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Cytogenetics of *Strumigenys louisianae* Roger, 1863 (Formicidae: Myrmicinae) from North-eastern Amazonia shed light on a difficult species complex

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Highlights

- *S. louisianae* from the Amazon rainforest showed an intriguing karyotype: $2n=26$.
- The karyotype of the Amazonian *S. louisianae* contrasts with an already described one.
- Morphological and karyotype variations put to question the limits of the taxon.
- Morphology of the Amazonian *S. louisianae* is similar to the type locality in the USA.
- After our findings, we encourage reevaluation of junior synonyms of *S. louisianae*.

Abstract

Cytogenetic techniques provide powerful insights on species-rich taxa—such as ants—allowing better understanding of their biodiversity. Some hints on evolutionary paths can be observed through comparative populational cytogenetics among different ant groups. In this study, the karyotype of *Strumigenys louisianae* Roger from the Amazon rainforest is described and showed diploid chromosome number of 26 chromosomes. This configuration intriguingly contrasts with the already described karyotype for this species from the Atlantic rainforest with only $2n = 4$ chromosomes. 18S rDNA site were detected on the pericentromeric region of the long arm of a metacentric pair and co-localizing with GC-rich chromatin. Recurrent synonymizations of *S. louisianae* may not reflect the species status of this taxon. The karyotypic differences and the observable morphological variation between the populations of both localities corroborates the idea of a species complex within *Strumigenys louisianae*. The morphology of *S. louisianae* from the Amazonian region is similar to that from the United States, the type locality. On the other hand, specimens from the Atlantic rainforest are more similar to the junior synonym *S. unidentata* Mayr. This study reinforces the need of taxonomical revision in *Strumigenys louisianae* by means of integrative taxonomy approaches.

Keywords: chromosome, karyotype, biodiversity, trap-jaw ants, karyomorphometry, 18S rDNA.

1. Introduction

The use of cytogenetics in the Formicidae has been highlighted in different studies, including chromosomal evolution, phylogeny, endangered species, chromosomal structure, and in taxonomic issues (Mariano et al., 2012; Cristiano et al., 2013; Santos et al., 2016; Correia et al., 2016; Huang et al., 2016; Cristiano et al. 2020; Hirai, 2020). There are more than 800 ant species karyotyped so far, most of which are from the Neotropical region, particularly, the myrmecofauna of the Brazilian Atlantic rainforest (reviewed by Lorite and Palomeque, 2010; Cardoso et al. 2018; Mariano et al., 2019).

The use of classical cytogenetics alone (see discussion in Liehr, 2017), which makes use of a single dye, has shown its importance in the detection and comprehension of diversity in different organisms (Petitpierre, 2011; Di-Nizo, 2017; Zacharopoulou et al., 2017; Cioffi et al., 2018) including ants (Mariano et al., 2012, 2019; Santos et al., 2018). The chromosome number in Formicidae ranges from $n = 1$ in the Australian bulldog ant *Myrmecia croslandi* Taylor, 1991 (Crosland & Crozier, 1986) to $n = 60$ in the giant ant of the Atlantic rainforest *Dinoponera lucida* Emery, 1901 in Brazil (Mariano et al. 2008).

Variations in chromosome number have been observed among different ant groups including species with high morphological similarities. The great variations in chromosome number in the Australian jack-jumper ants of the *Myrmecia pilosula* complex ($2n = 2-32$) have helped in defining the six species currently recognized for the complex (Imai et al., 1994; Taylor, 2015). Other interesting examples have been observed in *Neoponera apicalis* (Latreille 1802) and *N. verenae* Forel, 1922, with extensive variations in chromosome number among populations of the same species (Mariano et al., 2012). Karyotypic populational studies in ants although scarce have brought novelties and important insights in ant evolution (Velasco et al., 2014; Aguiar et al. 2017; Micolino et al., 2019a, b; Teixeira et al., 2020).

Karyological information is available for some Indomalayan and Australian species of the genus *Strumigenys* Smith, 1860, ranging from $2n=16$ to $2n = 44$ (reviewed by Lorite and Palomeque, 2010; Cardoso et al. 2018). Cytogenetic data in the Neotropics are restricted to two species (Table 1),

Strumigenys diabolus Bolton, 2000 from the Amazon rainforest, $2n = 40$ (Aguilar et al., 2020) and *Strumigenys louisianae* Roger, 1863 from Atlantic rainforest with $2n = 4$, the latter having all metacentrics and corresponding to the smallest chromosome number observed in Myrmicinae (Alves-Silva et al., 2014).

Strumigenys louisianae shows a wide geographic distribution in the New World, ranging from southern United States until northern Argentina (Bolton, 2000; Wetterer, 2014). The species shows great morphological variation concerning size, pilosity, sculpturing, and even color, strongly indicating it represents a complex of different species (Bolton, 2000). To date, a total fourteen species, subspecies and variations are synonymized under *S. louisianae* (Brown 1953, 1961; Bolton et al., 2008).

In this study, we described the karyotype of *S. louisianae* from Oiapoque, state of Amapá, Brazil, in north-eastern Amazonia and compared it with the available karyotype from the population of the Atlantic rainforest (Alves-Silva et al., 2014). We also highlighted morphological differences between the two populations and discussed the taxonomic importance of our results.

2. Materials and methods

An ant colony of *S. louisianae* was collected in Oiapoque, state of Amapá in Brazil (3.84151, -51.84112) in October 2013 (Figure 1). The sampling authorization in Brazil had the accession number 32459 ICMBio/SISBIO. Specimens from the colony, three workers (UFV-LABECOL-000100, UFV-LABECOL-001548, UFV-LABECOL-007492) and a dealate queen (UFV-LABECOL-000100), were deposited in Coleção Entomológica do Laboratório de Sistemática de Coleoptera (CELC), Universidade Federal de Viçosa, Brazil.

Images of the specimens of *S. louisianae* were made in a Leica S8APO with a 2x auxiliary lens with a Canon 1100D attached. Acquired images were stacked in Zerene software (Zerene Systems LLC). Images enhancement were made in GIMP 2.10.20 (Kimball & Mattis, 1996) and scale bars were added in ImageJ (Schneider et al., 2012) through measurements taken from the specimen during image acquisition.

Table 1

Cytogenetics of Neotropical *Strumigenys*. Localities of collection sites, diploid (2n) chromosome numbers, diploid karyotypic formulae

Ant species	Locality	2n	Karyotypic formula	References
<i>Strumigenys diabolus</i> Bolton, 2000	Sinnamary, FG	40	4m + 10st + 26a*	Aguiar et al. (2020)
<i>Strumigenys louisianae</i> Roger, 1863	Viçosa-MG, BR	4	4m	Alves-Silva et al. (2014)
<i>Strumigenys louisianae</i> Roger, 1863	Oiapoque-AP, BR	26	12m + 12sm + 2st	This study

FG: French Guiana; BR: Brazil; MG: state of Minas Gerais; AP: state of Amapá.

m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric.

* The karyotypic formula of *S. diabolus* is presented based on Fig. 5a by Aguiar et al. (2020) because the karyotype is incorrectly written in the table.



Fig. 1. Map with the localities of cytotogenetically studied populations of *Strumigenys louisianae* in the Amazon rainforest (1) at Oiapoque, state of Amapá (this study) and in the Atlantic rainforest (2), at Viçosa, state of Minas Gerais (Alves-Silva et al. 2014), in Brazil.

Mitotic metaphase chromosomes were obtained from the cerebral ganglia after meconium elimination, according to Imai et al. (1988). The definition of chromosome number and morphology of metaphases was done using conventional 4% Giemsa staining. Karyomorphometric analyses were conducted using eight metaphases well spread, with similar degree of condensation, and evident centromeres. The measurements were obtained in micromeres for short arms (p), long arms (q), and total length (TL = p+q). The relative length was obtained by $RL = TL \cdot 100 / \Sigma TL$ and given in percentage of the total genome length. Chromosomes were arranged in order of decreasing size and based on the ratio of the chromosomes arm lengths ($r = \text{long arm} / \text{short arm}$), according to the classification proposed by Levan et al. (1964), with minor modification on chromosome nomenclature with $r > 7$. The chromosomes were classified as m = metacentric ($r = 1-1.7$), sm = submetacentric ($r = 1.7-3$), st =

subtelocentric ($r = 3-7$) and $a =$ acrocentric ($r > 7$). Chromosomes were organized using Adobe Photoshop® 21.1.1 and measured using Image Pro Plus®.

The detection of GC- and AT-rich regions was performed using the fluorochromes CMA₃ and DAPI (Schweizer, 1980). The detection of 18S rDNA clusters by fluorescence *in situ* hybridization (FISH) technique was done following Pinkel et al. (1986). The 18S rDNA probe was obtained via polymerase chain reaction (PCR) amplification employing rDNA primers 18SF1 (5'-GTC ATA GCT TTG TCT CAA AGA-3') and 18SR1.1 (5'-CGC AAA TGA AAC TTT AAT CT-3') designed for the bee *Melipona quinquefasciata* Lepeletier, 1836 (Pereira, 2006). Genomic DNA of the ant *Camponotus rufipes* (Fabricius, 1775) was used as a template in the PCR reactions. 18S rDNA probes were labeled maintaining the conditions for PCR amplification (Pereira 2006) by an indirect method using digoxigenin-11-dUTP (Roche, Mannheim, Germany). FISH signals were detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

The metaphases were observed and photographed using an Olympus BX60 microscope, linked to a Q Color 3 Olympus® image capture system. The filters WB (450–480 nm), WU (330–385 nm), and WG (510–550 nm) were used for analyzing CMA₃, DAPI, and rhodamine respectively.

3. Results and discussion

Strumigenys louisianae from the Amazon rainforest showed $2n = 26$ chromosomes and karyomorphometrical analysis revealed that the karyotype consists of m , sm and st chromosomes with diploid karyotypic formula $12m + 12sm + 2st$ (Table 2, Figure 2). The species presented a quite distinct chromosome number from that of the Atlantic rainforest population ($2n = 4$, all metacentrics, see Alves-Silva et al., 2014). The chromosome size of the largest chromosome pair (the single st pair) showed its size 2.5x larger than the second largest chromosome pair (1st m pair) corresponding to a notable size difference in the karyotype (Table 2, Figure 3).

GC-rich blocks co-localized with 18S rDNA sites were located on the pericentromeric region of the long arm of the second metacentric pair (Figure

Table 2

Karyomorphometric analyses of the ant *Strumigenys louisianae* $2n = 26$ from Oiapoque, state of Amapá, Brazil,

Chromosome	Chromosome arm*		Total length (p+q)	Relative Length (%)	Arm Ratio (q/p)	Chromosome morphology
	Short (p)	Long (q)				
1	1,29±0,27	1,59±0,22	2,89±0,47	4,84	1,26±0,18	m
1	1,23±0,19	1,59±0,35	2,82±0,52	4,73	1,30±0,18	m
2	1,17±0,25	1,65±0,25	2,81±0,43	4,72	1,45±0,27	m
2	1,08±0,25	1,63±0,28	2,71±0,46	4,54	1,55±0,27	m
3	1,10±0,16	1,22±0,13	2,32±0,28	3,89	1,11±0,06	m
3	1,02±0,14	1,20±0,17	2,22±0,29	3,72	1,19±0,12	m
4	0,94±0,15	1,09±0,17	2,03±0,31	3,40	1,16±0,11	m
4	0,91±0,15	1,11±0,08	2,01±0,21	3,38	1,25±0,19	m
5	0,62±0,05	0,96±0,14	1,58±0,18	2,65	1,55±0,19	m
5	0,60±0,08	0,94±0,13	1,54±0,18	2,59	1,57±0,19	m
6	0,58±0,10	0,84±0,10	1,42±0,20	2,38	1,46±0,11	m
6	0,59±0,06	0,82±0,10	1,41±0,15	2,36	1,40±0,11	m
7	0,63±0,08	1,28±0,17	1,91±0,21	3,21	2,04±0,27	sm
7	0,60±0,10	1,20±0,10	1,81±0,17	3,03	2,02±0,25	sm
8	0,58±0,07	1,20±0,14	1,78±0,18	2,98	2,08±0,26	sm
8	0,59±0,11	1,15±0,15	1,74±0,22	2,91	2,00±0,40	sm
9	0,51±0,08	1,08±0,11	1,60±0,15	2,68	2,14±0,32	sm
9	0,52±0,08	1,02±0,13	1,55±0,19	2,60	1,99±0,29	sm
10	0,50±0,11	1,05±0,10	1,55±0,18	2,61	2,18±0,41	sm
10	0,51±0,10	1,04±0,09	1,54±0,17	2,59	2,10±0,35	sm
11	0,50±0,05	0,98±0,14	1,47±0,16	2,47	1,98±0,35	sm
11	0,48±0,05	0,92±0,15	1,40±0,18	2,35	1,93±0,26	sm
12	0,44±0,06	0,94±0,12	1,39±0,15	2,33	2,16±0,27	sm
12	0,43±0,05	0,92±0,14	1,35±0,16	2,26	2,20±0,40	sm
13	1,64±0,25	5,79±0,82	7,44±1,04	12,47	3,54±0,28	st
13	1,59±0,32	5,75±0,74	7,34±1,03	12,31	3,67±0,43	st
Total			59,61	100		

* chromosome length obtained in micrometers (μm) from eight metaphases
m = metacentric, sm = submetacentric, st = subtelocentric.

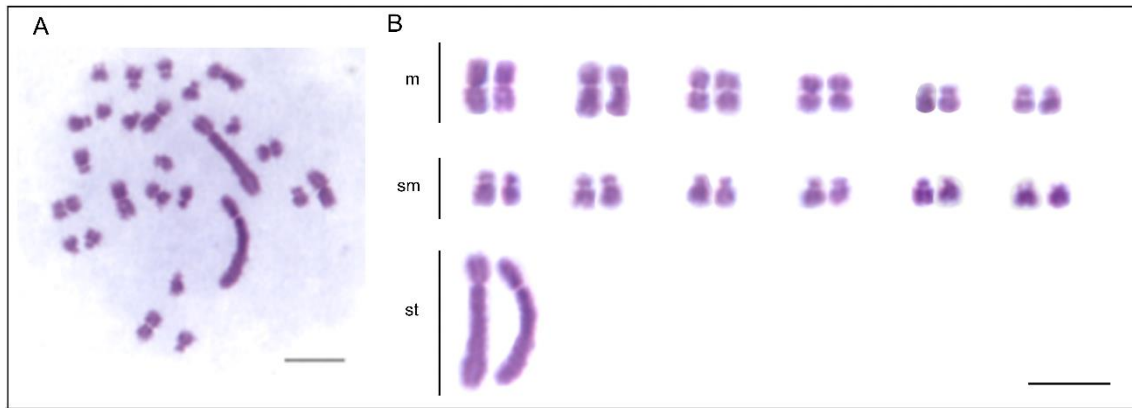


Fig. 2. Giemsa staining of A) metaphase and B) karyotype of the ant *Strumigenys louisianae* from Amazonia. m = metacentric, sm = submetacentric, st = subtelocentric. Scale bar = 5 μ m.

4). In the Atlantic rainforest sample, the pattern of GC-rich regions was detected also in a single chromosome pair in the interstitial region of the short arm of the largest chromosome pair (Alves-Silva et al., 2014). The presence of a single chromosome pair bearing GC-rich and 18S rDNA sites is commonly observed in ants (Teixeira et al., 2021) indicating that GC-rich blocks of the Atlantic rainforest population should correspond to rDNA clusters. The single rDNA site of *S. louisianae* follows the pattern of the majority of the ants analyzed so far (Teixeira et al., 2021). The complementary negative DAPI result is useful to detect the exact chromosome pair involved in the 18S rDNA blocks (Figure 4c).

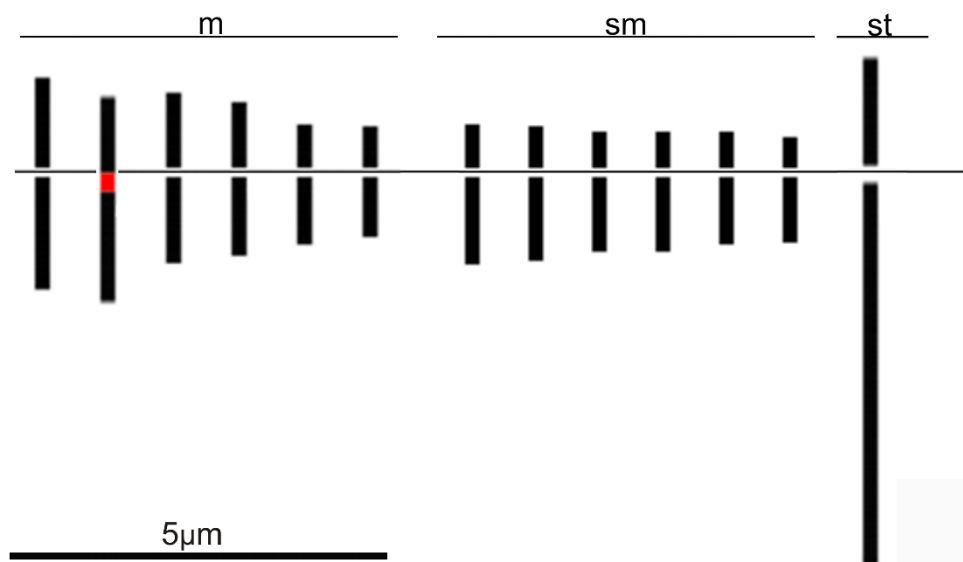


Fig. 3. Ideogram of the chromosomes of the ant *Strumigenys louisianae* ($n = 13$) from Oiapoque, north-eastern Amazonia. Red block corresponds to 18S rDNA sites co-localized with GC-rich region.

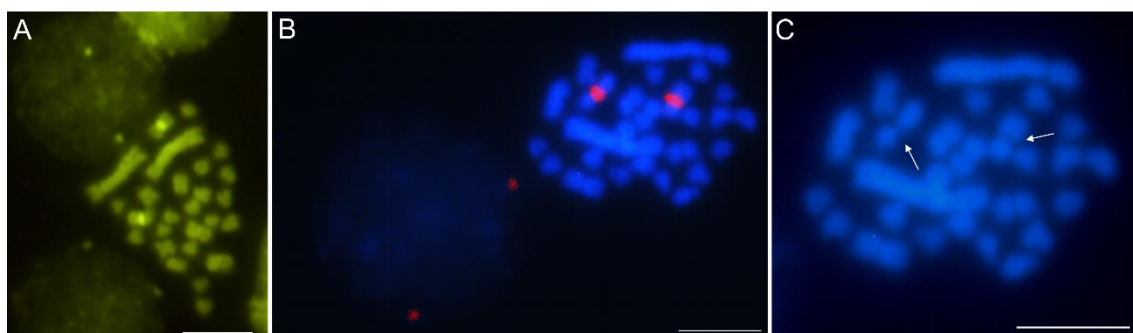


Fig. 4. Metaphases of *Strumigenys louisianae* from Oiapoque, north-eastern Amazonia after A) fluorochrome staining with CMA₃ for detection of GC-rich regions, B) physical mapping of ribosomal DNA using the probe 18S rDNA (red blocks) in interphasic nucleus and metaphase, and C) DAPI staining highlighting the AT negative pericentromeric region of the second chromosome pair. Scale bar = 5 μ m.

Strumigenys louisianae is one of the most sampled *Strumigenys* species in the New World and has a wide distribution (Bolton, 2000), but a few records are available from the Brazilian Amazon rainforest (Wetterer, 2014). Records of the species were absent in the state of Amapá, and there is a single report from a relatively close locality in French Guiana, Petit Saut dam (05.061, -43.043) (Wetterer 2014, personal communication). In the present study, it was collected by an active search in the bark of a fallen tree.

The synonymizations of various *taxa* under *S. louisianae* (Brown 1961) may not reflect the taxonomic unit of the species. Bolton (2000) suspected that *S. louisianae* could represent many genuine species. In this scenario, the wide distribution could be explained by the fact it is not of a single, but of many different species. The wide cytogenetic difference and morphological variation between both localities corroborates a species complex.

The morphology of *S. louisianae* from the Amazonian rainforest ($2n = 26$) links it to the populations from southern United States, the type locality of the species. This Nearctic-Neotropic form has relatively larger compound eyes; robust mandibles; ground pilosity on head being the “typical” spoon-shaped, ovoid and not thin; and generally a small smooth patch laterally on mesosoma, restricted to the mesopleuron (Fig. 5, A and C). Moreover, the alate queen is known and no intercaste is known for that population (Chaul et al., in prep.).

The population from the Atlantic rainforest ($2n = 4$) (Alves-Silva et al. 2014) on the other hand, is one of the most common morphotypes of *Strumigenys* in the locality where the karyotyped colony was found. Morphologically similar populations occur in a few other localities in the state of Minas Gerais, as well as other states from southern Brazil. It more closely matches the morphology of the syntype of the junior synonym *S. unidentata* Mayr, 1887 than that of the types of *S. louisianae* Roger, 1863 itself. This population has relatively smaller compound eyes; somewhat fragile mandibles; ground pilosity on head composed of thin spoon-shaped hairs; and larger smooth patch laterally on the mesosoma (Fig. 5, B and D). It also has a smaller body size in workers and has an intercaste form, which are probably the only reproductive individuals in the colony as no alate queens have ever been found. This population with $2n = 4$ chromosomes represents one of many populations occurring in sympatry along the Brazilian Atlantic rainforest, which harbour the greatest morphological diversity of the *Strumigenys louisianae* complex (Chaul et al., in prep.). The great differences in karyotype between the two populations further endorse the “complex of various species” hypothesis instead of a “plastic species” hypothesis (Bolton 2000). The different karyotypes of *S. louisianae* highlight the importance of cytogenetic data from distant populations, essential for the understanding of chromosome evolution of widely distributed taxa as observed for other several ant groups (Mariano et al., 2012; Micolino et al., 2019a; Teixeira et al., 2020).

The comparison between the karyotypes of both populations showed unequivocal discrepancy and the inclusion of additional localities associated with morphological data is necessary to better understand *S. louisianae* complex, since intermediary chromosome numbers could be observed. However, considering only the two already known chromosome numbers, it is unlikely that they properly pair producing multivalents during meiosis.

Considering the karyotype of both populations, more variation is expected in the Amazon population ($2n = 26$) since the small chromosome number in the Atlantic rainforest population ($2n = 4$) reduces, in theory, genetic variability inside the population due smaller linked groups. The “increases in the number of chromosomes increase the possible genotypes due to independent

assortment during meiosis" (Ross et al., 2015, see also discussion in Santos et al., 2016). The increase of chromosome number also favors the rates of recombination which make possible the combination of alleles in a same zygote (Ross et al., 2015). By contrast, there is significantly more morphological variation within the Atlantic rainforest populations than those from north South America, Central and North America, which could alternatively suggest a greater chromosomal variation in the Atlantic rainforest populations than in all the remaining populations.

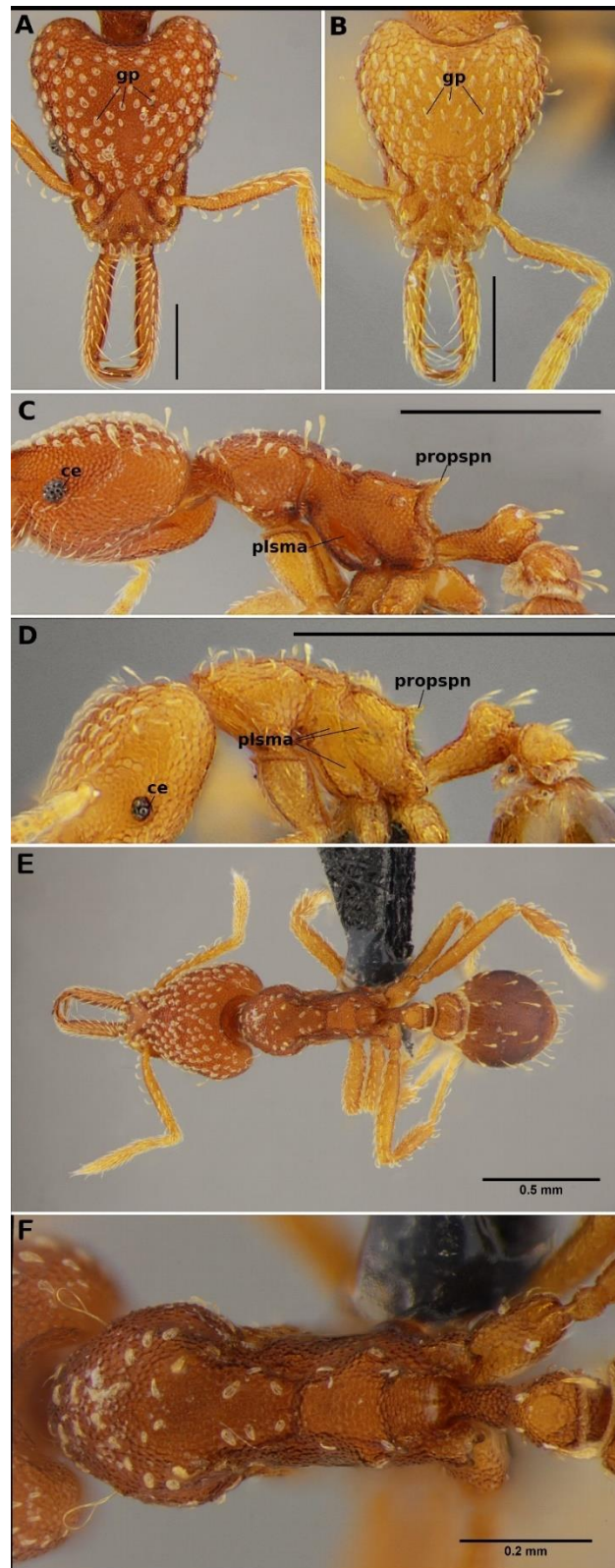


Fig. 5. Main morphological differences between the two *Strumigenys louisianae* populations with known karyotypes. A and C, full-face view and profile, respectively, of a worker from the Amapá population and B and D, the same for a worker from Minas Gerais. E and F, Dorsal view of a worker from the Amapá

population highlighting the humeral hair. Abbreviations: ce, compound eye; gp, ground pilosity; plsma, pleural smooth area; propspn, propodeal spine. Scale bars 0.2 mm in A and B and 0.5 mm in C and D.

The data highlights the need for research in geographic intermediary populations since the use of cytogenetics in species delimitation may be useful in an integrative approach (see Schlick-Steiner et al., 2010) of the *S. louisianae* complex. Considering that the population approach in ants is rarely embraced by cytogenetic studies, the increase of karyological data from Amazonian species will be valuable in increasing the knowledge of ant biodiversity. This study highlights the use of classical cytogenetics in species delimitation of *S. louisianae* in an integrative approach.

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