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Exploratory analysis of molecular traits of the mitochondrial DNA of leafcutting ants to infer taxonomic characters towards an integrative taxonomy

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Abstract

Accurate systematic classification of biodiversity is fundamental for ecological and evolutionary studies, especially in a world where biodiversity is increasingly reduced and threatened. In the present study we propose using exploratory analysis of genetic markers to obtain additional information from the comparison of sequences among species as molecular traits (MTs). These molecular traits can in turn provide independent information for integrative taxonomy, aiding genus-level circumscriptions. Therefore, we use the leafcutting ant genera *Amoimyrmex* Cristiano et al., 2020, *Atta* Fabricius, 1804 and *Acromyrmex* Mayr, 1865 as models to evaluate a mitochondrial genome fragment regularly applied in phylogenetic and evolutionary studies. Bioinformatic analysis revealed shared structural evidence between species that can serve as diagnostic characters, distinguishing them from other species and supporting the classification of the three genera of leafcutting ants. The molecular features of the mitogenome segments, along with other traits such as chromosome number, karyotype features, molecular phylogeny, and morphological data can be used in an integrative framework to access biodiversity and purpose taxonomic hypothesis.

Keywords

DNA barcode, gene length, genus, insects, integrative taxonomy, mtDNA, phylogenetic systematics

Introduction

Leafcutting ants form a clade within the fungus-farming ants (tribe Attini, subtribe Attina) and are considered the primary herbivores of the Neotropics. They represent a mutualistic group of ants that engage in a relationship with symbiotic basidiomycete fungi, farming them inside their nests. The ants provide nourishment and protection to the fungi, while specialized parts of the fungi serve as the main source of nutrition for the colony (Hölldobler and Wilson 2010). Currently, leafcutting ants are classified into four genera: *Atta* Fabricius, 1804, *Acromyrmex* Mayr, 1865, and *Amoimyrmex* Cristiano et al., 2020 (Cristiano et al. 2020) and *Pseudoatta* Gallardo, 1916 (Bolton 2024). Previously, *Amoimyrmex* was part of *Acromyrmex* until an integrative taxonomic approach grouped three species into this new genus (Cristiano et al. 2013). In the phylogenetic tree, leafcutting ants assemble in a clade where *Amoimyrmex* is the sister group to *Atta* + *Acromyrmex*, which split off by a long branch (Cristiano et al. 2020). It is important to note that while *Pseudoatta* is still regarded as a valid name (Bolton 2024), it should undoubtedly be synonymized with *Acromyrmex*.

Considering molecular phylogenetic relationships alone, two paths could have been taken for classifying leafcutting ants. The first would be to gather all leafcutting ants into a single taxonomic genus, while the second, which was ultimately chosen, was to divide them into three genera. This decision could be a point of debate between lumpers and splitters (Silva et al. 2019). Evidently, several other pieces of evidence were considered to justify the current systematics, such as morphology, karyotypes, colony size, and other biologically divergent features among the three genera (Cristiano et al. 2020).

Ant systematics has advanced significantly over the last decade due to improvements in molecular methods, making it increasingly accessible and widely applied. New ant subfamilies, genera, and species have been proposed based on molecular phylogenies associated with morphology (Rabeling et al. 2008; Sosa-Calvo et al. 2013; Cristiano et al. 2020; Camacho et al. 2022). For instance, the integration of morphology and other independent data, such as karyotype information, has helped resolve the phylogenetic relationships among species of historically challenging taxonomic groups (Taylor 2015). The difficulty in morphological identification has often led to the use of molecular datasets to assist in the morphological analysis for identifying and characterizing different taxa (Roos et al. 2010).

The advancement and accessibility of genetic and genomic data have significantly impacted species delimitation, allowing for the definition and naming of biological organisms at all taxonomic levels. Historically, DNA Sanger sequencing of specific DNA markers was the primary method used in molecular-based research. Among these markers, mitochondrial DNA (mtDNA) has been the most frequently employed to assess molecular diversity (Antil et al. 2023). Specifically, the cytochrome c oxidase subunit I (*cox1*) gene has been used as a standardized molecular index in DNA barcoding, providing a powerful taxonomic tool for identifying and discovering species in animals (Herbert et al. 2003). Although DNA barcoding initially faced several controversies, advancements in DNA technologies, such as metabarcoding (including community DNA and environmental DNA), have established it as an essential tool

in contemporary biodiversity studies on ants and other invertebrates (Ng'endo et al. 2013; Antil et al. 2023; Vuataz et al. 2024).

A major difficulty with DNA barcoding is that using a single short piece of mitochondrial DNA may not be universally applicable among metazoans due to variable mutation rates (DeSalle et al. 2005). This raises concerns about the lack of realistic cut-offs for genetic distances used to differentiate species (Jansen et al. 2009), which may result in species overestimation in some circumstances (Heterick et al. 2017). However, alternative approaches to using DNA barcoding in systematics have been proposed and successfully applied to ants (DeSalle et al. 2005). One such approach, known as character-based DNA barcoding, utilized the four DNA states in a layered framework to define character attributes allowing species, genus and subfamily delimitation (Paknia et al. 2015).

Mitochondrial DNA (mtDNA) remains an essential genetic marker in the study of ant biology, particularly in population studies, species delimitation, and understanding evolutionary relationships (Fisher and Smith 2008; Ng'endo et al. 2013; Paknia et al. 2015; Rasool et al. 2019). Due to its relatively rapid mutation rate and maternal inheritance, mtDNA provides valuable insights into the genetic diversity and phylogenetic structure of ant populations (Cardoso et al. 2015; Cristiano et al. 2016). For example, *cox1* sequences were utilized to delimit species in the fungus-farming ant genus *Sericomyrmex* Mayr, 1865, in the absence of genomic data (Ješovnik et al. 2017). The authors further emphasize that the consistent clustering of taxa within species establishes *cox1* as a reliable and effective tool for species identification through DNA barcoding. While the *cox1* gene has been the most popular marker, other mtDNA fragments, sometimes longer target fragments to avoid nuclear paralogues, have also been utilized (Seal et al. 2011; Cristiano et al. 2014; Cardoso et al. 2018).

With the advancement of massive DNA technologies, more and more ant mitochondrial genomes have become available (Vieira and Prosdocimi 2019). The sequence and structure of these genomes provide evolutionary and comparative genomic information as well as insights into the molecular evolution of ant mitogenomes (Ruiz-Mena et al. 2022). Variations in mitochondrial gene content and order have been used to resolve relationships of related species based on shared derived properties (Rawlings et al. 2001; Dowton et al. 2003). These advancements underscore the continued importance of mtDNA in advancing our understanding of ant biology and evolution. Indeed, length differences identified in various animal mitochondrial 12S and 16S rRNA genes have been suggested for intra- and interspecies identification (Yang et al. 2014). Also, mitochondrial gene order was used as evidence for species delimitation in plathelminths (Wang et al. 2022).

Here, we take advantage of the availability of mtDNA sequences in public databases to propose the use of structural gene information as diagnostic characters for genus-level evaluation. We use the well-defined leafcutting ant genera as a model. We retrieve sequences of three adjacent mtDNA markers that are frequently used in molecular studies (*cox1*-tRNA^{Leu}-*cox2*) to evaluate structural gene characters. Our aim is to go further than the nucleotide sequence *per se* (i.e., the order of adenine, thymine, cytosine, and guanine) to provide useful insights for delimiting the recent genus-level systematics of leafcutting ants.

Materials and methods

A preliminary search was performed in GenBank using the following terms: (cytochrome c oxidase [All Fields] AND ("Formicidae"[Organism] OR Formicidae [All Fields]). We searched for species that had the complete sequence of the *cox1*-tRNA^{Leu}-*cox2* genomic segment to allow for the most inclusive study, and 31 species of leafcutting ants were selected. In addition to Sanger sequences, we also looked for the target mitochondrial markers in genomic data, including third-party annotations, complete mitogenomes, and partial mitogenomes deposited in the NCBI.

The sequences were then aligned using the MEGA X software (Kumar et al. 2018). We used the mitogenome of *Solenopsis saevissima* (Smith, 1855) as the reference (GenBank accession number: NC014672) for aligning the leafcutting ant sequences. *Wasmannia auropunctata* (Roger, 1863), *Mycetophylax simplex* Emery, 1808, and *Trachymyrmex arizonensis* (Wheeler, 1907) were used as outgroups to polarize the molecular features.

The alignment was visually inspected, comparing it with the reference genome. Gaps (-) were added to the alignment when likely deletions/insertions were identified according to the reading frame. Unavailable sites in incomplete sequences were coded with (?). The genomic region analyzed here includes: *cox1* – intergenic spacer 1 – tRNA^{Leu} – intergenic spacer 2 – *cox2*. Each of these regions was then analyzed separately.

Simple statistical analyses were performed using MEGA X software, including base compositions, transitions and transversions, and p-distance. Reading-frame shifts, and amino acid comparisons were conducted in Geneious Prime (Kearse et al. 2012). The determination of tRNA^{Leu} was assisted by using tRNAScan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/), which estimated the size and putative structure, and base pairing across the studied species.

Results

Our search in the GenBank database recovered more than 11,000 sequences mentioning cytochrome c oxidase genes, but only a few entries (31 queries) met our criteria of covering the *cox1-cox2* mtDNA region. Thus, our alignment comprises 31 specimens of ants from the Myrmicinae subfamily, including the leafcutting ants from the genera *Amoimyrmex*, *Atta*, *Acromyrmex*, and some outgroups (Table 1). Genomic sequences of mtDNA obtained through high-throughput sequencing provided the complete *cox1*-tRNA^{Leu}-*cox2* region, while the Sanger dideoxy method yielded nucleotide sequences of varying lengths. The size of the *cox1*-tRNA^{Leu}-*cox2* region ranges from 2,286 to 2,570 nucleotides, for *Wasmannia auropunctata* and *Acromyrmex lundi* (Guérin-Méneville, 1838), respectively. Our alignment matrix covers 2,607 nucleotide sites, with gaps due to the variation in gene length and intergenic spacers.

The mean proportion of adenine/thymine and guanine/cytosine of protein-coding genes by genus is shown in Table 2. The base composition of *cox1* and *cox2* are biased toward AT.

Table 1. Genbank accession numbers of molecular data used in the present study.

	Accession #	Species	Type
Out-groups:			
	NC_014672.1	*Solenopsis invicta*	Genome
	NC_030541.1	*Wasmannia auropunctata*	Genome
	BK012272.1	*Mycetophylax simplex*	Genome
	BK012135.1	*Trachymyrmex arizonensis*	Genome
Leafcutting ants:			
	BK012227.1	*Amoimyrmex striatus*	Genome
	MG753551.1	*Amoimyrmex striatus*	Nucleotide
	BK012716.1	*Atta texana*	Genome
	EU848060.1	*Atta colombica*	Nucleotide
	EU156273.1	*Atta cephalotes*	Nucleotide
	AF016018.1	*Atta sexdens rubropilosa*	Nucleotide
	AF016019.1	*Atta sexdens sexdens*	Nucleotide
	EU165430.1	*Atta mexicana*	Nucleotide
	EU848062.1	*Atta texana*	Nucleotide
	EU848122.1	*Atta laevigata*	Nucleotide
	EU165431.1	*Atta insularis*	Nucleotide
	BK012454.1	*Acromyrmex lundi*	Genome
	BK012312.1	*Acromyrmex heyeri*	Genome
	BK012808.1	*Acromyrmex octospinosus*	Genome
	BK012482.1	*Acromyrmex versicolor*	Genome
	AY265962.1	*Acromyrmex subterraneus*	Nucleotide
	AY265963.1	*Acromyrmex echinatior*	Nucleotide
	AY265964.1	*Acromyrmex balzani*	Nucleotide
	AY265965.1	*Acromyrmex octospinosus*	Nucleotide
	AY265966.1	*Acromyrmex insinuator*	Nucleotide
	AY265967.1	*Acromyrmex lundi*	Nucleotide
	AY265968.1	*Acromyrmex rugosus*	Nucleotide
	AY265969.1	*Acromyrmex coronatus*	Nucleotide
	AY265970.1	*Acromyrmex crassispinus*	Nucleotide
	AY265971.1	*Acromyrmex heyeri*	Nucleotide
	AY265972.1	*Pseudoatta* sp. SS-2003	Nucleotide

Table 2. Nucleotide base proportions of the mtDNA genes *cox1* and *cox2* among leafcutting ant genera.

Gene	Genus	Mean AT%	Mean GC%	Codon Position 1 AT%	Codon Position 2 AT%	Codon Position 3 AT%
cox1	*Amoimyrmex*	65.44%	34.56%	61.53%	63.53%	71.24%
	Atta	70.47%	29.53%	63.84%	61.31%	86.24%
	Acromyrmex	68.88%	31.12%	63.20%	64.28%	79.15%
cox2	*Amoimyrmex*	69.12%	30.88%	64.94%	67.97%	74.46%
	Atta	76.66%	23.34%	70.90%	73.27%	85.84%
	Acromyrmex	74.02%	25.98%	73.40%	70.37%	78.31%

We could evaluate the *cox1*-tRNA^{Leu}-*cox2* mtDNA region among leafcutting ants in its entirety due to the availability of extensive genomic data. In contrast, to include a higher number of species in our analysis, data from standard sequencing (Sanger) cover a greater diversity of species (Fig. 1a). When comparing specimens across our

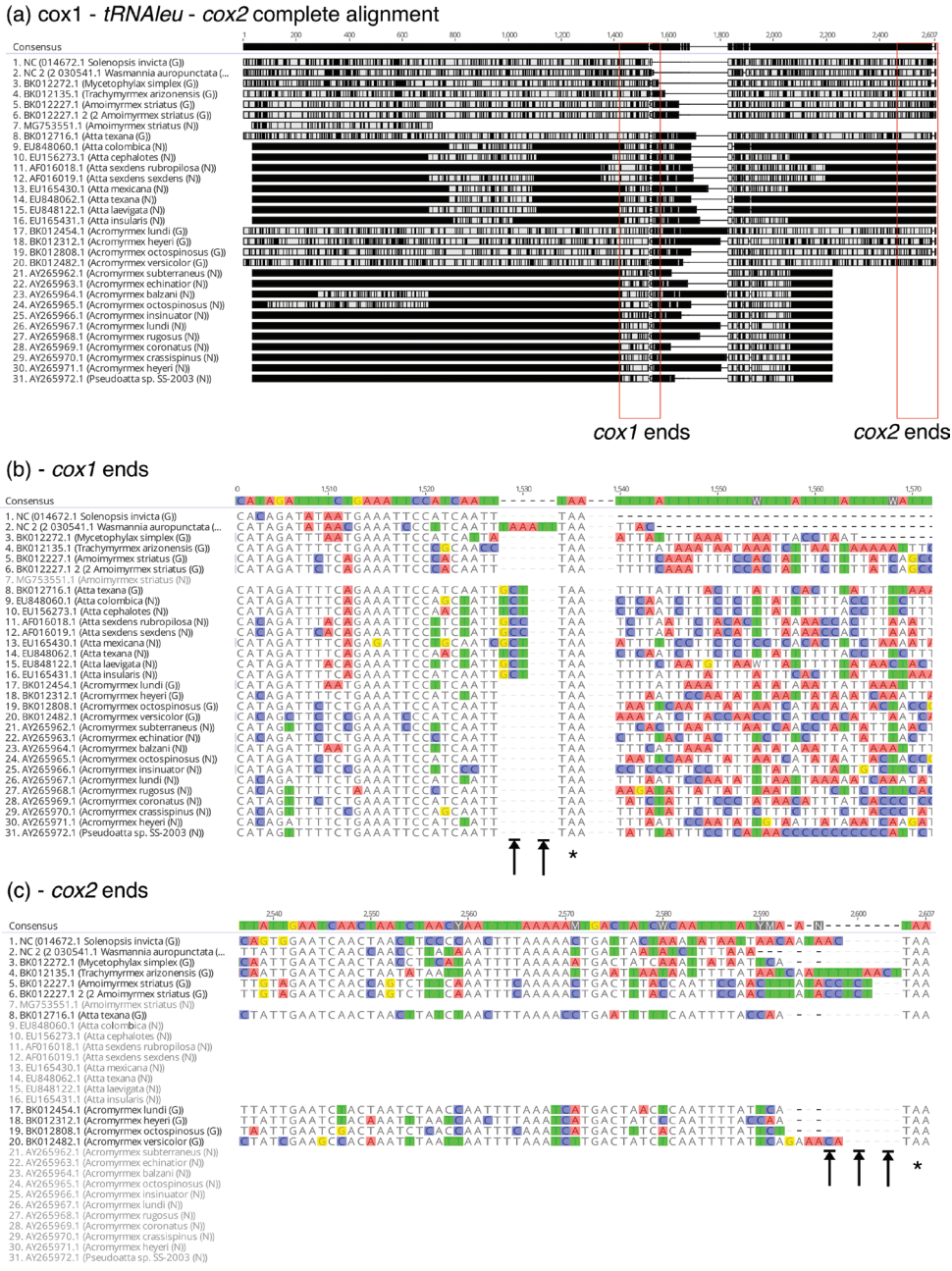

Figure 1. Alignment of the genomic region studied **a** complete alignment (*cox1*-tRNA^{Leu}-*cox2*) compared to the reference genome, indicating specific sites **b** depiction of the end region of Cytochrome Oxidase subunit I **c** Depiction of the end region of Cytochrome Oxidase subunit II. Arrows indicate the differences observed in the end regions of *cox1* and *cox2*. Asterisks denote the stop-codon. Bases in grey represent invariable nucleotides when compared to the consensus. Colors differentiate each nucleotide. (G) and (N) after the name of specimen indicates whether data source was genome or nucleotide sequence, respectively.

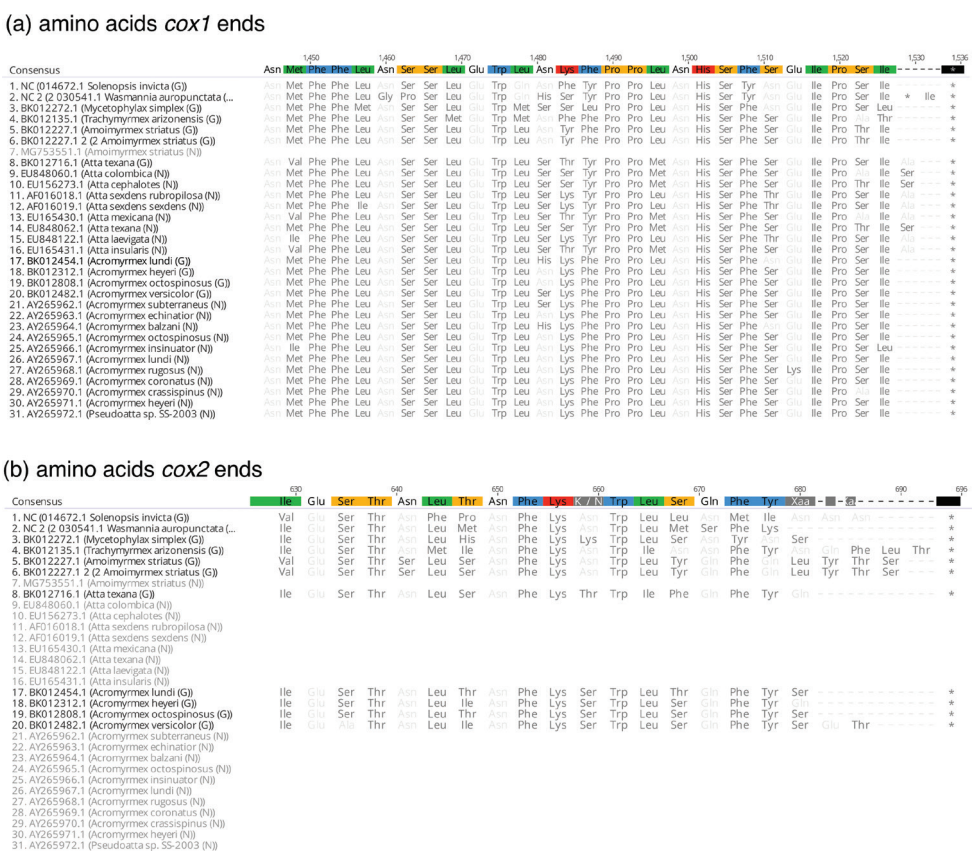

Figure 2. Translated section of the end of Cytochrome Oxidase subunit I and II showing the extra amino acids Serine and Alanine in *Atta*. The duplicated ends in myrmicine out-group *Wasmannia auropunctata* are depicted. Asterisks represent the stop codon. Amino acids in grey represent invariable ones when compared to the consensus.

DNA matrix, the sizes of *cox1* and *cox2* differ among genera, with *cox1* being shorter in *Amoimyrmex* and *Acromyrmex* and three nucleotides longer (one codon) in *Atta* species (Fig. 1b). Conversely, *cox2* is shorter in *Atta* and *Acromyrmex* compared to *Amoimyrmex* (Fig. 1c). Compared to outgroups, leafcutting ants show a complete TAA stop codon, with the last amino acid before the stop codon being Isoleucine (Ile). There is a noticeable duplication at the end of *cox1* in *Wasmannia auropunctata*, involving the Isoleucine codon and the stop codon (Fig. 2a). Similarly, *Atta* species share an insertion of three nucleotides just before the stop codon, while *Amoimyrmex* and *Acromyrmex* do not exhibit this insertion, aligning with the outgroups for this molecular trait. Regarding *cox2*, we only obtained complete sequences from the genomic data, which reduced the number of species compared from 31 to 11 (Fig. 2b). In *Amoimyrmex*, the *cox2* gene is nine nucleotides longer (three codons) just before the stop codon compared to *Atta* and most *Acromyrmex* species studied here. An exception is *Acromyrmex versicolor*, which has an additional six nucleotides (two codons) before the stop codon (Fig. 2b). These findings

Figure 3. Sequences of the intergenic spacer between *cox1* and tRNA^{Leu}. Similarly, *Atta* and *Acromyrmex* share longer intergenic spacers compered to *Amoimymex* suggesting an apomorphy in of the clade *Atta+Acromyrmex*. Colors correspond to each nucleotide and highlight the high proportion of adenine (red) and thymine (green).

suggest that insertions and deletions have occurred across leafcutting ant lineages compared to outgroups; however, further analysis will be necessary to confirm this pattern. The typical stop codon (TAA) is consistent across all evaluated species. Table 3 shows the p-distances between different leafcutting ant genera. Following the commonly used cut-off for taxonomic discrimination, all values range between 2 and 3%.

There is an intergenic spacer between *cox1* and tRNA^{Leu} in all leafcutting ants, whereas the outgroups show no intergenic spacer, as seen in *Solenopsis saevissima*, or only a few nucleotides (four) in *Wasmannia auropunctata* (Fig. 3). A notably long intergenic spacer is an interesting molecular trait shared by *Acromyrmex* and *Atta*, extending up to 285 nucleotides (Fig. 3). In contrast, the intergenic spacer between *cox1* and tRNA^{Leu} in *Amoimyrmex* is as short as in the outgroups. Considerable variation was observed in tRNA^{Leu} across leafcutting ants compared to the outgroups (Fig. 4). *Acromyrmex* species have tRNA^{Leu} genes that are slightly longer than those in the other leafcutting ants. Considering the phylogenetic relationships among leafcutting ants and outgroups, insertions in the TyC and DHU loops appear to be a novel feature in *Acromyrmex* species (Figs 4, 5).

Table 3. Mean uncorrected pairwise genetic distance (p-distance; %) between leafcutting ant genera based on mtDNA *cox1* nucleotide sequence analysis above the diagonal and *cox2* bellow the diagonal.

	Amoimyrmex	***Atta***	***Acromyrmex***
Amoimyrmex	–	2.89	0.260
Atta	0.390	–	0.280
Acromyrmex	0.345	0.359	–

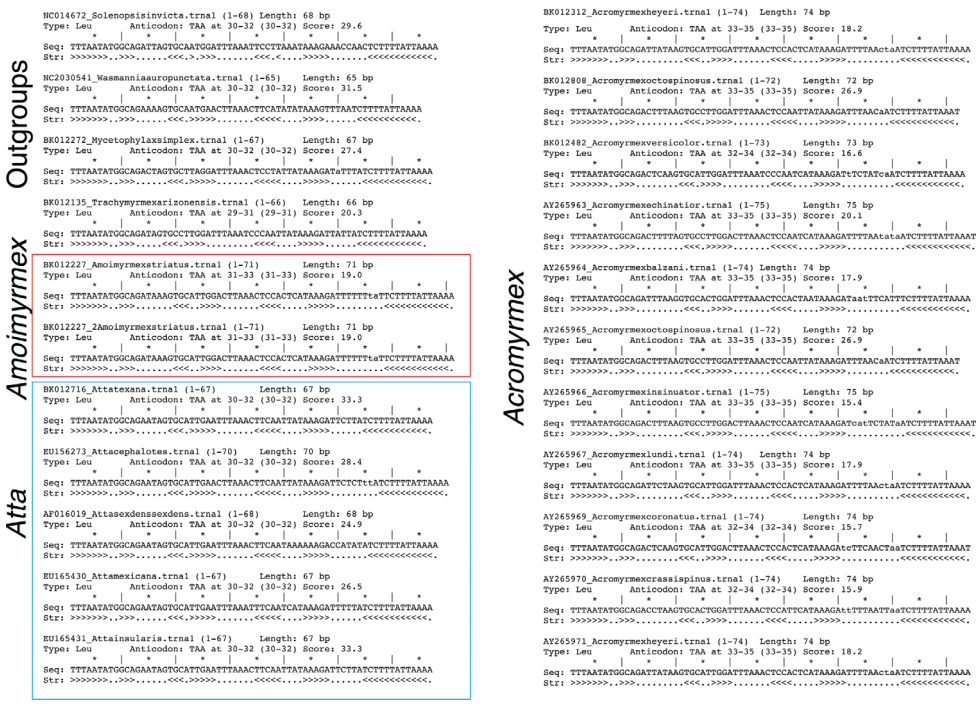

Figure 4. tRNA^{Leu} sequences of leafcutting ants studied. The length and score of the predicted secondary structure are given, as well as the position of the anticodon.

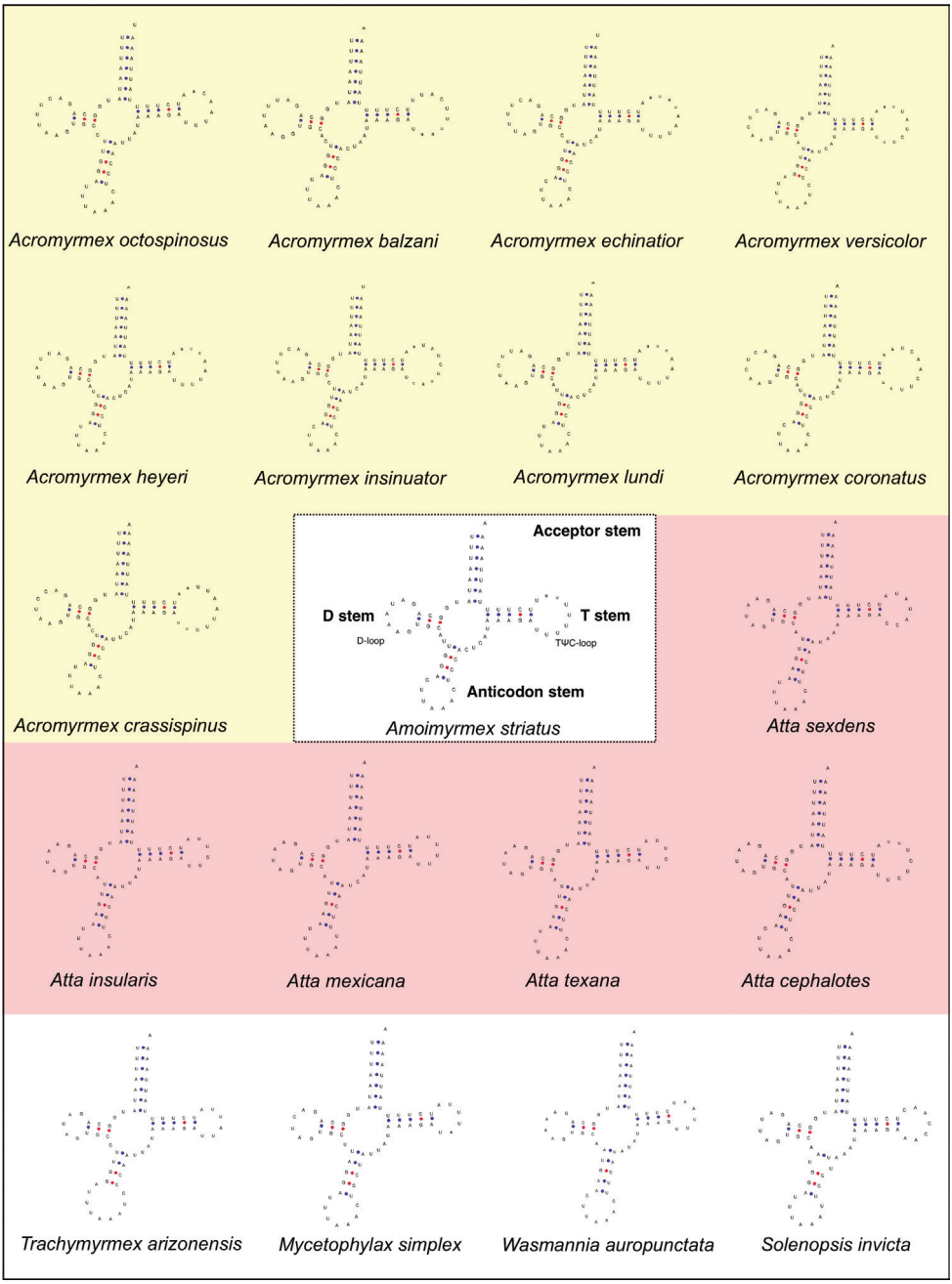

Figure 5. Secondary clover-leaf structure of $tRNA^{Leu}$ in leafcutting ants. Representatives of *Acromyrmex* species studied are shaded in yellow, representatives of *Atta* species studied are shaded in red, and *Amoimyrmex* is in the center. Slight variations are observed in the size of the D-loop and TψC-loop. *Acromyrmex* harbors sequences with up to eight nucleotides. The names of the stems and loops are indicated.

Discussion

In this study, we analyzed the mitochondrial genomic segment routinely used in phylogenetic studies that includes the barcode region (*cox1*). We clearly demonstrate that there are molecular traits, besides nucleotide differences shared among congeners, that can be used in systematic studies to contribute to integrative taxonomy. Generally, we use DNA sequence matrices under an evolutionary substitution model to estimate a phylogenetic hypothesis. However, some structural genomic traits may be significant and additionally used to distinguish species or genera. Here, *cox1*-tRNA^{Leu}-*cox2* showed shared differences and similarities in their nucleotide sizes and leaf structure, aligning with the most recent taxonomic hypothesis proposed for leafcutting ants by Cristiano et al. (2020). *Atta* species share an insertion of three nucleotides just before the stop codon of the *cox1* gene, which is not present in *Amoimyrmex* and *Acromyrmex*. Additionally, the *cox2* ends in *Amoimyrmex* are three codons longer than those in *Atta* and most *Acromyrmex*.

Despite recognized limitations, such as uniparental inheritance and nuclear paralogues (Cristiano et al. 2014), mitochondrial DNA (mtDNA) markers remain valuable in phylogenetic research due to their significant advantages. They allow for rapid and cost-effective species identification and comparison, making them accessible to a broad research community (Srivathsan et al. 2021). The high mutation rate of mtDNA provides extensive genetic diversity, which is particularly useful for distinguishing closely related species. Additionally, the relatively compact and conserved structure of mtDNA facilitates analysis and alignment. Although mtDNA markers alone may not capture the full complexity of lineage relationships, they continue to play an important role in phylogenetics when combined with nuclear markers and other complementary techniques (e.g., microsatellites). Our exploratory analysis aims to examine insertions, deletions, and other structural mtDNA parameters to differentiate leafcutting ant genera, aligning with the character-based DNA barcoding approach suggested by Paknia et al. (2015). This approach, which identifies diagnostic nucleotide sites, has been successfully applied in ants (Paknia et al. 2015) and other insects (Damm et al. 2010), aiding not only in species-level identification but also at higher taxonomic levels, such as the genus level. As a result, we feel that explaining our research methodology can serve as an additional resource for developing ant systematics, analogous to the role of cytogenetics. Karyotype data, for example, has been utilized as a morphological trait to help in species identification (Gokhman 2006; Schlick-Steiner et al. 2010; Cristiano et al. 2020).

Integrative taxonomy has emerged as a multidisciplinary approach that integrates several fields of study, such as morphology, cytogenetics, embryology, molecular genetics, and more (Schlick-Steiner et al. 2010), to improve the taxonomy and systematics of biological entities (Dayrat 2005). By following the cumulative framework (see Padial et al. 2010), leaf-cutting ants were divided into three genera (Cristiano et al. 2013; Cristiano et al. 2020). Morphological, molecular, cytogenetic, and ecological data were

combined to reclassify three species previously placed in *Acromyrmex* into the new genus *Amoimyrmex* (Cristiano et al. 2020). Here, we propose using additional information extracted from the comparison of sequences among species as molecular traits (MTs). These MTs can provide independent information for integrative taxonomy, aiding in species identification and genus-level circumscriptions.

The AT-biased composition of all genes studied here is expected, as it is well-known that the mitochondrial genome of insects is AT-rich (Crozier and Crozier 1993; Simon et al. 1994). This mitochondrial trait has been extensively corroborated by genomic data (Gotzek et al. 2010; Cardoso et al. 2024). Interestingly, *Atta* spp. and *Acromyrmex* spp., which are sister clades, showed higher values of adenine and thymine percentages compared to *Amoimyrmex*.

Likewise, the size of gene spacers is phylogenetically correlated, being larger in *Atta* and *Acromyrmex* compared to *Amoimyrmex*. This is notable because larger mitochondrial genomes have been reported for *Atta* spp. (de Melo-Rodovalho et al. 2014; Barbosa et al. 2019), and the higher number of nucleotides is attributed to the intergenic spacers. In our study, we found such an intergenic spacer between *cox1* and $tRNA^{Leu}$, but no intergenic spacer was found between $tRNA^{Leu}$ and *cox2*, suggesting that longer intergenic spacers are not arbitrarily distributed across the genome. In fact, the mitogenome of the fungus-farming ant *Mycetophylax simplex* does not show longer intergenic spacers but instead has shorter genes (Cardoso et al. 2024).

We found slight differences in the $tRNA^{Leu}$ structure across leafcutting ants, such as in nucleotide sequence size, which in turn reflects the estimated clover-leaf structure. There is variation in the size of the D-loop and TψC-loop among species. The acceptor stem is preserved, and no variation was found in the variable loop, unlike in other ants such as *Camponotus atrox* Emery, 1925, which bears a very long variable loop (Kim et al. 2016). As expected, the anticodon loop is preserved, but the stem is shorter in *Amoimyrmex* and some *Acromyrmex* species, whereas it is always long in *Atta* species. This variation seems not to affect function since it is shared among some leafcutting ants. These small differences, ranging from 1 to 8 nucleotides in stems and loops, justify the variation in the number of nucleotide sites across leafcutting ant genera, with *Acromyrmex* having longer sequences. Variation in cloverleaf arrangement is known (Brennan and Sudaralingam 1976) but still underreported in ants despite its apparent value in evolutionary studies (see Gotzek et al. 2010).

Considering the molecular structural traits observed here, all of them corroborate the recent systematic hypothesis by Cristiano et al. (2020) for leafcutting ants. Although they fall into a large clade comprising the three genera, each genus displays relevant particularities and traits that justify the splitting of leafcutting ants into three genera. The molecular features of the mitogenome segments, along with other traits such as chromosome number, karyotype features, molecular phylogeny, and morphological data, support the distinction of *Amoimyrmex*, *Acromyrmex*, and *Atta* (Cristiano et al. 2020).

The widespread use of mtDNA in evolutionary and phylogenetic investigations at different taxonomic levels has demonstrated the value of this molecular marker for systematics even after the second and third sequencing technologies. The findings of

this study demonstrate that, despite certain limitations, mtDNA markers can serve as effective tools when employed with character-based barcoding and accompanied by other datasets. We demonstrated here that fine-scale structure and nucleotides sequence length of molecular markers can help in ant systematics. Clearly, this needs to be verified further by comparing different taxa, and it may differ depending on the taxa under study. Expanding this approach to other taxa may improve our understanding of comparative taxonomy, the phylogenetic relationships among ants, and the genomic processes underlying their evolution.

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Authors' contributions

DCC conceived and designed research. PON, DCC and MPC conducted experiments. DCC contributed new reagents or analytical tools. PON, NMT, DCC and MPC analyzed data. PON, DCC, MPC and NMT wrote the manuscript. All authors read and approved the manuscript.

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