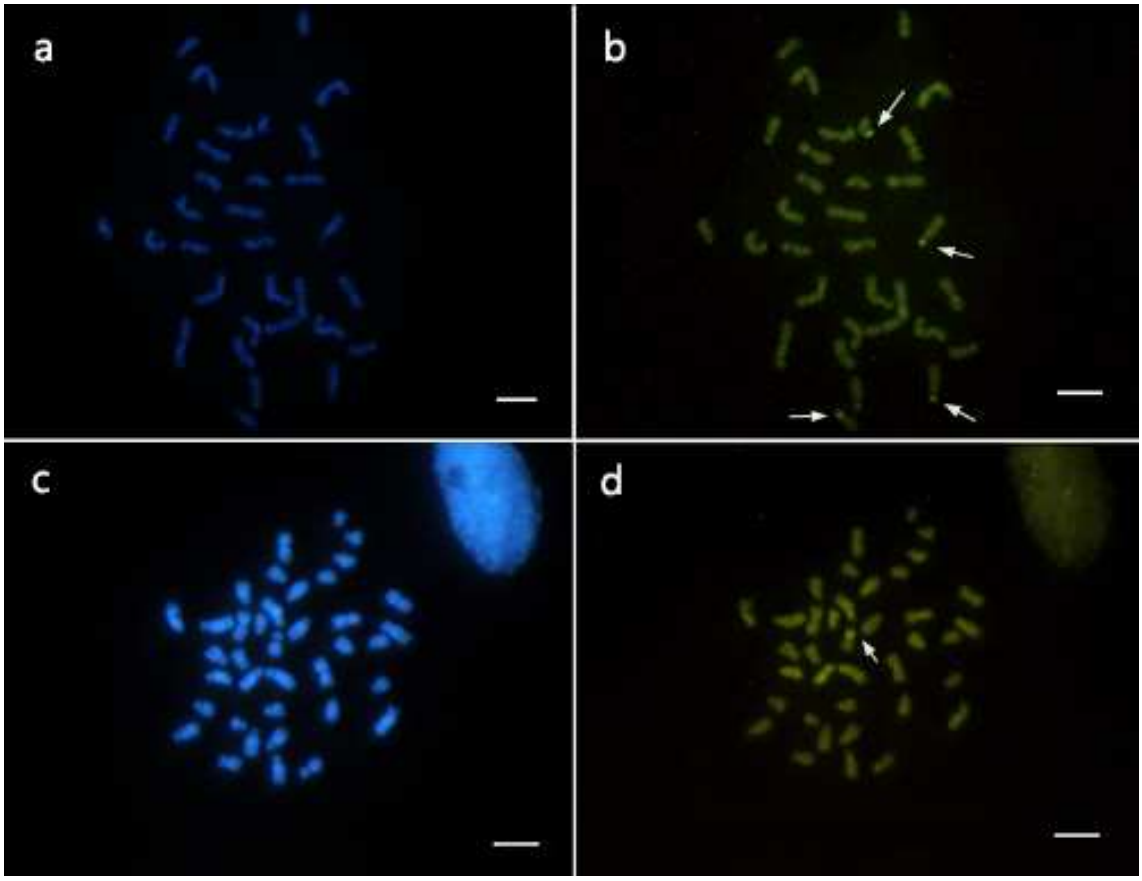

Figure 11: **Metaphases stained with the fluorochromes DAPI/CMA_3 of: a-b) *Solenopsis saevissima* (2n=32 chromosomes); c-d) *Solenopsis* sp. 13 (2n=38 chromosomes). Arrows point to the positive CMA_3 marks. The scale bar represents 5 µm**

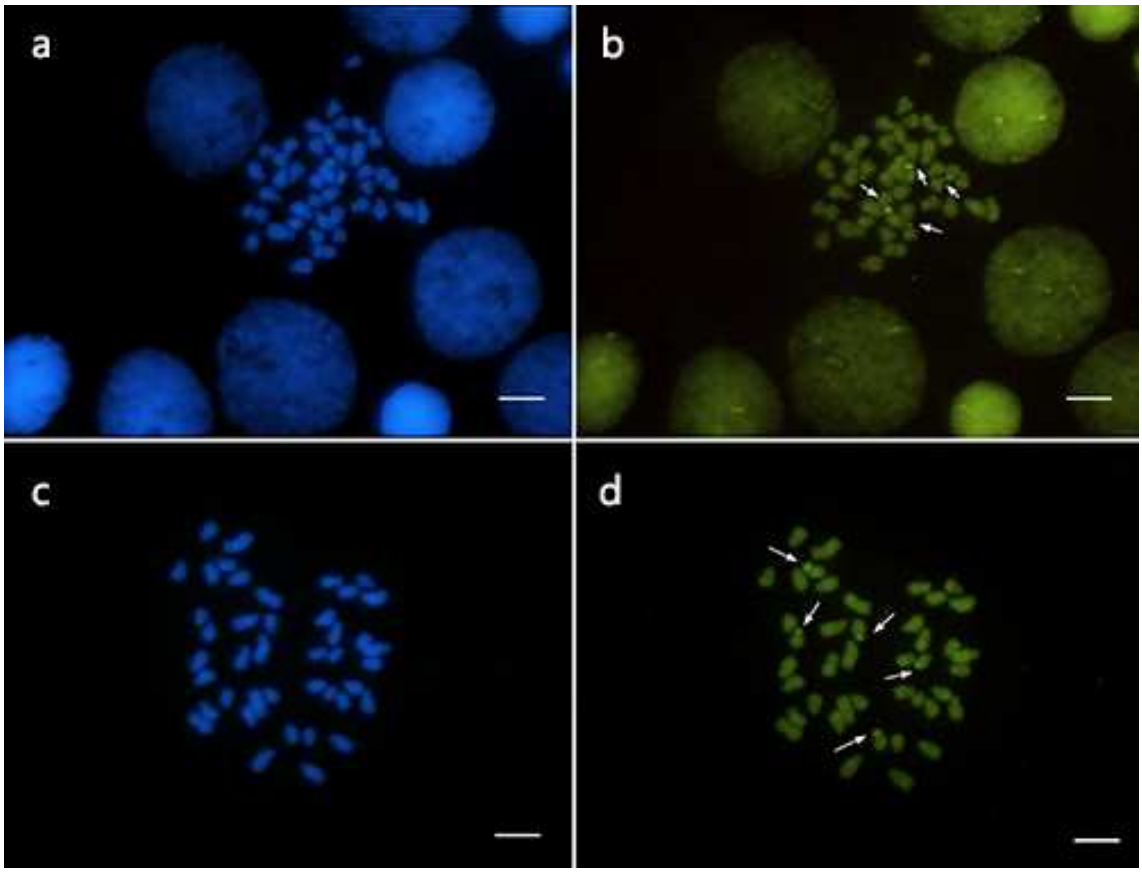

Figure 12: **Metaphases of *Solenopsis* sp. 2 (2n= 42 chromosomes) stained with the fluorochromes: a-c) DAPI and b-d) CMA_3. Arrows point to the positive CMA_3 marks. The scale bar represents 5 µm**

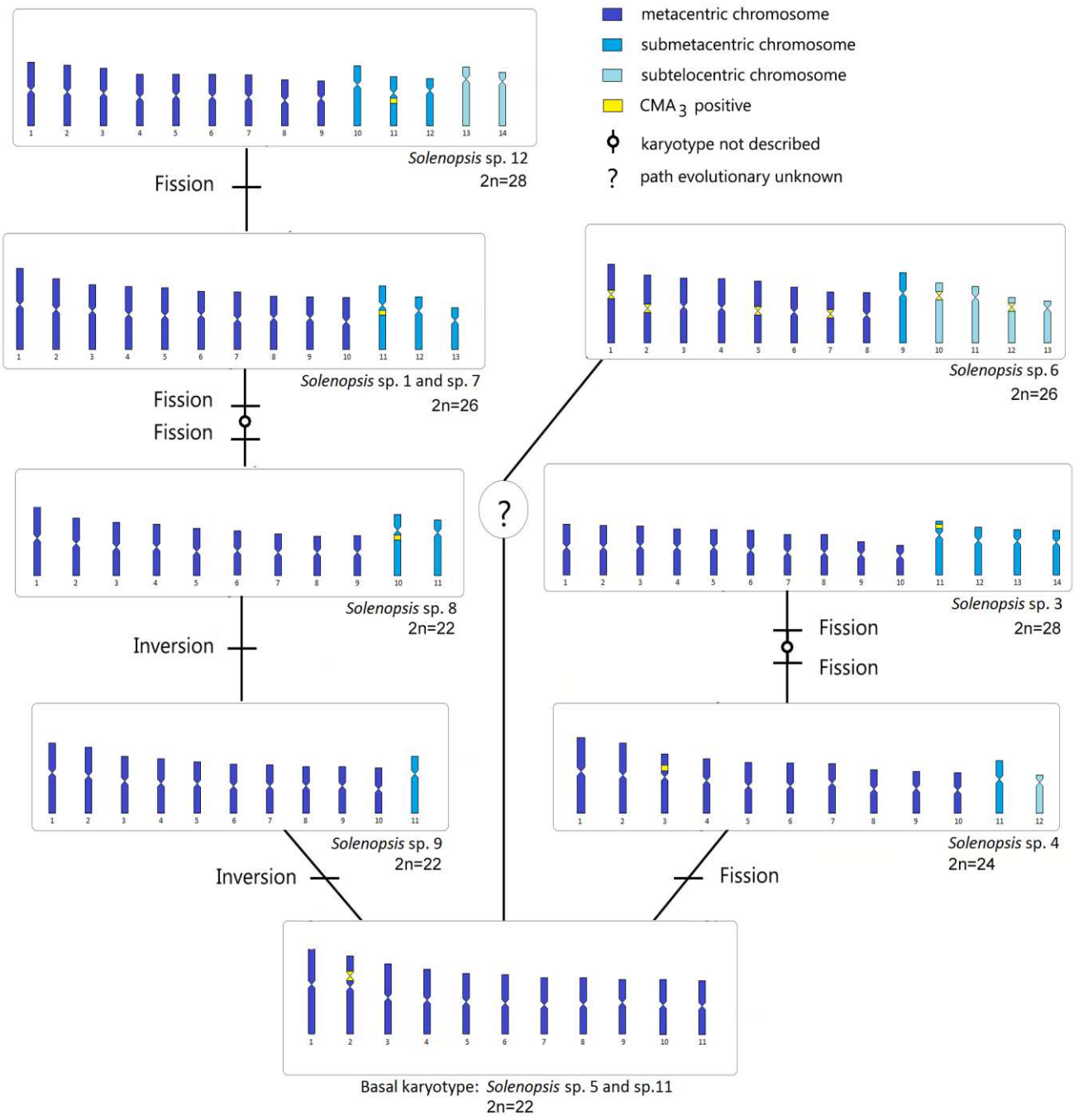

Figure 13: **Evolutionary network with the ideograms for each karyotype found in *Solenopsis* with 22 to 28 chromosomes, including data about number and position of CG-rich region (CMA_3 positive) and the possible rearrangements presents in the formation of the karyotypes;**

Capítulo II

Behavior of polyploid cells during the development of neural tissue in the ant genus *Solenopsis* Westwood, 1840 (Hymenoptera: Formicidae)

Behavior of polyploid cells during the development of neural tissue in the ant genus *Solenopsis* Westwood, 1840 (Hymenoptera: Formicidae)

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Abstract

During the research on cytogenetics of the genus *Solenopsis*, we observed the presence of polyploid cells in the cephalic ganglion. The aim of this study is to understand whether species of the *Solenopsis* genus naturally present polyploid cells, and if the number of polyploid cells keeps constant during development. For this, we determined the DNA ploidy of cephalic ganglion cells of four different development stages (larvae, young prepupae, old prepupae, and pupae) of *Solenopsis saevissima* workers based on the measurement of nuclear-DNA content by flow cytometry. The data show the presence of diploid and tetraploid cells, and these cells present different proportions depending on the development stage analyzed. The tetraploid cells have a decrease in representativity with the progress of the ant's development, suggesting that the polyploid cells are not permanent in the neural tissue and will disappear from this tissue of the adult ants. This is different from what happens with diploid cells that have a proliferation peak in young prepupae stage and then presents a significant progressive reduction in the cell number in division. This result brings to light various issues in several areas, such as, which would be the genes involved in the polyploidization and cell death in this tissue.

Key-words: cell death; polyploidization; fire ant; flow cytometry;

1. INTRODUCTION

Polyploidy is originated by multiplication of the complete genome of a species, creating an individual or a tissue with more than two copies of homologous chromosomes, or with polytene chromosomes (FOX & DURONIO 2013). Entire polyploid individuals are easily found in plants, such as wheat (*Triticum aestivum)* and alfalfa (*Medicago sativa*) (SCHIFINO-WITTMANN & DALL'AGNOL 2001), but are rare in animals (WHITE 1973, CLARK & WALL 1996). However, isolated polyploid cells can be found in somatic tissue of some animals, often during development stages, such as in the brain and hindgut of *Drosophila*, and in the liver of *Mus musculus* (FOX & al. 2010, UNHAVAITHAYA & ORR-WEAVER 2012, FOX & DURONIO 2013).

Different from other animal groups, in Hymenoptera, the ploidy number is responsible for sexual determination. In general, females are originated from fertilized eggs, while the males are originated from unfertilized eggs, in other words, the females are diploid and the males are haploid (HEIMPEL & BOER 2008). Beyond the variance of ploidy associated with sexual determination, in ants, the presence of polyploid cells was also described in some species, such as *Aphaenogaster osimensis* (IMAI & YOSIDA 1966)*, Linepithema humile* (ARON & al. 2003)*, Crematogaster* sp.*., Camponotus ligniperda* e *Pheidole pallidula* (CROZIER 1975).

The ant genus *Solenopsis*, commonly known as 'fire ants', comprises 195 described species (BOLTON 2016). This genus is mostly known by two species: *Solenopsis saevissima* and *S. invicta*, which are pests of economic importance in a global scale (DAVIS & al. 2001, NATTRASS & VANDERWOUDE 2001, ZHANG & al. 2007). During the research about cytogenetics of this genus (1st chapter of this thesis), we observed the presence of polyploid cells (see e.g., Figure 1) in the cephalic ganglion. In this research, like in the other articles that described polyploids in ants, was used a blocking cell division – colchicine (protocol by IMAI & al. (1988)). It is used commonly in cytogenetic research and to induce polyploidy in cells of plants and animals (EDWARDS 1958, SIGAL & al. 1999, GLOWACKA & al. 2010). Thus, the aim of this study is to understand whether species of the genus

Solenopsis naturally present polyploid cells, that is, without treatment with blocking cell division, and if the number of polyploid cells keeps constant during development. For this, we determined the ploidy of cephalic ganglion cells of different development stages of larvae and pupae of *Solenopsis saevissima* based on the measurement of nuclear-DNA content by flow cytometry.

2. MATERIAL AND METHODS

All analyses presented here were undertaken in a single nest, collected in Viçosa (20º45'23''S, 42º52'25''W), Minas Gerais, Brazil. The National Collecting Permit was issued to the first author of this study (SISBIO 46427-2). The nests were transported and kept in plastic containers until we obtained larvae at the desired stages for the protocols. For flow cytometry analyses, we used four different development stages of *S. saevissima*'s workers that we call larvae, young prepupae (after eliminating meconium), old prepupae, and pupae with red eyes (Figure 2), based on classification of Imai (1966) and IMAI & al. (1988). We replicated all techniques three times for each stage.

To prepare the nuclear suspension, the cerebral ganglia were extracted in physiologic solution (0.155 mM NaCl) and transferred to 100 µL OTTO-I lysis buffer (Otto, 1990) containing 0.1 M citric acid (Merck), 0.5% Tween 20 (Merck) and 50 µg mL−1 RNAse (Sigma-Aldrich), pH = 2.3 (LOUREIRO & al. 2006, TAVARES & al. 2010). The materials were macerated 10 times with a pestle in a tissue grinder. The suspensions were increased to 1.0 mL with the same buffer, filtered in a 30 µm nylon mesh (Partec), and centrifuged at 100 G in microcentrifuge tubes for 5 min. The pellet was incubated for 10 min in 100 µL OTTO-I lysis buffer and stained with 1.5 mL OTTO-I:OTTO-II (1:2) solution, supplemented with 75 µM 4'6-diamidino-2-phenylindole (DAPI – excitation/emission wavelengths: 350/470). The nuclear suspensions were filtered through 20 µm diameter mesh nylon filter (Partec) and maintained in the dark for 30 min (TAVARES & al. 2010).

The material was analyzed by a Partec PAS flow cytometer (Partec). A high-pressure mercury lamp (HBO-100W) and filters (KG 1, BG 38, and XL 435) were used for analysis of the DAPI-stained nuclei. The equipment was calibrated for linearity and aligned with microbeads and standard solutions according to the

manufacturer's recommendations. FlowMax software (Partec) was used for data analyses. The nuclei peak G_0/G_1 was set to channel 100 and more than 10,000 nuclei were analyzed in each replica.

3. RESULTS

The histograms obtained in each developmental stage from flow cytometry analysis showed three distinct peaks with CVs ranging from 2.2% to 2.8%. The first one of each stage, aligned at the channel 100, represents the diploid cell number in the stage G_0/G_1. The second peak (channel 200) represents cells in two different cellular division stages: diploid cells in stage G_2 and tetraploid cells in G_0/G_1. The third peak (channel 400) stands for the tetraploid cells in cellular division, in stage G_2 (Figure 3).

The data show different proportions of diploid and tetraploid cells depending on the development stage analyzed. The tetraploid cells present a decrease in its representativity with the evolution of the ant development. This was observed with the progressive shrinkage of the third peaks in the histograms (Figure 3). These peaks represent the number of tetraploid cells in a proliferative state (peak G_2), the larvae showed 4.31% of the analyzed cells, prepupae (young) 2.41%, prepupae (old) 1.90%, and pupae 1.65% (Figure 3).

The behavior of the diploid cells is different. The prepupae (young) stage shows the lowest first peak with 61.25% of analyzed cells, and the largest second peak with 36.33%. This reveals that the top of the proliferative state of the diploid cells happens in the prepupae (young) stage (Figure 3b). Then, the proliferative diploid cells undergoes a reduction together with the tetraploids stationary cells, prepupae (old) with 33.29%, and pupae with 6.62% (second peak). In parallel, the increase in the stationary diploid cells, old prepupae with 64.81%, and pupae 91.73% first peak (Figure 3c and 3d, respectively), happens.

4. DISCUSSION

The results obtained in this paper by flow cytometry show that the polyploid cells in the neural tissue of *Solenopsis* and, likely, in the other species of the ants are natural (IMAI & YOSIDA 1966, CROZIER 1975, IMAI & al. 1977) and not caused by inhibits of the cellular cycle. Many inhibitors can be used to block cell cycle progression, such as the colchicine, an alkaloid extracted from *Colchicum autumnale* (CAPERTA & al. 2006). The colchicine inhibits the polymerization of the proteins of mitotic spindles stopping the division of cells for hours or even days (ALBERTS & al. 2014). The role of this substance in cytogenetics is an increase in the number of cells in metaphase suitable for analysis of chromosome morphology (IMAI & al. 1977), but a mistake in the use of this substance can cause cellular polyploidization and an equivocal about the obtained results.

The mechanism of cellular polyploidization that occurs during the development of neural tissue in *Solenopsis* is the endomitosis. This presents the cellular cycle with the phase G1, S and G2 complete, but only a partial M-phase. In this phase, the occurrence of anaphase b and cytokinesis is skipped, in other words, the sister chromatids are separated, but they keep within the same nucleus. We can find this mechanism in the formation of the megakaryocytes of the mammalian as well (RAVID & al. 2002). In total, there are three different mechanisms of cellular polyploidization, which have as results distinct polyploid cells. Beyond the endomitosis, there is the endocycle. This mechanism consists in the alternating between Synthesis (S) and Gap (G) phases and is found in most of *Drosophila*'s cells, being that this is responsible for the production the polytene chromosomes (SMITH & ORR-WEAVER 1991). The last of the three is responsible for formation, per example, of tetraploids hepatocytes. During the polyploidization of those cells, it happens that all phases until the nuclear division, but not cytokinesis, are being produced as multinucleate cells (SHER & al. 2013).

The polyploid cells in this tissue are not equally represented between the analyzed stages; this suffers a gradual reduction with the evolution of the morphological development (Figure 3). This suggests that the polyploid cells are

not permanent in the neural tissue and, possibly, will not be present in this tissue of the adult ants. This cellular behavior of temporal polyploidization is common in many tissues that, during its development, needs to sustain the mass production of proteins or high metabolic activity that are required for embryogenesis (LEE & al. 2009). There are many examples of cells adopting this mechanism as part of terminal differentiation to support a specialized function. In some cases, endoreplication is not used directly to provide gene products to the embryonic tissues. In rodents, for example, the extraembryonic cells trophoblast giant increased gene expression through polyploidy to supply the energy necessary for uteral implantation of the fertilized egg (RAVID & al. 2002, LEE & al. 2009).

Like as in *Solenopsis*, the presence of tissue polyploid only during the early stages of morphological development happens in other animals and plants. In *Drosophila*, most of the cells of the larvae enter the process of the endoreplication and increase its ploidy level, being the imaginal cell and nerve cells, the unique who keeps diploid (SMITH & ORR-WEAVER 1991, KRESS 1996). These cells became polyploid in the beginning of the larval stage, and kept it until the end of the prepupae stage (SMITH & ORR-WEAVER 1991). The progressive replacement of polyploid for diploid cells observed in *Solenopsis* is similar to what happens in *Drosophila*'s larval midgut. This tissue has polyploid larval epithelial cells and, adjacent to them, there are islands of the diploid imaginal cells. These diploid cells will proliferate during early prepupal development (JIANG & al. 1997), like we observed in neural tissue of *S. saevissima*, that present one peak of proliferation of the diploid cells in young prepupae (Figure 3b). This data confirms that the young prepupae stage is the most appropriate to cytogenetics researches, as suggest by IMAI (1966). The new diploid cells give rise to the adult midgut in *Drosophila*, and, possibly, to the brain in *Solenopsis*. Concomitant to the proliferation of diploid cells, the polyploid cells enter into the cell death cycle that can happen for two ways: by apoptosis or autophagy (JIANG & al. 1997, BAEHRECKE 2003). Programed cell death plays an important role for the maintenance of homeostasis in organisms, being directly involved in control of the cell number, remove abnormal cells, and eliminate unneeded cells and tissues (FUCHS & STELLER 2011).

Therefore, the utilization of the flow cytometry confirmed the presence of tetraploid cells in the neural tissue of *Solenopsis* workers in development. The results suggest that these cells are not permanent and disappear around the end of neural tissue development, being possibly the fully diploid adult brain. This data brings to light various issues in several areas, such as: which would be the genes involved in the polyploidization and death of cells in this tissue; if there is a special organization of the polyploid and diploid cells, and what would be; the other tissues have these behaviors in *Solenopsis* development, or is it exclusive to neural tissue, among others questions.

Acknowledgments

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Pesquisa (CNPq) for the grants provided to APAS and FAFS. This research was supported by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG).

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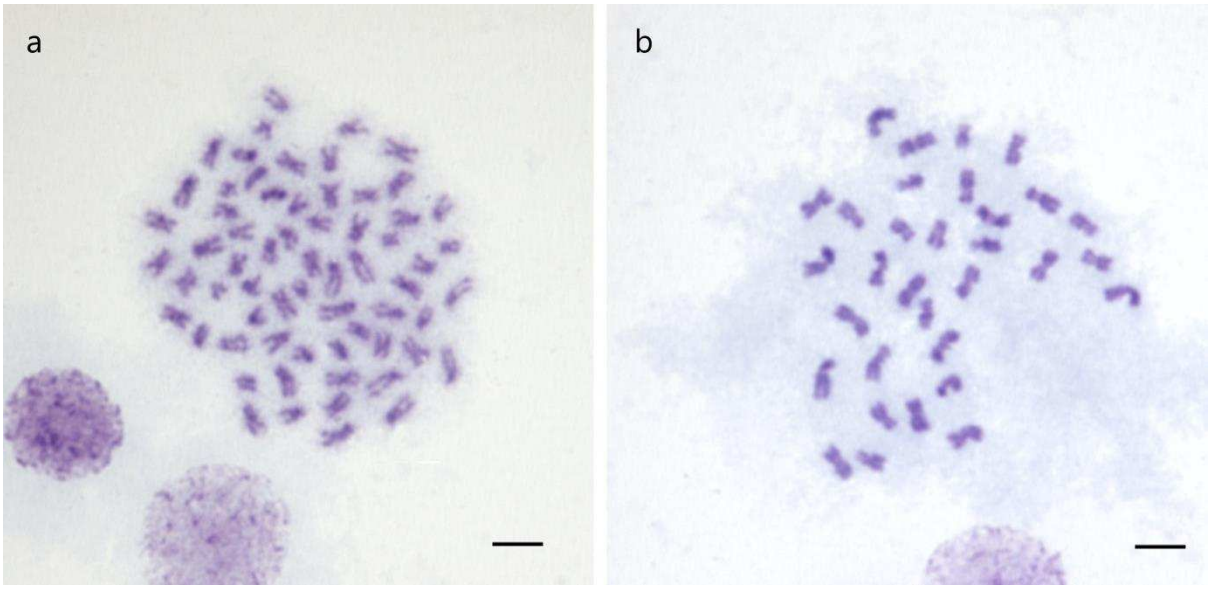

Figure 8: Metaphases observed in *Solenopsis saevissima* in a previous research of cytogenetic (1st chapter of this thesis) following the protocol of IMAI & al. (1988): a) tetraploid cell with 64 chromosomes; b) diploid cell with 32 chromosomes. The scale bar represents 5 µm;

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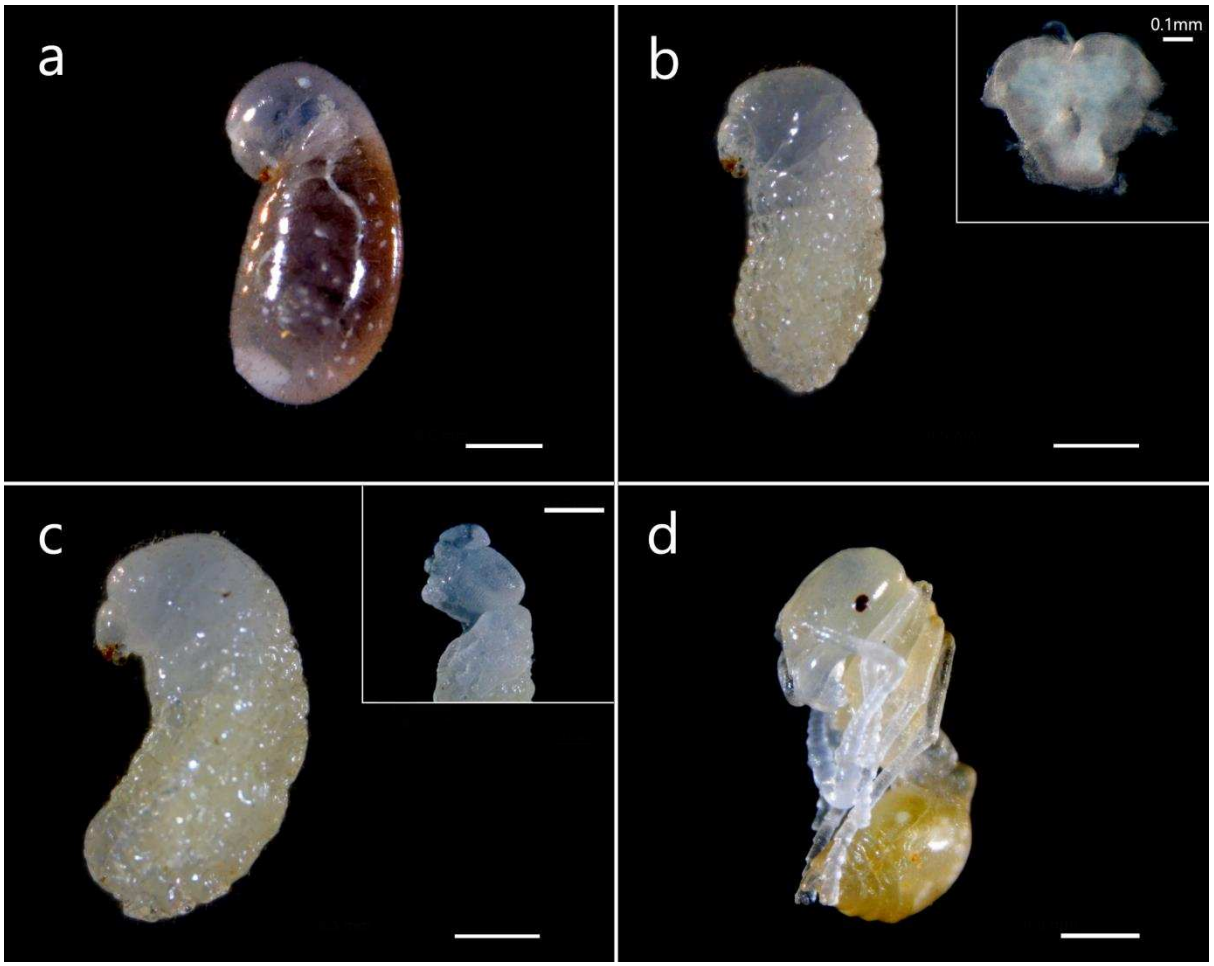

Figure 9: The development stage of the *Solenopsis saevissima* used in flow cytometry analyses**:** a) larvae stage; b) young prepupae stage (square upper right: the dissected cephalic ganglion); c) old prepupae stage (square upper right: the dissected old prepupae) and d) red eyes pupae. The scale bar represents 0.5 mm

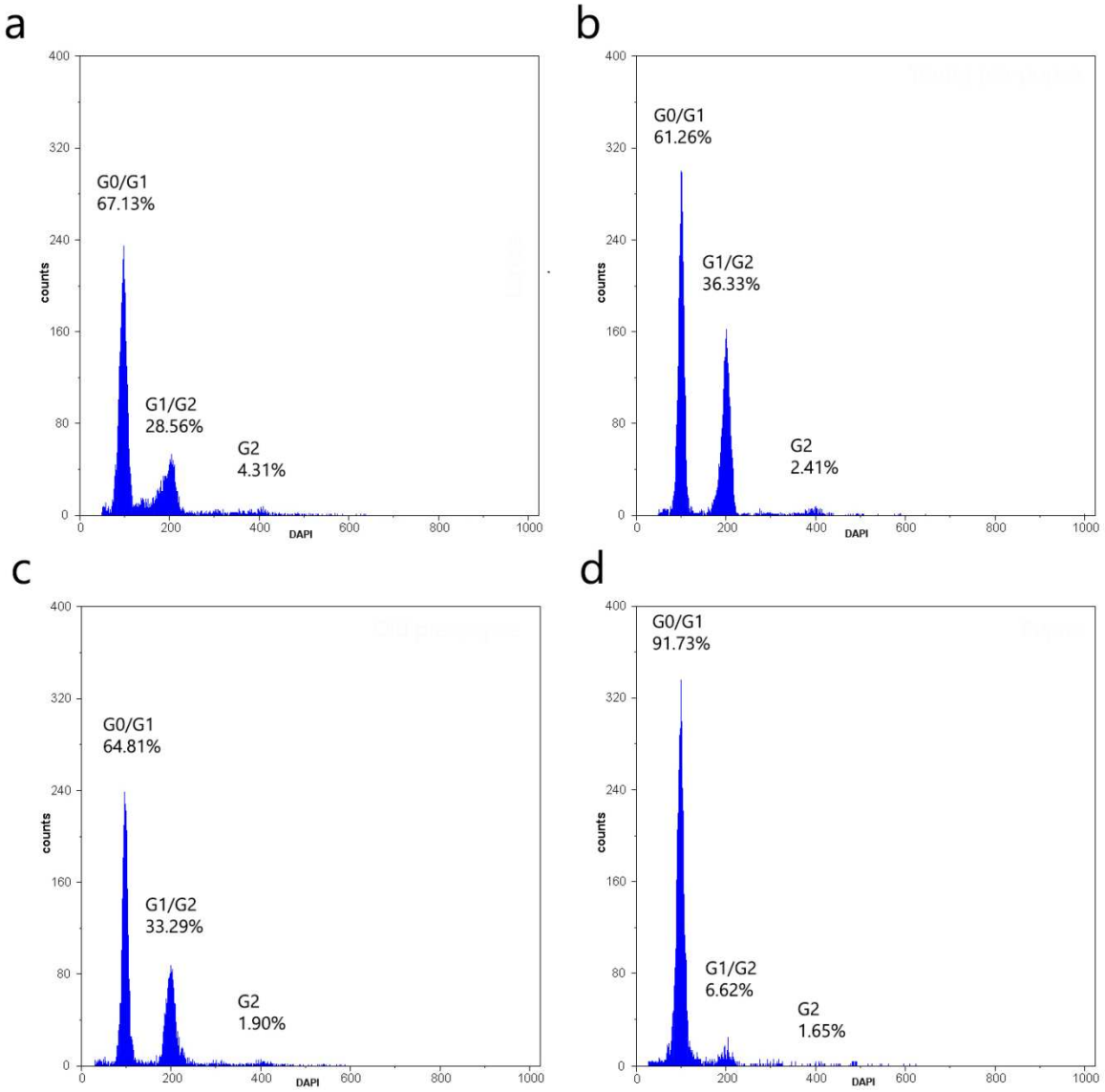

Figure 10: Histograms obtained by flow cytometry using cephalic ganglions of the 4 development stages of the *Solenopsis saevissima*: a) larvae; b) young prepupae; c) old prepupae; d) pupae. The percentages represent the average number of cells that are in the respective cellular cycle phase for each development stages. The first peak of the each histogram, aligned at the channel 100, represents the diploid cell number in the stage G0/G1. The second peak (channel 200) represents cells in two different cellular division stages: diploid cells in stage G2 and tetraploid cells in G0/G1. The third peak (channel 400) stands for the tetraploid cells in cellular division, in stage G2.

Conclusão geral

A utilização de algumas técnicas da citogenética clássica mostrou uma grande variação do número diploide para o gênero *Solenopsis*, além de sugerir uma evolução cariotípica complexa envolvendo vários tipos de rearranjos. Os resultados obtidos são de grande importância pra o entendimento da evolução cariotípica em Formicidae e Hymenoptera. Além disso, a Citometria de fluxo confirmou a presença de células tetraplóides durante o desenvolvimento do tecido neural das operárias de *Solenopsis*, abrindo várias questões em diversas áreas da genética e histologia.

Anexos

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PLOS ONE

The First Cytogenetic Data on *Strumigenys louisianae* Roger, 1863 (Formicidae: Myrmicinae: Dacetini): The Lowest Chromosome Number in the Hymenoptera of the Neotropical Region

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Abstract

In the present study, the first cytogenetic data was obtained for the ant species *Strumigenys louisianae*, from a genus possessing no previous cytogenetic data for the Neotropical region. The chromosome number observed was 2n = 4, all possessing metacentric morphology. Blocks rich in GC base pairs were observed in the interstitial region of the short arm of the largest chromosome pair, which may indicate that this region corresponds to the NORs. The referred species presented the lowest chromosome number observed for the subfamily Myrmicinae and for the Hymenoptera found in the Neotropical region. Observation of a low chromosome number karyotype has been described in *Myrmecia croslandi*, in which the occurrence of tandem fusions accounts for the most probable rearrangement for its formation. The accumulation of cytogenetic data may carry crucial information to ensure deeper understanding of the systematics of the tribe Dacetini.

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Introduction

The order Hymenoptera is one of the most diversified among insects and has interesting characteristics regarding its form of reproduction, sex determination and evolution of social behavior [1].

The Minimum Interaction Theory formulated by Imai et al. [2], is well accepted as an explanation for the chromosomal evolution of Formicidae. This theory is based on the occurrence of chromosomal rearrangements, where centric fission is the most frequent event. It postulates that there is a tendency for reduced chromosome size, and consequently an increase in chromosome number by means of fissions and a subsequent heterochromatin growth [2–4]. This process is evolutionarily favored by decreasing the interaction between the chromosomes,in particular, the deleterious translocations during meiosis. However, different chromosomal rearrangements have already been reported in ants, including centric fusions (reviewed in [4]) [5,6].

A wide variation in the chromosome number is observed in Hymenoptera, particularly in the family Formicidae, which includesextremes of variation in the order [4]. This variation ranges from n = 1 in *Myrmecia croslandi*, Australia [7], to n = 60 in *Dinoponera lucida*, Brazil [8]. Among the 750 ant species that have had their karyotypes described, 72 belong to the Neotropical region [4]. This region hosts approximately 3,100 described species and is considered one of the richest in ant species in the world [9].

Currently, the various synonyms related to the genus *Strumigenys* proposed by Baroni Urbani & De Andrade [10] are well accepted [11], although, the proposal of merging the tribes Phalacromyrmecini and Basicerotini into the tribe Dacetini is still controversial [12]. Commenting on this issue is not withinthe scope of this paper and assuming that the Basicerotini and Phalacromyrmecini continue to be ranked as separate tribes [12], the tribe Dacetini would include 203 Neotropical species with representatives from three genera: *Acanthognathus*, *Daceton* and *Strumigenys* [9,13–17]. The genus *Strumigenys* includes 194 species in the Neotropics, although none have been subjected to cytogenetic studies [4,9]. However, information regarding their chromosome number is available for a few species of this genus from southern Asia and Oceania: *S. doriae* (2n = 22), *S. friedae* (2n = 24) and *S. godeffroyi* (2n = 40, 44) [18–20], *Strumigenys* spp. (2n = 16; 2n = 38; n = 13), *S. mutica* (2n = 36) and *S. dohertyi*

(2n = 24) (in [4], as *Pyramica* spp., *P. mutica* and *P. dohertyi*, respectively).

Strumigenys louisianae distribution ranges from southern United States to Argentina. This species shows great morphological variation, including the density and intensity of the sculpture on the mesosoma, on the post-petiole and gaster and the shape and size of the spongiform appendages, possiblybeing that *S. louisianae* represents, in fact, a complex of species according to the morphological data available [13]. The uncertainty regarding the taxonomic status of *S. louisianae* warrants the need for further evidence to create a better understanding of the true boundaries of this taxa. Morphologically independent data like molecular and cytogenetics are of great value in this endeavor. In light of the absence of cytogenetic data for this species, the objective of this study was to present the first cytogenetic data for *S. louisianae*.

Materials and Methods

Cytogenetic studies were conducted on a *S. louisianae* colony collected in the '*Mata da Biologia*' secondary forest patch located at the Universidade Federal de Viçosa campus, Viçosa, Minas Gerais, Brazil (20°45′23″S, 42°52′25″W) in July 2013. The national collecting permit was issued for Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio to Luisa Antônia Campos Barros (SISBio: 32459-5).For this location specific permit was not required for the sampling and the species studied is neither an endangered nor protected species. The colony was maintained in a plastic container to obtain the larvae at an appropriate stage. One adult specimen was identified and photographed (Fig. 1) by Thiago Sanchez Ranzani da Silva and deposited in the Hymenoptera collection of the Museu de Zoologia, Universidade de São Paulo (MZUSP), Brazil.

The metaphases were obtained using the cerebral ganglion, according to Imai et al. [21]. More than 80 metaphases were analyzed from two individuals. Some metaphases were stained with 5% Giemsa, and 10 of these were measured for the classification of chromosome morphology as proposed by Levan et al. [22]. Characterization of the richness of the CG and AT base pairs along the chromosome was acquired using the fluorochromes Chromomycin A_3 (CMA_3) and 4′6- diamidino-2-phenylindole (DAPI), according to Schweizer [23].

Results and Discussion

Strumigenys louisianae presented 2n = 4 chromosomes (Fig. 2A), all metacentric and properly paired (mean of the arm ratio: first pair 1.61; second pair 1.04; as obtained from 10 metaphases). This species presents the lowest chromosome number among the Hymenoptera from the Neotropical region [4]. Data from this study also correspond to the lowest chromosome number ever recorded in the subfamily Myrmicinae. Previously, 2n = 8 chromosomes was considered the lowest number reported for this subfamily, which had been recorded for *Mycocepurus goeldii* [6] and *Mycocepurus* sp. [24]. Although a low number of cytogenetics studies were conducted on the Neotropical ant fauna, a great diversity rangeis observed,from the finding of 2n = 4 chromosomes in *S. louisianae* in this work to the highest number known for Hymenoptera, the 2n = 120 chromosomes found in *D. lucida* [8].

The occurrence of a species with a low chromosome number and being phylogenetically similar to others with higher numbers is not unique to the genus *Strumigenys*. A similar case is evident in the ant *Myrmecia croslandi* (Formicidae: Myrmicinae)in which 2n = 2 chromosomes were observed in the females, whereas in the males, which are haploid, the presence of n = 1 chromosome was found [25]. Although fission plays an important role in the evolution of the karyotype in Formicidae, some centric fusions occasionally occur as a mechanism for heterochromatin elimination, especially in those karyotypes presenting pseudo-acrocentric chromosomes [21]. A better supported interpretation, based on the cytogenetic and molecular data, for the emergence of the karyotype 2n = 2 chromosomes in *M. croslandi* suggests that this karyotype originated from the fusion of the chromosomes of the karyotype 2n = 4 occurring in individuals of the same species, where the intermediate karyotype 2n = 3 is known and possibly originated from the female gametes of *M. croslandi* with n = 2 chromosomes and the male with n = 1 chromosome or vice versa [25,26]. *Pheidole nodus* presents chromosomal polymorphism in which its chromosomal number varies from n = 17 to n = 20, and an ancestral karyotype of n = 18; the other three karyotypes result probably either from centric fusion (n = 17) and fission (n = 19 and n = 20) [27]. Fusion type chromosomal rearrangements were also suggested recently in the evolution of the genus *Mycetophylax* [5]. Another example is the social parasite *Acromyrmex ameliae* that

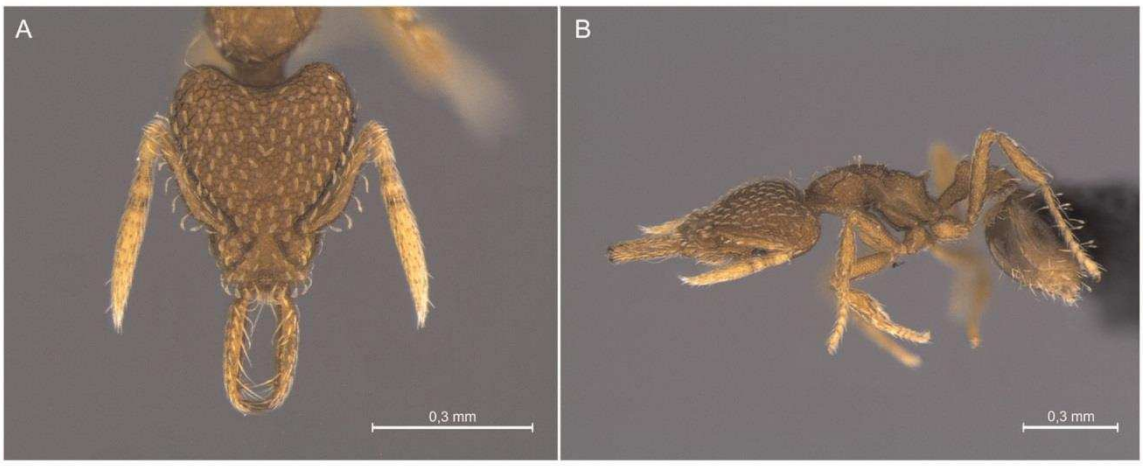

Figure 1. *Strumigenys louisianae* images: A) frontal view of the head, B) lateral view.
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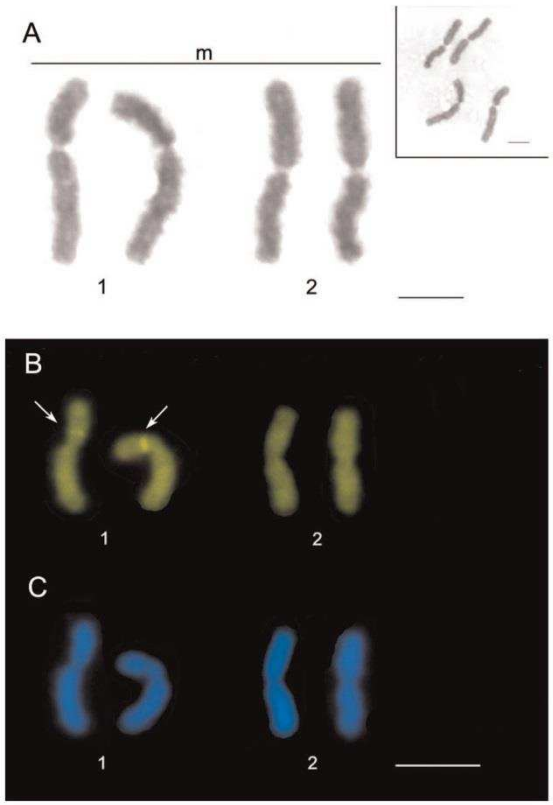

Figure 2. Metaphase and karyotype of the 2n = 4 chromosomes in *Strumigenys louisianae*; Giemsa staining (A) Karyotype with CMA_3 (B) and DAPI staining (C). Arrows indicate the positive marks for CMA_3. m = metacentric. Bar: 5 μm.
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presents a distinct chromosome number of 2n = 36 rather than the 2n = 38 chromosomes, found in all other members of the genus *Acromyrmex* (2n = 38), indicating a centric fusion of two pairs of chromosomes (unpublished data). The same rearrangement has been suggested for vertebrate species, such as *Muntiacus muntjak* (Cervidae) during the formation of the karyotype 2n = 6 chromosomes in females and 2n = 7 in males from the karyotype 2n = 46 of *Muntiacus reevesi*. For the formation of the karyotype 2n = 6 it was suggested that at least 20 tandem fusions had to occur in the karyotype 2n = 46 [28–30]. With the cytogenetic data obtained to date for the genus *Strumigenys* (2n = 16 to 2n = 40, reviewed in [4]) it is believed that tandem fusions are possibly the ones responsible for the formation of the karyotype 2n = 4 observed in *S. louisianae*. It could have occurred as a mechanism of heterochromatin elimination, since the heterochromatic blocks were not evident in the chromosomes of this species on using the Giemsa staining protocol as proposed by [31].

The fluorochrome CMA_3 showed the presence of a block rich in GC base pairs in the interstitial region of the short arm of the largest chromosome pair, where this is the first data recorded on banding in this genus (Fig. 2B). The CMA_3 was used in some ant species and it revealed markings on a chromosome pair in *Dinoponera lucida* [8], *Azteca trigona* [32], and *Tapinoma nigerrimum* [33] corresponding to the Nucleolar Organizer Regions (NORs). This correlation was confirmed by the FISH and/or NOR banding technique. The correlation between the Nucleolar organizer regions (NORs) and GC-rich regions is a very common occurrence in Hymenoptera [34]; therefore, the banding with the CMA_3 may contribute to the identification of the NORs, especially for the single NORs. These regions are considered conserved and found in specific locations for each species. As a result of this specificity, the description of the number and position of this region in the chromosomes can be reliably used in taxonomic and phylogenetic studies [35].

The fluorochrome DAPI nonspecifically marked the chromosomes of *S. louisianae;* however, the AT-rich regions were not observed (Fig. 2C). Some species among Hymenoptera present these markings, including some bees [34,36] and the little fire ant *Wasmannia auropunctata* [35]. These markings are mainly present in the centromeric regions [37], although in *W. auropunctata* the DAPI rich regions were observed in the pericentromeric region in most chromosomes [35].

Morphology and Molecular Genetics are the most commonly used tools by systematists to reconstruct phylogeny. In this context, cytogenetics play an important role acting as another independent source of evidence that can strengthen ideas on the evolution of particular groups. Further cytogenetic data can bring evidence to the many synonyms that resulted in the current hyperdiverse genus *Strumigenys* [10–38] and shed light on the understanding of the evolution of the various mandible forms found in this genus. Moreover, data of this kind have the potential to test the contradictory concepts on the tribal rank of the Dacetini, Basicerotini and Phalacromyrmecini [10,38–40], none of which are fully supported by the current molecular evidence [41,42].

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Author Contributions

Conceived and designed the experiments: APAS LACB JCMC SGP. Performed the experiments: APAS LACB. Analyzed the data: APAS LACB JCMC SGP. Contributed reagents/materials/analysis tools: APAS LACB JCMC SGP. Wrote the paper: APAS LACB JCMC SGP.

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General Protocol of FISH for Insects

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Abstract

The class Insecta comprises a widely distributed and diverse group of organisms. This diversity also extends to cytogenetic data, such as chromosome number, sex determination systems and peculiar kinds of chromosomes. Information on fluorescence in situ hybridization applied in insects is available, and the approach has been increasingly used over recent years. However, corresponding data is still scarce for some groups. In this chapter, we provide a detailed FISH protocol with varied use in insects, including reagent preparation instructions, adaptations and discussion. We also provide information about homemade C_0t1 DNA.

Keywords C_0t1 DNA, Molecular cytogenetics, FISH protocol for insects, Class Insecta

1 Introduction

The class Insecta harbours more than half of the described eukaryote species on Earth. The estimates of the number of insect species already described range from 720,000 [1] to around one million species [2]; however, real numbers of these species can range from around 5 to 6 million [3]. Insects are distributed over 29 orders and can be considered cosmopolitan, being found from freshwater streams up to very dry deserts.

The taxonomic diversity observed can be extended to the cytogenetic data of the group. The diploid number varies between $n = 1$ in *Myrmecia croslandi* (Hymenoptera: Formicidae) [4] and $n = \sim 224–226$ in *Polyommatus* (*Plebicula*) *atlanticus* (Lepidoptera: Lycaenidae) [5]. Moreover, all types of sex determination systems of the animal kingdom are represented in insects. For example, the sex determination "ratio of X chromosomes to sets of autosomes", where females are 2X:2A and males are X:2A (A is a haploid group of autosomes), is what determines the sex in some orthopterans and dipterans [e.g. crickets, grasshopper and some

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population of *Drosophila* (chapter by Amanda Larracuente "FISH in *Drosophila*")]. The XY and ZW sexual system can be found in the genus *Musca* and the lepidopterans, respectively (e.g. butterflies and moths). The order Hymenoptera organizes its sex determination by haplodiploidy, where females are diploid originated from fertilized eggs and males are haploid, originated from unfertilized eggs [6]. Beyond these, some insects still present polytene, lampbrush and holocentric chromosome.

In 1977, the first non-radioactive in situ hybridization was performed using labelled probes by indirect immunofluorescence. This protocol was applied on polytene chromosomes, in *Drosophila melanogaster* using the 5S rRNA probe [7]. Before this improvement in the protocol, radioactively labelled RNA-DNA hybrids were exclusively used and visualized using the autoradiographic method. That method was slow and it was necessary to wait several days to visualize the results [8].

Currently, the fluorescence in situ hybridization (FISH) is a tool used in many groups of insects, from studies focussing on insects of medical and agricultural interest (e.g. methods for introduction of exogenous genes) to descriptive and evolutionary research, using specific probes (e.g. 18S and repetitive DNA [9, 10]). Despite its increasing application during the last 30 years, in some groups such as the ants, which already include 13,986 described species [11], only 23 species have been studied by FISH, all of them using ribosomal DNA and/or telomeric probes [12–20].

In this chapter, we provide a detailed FISH protocol with varied use in insects, including reagent preparation instructions, adaptations and discussion. We also provide information about homemade C_0t1 DNA.

2 Materials

2.1 Homemade C_0t DNA

- Autoclaved Milli-Q water
- S1 nuclease and 10 × S1 nuclease buffer (Ref. M5761, Promega)
- 2-Propanol ACS reagent, ≥99.5 % (e.g. Sigma; stored at room temperature = RT)
- Sodium acetate solution (3 M, ph 5.2; e.g. Merck; stored at RT)
- TE buffer solution (e.g. Promega; stored at RT)
- PureLink® Genomic DNA Mini Kits (Ref. K1820-02, Invitrogen; stored at 15 to 30 °C)

2.2 FISH Probe Labelling

- DIG-Nick Translation Mix (Ref. 11 745 816 910, Roche Diagnostics; stored at −15 to −25 °C).

- EDTA 0.5 M (e.g. Merck; stored at RT).
- Ethanol 100 % (e.g. Merck; stored at RT).
- Hybridization buffer: dissolve 2 g dextran sulphate in 10 ml 50 % deionized formamide/2 × SSC/50 mM phosphate buffer for 3 h at 70 °C. pH adjusted to 7 with phosphate buffer or hydrochloric acid destabilizes buffer solution. Aliquot and store at −20 °C.
- Autoclaved Milli-Q water.
- Sodium acetate solution (3 M, ph 5.2; e.g. Merck; stored at −20 °C).
- Dextran sulphate (Ref. 67578, Sigma; stored at RT).
- Formamide (Ref. F7503, Sigma).

2.3 RNAse and Pepsin Pretreatment

- RNAse stock (Ref. 10 109 142 001, Roche Diagnostics GmbH; stored at 2–8 °C).
- RNAse solution: add 0.05 vol. RNAse at 10 mg ml^{-1} in 0.95 vol. of 2 × SSC and mix well; make fresh as required.
- 20 × SSC = saline sodium citrate (Ref. 15557-036, Gibco BRL; store at RT) or homemade: dilute 175.6 g of NaCl (Ref. S3014, Sigma) and 88.2 g of sodium citrate (Ref. 6132-04-3, Sigma) in 1 l of Milli-Q water. If necessary, use HCl to reach pH 7.0. Set up 0.4 ×, 1 × and 2 × before use.
- PBS 10 × = phosphate-buffered saline (Ref. L1825, Biochrom; stored at RT) or homemade: dilute 75.8 g NaCl (Ref. S3014, Sigma), 9.93 g Na_2HPO_4 (Ref. S7907, Sigma) and 4.14 g NaH_2PO_4 (Ref. S8282, Sigma) in 1 l of Milli-Q water.
- Ethanol 70 %, 95 % and 100 % each (e.g. Merck; stored at RT).
- Pepsin stock (Ref. P7012, Sigma).
- Pepsin solution: mix 10 µl of 1 M HCl and 2.5 µl vol. 20 ng ml^{-1} pepsin in 990 µl of Milli-Q water; make fresh as required.
- Postfix solution: mix 5 ml of 2 % paraformaldehyde (e.g. Merck), 4.5 ml of 1 × PBS and 0.5 ml 1 M $MgCl_2$; store at 4 °C.

2.4 Denaturation

- Denaturation buffer: 0.7 vol. of formamide, 0.2 vol. of Milli-Q water and 0.1 vol. 20 × SSC; store at 4 °C.
- Formamide (Ref. F7503, Sigma).
- Ethanol 70 % (at −20 °C), 95 % and 100 % (e.g. Merck; stored at RT).

2.5 Washing Slide

- Washing buffer: 0.2 vol. of 20 × SSC, 0.8 vol. Milli-Q water and 0.05 vol. of Tween 20.

- Tween 20 = polyoxyethylene-sorbitan monolaurate (Ref. 10670-1000, Sigma; stored at RT).
- Ethanol 70 %, 95 % and 100 % each (e.g. Merck; stored at RT).
- Marvel solution: dilute 0.1 g of milk powder Marvel in 2 ml of washing buffer (can use milk powder Molico®).
- Anti-digoxigenin-rhodamine (Ref. 11 207 750 910, Roche Diagnostics GmbH; stored at 2–8 °C).
- Antibody solution: 10 μl of anti-digoxigenin-rhodamine (200 μl ml^{-1}) and 990 μl of Marvel solution, make fresh as required.
- Fluoroshield™ with DAPI (Ref. F6057, Sigma; stored at 2–8 °C) or homemade: dissolve 1.5 μl of 1 M DAPI stock solution (4,6-diamidino-2-phenylindole · 2HCl: Ref. 124653, Merck) in 1 ml antifade VECTASHIELD (Ref. H1000, Vector Laboratories/Biozol; stored at 4 °C).

3 Methods

3.1 Homemade C_0t DNA

The C_0t-1 DNA is an unlabelled repetitive DNA fraction that is used to block nonspecific hybridization in FISH assays. Its use allows better and clearer results in some probes, such as genomic bacterial artificial chromosome (BAC) clone or whole chromosome painting.

1. Extract ~500 μg of the genomic DNA from the species of interest using PureLink® Genomic DNA Mini Kits, and dilute to 100–1,000 ng μl^{-1} DNA solution in Milli-Q water.
2. For denaturation and fragmentation of the DNA, use a heat block preheated at 120 °C for 2 min and 30 sec, using 0.5 μl tubes with safe-lock (*see* **Note 1**).
3. Reassociate the DNA at 60 °C for 15–150 min (*see* **Note 2**). Then place the tube with DNA on ice for 2 min.
4. Transfer the DNA tube to 42 °C and add preheated 10 × S1 nuclease buffer and S1 nuclease, then incubate for 1 h (*see* **Note 3**).
5. Precipitate DNA by adding 0.1 vol. of 3 M sodium acetate and 1 vol. of 2-propanol. Centrifuge at 14,000 rpm for 20 min at 4 °C.
6. Discard the supernatant, and dry the DNA pellet at room temperature (RT).
7. Add 100 μl DNA in 70 % ethanol, shake for 30 s and centrifuge again at 14,000 rpm for 10 min at 4 °C.
8. Discard the supernatant carefully, and dry the pellet.
9. Dissolve the DNA pellet in 50–100 μl of TE buffer solution.

10. Measure the DNA concentration and visualize by gel electrophoresis (*see* **Note 4**).

3.2 Self-Labelled and Denaturation Probes for FISH

The probe DNA can be obtained by flow sorting, microdissection or PCR (chapter by Fengtang Yang et al. "Generation of Paint Probes from Flow-Sorted and Microdissected Chromosomes"; chapter by Nadezda Kosyakova et al. "FISH-Microdissection"; chapter by Thomas Liehr "Homemade Locus-Specific FISH Probes: Bacterial Artificial Chromosomes") and labelled directly (e.g. SpectrumOrange, SpectrumGreen, TexasRed) or indirectly (e.g. biotin, digoxigenin). In our lab, we use indirect labelling, but there are available Nick translation kits for direct and indirect labelling.

3.2.1 Indirect Labelling Probes

1. Put in a microtube 3.5 μl of the probe DNA (recommended concentration 50 ng μl^{-1}) in 12.5 μl of Milli-Q water, and add 4 μl of the DIG-Nick Translation Mix. Mix and spin briefly.
2. Incubate for 90 min at 16 °C.
3. To stop the reaction, add 1 μl of 0.5 M EDTA and put at 65 °C for 10 min.
4. Store at −20 °C.

3.2.2 Denature Probe Solution for FISH

If it is not necessary to use C_0t DNA:

1. For each slide use 2 μl of the labelled probe diluted in 18 μl of hybridization buffer.
2. Denature for 5 min at 85 °C, and then keep the solution in ice or at 4 °C until use.

If it is necessary to use C_0t DNA:

1. Precipitate the labelled probe and C_0t DNA together using 1 vol. of 2-propanol and 0.1 vol. of sodium acetate (3 M, pH 5.2). For better results the use of proportions higher than 1:20 probe to C_0t1 DNA fraction is recommended.
2. Centrifuge at 14,000–15,000 rpm for 20 min at 4 °C, discard the supernatant and dry the DNA pellet at RT.
3. Dilute the pellet in 20 μl of hybridization buffer.
4. Denature the probe solution at 85 °C for 5 min. Then, the prehybridization step is done at 37 °C for 30 min.
5. Keep the solution in ice or at 4 °C until it is used.

3.2.3 RNAse and Pepsin Pretreatment

Most of the protocols in insects use RNAse and pepsin treatment, followed by postfixation, to reduce the background. However, in FISH made in ants and wasps, we see no significant change in the background amount and less degraded chromosomes when we skipped the RNAse and pepsin treatment, thus starting the procedure at step 4.

1. Put 100 μl RNAse solution on slide and cover with coverslip, and then incubate in humid chamber at 37 °C for 1 h.
2. Remove the coverslip and wash in 2 × SSC for 5 min and air-dry.
3. Add 50 μl of 0.005 % pepsin and cover with coverslip for 10 min at RT.
4. Wash in 1 × PBS for 5 min at RT.
5. Incubate the slide in 100 μl of postfix solution under coverslip for 10 min at RT.
6. Remove the coverslip and wash for 5 min in 1 × PBS.
7. Dehydrate slides in ethanol series (70 %, 95 % and 100 %, 3 min each one) and air-dry.

3.3 Denaturation

1. Put 100 μl of denaturation buffer on slide and cover with coverslip, and then denature on preheated hotplate at 73 °C for 3 min.
2. Remove the coverslip and place the slide in 70 % ethanol at −20 °C for 3 min; after, pass the slide through ethanol series, 95 % and 100 %, for 3 min each one at RT (*see* **Note 5**). Air-dry.
3. Add 20 μl of probe solution, already denatured, on the slide and cover with coverslip. Incubate the slide overnight at 37 °C in a humid chamber (*see* **Note 6**).

3.4 Detect Probe and Washing Slides

When labelled indirectly, the use of antibody conjugated with fluorochrome is necessary (e.g. anti-digoxigenin or anti-biotin attached with Cy3, Cy5 or rhodamine). If you use direct labelling, there is no need to make detection steps of the probes, and skip steps 3 to 6 of this Section.

1. Take slide from humid chamber at 37 °C and remove coverslip, and then wash the slide in 100 ml of 0.4 × SSC at 65–68 °C for 3–4 min in a Coplin jar placed into a water bath.
2. Transfer the slides into 100 ml of 4 × SSC/0.2 % Tween for 5 min at RT in a shaker.
3. Add 100 μl Marvel solution under coverslip at 37 °C for 10–15 min in a humid chamber.
4. Remove coverslip and wash the slide in 4 × SSC/Tween for 2 min at RT.
5. The detection is made through 100 μl of antibody solution for each slide covered with coverslip, and incubate for 20–35 min at 37 °C in a humid chamber.

6. Remove the coverslip and put the slide in 4 × SSC/0.2 % Tween for 5 min on the shaker; repeat this step three times using a new solution in each one.
7. Rinse in Milli-Q water briefly, and pass through ethanol series (70 %, 95 % and 100 %) for 3 min each one at RT. Air-dry.
8. Apply 20 μl of Fluoroshield™ with DAPI and cover with coverslip.
9. Evaluate through microscope.

4 Notes

1. The fragmentation of DNA can be made by autoclaving; autoclave for 5 min at 121 °C (1 atm). To finish this step, it is important to take a sample and check in gel if the fragmentation worked well.
2. The reassociation time can be calculated following the formula $t = \frac{C_0 t X \times 4.98}{C_0}$ where t is the time of incubation, X is the fraction of C_0t ($C_0t1 = 1$; $C_0t2 = 2$, $C_0t3 = 3$, etc.) and C_0 is the concentration of initial DNA in μg μl^{-1} [21]. The C_0t1 fraction is the most commonly used in FISH.
3. The amount of the S1 nuclease and 10 × S1 nuclease buffer is associated with the quantity of DNA and with the final total volume, respectively. For each 1 μg of DNA, 1U of S1 nuclease is used, and the buffer represents 10 % of the total volume (e.g. for 500 μg of DNA + 5.6 μl S1 nuclease (89 U μl^{-1}) + 50 μl of 10 × S1 nuclease buffer + 444.4 μl of water, the final total volume is 500 μl).
4. As C_0t DNA are fragments of repetitive DNA, normally, with sizes between 50 and 500 bp, you should see in gel a long band with more DNA concentration around 200 bp size (use a 100 bp DNA ladder, e.g. New England Biolabs, to help identify the size).
5. It is important that 70 % ethanol is very cold in this step. Then, make sure that it has been placed under refrigeration at −20 °C some hours before starting the protocol.
6. The time of incubation can vary; while some probes show better results when incubated for 8 h (overnight), others will need up to 48 h. However, longer times can cause an increase of the background. To reduce background, if the probe was labelled directly, incubate the slide upside down only in this step; if the probe was labelled indirectly, incubate the slide upside down in this step and in the antibody solution step.

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