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Cytogenetics of Four Turtle Ants from the Amazon: First Steps Towards Understanding Karyotype Evolution in *Cephalotes*

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Ants of the genus *Cephalotes* have unique morphological traits, with morphotypes specifically adapted to arboreal habitats, earning them the common name “turtle ants”. In the Americas, 118 *Cephalotes* spp. have been identified, 64 in Brazil. Phylogenetic studies suggest 15 distinct groups of *Cephalotes* spp. In this context, cytogenetics emerges as a valuable tool for comprehending the diversification of these clades. This study used cytogenetics to characterize four *Cephalotes* spp. from the northernmost Brazilian state of Amapá. The number, morphology, and locations of specific DNA sequences varied. Most chromosomes were metacentric; *C. atratus* had a low chromosome number ($2n = 22$), compared to *C. minutus*, *C. pavonii*, and *C. simillimus* ($2n = 44$, $2n = 42$, and $n = 22$, respectively). Ribosomal genes were restricted to a single chromosome pair which were present within pericentromeric regions in *C. atratus* and *C. simillimus*, interstitial region in *C. pavonii* and telomeric region in *C. minutus*. The microsatellite $(GA)_n$ was clustered only in *C. atratus*; whereas in species with high chromosome numbers, this microsatellite was absent on one arm of several chromosome pairs. The telomeric $(TTAGG)_n$ motif was restricted to the chromosome ends in *C. atratus* as expected. Our findings suggest that centric fission, along with occasional chromosomal inversion events are principal mechanisms driving karyotype evolution in *Cephalotes* spp. Combining the cytogenetic data on *Procryptocerus* spp. we suggest that the putative ancestral karyotype of *Cephalotes* has 2n = 22 chromosomes. Cytogenetic analyses of a broader diversity of *Cephalotes* spp. are essential to elucidate whether fission events represent lineage-specific innovations or shared ancestral processes. They may provide deeper insights into the evolutionary dynamics underlying chromosomal architecture and its role in the diversification of these ecologically specialized turtle ants.

Keywords: Formicidae, Biodiversity, Chromosomes, rDNA, Microsatellites

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BACKGROUND

Arboreal ants are remarkably abundant in tropical forests because of their complex interactions with trees and other insect species (Davidson et al. 2003). *Cephalotes* Latreille, 1802 species are exclusively arboreal and restricted to the Neotropics (Andrade and Baroni Urbani 1999; Price et al. 2014; Guénard et al. 2017; Economo and Guénard 2025). These ants belong to the subfamily Myrmicinae; together with the genus *Procryptocerus* Emery, 1887, they form a clade within the Attini tribe (Ward et al. 2015). The soldier castes of *Cephalotes* ants exhibit remarkable morphological adaptations that highlight complex ecological interactions between these insects and the trees harboring their nests. These ants display four distinct head shapes, earning them the common name “turtle ants” (Andrade and Baroni Urbani 1999; Powell et al. 2020). In total, 118 *Cephalotes* species (Bolton 2026) are valid, and their phylogeny reveals diversification patterns that resulted in 15 main species groups (Price et al. 2016 2022). In Brazilian biomes, 64 species and 13 species groups have been reported (Oliveira et al. 2021). Recent findings suggest that *Cephalotes maculatus* (Smith, 1876) may represent a cryptic species complex (Rana 2023); this discovery sheds light on an unknown but significant aspect of the biodiversity of this genus.

Anthropogenic activities in the Amazon continue to fragment habitats, significantly threating *Cephalotes* spp. Monitoring their presence or absence across such landscapes provide valuable insights into the ecological consequences of habitat disruption, particularly the “edge effect” in Amazonian environments (Santos et al. 2023).

Effective taxonomic delimitation is essential for conservation; integrative taxonomy—an approach that synthesizes data from multiple biological disciplines—has been proven to be particularly powerful (Schlick-Steiner et al. 2010). Cytogenetics, in particular, contributes to advances in taxonomy, phylogeny, and conservation by revealing chromosomal structures and evolutionary patterns (Barros et al. 2015 2021b; Gokhman 2023). Chromosomal variation is remarkably broad in Formicidae, ranging from 2n = 2 to 2n = 120, driven by processes that alter both chromosome number and morphology (Imai et al. 1988; Mariano et al. 2015 2019).

Despite *Cephalotes* being an ecologically and morphologically diverse genus, cytogenetic data remain scarce and are available for only five species. Documented chromosome numbers include 2n = 22 in *C. atratus* (Linnaeus, 1758) (Mariano et al. 2019), 2n = 24 in *C. cordatus* (Smith, 1853) (Damasceno et al. 2024), and 2n = 44 in *C. angustus* (Mayr, 1862) (Mariano et al. 2019), *C. minutus* (Mayr, 1862) (Damasceno et al. 2024), and *C. pusillus* (Klug, 1824) (Cristiano et al. 2017) (Table 1). Cytogenetics, especially when integrated with molecular tools such as fluorescence *in situ* hybridization (FISH), offers detailed insights into gene arrangement and chromosomal architecture. These techniques enhance our understanding of the evolutionary relationships within and between ant lineages (Santos et al. 2016; Teixeira et al. 2020, 2021a; Barros et al. 2021a b).

The physical location of 45S rDNA sites is a common molecular marker as these sequences are conserved (Gokhman, 2023, Gokhman and Kuznetsova, 2024). Their distribution patterns in Formicidae have contributed to our understanding of karyotype evolution and address several taxonomic issues (Aguiar et al. 2017; Teixeira et al. 2021a b; Barros et al. 2021a b; Damasceno et al. 2024; Cardoso and Cristiano 2026a). In *Cephalotes*, two species from the Guiana Shield showed single pairs of chromosomes bearing rDNA genes (Damasceno et al. 2024).

Microsatellites consist of short tandemly repeated nucleotide motifs ranging from 1 to 6 bp. These sequences are highly mutable and distributed across the genomes of diverse organisms (Tóth et al. 2000; Ellegren 2004). Beyond a structural role, they are believed to influence gene regulation and contribute to genomic evolution (Tóth et al. 2000; Li et al. 2004).

Table 1. Summary of available cytogenetic data of *Cephalotes* ants

Species	2n/(n)[a]	Karyotype formulae 2n(n)	Col./Ind. Analyzed	Locality	Phylogenetic group*	Soldier morphotype [+]	References
Cephalotes atratus	22	20m+2sm [c]	2/20	Amapá[1]	atratus	No soldier	This study
	22	22M [b]	–	Minas Gerais[2]			Mariano et al. (2019)
Cephalotes angustus	44	38M+6A[b]	–	Bahia[2]	angustus + fiebrigi + bruchi	disc	Mariano et al. (2019)
Cephalotes cordatus	24	18m+6sm [c]	–	Amapá[1]	depresus	disc	Damasceno et al. (2024)
Cephalotes minutus	44	18m+18sm+8st [c]	2/12	Amapá[1]	laminatus + pusillus	dome	This study
	44	18m+22sm+4st [c]		Guiana Francesa[1]		dome	Damasceno et al. (2024)
Cephalotes pavonii	42	16m+18sm+8st [c]	2/11	Amapá[1]	depresus	disc	This study
Cephalotes pusillus	44	16m+18sm+10st [c]	–	Minas Gerais[2]	laminatus + pusillus	dome	Cristiano et al. (2017)
	44	26M+18A[b]	–	Minas Gerais[2]		dome	Mariano et al. (2019)
Cephalotes simillimus	(22)	(9m+9sm+4st) [c]	1/6	Amapá[1]	laminatus + pusillus	dome	This study

*Phylogenetic group according to Price et al. (2022). [+]Soldier morphotype according to Powell et al. (2020). [1]Amazonian Guiana Shield region; [2]Atlantic Forest Region. [a]2n, diploid, (n), haploid. [b]Terminology according to the nomenclature of Imai. [c]Terminology according to the nomenclature of Levan.

In insects, chromosomal distribution of microsatellites varies markedly across genera and species (Cunha et al. 2020; Jonika et al. 2020; Teixeira et al. 2022). Physical mapping of the $(GA)_n$ motif reveals a dispersed pattern across euchromatic regions of chromosomes among several ant species, including fungus-growing ants and *Crematogaster* Lund, 1831 (Barros et al. 2018; Teixeira et al. 2021a, b; Silveira et al. 2024). In contrast, the canonical telomeric repeat sequence $(TTAGG)_n$ is typically restricted to the termini of chromosomes in ants and most insects, but with a few exceptions (Kuznetsova et al. 2020). It serves as a robust molecular marker for investigating chromosomal rearrangements and karyotype evolution (Teixeira et al. 2021a; Barros et al. 2021a; Silveira et al. 2024).

Cephalotes ants play a vital role within Neotropical ecosystems due to their widespread presence, reflecting ecological versatility and resilience. Although restricted to this region, they exhibit low levels of endemism, suggesting successful adaptation to diverse arboreal habitats. Robust phylogenetic frameworks have helped elucidate the evolutionary history of *Cephalotes*. Their striking morphological diversity across lineages underscore the role of adaptive radiation in shaping their evolutionary pathways. Such variability reflects the influence of distinct environmental pressures, which drive the emergence of specialized morphotypes suited to particular ecological niches. In this context, understanding the evolutionary trends and patterns in *Cephalotes* karyotypes, requires co-analysis with their phylogenetic arrangement. Cytogenetic insights offer a powerful lens to explore chromosomal evolution, diversification, and speciation within this genus. These findings raise crucial questions for future taxonomic and evolutionary research, emphasizing the need for continued investigations into chromosomal architecture and its role in shaping the diversity of this remarkable group of ants.

MATERIALS AND METHODS

Colonies of four *Cephalotes* species were collected by active search in the northernmost municipality of Oiapoque (3°50'31.5"N, 51°50'05.9"W), Amapá state, Brazil (Table 1). Collections were authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/IBAMA) (SISBIO accession number 87049). Workers were analyzed and photographed at the Laboratório de Termitologia of Universidade Federal de Viçosa (UFV), using a Leica S8APO stereoscope connected to a camera. The Z-stacking imaging method was employed for this purpose. Subsequently, the specimens were deposited in the Coleção Entomológica do Laboratório de Coleoptera (CELC) at the UFV, Brazil, records: ANTWEB1047611, ANTWEB1047612 and

ANTWEB1047613. Adult individuals from *C. minutus* and *C. atratus* were identified by Dr. Jacques H. C. Delabie and vouchers were deposited in the myrmecological collection of the Centro de Pesquisas do Cacau (CPDC) at the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), at Bahia, Brazil, record #5882. Z-stacked images were used for taxonomic identification of a second colony of *C. minutus* and the species *C. pavonii* and *C. simillimus* by Dr. Aline Oliveira.

Images of mitotic metaphase spreads were obtained from the cerebral ganglia of larvae and testes from prepupal stages using a Leica KL 300 LED stereoscope. The cerebral ganglia and testes were treated with colchicine-hypotonic solution for 90 minutes (Imai et al. 1988). In accordance with Imai (2016) 10 mL of the colchicine-hypotonic solution consisted of 9.5 mL hypotonic solution (1g of Trisodium citrate dihydrate per 100 ml of distilled water) and 0.5 mL of colchicine stock solution (1mg of Colchicine per 1mL of distilled water). Subsequently, they were stained with 4% Giemsa for 30 minutes. To characterize the karyotypes, at least five individuals per species and 10 metaphases with clearly visible centromeres were used to plot the karyotypes. At least 50 metaphases were analyzed to determine the chromosome number. Chromosomes were measured and classified according to Levan et al. (1964). The karyotypes were assembled using Adobe Photoshop CS6 and measured using Image Pro Plus®.

Fluorescence *in situ* hybridization (FISH) was used to physically map highly repetitive sequences, including 45S rDNA, microsatellites $(GA)_n$, and telomeric motifs, within the chromosomes. To detect 45S rDNA genes, slides underwent indirect labeling following the protocol established by Pinkel et al. (1986). The probe for the 18S rDNA region was obtained through amplification of total DNA using PCR primers 18SF1 (5' GTC ATA GCT TTG TCT CAA AGA 3') and 18SR1.1 (5'-CGC AAA TGA AAC TTT TTT AAT CT-3') designed for the bee *Melipona quinquefasciata* Lepeletier, 1836 (Pereira 2006) and total DNA extracted from the ant *Camponotus rufipes* (Fabricius, 1775). The amplification conditions followed Pereira (2006). The 18S rDNA region probes were incorporated using digoxigenin-11-dUTP (Roche, Mannheim, Germany) for posterior indirect detection, and the signal was detected with anti-digoxigenin-rhodamine (Roche Applied Science) according to the manufacturer's instructions. To identify tandem repeat regions $(GA)_n$ microsatellites and $(TTAGG)_n$ telomeric motifs, the technique of Pinkel et al. (1986) was employed, with modifications made by Cioffi et al. (2010). The probes $(GA)_{15}$ and $(TTAGG)_6$, labeled with cyanine 3 (Cy3) at their 5' terminal end, as recommended by Sigma (St. Louis, MO, USA), were used for FISH. At least two slides from each technique were used for the detection of rDNA and microsatellite sequences for each species.

RESULTS

In this study four *Cephalotes* spp. were cytogenetically analyzed based on their morphotypes and phylogenetic groups (Table 1). The observed chromosome numbers were 2n = 22 in *C. atratus*, 2n = 42 in *C. pavonii* (Latreille, 1809), 2n = 44 in *C. minutus*, and *C. simillimus* (Smith, 1876) with *n* = 22 (haploid) (Fig. 1). Each of them demonstrated a single pair of chromosomes bearing 45S rDNA sites, whose locations varied among species (Fig. 2). In *C. atratus*, the sites were detected in the pericentromeric region of a metacentric pair and in *C. pavonii* within the interstitial region of the long arm of a metacentric pair. In contrast, in *C. minutus*, they were mapped on the short arm of a submetacentric pair, and in male *C. simillimus*, on the pericentromeric region of the short arm of a submetacentric chromosome. No clear evidence of NOR heteromorphisms was detected, and the minor size differences observed between homologs were most consistent with FISH-related artifacts or slight variation in chromosomal condensation. Mapping of $(GA)_n$ microsatellites revealed distinct variations among the four species analyzed (Fig. 3). In *C. minutus*, *C. pavonii*, and *C. simillimus*, these sequences were predominantly restricted to the long arms of the chromosomes. In contrast, *C. atratus* exhibited a distinct pattern, with $(GA)_n$ clusters concentrated at the centromeric regions of eight chromosomes. Furthermore, telomeric sequences were consistently mapped to the termini of all *C. atratus* chromosomes (Fig. 4), confirming their expected positions.

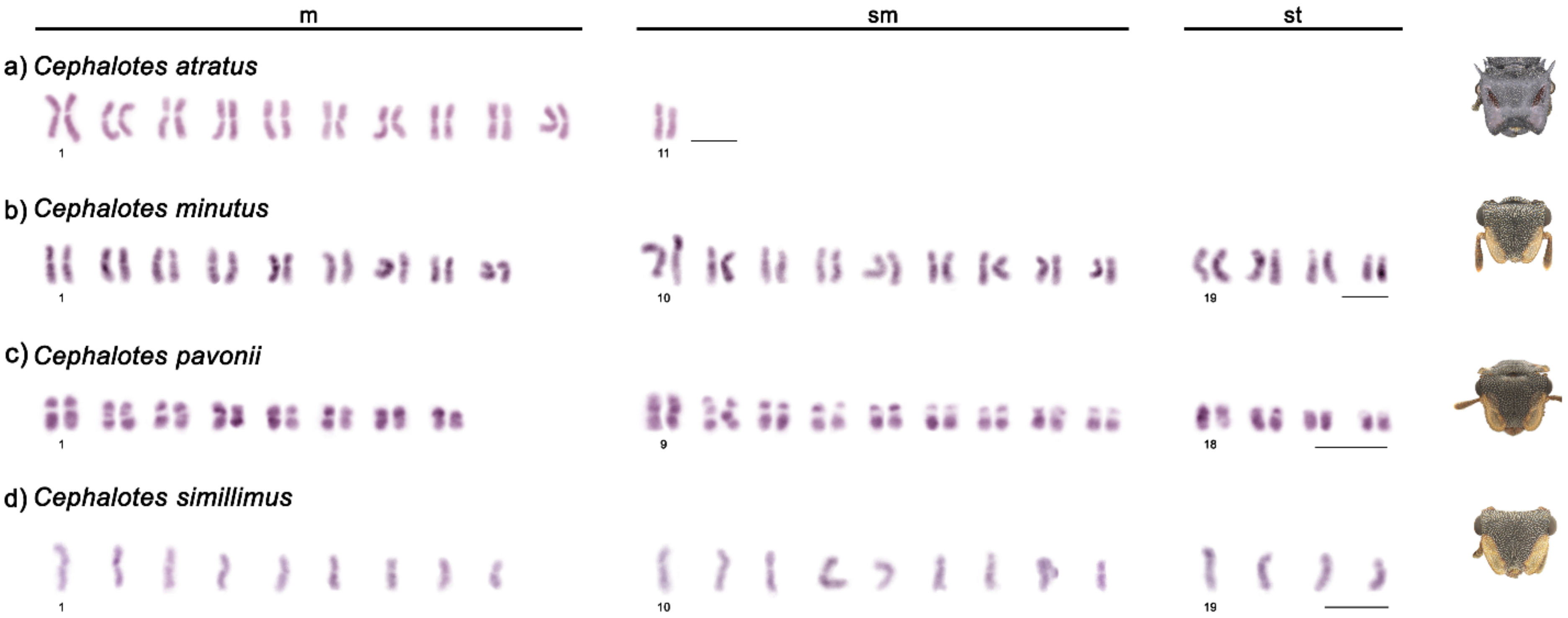

Fig. 1. Karyotypes of *Cephalotes* species stained with DAPI and the heads of their worker ants. The karyotype of *C. simillimus* is haploid (male). Scale bars = 5 µm. The bars refer to the chromosomes.

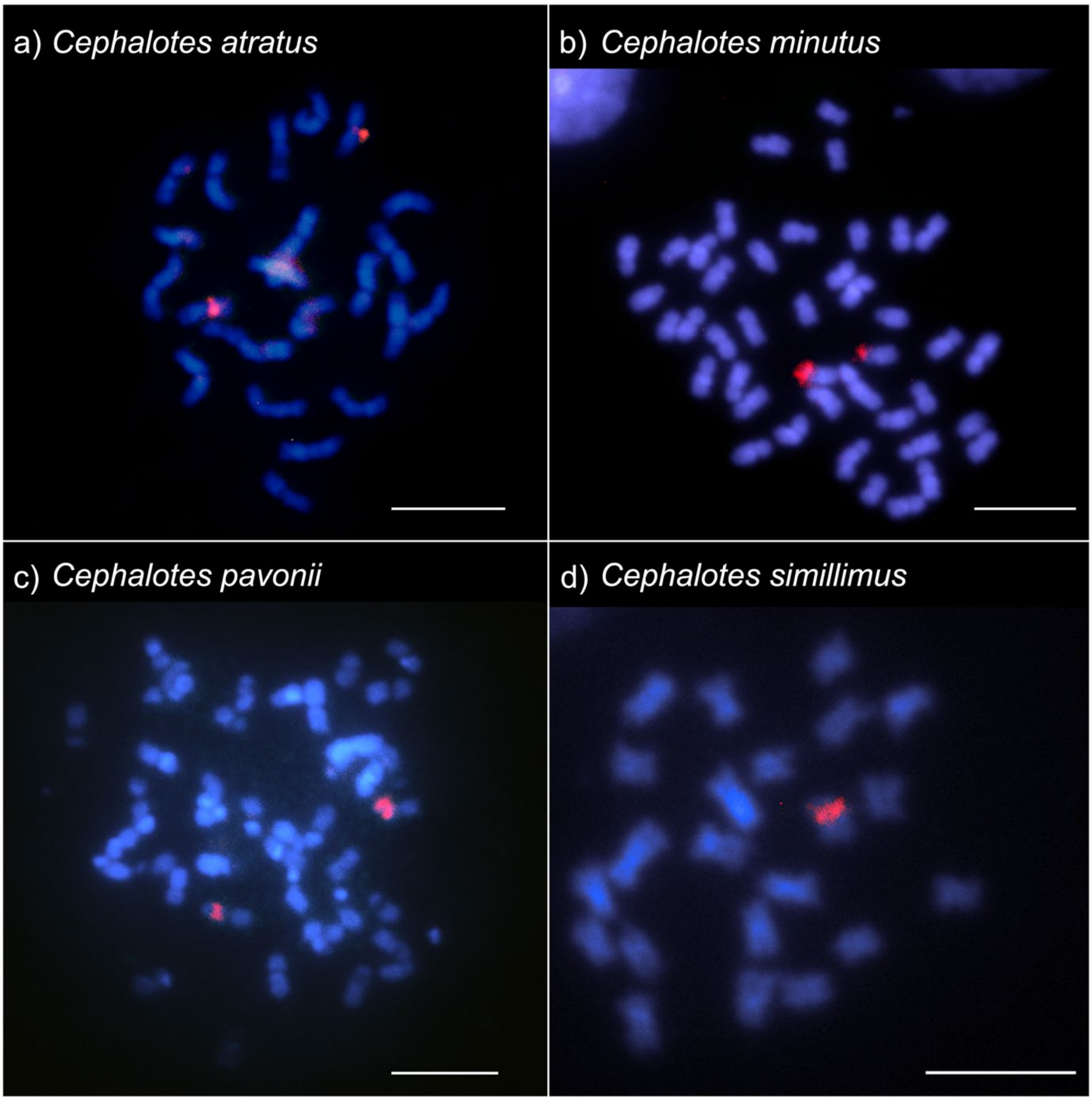

Fig. 2. Physical mapping of 45S rDNA genes (red regions) in the mitotic metaphases of *Cephalotes.* The mitotic metaphasis of *C. simillimus* presented here is haploid (male). Scale bars = 5 μm

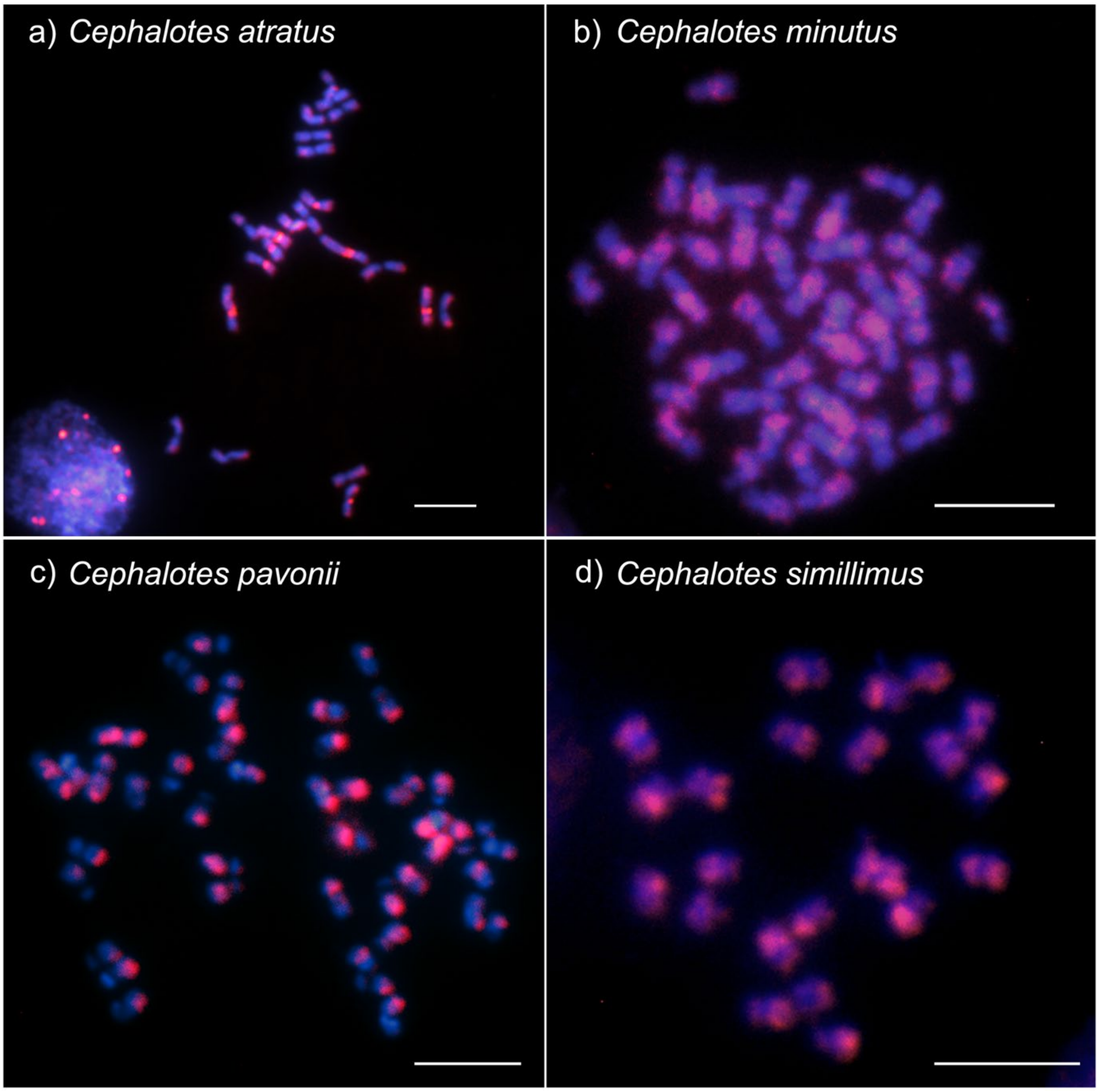

Fig. 3. Physical mapping of $(GA)_n$ microsatellites (red regions) in the mitotic metaphases of *Cephalote.* The mitotic metaphasis of *C. simillimus* presented here is haploid (male). Scale bars = 5 μm.

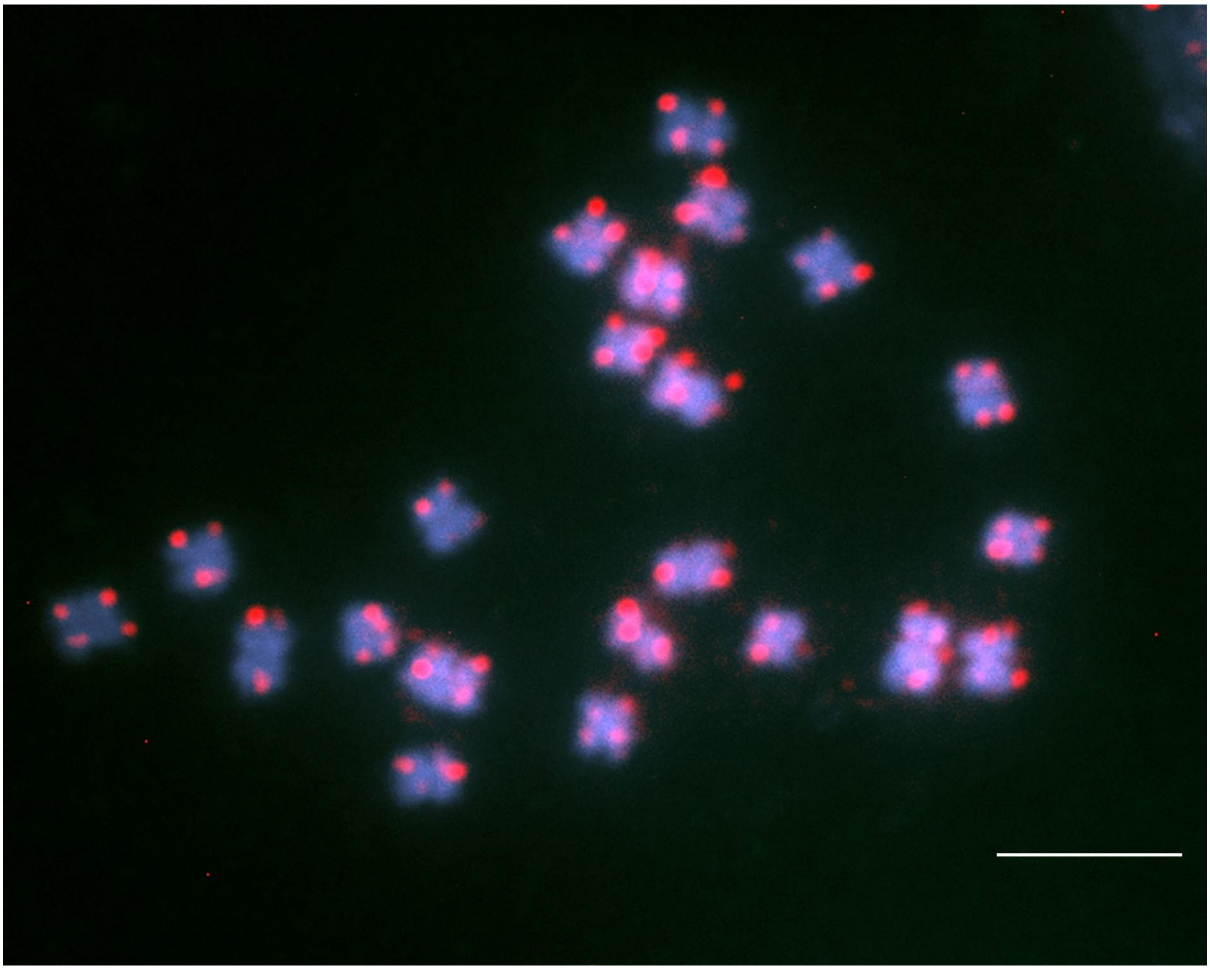

Fig. 4. Physical mapping of insect (TTAGG)$_n$ telomeric sequences (red regions) in *Cephalotes atratus*. Scale bars = 5 µm.

DISCUSSION

All four turtle ant species analyzed in this study inhabit the Amazon biome. However, *C. simillimus* was still unknown in the state of Amapá (Guénard et al. 2017; Franco et al. 2019), suggesting that these species are relatively common and that their nests can be readily located through active searching. Although the number of *Cephalotes* spp. analyzed cytogenetically remains limited, they clearly fall into two distinct chromosomal groups with lower or higher chromosome numbers (2n = 22–24 and 2n = 42–44, respectively) (Table 1), a bimodal pattern. With a few exceptions, certain genera within Myrmicinae exhibited unimodal chromosome numbers. For example, *Atta* Fabricius, 1804 typically has 2n = 22; *Acromyrmex* Mayr, 1865 has 2n = 38; and *Pheidole* Westwood, 1839 has 2n = 20 (Aguiar et al. 2020; Barros et al. 2014 2015 2016 2021a; Lorite and Palomeque 2010).

Workers of *C. atratus* from Oiapoque, in the Amazon rainforest showed 2n = 22 chromosomes, similarly to a population located 2,500 km away from southeastern Brazil (Mariano et al. 2019). Differences in karyotype formulas likely stem from variations in chromosomal classification methods. Unlike the previous study, which categorized the chromosomes according to Imai et al. (1994) nomenclature, in this study the chromosomes were classified solely on the position of the centromere position following Levan et al. (1964). This approach avoids additional complexities associated to the heterochromatic patterns found in the Imai et al. (1994) nomenclature.

The molecular phylogeny proposed by Price et al. (2022) assigns *C. atratus* to the atratus clade, considered the earliest lineage to diverge within this genus. *Procryptocerus* is recognized as a sister genus of *Cephalotes*. Of the 44 *Procryptocerus* species (Bolton, 2025), cytogenetic data are available for only four, all exhibiting low chromosome numbers. These include *P. alderzi* (Mayr, 1887) with 2n = 16 chromosomes, and *P. convergens* (Mayr, 1887), *P. regularis* (Emery, 1888) (Mariano et al. 2019), and *P. hylaeus* Kempf, 1951 (Leitão, unpublished data), all with 2n = 22 chromosomes. Despite the need for more comprehensive cytogenetic sampling in *Procryptocerus* and *Cephalotes*, evidence consistently points toward a karyotype with low chromosome number – such as 2n = 22 – as the hypothesized plesiomorphic condition of *Cephalotes* (Fig. 5). Such a pattern aligns with broader trends observed within Formicidae, suggesting that an increase in chromosomal numbers followed by a diversification of the genus potentially shaped subsequent karyotypic evolution (Cardoso and Cristiano 2025 2026b). Under this scenario, higher chromosome numbers in phylogenetically more derived lineages could be due to chromosomal fissions.

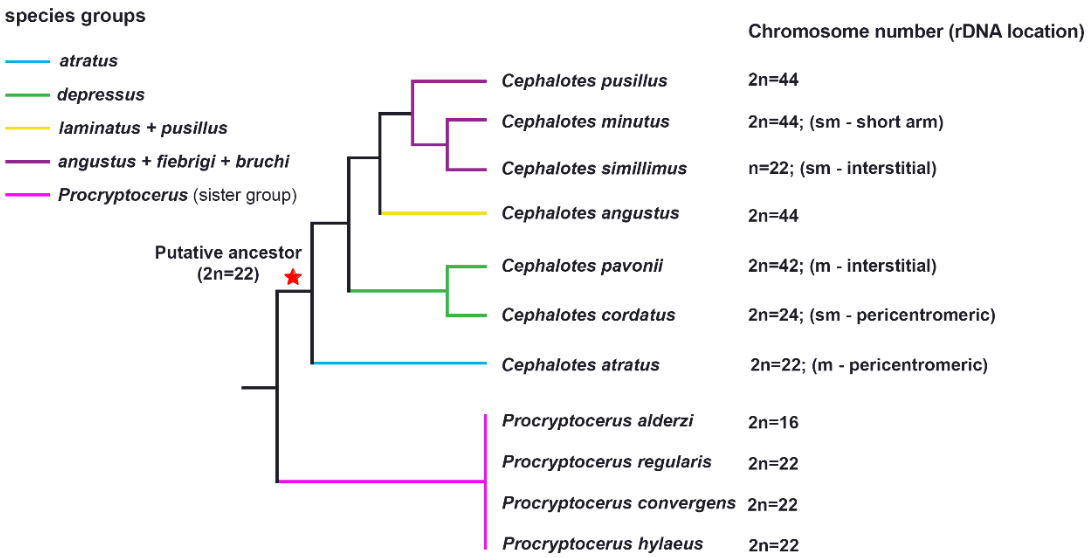

Fig. 5. Phylogenetic relationships among turtle ant species established based on the available cytogenetic data—chromosome number and *18S rDNA* localization—showing *Procryptocerus* as a

monophyletic sister lineage to *Cephalotes*. The red star indicates the putative ancestral karyotype of the genus *Cephalotes*. Phylogeny was based on the molecular analysis of Price et al. (2022).

C. minutus and *C. simillimus* are members of the laminatus + pusillus clade and share a proximal phylogenetic relationship (Price et al. 2022), which is reflected in their karyotypes. In the present study only the males of *C. simillimus* were analyzed, their haploid karyotype (*n* = 22) matches the diploid configuration observed in *C. minutus* (2n = 44). However, the karyotypic formula of the *C. minutus* population from French Guiana demonstrated a divergence, with a distinct proportion of submetacentric and subtelocentric chromosomes (Damasceno et al. 2024). This variation may be linked to differences in chromosomal condensation, especially considering the phylogenetic closeness and karyotypic similarity between *C. minutus* and *C. simillimus*. Expansive cytogenetic analyses spanning multiple populations of the two species, integrated with phylogenetic data, may provide valuable insights into their phylogenetic relationships. The presence of cryptic species complexes within *Cephalotes*, as evidenced by *C. maculatus* (Rana 2023), highlights the need for a closer examination of the relationship between *C. minutus* and *C. simillimus*.

The karyotypic configuration of *C. cordatus*, characterized by 2n = 24 chromosomes and composed of metacentric and submetacentric elements (Damasceno et al. 2024), suggests a cytogenetic affinity with *C. atratus*. However, *C. cordatus* belongs to depressus clade, which is phylogenetically distant from the atratus clade (Price et al. 2022), suggesting that these similarities may not reflect a recent common ancestry.

Interestingly, *C. cordatus* is phylogenetically closer to *C. pavonii* (Price et al. 2022), despite the latter possessing a markedly different karyotype with 2n = 42 chromosomes. Comparisons with other *Cephalotes* spp. (2n = 44) revealed that *C. pavonii* lacked a pair of metacentric chromosomes (Fig. 1c), a feature that may indicate to chromosomal fission. These observations support the hypothesis that the increase in chromosome number through fission may have occurred independently across different *Cephalotes* lineages.

Concerning rDNA distribution patterns, in the giant turtle ant *C. atratus*, low chromosome number and telomeric positioning of 45S rDNA sites within a single chromosome pair were observed (Fig. 2a); a similar configuration was detected in *C. cordatus* (Damasceno et al. 2024). Notably, *C. pavonii*, a member of the depressus clade and proximally related to *C. cordatus*, displayed interstitial 45S rDNA localization (Fig. 2c), despite its markedly higher chromosome number. In *C. simillimus*, the 45S rDNA genes were located within the pericentromeric region (Fig. 2d), contrasting with their position on the short arm of the *C. minutus* chromosome (Fig. 2b). The presence of a single pair of 45S rDNA sites within the pericentromeric or interstitial regions is considered a fundamental cytogenetic feature of ants (Teixeira et al. 2021a; Damasceno et al. 2024). Such an inference is likely due to a reduced probability of rDNA site dispersion when positioned near the centromere, as such regions within the nuclear architecture are more structurally stable (Hirai et al. 2020).

The phylogenetic proximity between *C. cordatus* and *C. pavonii*, coupled with their divergent karyotypic formulas suggests that the interstitial position of 45S rDNA may represent a conserved ancestral cytogenetic trait within *Cephalotes*. To support this hypothesis, 45S rDNA genes have been physically mapped to specific regions within a single pair of chromosome in *P. hylaeus*—a member of the sister genus to *Cephalotes* (Leitão, unpublished data). Together, these findings suggest a interstitial/pericentromeric location of 45S rDNA as a plesiomorphic feature characteristic of the *Cephalotes* lineage, potentially retained among divergent clades despite extensive karyotypic evolution. Increasing cytogenetic data from *Cephalotes* and *Procryptocerus* may contribute to a better understanding of this issue.

In *C. simillimus*, the 45S rDNA genes were located within the pericentromeric region (Fig. 2d), contrasting with the terminal position observed in *C. minutus* (Fig. 2b). Such a variation suggests that the latter terminal location may represent a derived condition within this genus. While chromosomal fissions may represent rearrangements driving karyotypic evolution in *Cephalotes*, rDNA distribution patterns in *C. minutus* suggest that additional mechanisms, such as inversions, may also contribute to variations in the chromosomal location of these genes. Fission events are often accompanied by inversions and have been widely documented in Formicidae (Imai et al. 1988 1994; Teixeira et al. 2021b; Cardoso and Cristiano 2025).

The distribution patterns of microsatellites, particularly the $(GA)_n$ motif, offer valuable insights into the evolutionary dynamics of ant karyotypes (Teixeira et al. 2021a). Due to their rapid rate of evolution, microsatellites exhibit a high degree of polymorphism in the number of repeat units within a given canonical sequence (Li et al. 2004; Ellegren 2004; Jentzsch et al. 2008; Jonika et al. 2020). This variability enables the detection of interspecific and intergeneric differences through FISH, as demonstrated with multiple insect taxa (Travenzoli et al. 2019; Teixeira et al. 2022; Cunha et al. 2023). Although this is the first study to physically map microsatellites in *Cephalotes*, the data obtained for four of its species provide an initial framework for exploring patterns in chromosomal evolution.

Ants exhibit a widespread distribution $(GA)_n$ microsatellite within euchromatic regions of chromosomes. However, in certain cases, specific chromosomal segments bear this motif at a high density, resulting in clustered patterns. Such a pattern has also been observed in the pericentromeric regions of *Mycocepurus goeldii* (Forel, 1893) and *Sericomyrmex* sp. (Teixeira et al. 2021a). These configurations may be polymorphic across natural populations, as observed within the centromeric region of a metacentric chromosome pair in cytotype I of *Crematogaster erecta* (Silveira et al. 2024) and, the pericentromeric region of a chromosome pair among a few populations of *Mycetomoellerius holmgreni* (Wheeler, 1925) (Micolino et al. 2019).

In *C. atratus*, a clustered pattern of $(GA)_n$ was observed in the pericentromeric region of three metacentric chromosome pairs and one submetacentric pair (Fig. 3a). Additionally, this microsatellite was detected at the termini of several chromosomes. In contrast, *C. minutus*, *C. pavonii*, and *C. simillimus*—species with a larger number of chromosomes—did not exhibit clustered markings. Instead, the $(GA)_n$ motif was dispersed throughout the entire length of chromosomes (Fig.s 3b–d), with a notable absence in one arm of certain chromosomes. These findings suggest that microsatellite distribution patterns may correlate with chromosomal architecture and evolutionary history, promising valuable insights into the mechanisms of karyotypic diversification within *Cephalotes* and other ant lineages.

The available cytogenetic data suggest that the putative ancestral karyotype in *Cephalotes* may have comprised approximately 2n = 22–24 chromosomes, predominantly metacentric in morphology. Additional karyotypic data from *Procryptocerus* and basal lineages of *Cephalotes* are crucial for refining these evolutionary inferences. Expansive cytogenetic sampling across these groups, including other lineages, may not only help clarify the ancestral chromosomal condition but also provide a stronger framework for interpreting the patterns in karyotype diversification observed within the genus. In contrast, several derived karyotypes exhibit nearly double the chromosome number, some of them subtelocentrics. According to the Minimal Interaction Theory, centric fission events precede an increase in pericentromeric heterochromatin blocks, which enhances chromosomal stability and may confer adaptive advantages (Imai et al. 1988 1994). Given that $(GA)_n$ microsatellites are typically enriched in noncoding regions, such as introns and intergenic spacers (Tóth et al. 2000), their presence within heterochromatic regions is expected. Thus, the $(GA)_n$-poor chromosome arms observed in *C. minutus*, *C. pavonii*, and *C. simillimus* may reflect post-fission stabilization processes.

The telomeric sequence $(TTAGG)_n$ was mapped to the termini of chromosomes in *C. atratus* (Fig. 4), marking the first such report of this motif in the genus. The conserved nature of this canonical sequence across ant taxa has been demonstrated (Kuznetsova et al. 2020; Barros et al. 2021a; Silveira et al. 2024; Teixeira et al. 2021a b; Wurm et al. 2011). Repetitive DNA containing the $(TTAGG)_n$ motif contribute to chromosomal integrity and facilitate attachment to the nuclear envelope (Kuznetsova et al. 2020; Hirai et al. 2020). Further physical mapping of telomeres and microsatellite sequences across additional species will be essential for elucidating the mechanisms underlying karyotype diversification in this genus.

CONCLUSIONS

This study provides the very first insight into the mechanisms driving karyotype evolution and diversification in *Cephalotes* turtle ants. Based on the data, it can be hypothesized that karyotypic changes among distinct lineages may be shaped by centric fission events, with chromosomal inversions also playing a significant role.

Expanding cytogenetic analyses that include additional turtle ant species—particularly those from underexplored phylogenetic groups—will be critical for unraveling chromosomal evolution within the genus. Given their broad geographic distribution and unique ecological interactions, population-level studies are especially valuable, particularly under contrasting environments such as savannas and humid forests. Cytogenetic data for *Cephalotes* spp. inhabiting the Cerrado biome are lacking and may be very promising. Future research into constitutive heterochromatin will be instrumental in testing the hypothesis that centric fission is a key driver of karyotypic evolution in this genus.

List of abbreviations

CELC, Coleção Entomológica do Laboratório de Coleoptera.
DAPI, (4',6-diamidino-2-phenylindole).
IBAMA, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis.
FISH, Fluorescence in situ hybridization.
rDNA, ribosomal DNA.
SISBio, Sistema de Autorização e Informação em Biodiversidade.
UFV, Universidade Federal de Viçosa.

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Availability of data and materials: *C. atratus* and *C. minutus* were deposited at Centro de Pesquisas do Cacau (CPDC/CEPLAC), Bahia, Brazil under the record #5882. *C. simillimus*, *C. minutus* and *C. pavonii* were deposited at the Coleção Entomológica do Laboratório de Coleoptera (CELC), at Universidade Federal de Viçosa under the records ANTWEB1047611, ANTWEB1047612 and ANTWEB1047613. The photographs used for identifying the species are available at the antweb website under the numbers: ANTWEB1047611, ANTWEB1047612 and ANTWEB1047613

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