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Karyotype structure and cytogenetic markers of *Amoimyrmex bruchi* and *Amoimyrmex silvestrii*: contribution to understanding leaf-cutting ant relationships

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Abstract: Leaf-cutting ants are considered the most important herbivores in terrestrial environments throughout the Neotropics. *Amoimyrmex* Cristiano, Cardoso, & Sandoval, 2020 is the sister clade of the remaining leaf-cutting ants from the genera *Atta* and *Acromyrmex*. *Amoimyrmex striatus* was the only species cytogenetically studied within the genus and shares the same chromosomal number as *Atta*, bearing 22 chromosomes, whereas *Acromyrmex* bears 38 chromosomes, with the exception of the social parasite *Acromyrmex ameliae* (2*n* = 36). Our objective here was to cytogenetically analyze the species of *Amoimyrmex bruchi* and *Amoimyrmex silvestrii*, as well as to describe the karyotype of these sister species, using an integrative approach using classical and molecular cytogenetics. We aimed to characterize the cytogenetic markers that contribute to the systematics and taxonomy of the genus. Our results showed that the karyotypes of these two species are very similar, with an identical chromosome number (2*n* = 22), chromosome morphology (2K = 20m + 2sm), and location of 18S rDNA and telomeric repeat TTAGG on the chromosomes. However, the microsatellite probe $GA_{(15)}$ showed variation across the species and populations studied. We suggest that both species diverged relatively recently and are unmistakably sisters because of the many shared characteristics, including the highly conserved karyotypes.

Key words: karyotype, chromosome number, cytogenetic markers, evolution, fungus-farming ants.

Résumé : Les fourmis coupe-feuille sont considérées comme les herbivores les plus importants dans les environnements terrestres à travers l'écozone néotropicale. L'*Amoimyrmex* Cristiano, Cardoso & Sandoval, 2020 formes un clade voisin des autres fourmis coupe-feuille des genres *Atta* et *Acromyrmex*. L'*Amoimyrmex striatus* est la seule espèce qui a fait l'objet d'études cytogénétiques au sein du genre et partage le même nombre de chromosomes avec le genre *Atta*, soit 22 chromosomes, tandis que le genre *Acromyrmex* en compte 38, si on fait exception de l'espèce parasite *Acromyrmex ameliae* (2*n* = 36). L'objectif de ce travail était de réaliser une caractérisation cytogénétique des espèces *Amoimyrmex bruchi* et *Amoimyrmex silvestrii* et d'en décrire le caryotype au moyen d'une approche intégrée faisant appel à des outils de cytogénétique classique et moléculaire. Les auteurs ont cherché à caractériser des marqueurs cytogénétiques qui contribuent à la systématique et à la taxonomie au sein de ce genre. Les résultats montrent que les caryotypes de ces deux espèces sont très similaires, avec un même nombre de chromosomes (2*n* = 22), une morphologie chromosomique semblable (2K = 20m + 2sm), ainsi qu'une localisation semblable des locus d'ADNr 18S et des répétitions télomériques TTAGG. Malgré cela, la sonde microsatellite $GA_{(15)}$ a montré une variation au sein de ces espèces et des populations étudiées. Les auteurs suggèrent que ces deux espèces ont divergé relativement

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récemment et sont indubitablement des espèces sœurs en raison du grand nombre de caractéristiques partagées, incluant des caryotypes fortement conservés.

Mots-clés : caryotype, nombre de chromosomes, marqueurs cytogénétiques, évolution, fourmis champignonnistes.

Introduction

Leaf-cutting ants comprise species from the genera *Acromyrmex* Mayr, 1865, *Atta* Fabricius, 1804, and now *Amoimyrmex* Cristiano, Cardoso, & Sandoval, 2020. They have an obligatory symbiosis with specific basidiomycete fungi that they grow for food in elaborate gardens. Worker ants provide fresh plant material to the fungus, which produces specialized hyphal tips (gongylidia) as the primary food source for the entire colony (Hölldobler and Wilson 2011). Approximately 20 million years ago, leaf-cutting ants evolved to become the dominant herbivores of terrestrial environments in the Neotropical region (Branstetter et al. 2017). This trait has led them to be of high economic importance because of the considerable losses they cause for a variety of crops (Hölldobler and Wilson 2011; Zanetti et al. 2014).

Despite the prominent karyotypic diversity within the Formicidae family, with chromosomes ranging from $2n = 2$ to $2n = 120$ (reviewed in Lorite and Palomeque 2010), each of the three genera of leaf-cutting ants has a mostly conserved chromosome number. The available cytogenetic data for species of the genus *Atta* display a regularity in both chromosome number and morphology, all with $2n = 22$. In contrast, species of *Acromyrmex* display predominantly $2n = 38$, but with great variability in the morphology of their chromosomes (reviewed in Cardoso et al. 2018*a*). Interestingly, *Amoimyrmex striatus* Roger, 1863 has the same karyotype number as the species of *Atta* ($2n = 22$) and includes most metacentric chromosomes, although it differs in the GC-rich block pattern (Cristiano et al. 2013). This iconic species also shares morphological characteristics with both genera: three pairs of spines on the promesonotum, a trait shared with *Acromyrmex* (there are only two pairs of spines in *Atta*), and a smooth gastral tergum, shared with *Atta* (*Acromyrmex* has a tuberculate gaster) (Mayhé-Nunes 1991). Phylogenetic analyses have indicated that the *Am. striatus* clade is the sister group of the remaining leaf-cutting ants (Cristiano et al. 2013; Branstetter et al. 2017). This is well supported by karyotypic information, where the ancestral number of leaf-cutting ants is $2n = 22$ (Pereira et al. 2018). All these data have resulted in the description of the new leaf-cutting genus *Amoimyrmex* Cristiano, Cardoso, & Sandoval, 2020 (Cristiano et al. 2020).

Amoimyrmex is restricted to grassland habitats in subtropical and temperate zones of southern South America, occurring on the southern Brazilian coast (known as the restinga ecosystem), sandy soils across the Pampas and Chaco, and from the southernmost part of Paraguay to the temperate savannas of the Low Monte in Argentina (Kempf 1972; compiled in Janicki et al. 2016). Recent paleodistribution analyses have identified that the potential distribution of the species may have gone through oscillations arising from the last glacial period, from which it would have expanded dramatically from the Pampas to the grasslands of Argentina (Cristiano et al. 2016). Thus, it is assumed that *Amoimyrmex* has been strongly influenced by changes in the connections between the Amazon River Basin and the Parana River Basin, as well as the expansion and contraction of open and dry areas in the southern lowlands, disrupting populations and promoting diversification of *Amoimyrmex* (Cristiano et al. 2020).

Karyotype changes, including chromosome number and morphology, are speculated to promote species diversification events through chromosomal rearrangements, which may later result in barriers to gene flow (Rieseberg 2001; Faria and Navarro 2010). In ants, such considerations can occur at various levels, including intraspecific variations involving the maintenance of different karyotypes in the same population, many of them between sister species or closely related species (e.g., Imai et al. 1977; Crosland et al. 1988; Hirai et al. 1996; Cardoso et al. 2014; Micolino et al. 2019*a*). Intraspecific variation among populations has been identified for a range of ants and may help in the identification of cryptic species. For example, such findings were obtained by fluorescence in situ hybridization (FISH)-based molecular cytogenetics using microsatellites and (or) ribosomal DNA (rDNA) probes (Micolino et al. 2019*a*, 2019*b*), as well as standardized chromosome measurement analysis (i.e., karyomorphometry) associated with genome size (Cardoso et al. 2018*b*). Such comparative analysis of karyotype variations among geographically isolated populations could help to understand the evolution of the distribution of particular species or populations. Natural species obviously do not respect the boundaries established by mankind, but because of traditionally dubious taxonomy and nesting sites that differ only slightly, it is worth examining cytogenetically other species of *Amoimyrmex* from the Argentine Chaco while seeking a minor distinction from their putative, older Brazilian relatives. Therefore, karyotype description of any of these lineages would shed light on the evolution of the group and may provide valuable indications of kinship and further evidence of its systematics and taxonomy.

The aim of this study was to analyze cytogenetically *Amoimyrmex bruchi* Forel, 1912 and *Amoimyrmex silvestrii* Emery, 1905 from the Chaco biome in Argentina, as well as to describe the hitherto unknown karyotype of the species, looking for cytogenetic markers that differentiate

the lineages. To this end, we used a karyomorphometric approach and FISH chromosomal mapping with ribosomal, telomeric, and microsatellite probes.

Materials and methods

Colony sampling

The colonies of *Am. silvestrii* were collected along the Argentine Chaco at the following sampling points within the Córdoba Province: four colonies in Tanti (–31.3505, –64.5338) – population A, one colony in Ruta Nacional 38/Ischilín (–30.6893, –64.6452) – population B, and two colonies in Punilla (–31.3482, –64.5345) – population C. Three colonies of *Am. bruchi* were collected around the city of Chamical in La Rioja Province, Argentina (–30.3749, –66.2809). All sampled colonies were under the domain of the Dry Chaco ecoregion, set in areas of tropical and subtropical grasslands, savannas, and shrublands, according to Dinerstein et al. (2017). A total of 62 *Am. silvestrii* and 31 *Am. bruchi* were sampled.

Chromosome preparations

Metaphase chromosomes were obtained using brain ganglia of prepupal larvae dissected in colchicine hypotonic solution (0.005%) according to Imai et al. (1988), with modifications described by Cardoso et al. (2012). We obtained 31 and 62 slides for *Am. bruchi* and *Am. silvestrii*, respectively. The metaphases were conventionally stained with 4% Giemsa® solution diluted in Sørensen's buffer (pH 6.8) to determine chromosome number and morphology. The karyotype structure was described by the chromosomal arm ratio proposed by Levan et al. (1964) and classified according to their centromeric position: metacentric (m), submetacentric (sm), subtelocentric (st), and telocentric (t). In total, 60 and 120 metaphases of *Am. bruchi* and *Am. Silvestrii*, respectively, were analyzed. The 10 best metaphases stained with Giemsa with chromosomal integrity, identical condensation, and non-overlapping and evident centromeres were measured using Image Pro Plus® software 4.5 (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were evaluated according to the following characteristics: total length (TL), long arm length (L), short arm length (S), long arm to short arm ratio (r = L/S), relative length of chromosomes (RL), and centromeric index (S/[L + S]), as specified in the standard protocol by Cristiano et al. (2017) and Peruzzi and Eroglu (2013). Karyotypes were assembled using Corel® PHOTO-PAINT software v.22 (Corel Corp, Ottawa, ON, Canada).

Fluorescence in situ hybridization (FISH)

The FISH procedure for mapping repetitive DNA sequences through 18S rDNA, telomeric $TTAGG_{(6)}$, and microsatellite $GA_{(15)}$ probes was performed according to the partial description by Kubat et al. (2008), with the appropriate modifications specified and detailed by Micolino et al. (2019*b*). The $TTAGG_{(6)}$ and $GA_{(15)}$ probes were labeled a priori, with Cy3 at the 5′ end during synthesis (Sigma, St. Louis, MO, USA), while the 18S rDNA probe was obtained by polymerase chain reaction (PCR) amplification (see Micolino et al. 2019*b*). In summary, the process involved several washes with saline solutions, followed by denaturation with formamide and dehydration with ethanol until hybridization of the FISH probe. After overnight maintenance, the chromosome slides were washed again, dehydrated to remove the excess probe, and then put through the final assembly process in DAPI antifading solution (DAPI Fluoroshield, Sigma–Aldrich). The final step consisted of analyzing the chromosome slides under an Olympus BX53 epifluorescence microscope (Olympus Corporation, Tokyo, Japan) using WU (330–385 nm) and WG (510–550 nm) filters for DAPI and rhodamine, respectively. Chromosome images were obtained using an MX10 digital camera (Olympus Corporation, Tokyo, Japan) attached to the microscope and CellSens software (Olympus Corporation, Tokyo, Japan). Subsequently, the metaphase photos were edited using Adobe Photoshop CC® software (Adobe Systems Inc., San Jose, CA, USA).

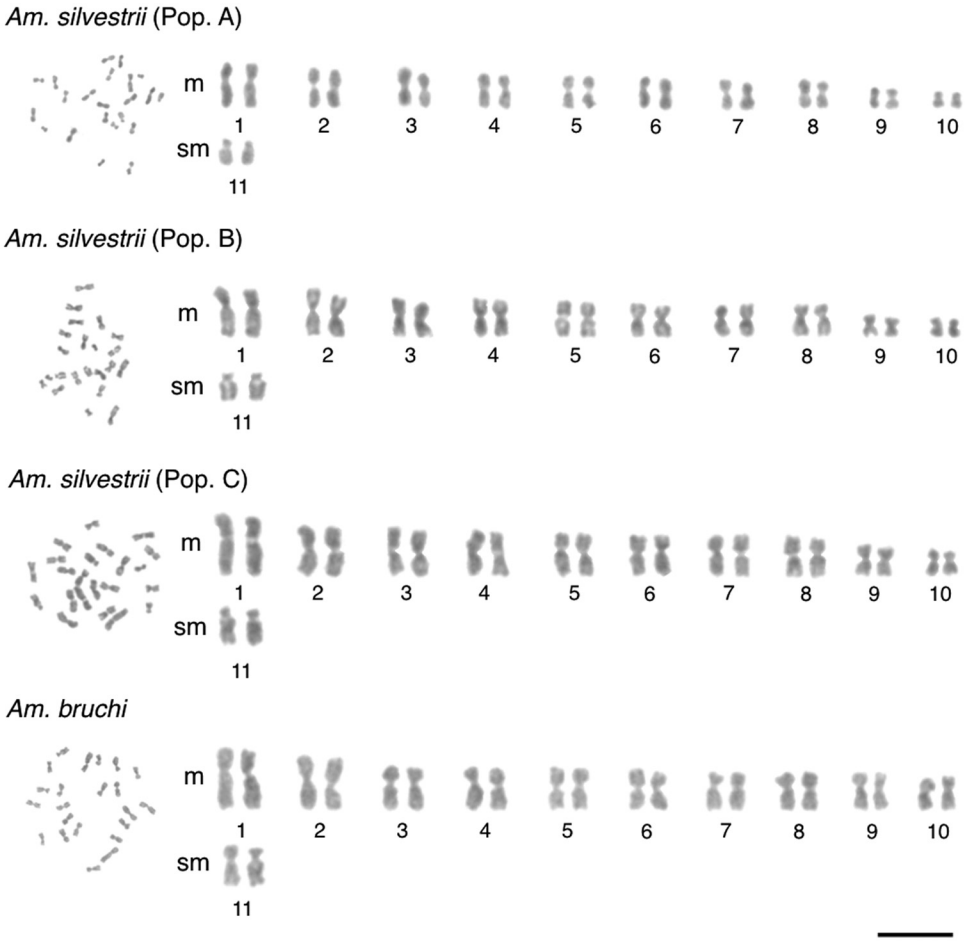
Results

The studied populations of *Am. silvestrii* displayed metaphases with a diploid chromosome number of $2n$ = 22 consisting of 10 metacentric chromosomal pairs and one submetacentric pair, with a karyotype formula of 2K = 20m + 2sm and fundamental number of NF = 44 (Fig. 1). Likewise, the *Am. bruchi* karyotype displayed the same characteristics as *Am. silvestrii* (Fig. 1). Karyomorphometric data for *Am. silvestrii* chromosomes in this work were as follows: the total chromosome length for population A ranged from 3.88 ± 0.48 to 2.33 ± 0.33 μm and centromeric index ranged from 0.30 ± 0.02 to 0.48 ± 0.02 μm (Table S1[1]), while the total length of all chromosomes was 59.35 μm (Table 1); population B presented a total chromosome length ranging from 3.99 ± 0.58 to 2.38 ± 0.35 μm, centromeric index ranged from 0.31 ± 0.00 to 0.49 ± 0.00 μm (Table S2[1]), and a total length of all chromosomes of 60.35 μm (Table 1); in population C, the total length of chromosomes ranged from 4.05 ± 0.63 to 2.34 ± 0.42 μm, centromeric index ranged from 0.31 ± 0.00 to 0.49 ± 0.01 μm (Table S3[1]), while the total length of all chromosomes was 59.35 μm (Table 1). In *Am. bruchi*, chromosome measurements ranged from 3.98 ± 0.52 to 2.61 ± 0.29 μm, centromeric index ranged from 0.32 ± 0.03 to 0.48 ± 0.02 μm (Table S4[1]), with the total length of all chromosomes being 60.29 μm (Table 1).

FISH chromosomal mapping was performed for the three Argentine populations of *Am. silvestrii* and the *Am. bruchi* and displayed no significant differences in either the number or brightness of the 18S and TTAGG

[1]Supplementary data are available with the article at https://doi.org/10.1139/gen-2021-0044.

Fig. 1. Conventional staining of mitotic metaphases and their respective karyotypes assembled by morphological similarity of three populations of *Amoimyrmex silvestrii* from the Argentine Chaco (Pop. A – Córdoba; Pop. B – Ischilín; and Pop. C – Punilla), as well as *Amoimyrmex bruchi*. m, metacentric chromosomes; sm, submetacentric chromosomes. Scale bar = 5 μm.



probes. The probe for the 18S rDNA cluster was labeled on only one chromosomal pair in all samples, located in the interstitial region of the long arms of the second pair of metacentric chromosomes (Fig. 2). The $TTAGG_{(6)}$ telomeric repeat distribution pattern was restricted to the ends of both arms in all chromosomal pairs. In addition, no signals for the interstitial telomeric sites (ITS) were observed (Fig. 2). The $GA_{(15)}$ microsatellite probe provided a rich band pattern in the subtelomeric regions of some specific chromosomes, extending more interstitially across both chromosomal arms, with some signals remarkably stronger than others. Weaker markings could be observed on smaller chromosomes, suggesting a shortage of this repeat. In general, the $GA_{(15)}$ probe produced a scattered distribution along the chromosomes with a different number of markings between *Am. silvestrii* population A versus B and C. For population A, all chromosomes had microsatellite markings, whereas for populations B and C, there was a small metacentric chromosome negative for $GA_{(15)}$. Yet, *Am. bruchi* presented two metacentric chromosomes that were negative for $GA_{(15)}$. This pattern was recurrent in all the samples analyzed (Fig. 2).

Discussion

The results of our comparative cytogenetic analysis showed that the diploid number of chromosomes in representatives of the three *Am. silvestrii* populations, as well as in the *Am. bruchi* population, was invariably $2n = 22$. Karyomorphometric analysis revealed that the karyotype in all samples consisted of 10 metacentric pairs and one submetacentric pair. We have described, for the first time, the karyotype of *Am. silvestrii* and *Am. bruchi*, a prospective sister lineage that belongs to what we call leaf-cutting ants. Recent phylogenetic analyses support this degree of closeness (Cristiano et al. 2020). We also demonstrated the karyotypes of *Am. silvestrii* and *Am. bruchi* were very similar, showing no differences in our cytogenetic surveys. It seems that the diversification in *Amoimyrmex* has not been accompanied by substantial chromosomal rearrangements. We therefore suggest that both species have diverged relatively

Table 1. Karyomorphometric analyses of the chromosomes from three different populations of *Amoimyrmex silvestrii* (Pop. A – Córdoba; Pop. B – Ischilín; and Pop. C – Punilla) and the *Amoimyrmex bruchi* species.

Chromosome (homologous)	*Am. silvestrii* Pop. A TL (μm)	*Am. silvestrii* Pop. B TL (μm)	*Am. silvestrii* Pop. C TL (μm)	*Am. bruchi* TL (μm)	Classification
1	3.88 ± 0.48	3.99 ± 0.58	4.05 ± 0.63	3.98 ± 0.52	Metacentric
(1)	3.76 ± 0.51	3.90 ± 0.59	3.85 ± 0.61	3.89 ± 0.51	Metacentric
2	3.43 ± 0.47	3.57 ± 0.44	3.44 ± 0.51	3.56 ± 0.47	Metacentric
(2)	3.30 ± 0.43	3.43 ± 0.38	3.31 ± 0.50	3.37 ± 0.51	Metacentric
3	3.12 ± 0.39	3.17 ± 0.44	3.12 ± 0.42	2.96 ± 0.42	Metacentric
(3)	3.02 ± 0.36	3.04 ± 0.41	3.01 ± 0.42	2.86 ± 0.44	Metacentric
4	2.97 ± 0.38	2.94 ± 0.38	2.97 ± 0.40	2.77 ± 0.39	Metacentric
(4)	2.90 ± 0.40	2.90 ± 0.38	2.89 ± 0.41	2.70 ± 0.37	Metacentric
5	2.86 ± 0.40	2.87 ± 0.36	2.81 ± 0.41	2.66 ± 0.37	Metacentric
(5)	2.79 ± 0.40	2.82 ± 0.35	2.76 ± 0.36	2.61 ± 0.38	Metacentric
6	2.74 ± 0.38	2.76 ± 0.33	2.70 ± 0.34	2.58 ± 0.37	Metacentric
(6)	2.72 ± 0.38	2.72 ± 0.35	2.65 ± 0.35	2.53 ± 0.35	Metacentric
7	2.67 ± 0.39	2.65 ± 0.36	2.59 ± 0.35	2.49 ± 0.34	Metacentric
(7)	2.61 ± 0.38	2.60 ± 0.38	2.55 ± 0.36	2.44 ± 0.34	Metacentric
8	2.48 ± 0.33	2.47 ± 0.29	2.46 ± 0.34	2.40 ± 0.32	Metacentric
(8)	2.37 ± 0.26	2.32 ± 0.26	2.31 ± 0.37	2.36 ± 0.31	Metacentric
9	1.94 ± 0.24	2.01 ± 0.32	2.01 ± 0.25	2.31 ± 0.33	Metacentric
(9)	1.82 ± 0.19	1.83 ± 0.34	1.86 ± 0.26	2.27 ± 0.33	Metacentric
10	1.63 ± 0.22	1.64 ± 0.24	1.69 ± 0.26	2.20 ± 0.33	Metacentric
(10)	1.57 ± 0.21	1.54 ± 0.25	1.52 ± 0.20	1.96 ± 0.34	Metacentric
11	2.44 ± 0.33	2.53 ± 0.38	2.46 ± 0.37	2.78 ± 0.30	Submetacentric
(11)	2.33 ± 0.33	2.38 ± 0.35	2.34 ± 0.42	2.61 ± 0.29	Submetacentric
∑(KL)	59.35	60.08	59.35	60.29	

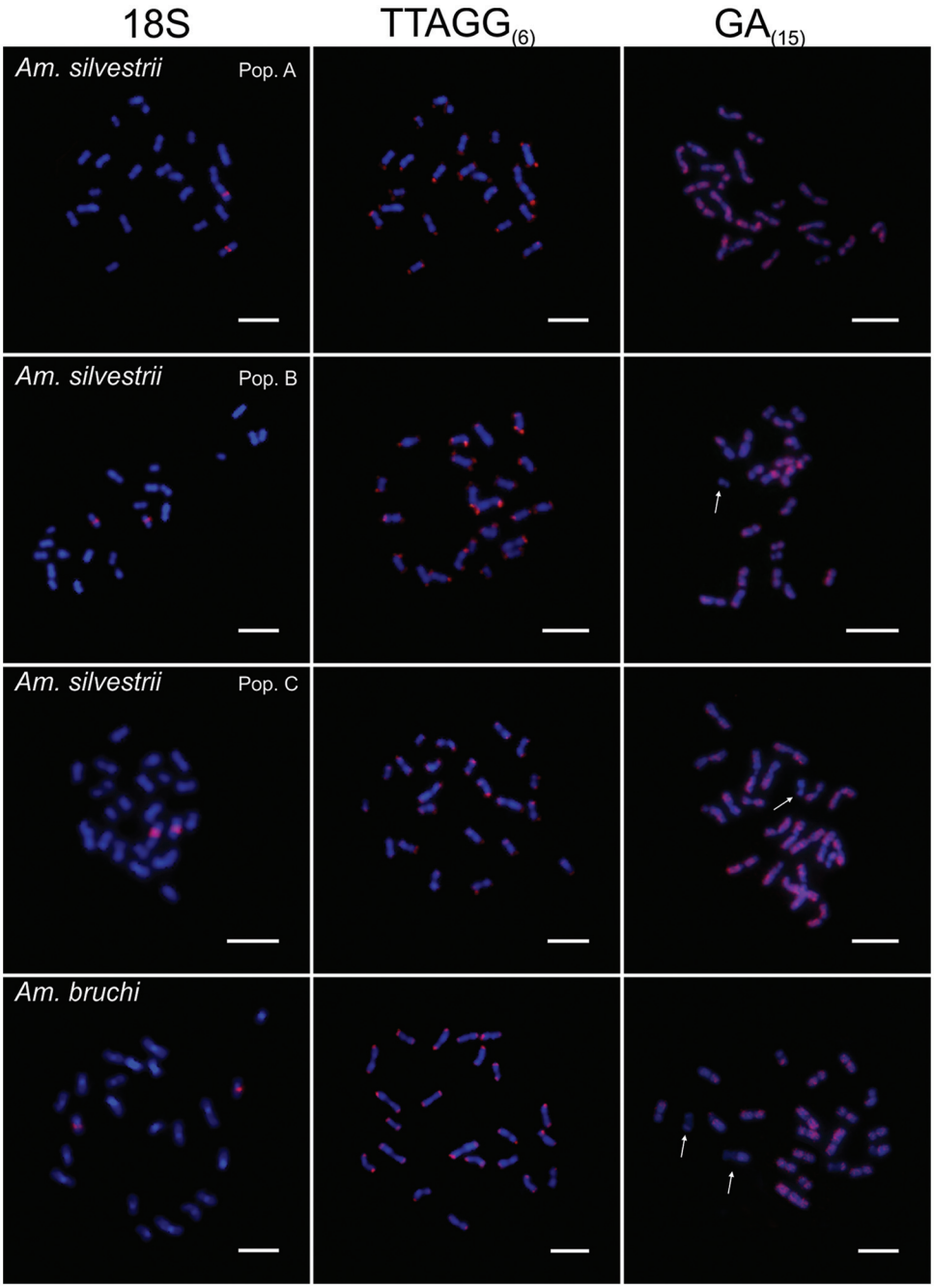
Note: The chromosome total length (TL) and karyotype length (KL) of each sample have been studied here.

recently, owing to many shared and conserved characteristics, including their karyotypes.

The results of karyotype length in species of the genus *Amoimyrmex* corroborate the previous findings of Pereira et al. (2018) and differ a little from those of Cristiano et al. (2013), which ranged from 5.78 ± 0.15 to 1.77 ± 0.05 μm and a total length of 78.67 μm. A possible explanation for this incongruence is that the karyomorphometric protocol used by our group has undergone successive improvements and standardizations, making it increasingly close to the appropriate one, which further corroborates genome size estimation by flow cytometry (see Cristiano et al. 2017; Cardoso et al. 2018*b*). The karyotype of *Am. silvestrii* and *Am. bruchi* showed pronounced structural and numerical similarities with *Am. striatus*, including the length of the chromosomes. Population-based chromosomal studies may reveal often unreached intraspecific diversity either by morphological means (e.g., cryptic species), molecular means (e.g., weak phylogenetic signal), or other methods (e.g., Talavera et al. 2013; Lukhtanov and Shapoval 2017). For example, a karyomorphometric analysis of *Mycetomoellerius holmgreni* Wheeler, 1925 fungus-farming ant populations revealed significant differences in karyotype length, suggesting that this ant species might be undergoing centromere drive (Cardoso et al. 2018*b*). In a further analysis of these populations, cytogenetic markers by means of microsatellite probes were population specific, a finding that corroborates the potential restriction of gene flow among the studied populations of *M. holmgreni* (Micolino et al. 2019*b*).

Our results for the physical mapping of the 18S rDNA cluster and the TTAGG telomeric motif are consistent with the previously described data for *Am. striatus* in the Brazilian ecosystems of the restingas and the Pampas (Teixeira et al. 2017; Pereira et al. 2018). An interesting cytogenetic feature of *Am. striatus* is the location of the 18S rDNA cluster, identified in the interstitial region of a pair of metacentric chromosomes, similar to those found in species of *Atta* (Teixeira et al. 2017). Likewise, the location of the 18S rDNA cluster in *Am. silvestrii* and *Am. bruchi* is indistinguishable from that of *Am. striatus*. rDNA is one of the most conserved fractions of the eukaryotic genome, and ribosomal RNA genes have changed minimally throughout evolutionary history (Raskina et al. 2008). Despite this conservation, rDNA is a strong source of genome instability, and rDNA dynamics are an indicator of significant intra-genomic processes (Raskina et al. 2004). Therefore, it is apparent that *Am. striatus* would have the ancestral karyotype structure of the clade of leaf-cutting ants, in terms of the number and location of the 18S rDNA on the chromosomes. Clearly, this assumption is supported by molecular data (e.g., Cristiano et al. 2013, 2020; Pereira et al. 2018), although it is noteworthy that an integrative approach (cytogenetic and molecular data) involving

Fig. 2. DAPI-stained mitotic metaphases from three populations of *Amoimyrmex silvestrii*, as well as *Amoimyrmex bruchi*. Left column: fluorescence in situ hybridization (FISH) mapping of the ribosomal DNA (rDNA) 18S cluster (in red). Center column: FISH mapping of the $TTAGG_{(6)}$ telomeric motif (in red). Right column: FISH mapping of the microsatellite $GA_{(15)}$ (in red). Scale bar = 5 μm.



extensive sampling would support such a point remarkably well.

The TTAGG telomeric repeat is considered to be the ancestral motif of insects (Kuznetsova et al. 2020). In general, the order Hymenoptera has the TTAGG repeat retained in its representatives, although it has been suggested that it was putatively lost in the ancestor Apocrita with at least two subsequent independent recoveries (Menezes et al. 2017; Gokhman and Kuznetsova 2018). One of them has occurred in Formicidae and has been increasingly identified in ant telomeres (e.g., Lorite et al. 2002; Pereira et al. 2018; Micolino et al. 2019*a*, 2019*b*). One application of telomeric probes has been in the recognition of fusion rearrangements, which can also identify population polymorphisms (Ruiz-Herrera et al. 2008). Preliminary analysis with the $TTAGG_{(6)}$ probe in *Am. striatus* revealed no interstitial telomeric signals, indicating that $2n$ = 22 is the most likely

ancestral karyotype of the leaf-cutting ants and is a plesiomorphic feature shared between *Am. striatus* and species of *Atta* (Pereira et al. 2018). We observed markings for the TTAGG motif exclusively at the ends of chromosomes in all samples of *Am. silvestrii* and *Am. bruchi* analyzed in this study, suggesting their putative conservation within these ant lineages.

The FISH mapping results for the microsatellite $GA_{(15)}$ suggest that there is distinguishable variation among samples, depicting subtly stronger markings on some chromosomal pairs. Such accumulation of these repeated sequences was observed in the subtelomeric region in both chromosomal arms. However, *Am. bruchi* showed two chromosomes without markings, compared to only one in *Am. silvestrii*, indicating a different pattern of microsatellite accumulation in these chromosomes. Such differential markers of the $GA_{(15)}$ probe can be used to differentiate between both species. Indeed, our cytogenetic data agree with the morphological and molecular data from Cristiano et al. (2020).

In fact, microsatellite repeats can be arranged in well-defined clusters on the chromosomes. For example, 15 species of stingless bees of the genus *Melipona* displayed $GA_{(15)}$ repeats in predominantly telomeric blocks (Travenzoli et al. 2019). Intraspecific cytogenetic polymorphisms have recently been found in the ant *M. holmgreni*, showing distinct, well-defined blocks of the microsatellite $GA_{(15)}$ in populations situated to the north and south of their occurrence area, denoting a potential geographic and (or) reproductive isolation between them (Micolino et al. 2019*b*). Moreover, birds generally have a preferential accumulation of microsatellite repeats restricted to the centromeric and telomeric regions. In particular, woodpeckers showed different distribution patterns of microsatellite sequences on the Z sex chromosome, including the dinucleotide $GA_{(15)}$ (de Oliveira et al. 2017). In contrast, many studies have shown such entirely scattered repeats in both heterochromatic and euchromatic regions on chromosomes (e.g., Cioffi et al. 2011; Palacios-Gimenez et al. 2015; Cunha et al. 2016). In summary, these findings suggest the particularly dynamic nature of microsatellite sequences, making them good cytogenetic markers, mainly when their occurrence is non-dispersive across chromosomes.

As each organism presents its own karyotype, the chromosome number and morphology are of interest for taxonomic studies, because closely related species tend to have karyotypes that are more similar than phylogenetically distant ones (Sumner 2003). This was seen in *Am. silvestrii* and *Am. bruchi*, whose karyotype number and structure were identical to those of *Am. striatus*. Furthermore, these species strongly resemble each other morphologically, making it difficult to identify and differentiate between them. The very similar karyotypes of the two species confer an additional characteristic in which closely related ant species can diverge without deep chromosomal changes during the speciation process. As no structural changes at the chromosomal level were identified, we suggest that perhaps changes related to genome function or regulation are more linked to divergence; this is a topic for further research.

The recognized chromosomal properties of the *Amoimyrmex* resemble karyotypic data in *Trachymyrmex* spp. (all karyotyped species have $2n = 20$), mainly due to the predominance of metacentric chromosomes and the absence of acrocentric chromosomes (reviewed in Cardoso et al. 2018*a*). Considering the phylogenetic position of these two clades (Cristiano et al. 2020), we suggest a likely evolutionary trajectory focusing on chromosome changes. The karyotype differentiation of species of *Amoimyrmex* towards species of *Trachymyrmex* is determined by the difference of one extra chromosomal pair and the presence of a pair of submetacentric chromosomes. Hence, there could have been a chromosomal fission in the ancestral karyotype, followed by a pericentric inversion that changed the karyotype to its current state of $2n = 22$, comprising 10 pairs of metacentric chromosomes and one submetacentric pair. A similar scenario regarding the role of chromosomal inversions in the lineage diversification process has been assumed for closely related species of the genus *Mycetomoellerius* (Micolino et al. 2020). Overall, we have provided new karyotype data for *Amoimyrmex* and further cytogenetic evidence that differentiates *Amoimyrmex* from the remaining leaf-cutting ants. Such data may be useful for future comparative cytogenetic analyses. We have also provided insights into the possible phylogenetic relationships hitherto unavailable.

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References

Branstetter, M.G., Ješovnik, A., Sosa-Calvo, J., Lloyd, M.W., Faircloth, B.C., Brady, S.G., and Schultz, T.R. 2017. Dry habitats were crucibles of domestication in the evolution of

agriculture in ants. Proc. R. Soc. B Biol. Sci. **284**: 20170095. doi:10.1098/rspb.2017.0095. PMID:28404776.

Cardoso, D.C., Cristiano, M.P., Barros, L.A.C., Lopes, D.M., and Pompolo, S.G. 2012. First cytogenetic characterization of a species of the arboreal ant genus *Azteca* Forel 1978 (Dolichoderinae Formicidae). Comp. Cytogenet. **6**: 107–114. doi:10.3897/compcytogen.v6i2.2397. PMID:24260655.

Cardoso, D.C., Pompolo, S.G., Cristiano, M.P., and Tavares, M.G. 2014. The role of fusion in ant chromosome evolution: insights from cytogenetic analysis using a molecular phylogenetic approach in the genus *Mycetophylax*. PLoS ONE, **9**: e87473. doi:10.1371/journal.pone.0087473. PMID:24489918.

Cardoso, D.C., Hg, S., and Cristiano, M.P. 2018*a*. The Ant Chromosome database – ACdb: an online resource for ant (Hymenoptera: Formicidae) chromosome researchers. Myrmecol. News, **27**: 87–91.

Cardoso, D.C., Heinze, J., Moura, M.N., and Cristiano, M.P. 2018*b*. Chromosomal variation among populations of a fungus-farming ant: implications for karyotype evolution and potential restriction to gene flow. BMC Evol. Biol. **18**: 146. doi:10.1186/s12862-018-1247-5. PMID:30241462.

Cioffi, M.B., Kejnovsky, E., and Bertollo, L.A.C. 2011. The chromosomal distribution of microsatellite repeats in the genome of the wolf fish *Hoplias malabaricus* focusing on the sex chromosomes. Cytogenet. Genome Res. **132**: 289–296. doi:10.1159/000322058. PMID:21099206.

Cristiano, M.P., Cardoso, D.C., and Fernandes-Salomão, T.M. 2013. Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger 1863) and other congeneric species: taxonomic implications. PLoS ONE, **8**: e59784. doi:10.1371/journal.pone.0059784. PMID:23527267.

Cristiano, M.P., Cardoso, D.C., Fernandes-Salomão, T.M., and Heinze, J. 2016. Integrating paleodistribution models and phylogeography in the grass-cutting ant *Acromyrmex striatus* (Hymenoptera: Formicidae) in southern lowlands of South America. PLoS ONE, **11**: e0146734. doi:10.1371/journal.pone.0146734. PMID:26734939.

Cristiano, M.P., Pereira, T.T.P., Simões, L.P., Sandoval-Gómez, V.E., and Cardoso, D.C. 2017. Reassessing the chromosome number and morphology of the turtle ant *Cephalotes pusillus* (Klug 1824) using karyomorphometrical analysis and observations of new nesting behavior. Insects, **8**: 114. doi:10.3390/insects8040114. PMID:29065555.

Cristiano, M.P., Cardoso, D.C., Sandoval-Gómez, V.E., and Simões-Gomes, F.C. 2020. *Amoimyrmex* Cristiano, Cardoso & Sandoval, gen. nov. (Hymenoptera: Formicidae): a new genus of leaf-cutting ants revealed by multilocus molecular phylogenetic and morphological analyses. Aust. Entomol. **59**: 643–676. doi:10.1111/aen.12493.

Crosland, M.W.J., Crozier, R.H., and Imai, H.T. 1988. Evidence for several sibling biological species centred on *Myrmecia pilosula* (F SMITH) (Hymenoptera: Formicidae. Aust. J. Entomol. **27**: 13–13. doi:10.1111/j.1440-6055.1988.tb01136.x.

Cunha, M.S., Reis, V.J.C., and Dergam, J.A. 2016. Closely related syntopic cytotypes of *Astyanax taeniatus* (Jenyns 1842) from the Upper Piranga River Upper Doce Basin in Southeastern Brazil. Zebrafish, **13**: 112–117. doi:10.1089/zeb.2015.1163. PMID:26771299.

De Oliveira, T.D., Kretschmer, R., Bertocchi, N.A., Degrandi, T.M., De Oliveira, E.H.C., Cioffi, M.B., et al. 2017. Genomic organization of repetitive DNA in woodpeckers (Aves Piciformes): implications for karyotype and ZW sex chromosome differentiation. PLoS ONE, **12**: e0169987. doi:10.1371/journal.pone.0169987. PMID:28081238.

Dinerstein, E., Olson, D., Joshi, A., Vynne, C., Burgess, N.D., Wikramanayake, E., et al. 2017. An ecoregion-based approach to protecting half the terrestrial realm. BioScience, **67**: 534–545. doi:10.1093/biosci/bix014. PMID:28608869.

Faria, R., and Navarro, A. 2010. Chromosomal speciation revisited: rearranging theory with pieces of evidence. Trends Ecol. Evol. **25**: 660–669. doi:10.1016/j.tree.2010.07.008. PMID:20817305.

Gokhman, V.E., and Kuznetsova, V.G. 2018. Presence of the canonical TTAGG insect telomeric repeat in the Tenthredinidae (Symphyta) suggests its ancestral nature in the order Hymenoptera. Genetica, **146**: 341–344. doi:10.1007/s10709-018-0019-x. PMID:29730744.

Hirai, H., Yamamoto, M.T., Taylor, R.W., and Imai, H.T. 1996. Genomic dispersion of 28S rDNA during karyotypic evolution in the ant genus *Myrmecia* (Formicidae). Chromosoma, **105**: 190–196. doi:10.1007/BF02509500. PMID:8781187.

Hölldobler, B., and Wilson, E.O. 2011. The leafcutter ants: civilization by instinct. W.W. Norton & Company, New York. 192 pp.

Imai, H.T., Crozier, R.H., and Taylor, R.W. 1977. Karyotype evolution in Australian ants. Chromosoma, **59**: 341–393. doi:10.1007/BF00327974.

Imai, H.T., Taylor, R.W., Crosland, M.W.J., and Crozier, R.H. 1988. Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. Jpn. J. Genet. **63**: 159–185. doi:10.1266/jjg.63.159. PMID:3273765.

Janicki, J., Narula, N., Ziegler, M., Guénard, B., and Economo, E.P. 2016. Visualizing and interacting with large-volume biodiversity data using client-server web-mapping applications: the design and implementation of antmapsorg. Ecol. Inform. **32**: 185–193. doi:10.1016/j.ecoinf.2016.02.006.

Kempf, W.W. 1972. Catálogo abreviado das formigas da região Neotropical. Studia Entomol. **15**: 3–344.

Kubat, Z., Hobza, R., Vyskot, B., and Kejnovsky, E. 2008. Microsatellite accumulation on the Y chromosome in *Silene latifolia*. Genome, **51**(5): 350–356. doi:10.1139/G08-024. PMID:18438438.

Kuznetsova, V., Grozeva, S., and Gokhman, V. 2020. Telomere structure in insects: a review. J. Zool. Syst. Evol. Res. **58**: 127–158. doi:10.1111/jzs.12332.

Levan, A., Fredga, K., and Sandberg, A.A. 1964. Nomeclature for centromeric position on chromosomes. Hereditas, **52**: 201–220. doi:10.1111/j.1601-5223.1964.tb01953.x.

Lorite, P., and Palomeque, T. 2010. Karyotype evolution in ants (Hymenoptera: Formicidae) with a review of the known ant chromosome numbers. Myrmecol. News, **13**: 89–102.

Lorite, P., Carrillo, J.A., and Palomeque, T. 2002. Conservation of (TTAGG)(n) telomeric sequences among ants (Hymenoptera, Formicidae). J. Hered. **93**: 282–285. doi:10.1093/jhered/93.4.282. PMID:12407216.

Lukhtanov, V.A., and Shapoval, N.A. 2017. Chromosomal identification of cryptic species sharing their DNA barcodes: *Polyommatus* (*Agrodiaetus*) *antidolus* and *P* (*A*) *morgani* in Iran (Lepidoptera Lycaenidae). Comp. Cytogenet. **11**: 759–768. doi:10.3897/compcytogen.v11i4.20876. PMID:29302296.

Mayhé-Nunes, A.J. 1991. Estudo de *Acromyrmex* (Hymenoptera Formicidae) com ocorrência constatada no Brasil: subsídios para uma análise filogenética. Ph.D. thesis, Universidade Federal de Viçosa Viçosa Brazil 122 pp.

Menezes, R.S.T., Bardella, V.B., Cabral-de-Mello, D.C., Lucena, D.A.A., and Almeida, E.A.B. 2017. Are the TTAGG and TTAGGG telomeric repeats phylogenetically conserved in aculeate Hymenoptera? Naturwissenschaften, **104**: 85. doi:10.1007/s00114-017-1507-z.

Micolino, R., Cristiano, M.P., Travenzoli, N.M., Lopes, D.M., and Cardoso, D.C. 2019*a*. Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. Sci. Rep. **9**: 18800. doi:10.1038/s41598-019-55135-5. PMID:31827151.

Micolino, R., Cristiano, M.P., and Cardoso, D.C. 2019*b*. Population-based cytogenetic banding analysis and phylogenetic relationships of the neotropical fungus-farming ant *Trachymyrmex holmgreni* Wheeler, 1925. Cytogenet. Genome Res. **159**: 151–161. doi:10.1159/000503913. PMID:31683269.

Micolino, R., Cristiano, M.P., and Cardoso, D.C. 2020. Karyotype and putative chromosomal inversion suggested by integration of cytogenetic and molecular data of the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888. Comp. Cytogenet. **14**(2): 197–210. doi:10.3897/CompCytogen.v14i2.49846. PMID:32431788.

Palacios-Gimenez, O.M., Carvalho, C.R., Soares, F.A.F., and Cabral-De-Mello, D.C. 2015. Contrasting the chromosomal organization of repetitive DNAs in two Gryllidae crickets with highly divergent karyotypes. PLoS ONE, **10**: e0143540. doi:10.1371/journal.pone.0143540. PMID:26630487.

Pereira, T.T.P., Reis, A.C.C.C., Cardoso, D.C., and Cristiano, M.P. 2018. Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comp. Cytogenet. **12**: 13–26. doi:10.3897/CompCytogen.v12i1.21799. PMID: 29362670.

Peruzzi, L., and Eroglu, H. 2013. Karyotype asymmetry: again, how to measure and what to measure? Comp. Cytogenet. **7**(1): 1–9. doi:10.3897/compcytogen.v7i1.4431. PMID:24260685.

Raskina, O., Belyayev, A., and Nevo, E. 2004. Quantum speciation in Aegilops: molecular cytogenetic evidence from rDNA cluster variability in natural populations. Proc. Natl. Acad. Sci. U.S.A. **101**: 14818–14823. doi:10.1073/pnas.0405817101. PMID:15466712.

Raskina, O., Barber, J.C., Nevo, E., and Belyayev, A. 2008. Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. Cytogenet. Genome Res. **120**: 351–357. doi:10.1159/000121084. PMID:18504364.

Rieseberg, L.H. 2001. Chromosomal rearrangements and speciation. Trends Ecol. Evol. **16**: 351–358. doi:10.1016/S0169-5347(01)02187-5. PMID:11403867.

Ruiz-Herrera, A., Nergadze, S.G., Santagostino, M., and Giulotto, E. 2008. Telomeric repeats far from the ends: mechanisms of origin and role in evolution. Cytogenet. Genome Res. **122**: 219–228. doi:10.1159/000167807. PMID:19188690.

Sumner, A.T. 2003. Chromosomes: organization and function. Blackwell Publishing Company, Malden, Mass. 287 pp

Talavera, G., Lukhtanov, V.A., Rieppel, L., Pierce, N.E., and Vila, R. 2013. In the shadow of phylogenetic uncertainty: the recent diversification of *Lysandra* butterflies through chromosomal change. Mol. Phylogenet. Evol. **69**: 469–478. doi:10.1016/j.ympev.2013.08.004. PMID:23954756.

Teixeira, G.A., Barros, L.A.C., Aguiar, H.J.A.C., and Pompolo, S.G. 2017. Comparative physical mapping of 18S rDNA in the karyotypes of six leafcutter ant species of the genera *Atta* and *Acromyrmex* (Formicidae: Myrmicinae). Genetica, **145**: 351–357. doi:10.1007/s10709-017-9970-1. PMID:28623426.

Travenzoli, N.M., Lima, B.A., Cardoso, D.C., Dergam, J.A., Fernandes-Salomão, T.M., and Lopes, D.M. 2019. Cytogenetic analysis and chromosomal mapping of repetitive DNA in *Melipona* species (Hymenoptera Meliponini). Cytogenet. Genome Res. **158**: 213–224. doi:10.1159/000501754. PMID:31352441.

Zanetti, R., Zanuncio, J., Santos, J., da Silva, W., Ribeiro, G., and Lemes, P. 2014. An overview of integrated management of leaf-cutting ants (Hymenoptera: Formicidae) in Brazilian forest plantations. Forests, **5**: 439–454. doi:10.3390/f5030439.