PERPETUA ONYINYECHI NDUBUISI

MITOCHONDRIAL DNA MARKERS AS TAXONOMIC CHARACTERS AND EVIDENCE OF INDEPENDENT EVOLUTION IN LEAF-CUTTING ANTS GENERA

Dissertation submitted to the Ecology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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Co-advisers: Natália Martins Travenzoli
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Tell me and I forget Teach
me and I remember
Involve me and I learn
(Benjamin Franklin)

ABSTRACT

NDUBUISI, Perpetua Onyinyechi, M.Sc., Universidade Federal de Viçosa, October, 2023. **Mitochondrial DNA markers as taxonomic characters and evidence of independent evolution in leaf-cutting ants genera.** Adviser: Danon Clemes Cardoso. Co-advisers: Natália Martins Travenzoli and Maykon Passos Cristiano.

Ants are among the most ecologically successful insects due to their diversity and occurrence in diverse habitats and terrestrial ecosystems. Ants manifest a diversity of biological attributes and ecological functions from soil modification, participation in nutrient cycling and diversity of biological interactions. In the Neotropics, fungus-growing ants stand out, establishing a symbiotic relationship with Basidiomycete fungi that are maintained, nourished and consumed by these ants. Fungi, on the other hand, receive protection, nutrition and are dispersed together with them. More than 200 species are known and occur from the southern United States to Argentina, with the exception of Chile. The most conspicuous and considered herbivores in the Neotropics are the leaf-cutting ants, which stand out because they exclusively use fresh cut plant material to nourish the symbiotic fungus. Two genera brought together more than 52 species and another 22 subspecies called Atta and *Acromyrmex*. However, some species did not fit the morphological descriptions of the two genera and were relocated to a new genus called *Amoimyrmex*. Furthermore, cytogenetic, and molecular data showed such taxonomic modification. However, there are still discussions about whether to keep the three genres or combine them into one. Here, we compare a segment of the mitochondrial genome that includes DNA barcode with the aim of evaluating whether structural molecular traits from molecular sequences can be used in an integrative taxonomic approach. In this study we evaluated the mitochondrial sequence of cytochrome oxidase 1 – tRNA Leucine – Cytochrome oxidase 2 of leaf-cutter ant species obtained from GenBank. In our comparative analysis we found that several molecular traits corroborate each of the leaf-cutter ant genera, such as shared insertions and deletions in the terminal portion of the genes, variation in the size of gene spacers and tRNALeu. Such data are taxonomically informative and can be used in an integrative taxonomic approach.

Keywords: Ants. Mitochondrial genome. Leafcutter-ants. Molecular markers.

RESUMO

NDUBUISI, Perpetua Onyinyechi, M.Sc., Universidade Federal de Viçosa, outubro de 2023. **Marcadores de DNA mitocondrial como caracteres taxonômicos e evidências de evolução independente em gêneros de formigas cortadeiras.** Orientador: Danon Clemes Cardoso. Coorientadores: Natália Martins Travenzoli e Maykon Passos Cristiano.

As formigas estão entre os insetos de maior sucesso ecológico devido sua diversidade e ocorrência em diversos habitats e ecossistemas terrestres. As formigas manifestam uma diversidade de atributos biológicos e funções ecológicas desde a modificação do solo, participação na ciclagem de nutrientes e diversidade de interação biológicas. No Neotrópico, destacam-se as formigas cultivadoras de fungos que estabeleceram uma relação simbiótica com fungos Basidiomycetos que são mantidos, nutridos e consumidos por estas formigas. Os fungos, em contrapartida, recebem proteção, nutrição e são dispersos juntos com elas. Mais de 200 espécies de formigas cultivadoras de fungos são conhecidas e ocorrem desde o sul dos Estados Unidos até a Argentina, com exceção do Chile. As formigas mais conspícuas e consideradas herbívoras do Neotrópico são as cortadoras de folhas que se destacam pois usam exclusivamente material vegetal fresco cortado para nutrir o fungo simbionte. Dois gêneros, *Atta* e *Acromyrmex,* possuem mais de 52 espécies descritas e mais 22 subespécies. Entretendo, algumas espécies não se enquadram nas descrições morfológicas dos dois gêneros e foram realocados em um novo gênero denominado *Amoimyrmex*. Além disso, dados citogenéticos e moleculares corroboraram tal modificação taxonômica. Entretanto, há ainda discussões acerca da validade taxonômica dos três gêneros. Neste trabalho, seguimentos do genoma mitocondrial que inclui o *DNA barcode* foi comparado entre espécies dos três gêneros com o objetivo de avaliar se traços moleculares estruturais nas sequencias de DNA podem ser utilizados complementarmente em uma abordagem taxonômica integrativa. Neste estudo avaliamos o seguimento mitocondrial do citocromo oxidase 1 (COI)– tRNA Leucina – Citocromo oxidase 2 (COII) de trinta e um espécies de formigas cortadeiras obtidas no GenBank. Em nossa análise comparativa verificamos que vários traços moleculares corroboram a existência dos três gêneros de formigas cortadoras de folhas, tais como, inserções e deleção compartilhadas na porção terminal dos genes, variação no tamanho de espaçadores gênico e tRNALeu. Tais dados podem ser utilizados em uma abordagem taxonômica integrativa.

Palavras-chave: Formigas. Formigas cortadeiras. Genoma mitocondrial. Marcadores moleculares.

LIST OF ILLUSTRATIONS

SUMMARY

INTRODUCTION

ANTS DIVERSITY AND MOLECULAR MARKERS

Ants (Formicidae: Hymenoptera) are one of the most species-rich insect families, with more than 16,000 described species (Bolton, 2021). Formicidae is divided into 21 subfamilies, of which 17 contain extant taxa and 358 genera, while four are exclusively fossil, and more than 16,000 species worldwide (cat.org), with many others yet to be described. Ants originated in the early Cretaceous, occupying the most terrestrial ecosystems and acquiring a global ecological footprint (a reference should be cited here). They can be regarded as the most significant family of soil insects due to the very large influence they have on soil structure. For instance, their influence on soil structure is particularly important in deserts, where earthworm densities are extremely low. Ants are ecosystem engineers, moving large volumes of soil (Hölldobler and Wilson, 1990). As eusocial insects, ants display a wide range of social behaviors, foraging habits, and associations with other organisms, which has generated scientific and public interest (Ward, 2007).

Ants are numerous, diverse, and widely distributed from arctic to tropical ecosystems, occupying virtually all major terrestrial habitats except for tundra and cold, ever-wet forests (Whitford, 2000). Ant communities contain many species, even in desert areas, and local species diversity is especially large in tropical areas (Whitford, 2000). They are major predators of small invertebrates, and their activities reduce the abundance of other predators such as spiders and carabid beetles (Wilson, 1987). Thus, they often act as keystone predators, such as weaver ants from the genus *Oecophylla*, or even herbivores like the leaf-cutting ants in the Neotropics (Higashi, 1979).

Certain plants require ants for protection, nutrient recycling, and seed dissemination (myrmecochory) (LaSalle and Gauld, 1993), and several creatures use ants as a food source, from various arthropods to mammals (Xenarthra) (Alonso and Agosti, 2000). Ants are associated with numerous organisms, including vertebrates that require them for their survival. Such interaction can be seen between army ants and ant birds, which follow the raiding trails of army ants (Mehdiabadi et al., 2010). Ants are especially notable among the insects for their ecological dominance as predators, scavengers, and indirect herbivores (Hölldobler and Wilson, 1990).

Being social insects, ants live in colonies with two female castes (workers and queens) and males, which present morphological and genetic differences (Lorite and Palomeque, 2010). The primary genetic variation between males and females is that workers and queens are diploid, whereas males are haploid.

Among ants, sub-tribe Attina (Formicidae) is a group of ants that is only found in the new world and has a particularly unusual morphology (Hölldobler and Wilson, 1990). They are a monophyletic group made up of 248 known species and an additional 31 subspecies in 20 genera (Bolton, 2021; Hanisch et al., 2022). The attine ants, which are fungus- farming, are splitted into two major clades named paleoattina and neoattina (Hölldobler and Wilson, 1990). Attine ants consist of the highly evolved fungus-farming ant species known as leaf-cutting ants, which are among the most ecologically successful species in terrestrial habitats (Hanisch et al., 2022).

The leaf-cutting ants stands-out as the ultimate ant-fungi symbiosis where the ants cut fresh vegetal sources to nourishment of the fungus and use the fresh plant material as substrate for fungiculture (Mehdiabadi and Schultz, 2009). Leaf cutting ants are very important in tropical and sub-tropical ecosystems and are the major herbivorous and sometimes considered as pests in cultivated fields in the Neotropics (Boulogne et al., 2014).

Ants’ diversity has also been studied with a cytogenetic point of view such as chromosome number and their morphologies. Over 780 ant species have been cytogenetically analyzed, although most of these studies have been made only to determine the chromosome number or the karyotype (Cardoso et al. 2018). Beginning in the 1990s, molecular data have come to play a central role in attempt to reconstruct the ant "tree of life" (Wangensteen and Turon, 2017). Molecular biology tools such as DNA sequencing have become prominent in studies of systematics and phylogenetic relationships of species. In recent decades, molecular tools have contributed to species delimitation, improved our knowledge concerning the evolutionary histories of several taxa, and helped assess community co-positioning and species richness in biodiversity and ecology studies (Wangensteen and Turon, 2017; Van der Loos and Nijland, 2021). DNA sequencing has been applied in the classification of life forms (Rodrigues et al., 2017). DNA sequences have been used as tools in supporting the morphological features and universally been accepted as molecular characters for phylogenetic tree reconstructions (Cardoso et al., 2018).

Mitochondrial DNA is the most popular marker of molecular diversity in the study of animals, fishes, and birds over the last three decades. They are mostly used in evolutionary studies due to their possession of strict maternal transmission in some animals (San et al., 2006) with high mutation rate due to limited repair system (Brown et al., 1979), conserved simple structure (Mandal et al.,2014), and can produce some signal regarding population history over brief time periods (Galtier et al., 2009).

Among the mitochondrial DNA markers, cytochrome c oxidase subunits I and II (CO I and CO II) have been identified as vital protein in most eukaryotic cells. These traits enable the construction of universal primers and simple recovery from tiny or degraded biological samples due to its high copy number in most cells and varying rates of evolution in different areas of mitochondrial DNA (Mandal et al.,2014). Lack of recombination in mitochondrial DNA and its relatively infrequent gene rearrangements makes it a choice for the population genetic studies (Mandal et al., 2014).

The mitochondrial cytochrome c oxidase subunit I (COI) gene has been adopted by the consortium for the barcode of Life (CBOL) for standard DNA barcoding of single animal specimens (San et al., 2006). The COI gene sequence is suitable for this role because its mutation rate is often fast enough to distinguish closely related species and conserve enough among conspecifics (San et al., 2006). Studies have demonstrated the efficiency of the COI barcode method for identifying species (Silva-Brandao et al., 2009), especially those considered to be morphologically similar (Ronque et al., 2015; Ramalho et al., 2015). Contrary to the primary objection raised by skeptics that COI sequence differences are too small to be detected between closely related species, more than 2% sequence divergence is typically detected between closely related animal species (Hebert, 2003), suggesting that the barcode is effective for many animals. This marker can be PCR amplified from most animals and associated database now boasts millions of taxonomically verified DNA sequences, applied in environmental monitoring, dietary studies, biodiversity surveys and evolution (Deagle et al., 2014). Based on their nucleotide substitution rates, mitochondrial gene sequence-based phylogenetic investigation is restricted to closely related species and have been used to resolve the phylogeny of distantly related species based on shared derived properties (Rawlings et al., 2001; Downton, 1992).

Thus, considering the genomic importance and as a feasible marker to evolutionary studies, there is a need to differentiate the leaf-cutting ants especially the newly defined *Amoimyrmex* using COI and COII to corroborate with the morphological studies on the new defined genera.

EVOLUTION OF ANTS

The family Formicidae belongs to the order Hymenoptera, which also includes sawflies, bees and wasps. Ants evolved from a lineage within the stinging wasps and are suggested to be sister group of Apoidea (Johnson et al., 2013). In 1966, E.O.Wilson and his colleagues identified the fossils remains of an ant (*Sphecomyrma*) that lived in the cretaceous period. The specimen, trapped in amber dating back to around 92 million years-ago, has features found in some wasps, but not found in modern ants (Wilson et al., 1967). *Sphecomyrma* was a ground forager, while *Haidomyrmex* and *Haidomyrmodes*, a related genus in subfamily Sphecomyrminae is reconstructed as active arboreal predators (Perrichot et al., 2008). Older ants in the genus *Sphecomyrmodes* have been found in 99 million years-old amber from Myanmar (Barden and Grimaldi, 2014).

Studies have suggested that ants arose up to 168 million years ago (Moreau et al., 2014). After the rise of flowering plants about 100 million years ago they diversified and assumed ecological dominance around 60 million years ago (Perrichot et al., 2014; Grimaldi and Agosti 2000; Wilson and Hölldobler, 2005). Some groups such as the Leptanillinae and Martilinae are suggested to have diversified from early primitive ants that were likely to have been predators underneath the surface of the soil (Barden and Grimaldi, 2012; Rabeling et al., 2008). During the Cretaceous period, a few species of primitive ants ranged widely on the Laurasian supercontinent. They were scarce in comparison to the populations of other insects, representing only about 1% of the entire insect population. Ants became dominant after adaptive radiation at the beginning of the Paleogene period (Wilson and Hölldobler, 1990).

By the Oligocene and Miocene period, ants had come to represent 20–40% of all insects found in major fossil deposits. Of the species that lived in the Eocene epoch, around one in 10 genera survived to the present. Genera surviving today comprise 56% of the genera in Baltic amber fossils (early Oligocene), and 92% of the genera in Dominican amber fossils (apparently early Miocene) (Grimaldi and Agosti, 2000; Philip, 2007). Ant species are less than 2% of the total number of insect species but make up one third of the insect biomass (Barden and Grimaldi, 2014).

Ant agriculture is hypothesized to have arisen in the wet forests of tropical South America approximately 55–65 million years ago (Ješovnik et al., 2016; Schultz et al., 2015).

Most subsequent attine agricultural evolution, including the domestication event that produced the ancestor of the higher attine cultivars, is likewise hypothesized to have occurred in South American rainforests because ant-cultivated fungi are thought to be native to such habitats and incapable of surviving elsewhere as free-living organisms (Mayhé-Nunes, 1998). Fungus- farming 'attina' ants (Formicidae: Myrmicinae: Attini: Attina) are a monophyletic subtribe of approximately 250 described New World species that cultivate fungi for food (Mehdiabadi et al., 2010).

The Attina subtribe, dates to 65 million years ago (MYA) and stands out for having leafcutting ants (Schultz and Brady, 2008; Branstetter et al., 2017). The old two genera of leaf- cutting ants within this tribe, the genus *Atta*, popularly known as "saúvas", and the genus *Acromyrmex*, known as "quenquéns" ants, are both neotropical (Fowler et al., 1989). The "saúvas" showed a great irradiation between 15MYA and 5MYA and are now widely distributed in the neotropics of South America, comprising 15 different species in Brazil (Fowler, 1995; Bacci et al., 2009). Although the ants are obligate symbionts, their fungal cultivars (Agaricaceae: Leucocoprineae and Pterulaceae: Pterula) vary in symbiotic commitment in a pattern that is highly correlated with ant and fungal phylogenies (Mueller et al., 1998). This 'out-of-the-rainforest' hypothesis is consistent with the observation that of all South American habitats (including deserts and seasonally dry habitats), wet Neotropical forests are home to the highest diversity and abundance of species in the fungal tribe Leucocoprineae, from which the ancestral attine cultivars arose (Guzmán,1992)

DISTRIBUTION AND DIVERSITY OF ANTS

Ants are found on all continents except Antarctica, and a few large islands, such as Greenland, Iceland, parts of Polynesia, and the Hawaiian Islands also lack native ant species (Thomas, 200; Schultz, 2000). Ants occupy a wide range of ecological niches and exploit many different food resources as direct or indirect herbivores, predators, and scavengers. Most ant species are omnivorous generalists, but a few are specialist feeders such as leafcutting ants (this sentence needs a reference). Their ecological dominance is demonstrated by their biomass: ants are estimated to contribute on average 15–20% (nearly 25% in the tropics) of terrestrial animal biomass, exceeding that of the vertebrates (Hölldobler & Wilson, 1990). Ants range in size from 0.75 to 52 millimeters (0.030–2.0 in), (Hölldobler &Wilson 1990; Shattuck, 1999) the largest species being the fossil *Titanomyrma giganteum*, the queen of which was 6 centimeters (2.4 inch) long with a wingspan of 15 centimeters (5.9 inch) (Schaal,2006).

Ants vary in color, most ants are red or black, but a few species are green, and some tropical species have a metallic luster. More than 16,000 species are currently known (Bolton 2021), with upper estimates of the potential existence of about 22,000, with the greatest diversity in the tropics. Taxonomic studies continue to resolve the classification and systematics of ants. The relative ease with which ants may be sampled and studied in ecosystems has made them useful as indicator species in biodiversity studies (Agosti et al., 2000; Johnson, 2007).

LEAFCUTTING ANTS

Leafcutting ants comprise 52 species from the genera *Atta*, *Acromyrmex* and *Amoimyrmex* that cultivate fungi as a food source upon which they obligately depend for nourishment (Schultz et al., 1998). These ants cut fresh vegetation to farm their fungi gardens. In the Neotropics, leafcutting ants are considered dominant herbivores, removing up to 15% of the standing leaf crop in their foraging areas (Urbas et al. 2007, Wirth et al. 2003). These insects have been acknowledged as being among the most "successful" species in tropical settings that have undergone anthropogenic modification (Leal et al. 2014) and inhabiting from forest to agricultural fields. They can be considered as key-stone species for natural ecosystems due to their impact as herbivores (Moreira et al., 2010).

Amoimyrmex is recognized as the ancestral genus within the evolutionary history of leafcutting ants (Cristiano et al., 2020), and described as a leafcutting ant specialized on monocots (Gonçalves 1961; Cristiano et al., 2020). *Amoimyrmex* is a small ground-nesting leaf- cutter ant species in southern South America. They can be found in locally plentiful amounts in the open habitats of Brazil's Atlantic Coast (Restingas), the Chaco, Pampas, and Campos Sulinos, as well as sand dune locations (Cristiano et al., 2020). Species of *Amoimyrmex* were previously thought to belong to the *Acromyrmex* subgenus *Moellerius* because of the absence of supraocular spines in the workers, their short and robust mandibles, and their use of grass (monocots) as a substrate for growing fungus (Emery, 1905; Gonçalves, 1961; Weber, 1972). These species were always placed apart from the other species of *Acromyrmex* due to their lack of tubercles on the first gastral tergite (Mayhé-Nunes 1991; Cristiano et al. 2013). Therefore, the new genus *Amoimyrmex* was suggested by

Cristiano et al. (2020).

PHYLOGENETICS

Phylogenetics is the study of evolutionary relationships and histories among or within a group of organisms. Phylogenetic analysis provides information that is useful for applied biology, systematic biology, molecular epidemiology, biodiversity, study of the evolution of genes, identification of gene functions, species history and species identification (Chang et al., 2004; Bentley and Parkhill, 2015; Baele et al., 2016; Horiike, 2016; Sene´s-Guerrero et al., 2016).

For phylogenetic analysis, DNA gene sequences, RNA sequences of functional RNA, or amino acid sequences of proteins are employed. In choosing the molecule for phylogenetic analysis, genes must be shared by all the given species and have the proper evolutionary rates because proteins have varied evolutionary rates (Miyata et al., 1980). Molecular phylogenetics has recently developed into a crucial tool for genome comparisons, including the classification of metagenomic sequences, gene identification, regulatory elements in newly sequenced non- coding RNAs, interpretation of both modern and ancient individual genomes, and reconstruction of ancestral genomes (Yang et al., 2012).

The phylogeny of ants has been the subject of several continuously improving reconstructions over the past 50 years, beginning with morphological data from living faunas (Grimaldi et al., 2000; Ward & Brady, 2003). Then moving forward with additional morphological data supplemented by fossil evidence and finally adding of DNA sequence data (Astruc et al., 2004; Baroni et al., 1992).

In the family Formicidae, ribosomal or mitochondrial DNA sequences have frequently been employed to establish evolutionary connections at the generic, tribe, or subfamily level (Abaur et al.,1995). In comparison to whole genome sequencing, it is more easily accomplished using a variety of reasonably conserved primers and provides more phylogenetic information compared to single or multi-gene. However, the problem of long-branch attraction/non-stationarity is brought on by the lineage-specifically heightened rates of nucleotide changes, which reduces the mitogenome's ability to resolve higher level insect phylogenetics.

OBJECTIVE

Comparing COI –tRNAleu- COII between species from the three genera (*Amoimyrmex*, *Atta*, and *Acromyrmex*) so as to provide new molecular information that corroborates the change in *Amoimyrmex* taxonomic status.

ARTICLE

Comparing COI –tRNAleu- COII between species from the three genera (*Amoimyrmex*, *Atta*, and *Acromyrmex*) so as to provide new molecular information that corroborates the change in *Amoimyrmex* taxonomic status.

Abstract

This study investigated the COI-tRNALEU-COII mitochondrial genome segment, which revealed common differences and similarities that supported the most current taxonomic hypothesis put forth for leafcutting ants. To find the most data on ant species, COI-tRNALeu- COII sequence was chosen after a preliminary search in GenBank using cytochrome c oxidase and Formicidae in all search fields. Statistical analysis was carried out using the software MEGA X. The result revealed that protein-coding genes have a base composition that is skewed toward AT. The simple AT means for *Amoimyrmex*, *Atta*, and *Acromyrmex* according to *cox1* were 65.44%, 70.47%, and 68.88%. Considering codon position of coding genes, the nucleotide adenine and thymine were most frequent in third position, followed by the second position. The nucleotide composition of the *cox2* exhibits similar pattern. *Amoimyrmex* and *Acromyrmex* had smaller sequence length among the DNA matrix, whereas *Atta* species had sequences that were three nucleotides longer in *cox1*, and *Atta* and *Acromyrmex* appeared to have longer sequences than *Amoimyrmex* in *cox2*. The leafcutting ants had a complete TAA stop codon and an intergenic spacer between *cox1* and tRNALeu, whereas the other groups showed no IGS as in outgroups *Solenopsis saevissima* or a few nucleotides (four) in *Wasmannia auropunctata*. Despite being closely related, the small variations in tRNALEU and coding mtDNA structure and nucleotide sequence size among leafcutting ants can be helpful in integrative taxonomy and evidence-based categorization of leafcutting genera.

Keywords: DNA barcode, mitochondrial genome, molecular markers, ants, leafcutter-ants

Introduction

Leaf-cutting ants are a sub-group of fungus growing ants (Ruggiero, 1994) and the primary herbivores of the neotropics (Cristiano et al., 2020). In neotropics, *Atta*, *Acromyrmex* and *Amoimyrmex* are the three genera of the leafcutters that are engaged with fungus symbiosis and cut fresh material from plant to nourishment these gardens inside the nests. These ants are among the most economically harmful species, severely harming fruits and vegetable crops, as well as personal gardens and natural areas (Boulogne et al., 2014). The studies of systematics, molecular phylogenies and phylogenetic relationships of species have become prominent with the aid of molecular biology tools (Cristiano et al., 2014).

Ant systematics has advanced over the last decade due to improvements in molecular methods and has become increasingly accessible. Studies revealed that molecular methods provide a better in-depth of understanding the variations and similarities among insects and evolutionary explanations among different species (Silva-Branda et al., 2009). The integration of molecular phylogenies and other independent data, such as karyotype information, has helped to resolve the phylogenetic relationships among species of historically problematic taxonomic groups (Taylor 2015). Eventually, difficulty in morphological identification has led to the use of molecular datasets to help morphological analysis for identifying and characterization of different taxa (CBOL, Consortium for the Barcode of Life).

The leading method utilized in molecular-based research is DNA sequencing, and mitochondrial DNA (mtDNA) is the most frequently employed genetic marker for gaining access to molecular diversity (Galtier et al., 2009; Cristiano et al., 2014). Among the mitochondrial DNA, COI is a gene that is often used as a DNA barcode to identify animal species and is suitable for this role because its mutation rate is often fast enough to distinguish closely related species and because its sequence is conserved among conspecifics (Hebert, 2003). Mitochondrial gene content and order variations have been used to resolve the phylogeny of distantly related species based on shared derived properties (Rawlings et al., 2001; Downton, 1992). Mitochondrial (mtDNA) genome sequence and structure provides evolutionary and comparative genomics information as well as information on molecular evolution and patterns of gene flow, phylogenetic and population genetics (Wilson et al., 2000; Savato et al., 2008).

Insect mitochondrial *cytochrome oxidase* I (Cox1 or COI) genes are used as a model to examine the within-gene heterogeneity of evolutionary rate and its implications for evolutionary analyses (Lunt et al., 1996). It is a double stranded molecule with a range of 14,503 bp (*Rhopalomyia pomum*) to 19,517 bp (*Drosophila melanogaster*) in size (Lewis et al., 1995). It consists of 37 genes encoding the large and small subunit ribosomal RNAs, 22 transfer RNAs and a regulatory element known as AT-rich region which plays important role in initiation of transcription and replication (Faure et al., 2006; Faure 2011). Among the protein coding genes, *cytochrome oxidase subunit* 1 is found to be the best molecular marker for evolutionary studies (Surajit De Mandal et al., 2014).

Studies revealed that character-based DNA barcoding, using short mitochondrial DNA fragments of the Cox1 gene, was introduced as a tool for rapid species identification or delimitation in ant surveys (Steiner et al., 2005; Alex Smith et al., 2005; Szalanskiet al., 2010; Ross et al., 2010) systematic revisions (Martins et al., 2014), effectiveness of the Cox1 approach for identifying species (Silva-Branda et al., 2009) and including species morphologically similar (Ronque et al., 2015). This approach is based on the fact that DNA sequences have undergone mutations that make them unique to each species (Herbert et al., 2003).

Here, we present molecular evidence for the Cox1 marker that distinguishes leafcutter ants and supports the taxonomic classification of *Amoimyrmex* as a sister group of the remaining leaf-cutting ants that split from their common ancestor (Cristiano et al., 2020). Hence, this study aims to use COI-tRNAleu-COII to provide molecular base information that corroborate the morphological classification of *Amoimyrmex*.

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 search for species that had the complete sequence of Cox1-tRNALeu-Cox2 genomic segment that would allow us the most inclusive study and 31 species were selected. Additional to Sanger sequences we also looked for the target mitochondrial markers in genomic data, as third part annotation, 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* as reference (GenBank accession number: NC014672) to aligning the leafcutting ant sequences. We use *Wasmannia auropunctata*, *Mycetophylax simplex*, *Trachymyrmex arisonensis* as out-groups to polarize the molecular features. The alignment was extensively inspected visually comparing with the reference genome. We added gaps (-) to the alignment when likely deletions/insertions were identified according to the reading-frame. Unavailable sites in incomplete sequences were coded with (?). We spliced the genomic region analyzed here into: **Cox1** – intergenic spacer 1 – **tRNALeu** – intergenic spacer 2 – **Cox2**. Then, each one of these regions were analyzed separately.

Simple statistical analysis was calculated in MegaX software, as bases compositions, transitions and transversion, *p*-distance. Reading-frame, shifts, and amino acid comparisons were done in GeniousPrime. The determination of tRNALeu was assisted by using tRNAScanSE (http://lowelab.ucsc.edu/tRNAscan-SE/) that estimated the size and putative structure, base paring across the studied species.

Results

Our screen in GenBank database recover more than 11 thousand sequences mentioning cytochrome c oxidase genes, but few entries meet our criteria of covering *Cox1*-*Cox2* mtDNA region. Thus, our alignment is comprised of 31 specimens of ants from Myrmicinae subfamily including the leafcutting ants from genera *Amoimyrmex*, *Atta*, *Acromyrmex*, and outgroups. Genomic sequences of mtDNA obtained by high throughput sequencing methods made available the complete *cox1*-*tRNA*Leu-*cox2* region whereas Sanger dideoxy method allowed the obtention of nucleotide sequences of various extents. *Cox1*-*tRNA*Leu-*Cox2* region size ranges from 2286 to 2570 nucleotides, for *Wasmannia auropunctata* and *Acromyrmex lundi*, respectively. Our alignment matrix cover 2607 nucleotide sites with gaps due to the variation of genes and intergenic spacers.

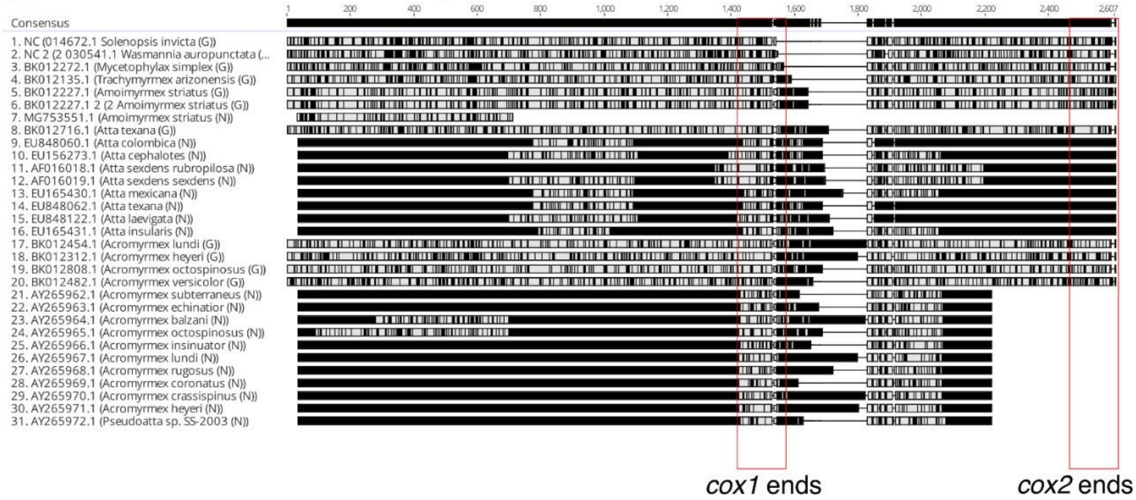
The base composition of the protein coding genes is biased to AT. *Cox1* show a simple AT mean of 65.44% in *Amoimyrmex*, 70.47% in *Atta* and 68.88% in *Acromyrmex*. Similarly, GC mean was 34.56% in *Amoimyrmex*, 29.53% in *Atta* and 31.12% in *Acromyrmex*. Considering each codon position: *Amoimyrmex* AT% was 61.53 to the first position, 63.53 to the second and 71.24 to the third position; *Atta* was 63.84 to the first position, 61.31 to the second and 86.24 to the third position, whereas to *Acromyrmex* was 63.20 to the first position, 64.28 to the second and 79.15 to the third position. Yet, *Cox2* gene show a nucleotide composition with a mean AT% value of 69.12% to *Amoimyrmex*, 76.66% to *Atta* and 74.02% to *Acromyrmex* and completely a mean GC% of 30.88%, 23.34%, 25.98; respectively. Concerning the codon position, *Amoimyrmex* first position show a mean AT% value of 64.94%, second position 67.97% and third position 74.46%; *Atta* first position show a mean AT% value of 70.90%, second position 73.27% and third position 85.84%; whereas *Acromyrmex* first position show a mean AT% value of 73.40%, second position 70.37% and third position 78.31%.

We could evaluate the *Cox1-tRNA*Leu-*Cox2* mtDNA region among leafcutting ants in their entirety due to availability of massive genomic data, but in contrast, to cover a higher number of species in our analysis, data from standard sequencing (Sanger) cover extensively more species (Figure 1a). When comparing specimens across our DNA matrix, the size of *Cox1* and *Cox2* differ among genera being shorter throughout *Amoimyrmex* and *Acromyrmex* and three nucleotides longer (one codon) across *Atta* species to *Cox1* (Figure 1b), whereas *Cox2* seems to be shorter in *Atta* and *Acromyrmex* than *Amoimyrmex* (Figure 1c). Compared to outgroups, leafcutting ants showed a complete TAA stop codon and the last amino acid just before the stop codon is Isoleucine (Ile). There is a clear duplication at the end of *Cox1* in *Wasmannia auropunctata* of the isoleucine codon and stop codon (Figure 2a). Likewise, *Atta* species seems to share an insertion of three nucleotides just before the stop codon. *Amoimyrmex* and *Acromyrmex* do not show the insertion being like the outgroups in this molecular trait. Concerning the *Cox2* we only get complete sequences from massive genomic data, which decrease the number of species compared (Figure 2b), however is possible conclude that insertions and deletions occur across leafcutting ants compared to the outgroups but without a clear pattern as the one observed in *Cox1*. The typical stop codon is shared in all species evaluated.

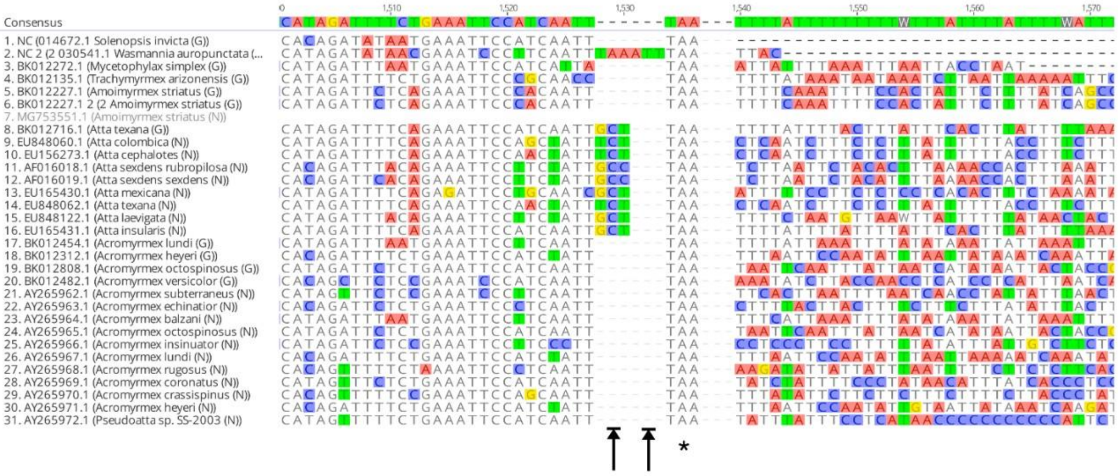
There is an intergenic spacer between *Cox1* and *tRNALeu* of all leafcutting ants whereas the outgroups show no IGS as in *Solenopsis saevissima* or few nucleotides (four) in *Wasmannia auropunctata* (Figure 3). Long intergenic spacer seems to be other interesting molecular trait shared by *Acromyrmex* and *Atta* that can reach as long as 285 nucleotides (Figure 3). Yet *Amoimyrmex* intergenic spacer *Cox1-tRNALeu* is as short as in the outgroups.

Considerable variation was observed between *tRNALeu* across leafcutting ants and compared to the outgroups (Figure 4). *Acromyrmex* species showed *tRNALeu* gene few bases longer than the remain leafcutting ants. Considering the phylogenetic relationship of leafcutting and the outgroups, insertions in the sequence of the TyC and DHU loops seems to be a novelty in *Acromyrmex* species (Figure 4, 5).

(a) cox1 - *tRNAleu* - *cox2* complete alignment



(b) - *cox1* ends



(c) - *cox2* ends

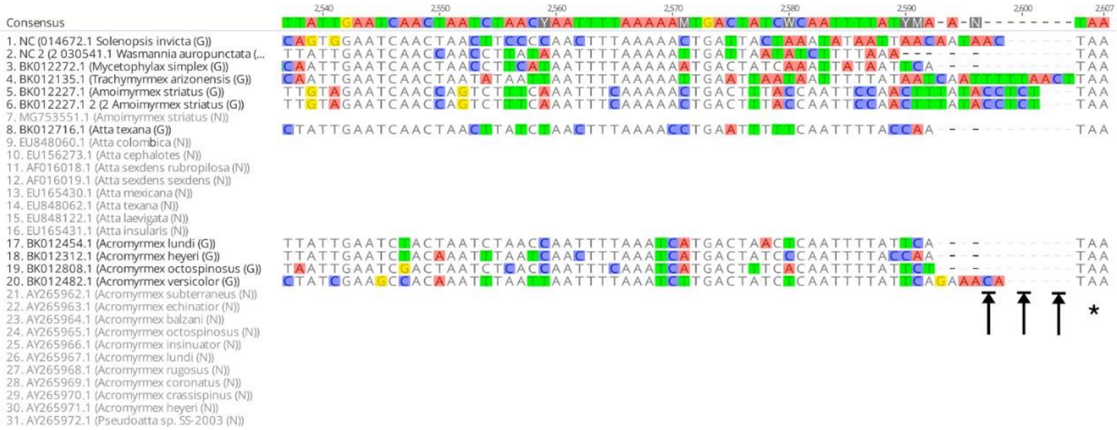


Figure 1: Alignment of genomic region studied (a) Complete alignment (COI-tRNAleu-COII) compared to the reference genome indication sitio sites. (b) Depicted Cytochrome oxidase one end`s region. (c) Depicted Cytochrome oxidase two end`s region. Arrows indicate the differences observed in the end regions of Cox1 and Cox2. Asterisks denote the stop-codon.

(a) amino acids *cox1* ends

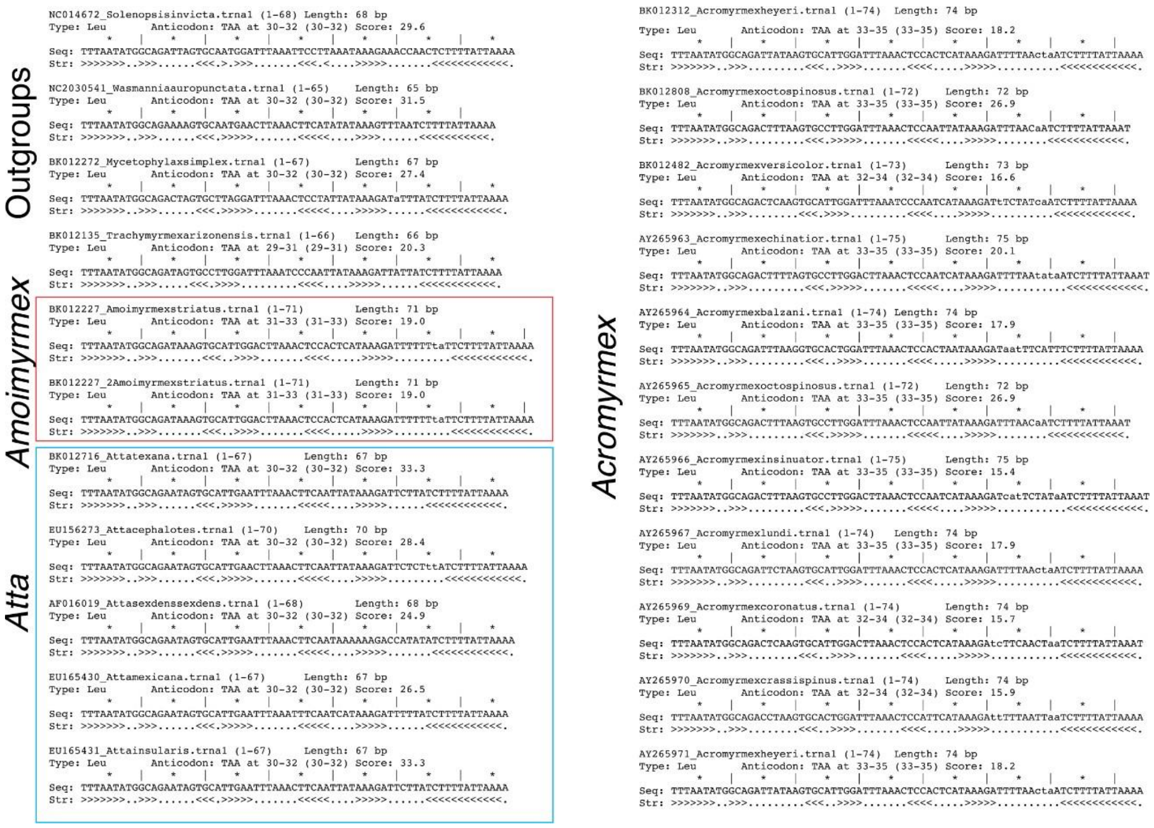
Consensus	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
1. NC (014672.1 Solenopsis invicta (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Gln	Asn	Phe	Tyr	Pro	Pro	Leu	Asn	His	Ser	Tyr	Asn	Glu	Ile	Pro	Ser	Ile	-	-	*
2. NC 2 (2 030541.1 Wasmannia auropunctata (...	Asn	Met	Phe	Phe	Leu	Gly	Pro	Ser	Leu	Glu	Trp	Gln	His	Ser	Tyr	Pro	Pro	Leu	Asn	His	Ser	Tyr	Asn	Glu	Ile	Pro	Ser	Ile	*	Ile	*
3. BK012272.1 (Mycetophylax simplex (G))	Asn	Met	Phe	Phe	Met	Asn	Ser	Ser	Leu	Glu	Trp	Met	Ser	Ser	Leu	Pro	Pro	Leu	Asn	His	Ser	Phe	Asn	Glu	Ile	Pro	Ser	Leu	-	-	*
4. BK012135.1 (Trachymyrmex arizonensis (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Met	Glu	Trp	Met	Asn	Phe	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ala	Thr	-	-	*
5. BK012227.1 (Amoimyrmex striatus (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Tyr	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Thr	Ile	-	-	*
6. BK012227.1 2 (2 Amoimyrmex striatus (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Tyr	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Thr	Ile	-	-	*
7. MG753551.1 (Amoimyrmex striatus (N))																															
8. BK012716.1 (Atta texana (G))	Asn	Val	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Thr	Tyr	Pro	Pro	Met	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	Ala	-	*
9. EU848060.1 (Atta colombica (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Ser	Tyr	Pro	Pro	Met	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ala	Ile	Ser	-	*
10. EU156273.1 (Atta cephalotes (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Ser	Tyr	Pro	Pro	Met	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Thr	Ile	Ser	-	*
11. AF016018.1 (Atta sexdens rubropilosa (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Lys	Tyr	Pro	Pro	Leu	Asn	His	Ser	Phe	Thr	Glu	Ile	Pro	Ser	Ile	Ala	-	*
12. AF016019.1 (Atta sexdens sexdens (N))	Asn	Met	Phe	Phe	Ile	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Lys	Tyr	Pro	Pro	Leu	Asn	His	Ser	Phe	Thr	Glu	Ile	Pro	Ser	Ile	Ala	-	*
13. EU165430.1 (Atta mexicana (N))	Asn	Val	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Thr	Tyr	Pro	Pro	Met	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ala	Ile	Ala	-	*
14. EU848062.1 (Atta texana (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Ser	Tyr	Pro	Pro	Met	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Thr	Ile	Ser	-	*
15. EU848122.1 (Atta laevigata (N))	Asn	Ile	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Lys	Tyr	Pro	Pro	Leu	Asn	His	Ser	Phe	Thr	Glu	Ile	Pro	Ser	Ile	Ala	-	*
16. EU165431.1 (Atta insularis (N))	Asn	Val	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Thr	Tyr	Pro	Pro	Met	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	Ala	-	*
17. BK012454.1 (Acromyrmex lundi (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	His	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Asn	Glu	Ile	Pro	Ser	Ile	-	-	*
18. BK012312.1 (Acromyrmex heyeri (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
19. BK012808.1 (Acromyrmex octospinosus (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
20. BK012482.1 (Acromyrmex versicolor (G))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
21. AY265962.1 (Acromyrmex subterraneus (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Ser	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
22. AY265963.1 (Acromyrmex echinatior (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
23. AY265964.1 (Acromyrmex balzani (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	His	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Asn	Glu	Ile	Pro	Ser	Ile	-	-	*
24. AY265965.1 (Acromyrmex octospinosus (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
25. AY265966.1 (Acromyrmex insinuator (N))	Asn	Ile	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Leu	-	-	*
26. AY265967.1 (Acromyrmex lundi (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
27. AY265968.1 (Acromyrmex rugosus (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Lys	Ile	Pro	Ser	Ile	-	-	*
28. AY265969.1 (Acromyrmex coronatus (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
29. AY265970.1 (Acromyrmex crassispinus (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ala	Ile	-	-	*
30. AY265971.1 (Acromyrmex heyeri (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*
31. AY265972.1 (Pseudoatta sp. SS-2003 (N))	Asn	Met	Phe	Phe	Leu	Asn	Ser	Ser	Leu	Glu	Trp	Leu	Asn	Lys	Phe	Pro	Pro	Leu	Asn	His	Ser	Phe	Ser	Glu	Ile	Pro	Ser	Ile	-	-	*

(b) amino acids *cox2* ends

Consensus	Ile	Glu	Ser	Thr	Asn	Leu	Thr	Asn	Phe	Lys	K / N	Trp	Leu	Ser	Gln	Phe	Tyr	Xaa	▪	a	-	-	■
1. NC (014672.1 Solenopsis invicta (G))	Val	Glu	Ser	Thr	Asn	Phe	Pro	Asn	Phe	Lys	Asn	Trp	Leu	Leu	Asn	Met	Ile	Asn	Asn	Asn	-	-	*
2. NC 2 (2 030541.1 Wasmannia auropunctata (...	Ile	Glu	Ser	Thr	Asn	Leu	Met	Asn	Phe	Lys	Asn	Trp	Leu	Met	Ser	Phe	Lys	-	-	-	-	-	*
3. BK012272.1 (Mycetophylax simplex (G))	Ile	Glu	Ser	Thr	Asn	Leu	His	Asn	Phe	Lys	Lys	Trp	Leu	Ser	Asn	Tyr	Asn	Ser	-	-	-	-	*
4. BK012135.1 (Trachymyrmex arizonensis (G))	Ile	Glu	Ser	Thr	Asn	Met	Ile	Asn	Phe	Lys	Asn	Trp	Ile	Asn	Asn	Phe	Tyr	Asn	Gln	Phe	Leu	Thr	*
5. BK012227.1 (Amoimyrmex striatus (G))	Val	Glu	Ser	Thr	Ser	Leu	Ser	Asn	Phe	Lys	Asn	Trp	Leu	Tyr	Gln	Phe	Gln	Leu	Tyr	Thr	Ser	-	*
6. BK012227.1 2 (2 Amoimyrmex striatus (G))	Val	Glu	Ser	Thr	Ser	Leu	Ser	Asn	Phe	Lys	Asn	Trp	Leu	Tyr	Gln	Phe	Gln	Leu	Tyr	Thr	Ser	-	*
7. MG753551.1 (Amoimyrmex striatus (N))																							
8. BK012716.1 (Atta texana (G))	Ile	Glu	Ser	Thr	Asn	Leu	Ser	Asn	Phe	Lys	Thr	Trp	Ile	Phe	Gln	Phe	Tyr	Gln	-	-	-	-	*
9. EU848060.1 (Atta colombica (N))																							
10. EU156273.1 (Atta cephalotes (N))																							
11. AF016018.1 (Atta sexdens rubropilosa (N))																							
12. AF016019.1 (Atta sexdens sexdens (N))																							
13. EU165430.1 (Atta mexicana (N))																							
14. EU848062.1 (Atta texana (N))																							
15. EU848122.1 (Atta laevigata (N))																							
16. EU165431.1 (Atta insularis (N))																							
17. BK012454.1 (Acromyrmex lundi (G))	Ile	Glu	Ser	Thr	Asn	Leu	Thr	Asn	Phe	Lys	Ser	Trp	Leu	Thr	Gln	Phe	Tyr	Ser	-	-	-	-	*
18. BK012312.1 (Acromyrmex heyeri (G))	Ile	Glu	Ser	Thr	Asn	Leu	Ile	Asn	Phe	Lys	Ser	Trp	Leu	Ser	Gln	Phe	Tyr	Gln	-	-	-	-	*
19. BK012808.1 (Acromyrmex octospinosus (G))	Ile	Glu	Ser	Thr	Asn	Leu	Thr	Asn	Phe	Lys	Ser	Trp	Leu	Ser	Gln	Phe	Tyr	Ser	-	-	-	-	*
20. BK012482.1 (Acromyrmex versicolor (G))	Ile	Glu	Ala	Thr	Asn	Leu	Ile	Asn	Phe	Lys	Ser	Trp	Leu	Ser	Gln	Phe	Tyr	Ser	Glu	Thr	-	-	*
21. AY265962.1 (Acromyrmex subterraneus (N))																							
22. AY265963.1 (Acromyrmex echinatior (N))																							
23. AY265964.1 (Acromyrmex balzani (N))																							
24. AY265965.1 (Acromyrmex octospinosus (N))																							
25. AY265966.1 (Acromyrmex insinuator (N))																							
26. AY265967.1 (Acromyrmex lundi (N))																							
27. AY265968.1 (Acromyrmex rugosus (N))																							
28. AY265969.1 (Acromyrmex coronatus (N))																							
29. AY265970.1 (Acromyrmex crassispinus (N))																							
30. AY265971.1 (Acromyrmex heyeri (N))																							
31. AY265972.1 (Pseudoatta sp. SS-2003 (N))																							

Figure 2: Translated section of the end of cytochrome oxidase 1 and 2 showing the extra amino acids Serine and Alanine in Atta. We depict the duplicating ends in *Wasmannia auropunctata*. Asterisks represents the stop-codon.

Figure 3: Sequences of the intergenic spacer between Cox1 and tRNALEU.



```
NC014672_Solenopsisinvicta.trnal (1-68) Length: 68 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 29.6
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGATTAGTGCAATGGATTTAAATTCCTTAAATAAAGAAACCAACTCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>.>>>.......<<<.<....>>>>>.......<<<<<<<<<<<<.

NC2030541_Wasmanniaauropunctata.trnal (1-65)    Length: 65 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 31.5
         *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGAAAAGTGCAATGAACTTAAACTTCATATATAAAGTTTAATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>......<<<<<<<<<<<.

BK012272_Mycetophylaxsimplex.trnal (1-67)       Length: 67 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 27.4
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGACTAGTGCTTAGGATTTAAACTCCTATTATAAAGATaTTTATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>>......<<<<<<<<<<<<.

BK012135_Trachymyrmexarizonensis.trnal (1-66)   Length: 66 bp
Type: Leu       Anticodon: TAA at 29-31 (29-31) Score: 20.3
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGATAGTGCCTTGGATTTAAATCCCAATTATAAAGATTATTATCTTTTATTAAAA
Str: >>>>>>>..>>>.....<<<.>>>>.........<<<<...>>>>>......<<<<<<<<<<<<.
```

```
BK012227_Amoimyrmexstriatus.trnal (1-71)        Length: 71 bp
Type: Leu       Anticodon: TAA at 31-33 (31-33) Score: 19.0
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGATAAAGTGCATTGGACTTAAACTCCACTCATAAAGATTTTTTtaTTCTTTTATTAAAA
Str: >>>>>>>..>>>.......<<<..>>>>.......<<<<.....>>>>>.........<<<<<<<<<<<<.

BK012227_2Amoimyrmexstriatus.trnal (1-71)       Length: 71 bp
Type: Leu       Anticodon: TAA at 31-33 (31-33) Score: 19.0
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGATAAAGTGCATTGGACTTAAACTCCACTCATAAAGATTTTTTtaTTCTTTTATTAAAA
Str: >>>>>>>..>>>.......<<<..>>>>.......<<<<.....>>>>>.........<<<<<<<<<<<<.
```

```
BK012716_Attatexana.trnal (1-67)        Length: 67 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 33.3
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGAATAGTGCATTGAATTTAAACTTCAATTATAAAGATTCTTATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>>......<<<<<<<<<<<<.

EU156273_Attacephalotes.trnal (1-70)    Length: 70 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 28.4
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGAATAGTGCATTGAACTTAAACTTCAATTATAAAGATTCTCTtATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>>.........<<<<<<<<<<<<.

AF016019_Attasexdenssexdens.trnal (1-68)        Length: 68 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 24.9
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGAATAGTGCATTGAATTTAAACTTCAATAAAAAAGACCATATATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>>.......<<<<<<<<<<<<.

EU165430_Attamexicana.trnal (1-67)      Length: 67 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 26.5
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGAATAGTGCATTGAATTTAAATTTCAATCATAAAGATTTTTATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>>......<<<<<<<<<<<<.

EU165431_Attainsularis.trnal (1-67)     Length: 67 bp
Type: Leu       Anticodon: TAA at 30-32 (30-32) Score: 33.3
         *    |    *    |    *    |    *    |    *    |    *    |    *
Seq: TTTAATATGGCAGAATAGTGCATTGAATTTAAACTTCAATTATAAAGATTCTTATCTTTTATTAAAA
Str: >>>>>>>..>>>......<<<.>>>>>.......<<<<<...>>>>>......<<<<<<<<<<<<.
```

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BK012312_Acromyrmexheyeri.trnal (1-74)  Length: 74 bp

Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 18.2
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGATTATAAGTGCATTGGATTTAAACTCCACTCATAAAGATTTTAActaATCTTTTATTAAAA
Str: >>>>>>>..>>>.........<<<..>>>>.......<<<<.....>>>>>..........<<<<<<<<<<<<.

BK012808_Acromyrmexoctospinosus.trnal (1-72)    Length: 72 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 26.9
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACTTTAAGTGCCTTGGATTTAAACTCCAATTATAAAGATTTAACaATCTTTTATTAAAT
Str: >>>>>>>..>>>.........<<<.>>>>>.......<<<<<....>>>>>........<<<<<<<<<<<<.

BK012482_Acromyrmexversicolor.trnal (1-73)      Length: 73 bp
Type: Leu       Anticodon: TAA at 32-34 (32-34) Score: 16.6
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACTCAAGTGCATTGGATTTAAATCCCAATCATAAAGATtTCTATcaATCTTTTATTAAAA
Str: >>>>>>>..>>>........<<<.>>>>.........<<<<...>>>>>..........<<<<<<<<<<<<.

AY265963_Acromyrmexechinatior.trnal (1-75)      Length: 75 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 20.1
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACTTTTAGTGCCTTGGACTTAAACTCCAATCATAAAGATTTTAAtataATCTTTTATTAAAT
Str: >>>>>>>..>>>.........<<<.>>>>>.......<<<<<....>>>>>...........<<<<<<<<<<<<.

AY265964_Acromyrmexbalzani.trnal (1-74) Length: 74 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 17.9
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGATTTAAGGTGCACTGGATTTAAACTCCACTAATAAAGATaatTTCATTTCTTTTATTAAAA
Str: >>>>>>>..>>>.........<<<..>>>>.......<<<<.....>>>>>..........<<<<<<<<<<<<.

AY265965_Acromyrmexoctospinosus.trnal (1-72)    Length: 72 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 26.9
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACTTTAAGTGCCTTGGATTTAAACTCCAATTATAAAGATTTAACaATCTTTTATTAAAT
Str: >>>>>>>..>>>.........<<<.>>>>>.......<<<<<....>>>>>........<<<<<<<<<<<<.

AY265966_Acromyrmexinsinuator.trnal (1-75)      Length: 75 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 15.4
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACTTTAAGTGCCTTGGACTTAAACTCCAATCATAAAGATcatTCTATaATCTTTTATTAAAT
Str: >>>>>>>..>>>.........<<<.>>>>>.......<<<<<....>>>>>...........<<<<<<<<<<<<.

AY265967_Acromyrmexlundi.trnal (1-74)   Length: 74 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 17.9
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGATTCTAAGTGCATTGGATTTAAACTCCACTCATAAAGATTTTAActaATCTTTTATTAAAA
Str: >>>>>>>..>>>.........<<<..>>>>.......<<<<.....>>>>>..........<<<<<<<<<<<<.

AY265969_Acromyrmexcoronatus.trnal (1-74)       Length: 74 bp
Type: Leu       Anticodon: TAA at 32-34 (32-34) Score: 15.7
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACTCAAGTGCATTGGACTTAAACTCCACTCATAAAGAtcTTCAACTaaTCTTTTATTAAAT
Str: >>>>>>>..>>>........<<<..>>>>.......<<<<.....>>>>>...........<<<<<<<<<<<<.

AY265970_Acromyrmexcrassispinus.trnal (1-74)    Length: 74 bp
Type: Leu       Anticodon: TAA at 32-34 (32-34) Score: 15.9
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGACCTAAGTGCACTGGATTTAAACTCCATTCATAAAGAttTTTAATTaaTCTTTTATTAAAA
Str: >>>>>>>..>>>........<<<..>>>>.......<<<<.....>>>>>...........<<<<<<<<<<<<.

AY265971_Acromyrmexheyeri.trnal (1-74)  Length: 74 bp
Type: Leu       Anticodon: TAA at 33-35 (33-35) Score: 18.2
         *    |    *    |    *    |    *    |    *    |    *    |    *    |
Seq: TTTAATATGGCAGATTATAAGTGCATTGGATTTAAACTCCACTCATAAAGATTTTAActaATCTTTTATTAAAA
Str: >>>>>>>..>>>.........<<<..>>>>.......<<<<.....>>>>>..........<<<<<<<<<<<<.
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Figure 4: tRNALEU sequences of leafcutting ants studied. The length and score of the predicted secondary structure are given, as well the position of anticodon.

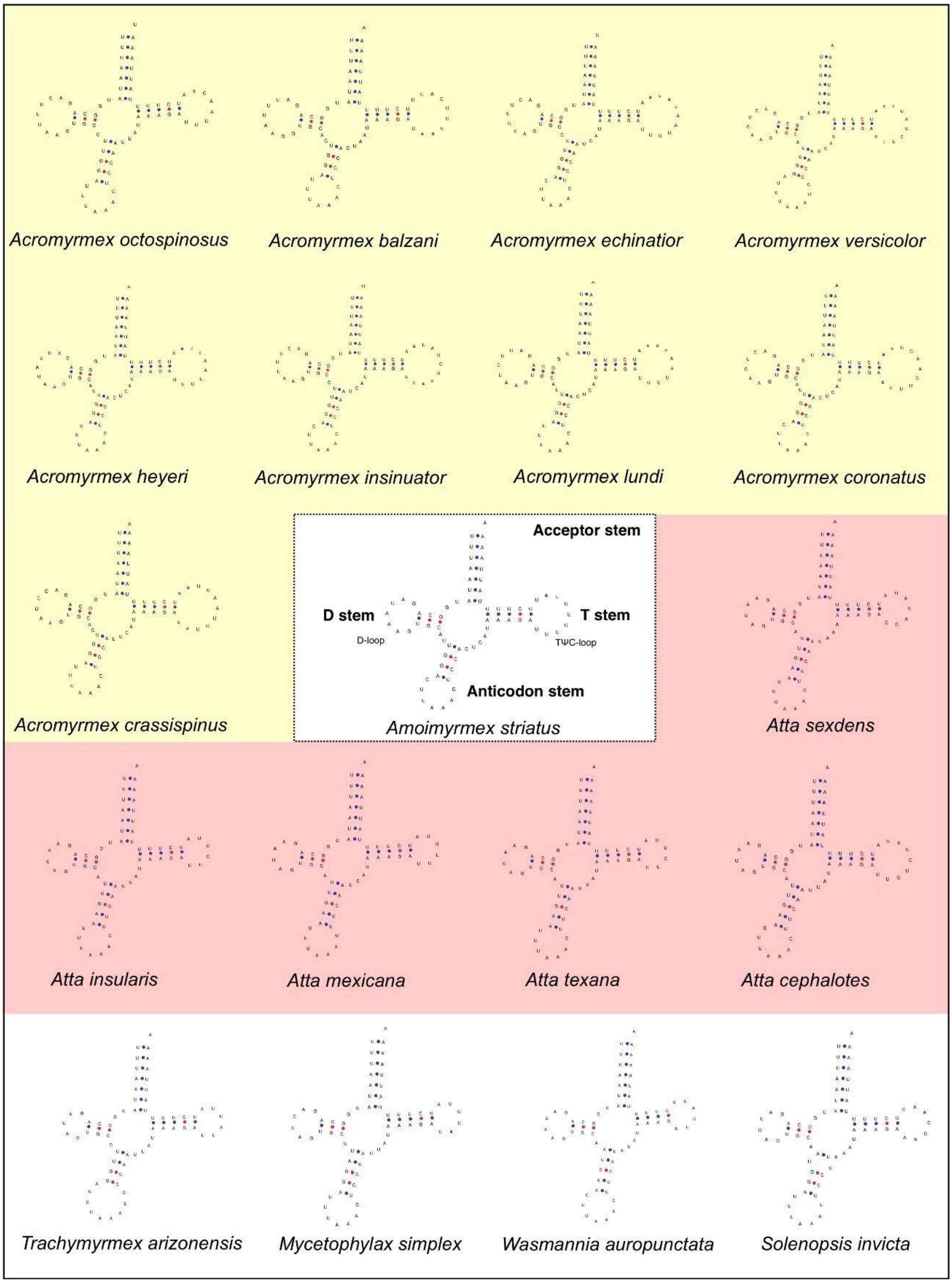

Figure 5: Secondary clover-leaf structure of tRNALEU of leafcutting ants. *Acromyrmex* species representative studied here in shaded yellow and *Atta* species representative studied here in shaded red, and *Amoimyrmex* in the center. The stems and loop names are given.

Discussion

In this study we analyzed the mitochondria genomic segment routinely used in phylogenetic studies that includes the barcode region. We clearly demonstrate that there are molecular traits besides the strictly nucleotide differences shared among congeners that can be used in systematic studies, contribution to the integrative taxonomy. In general, we use DNA sequences matrices underneath an evolutionary substitution model to estimate a phylogenetic hypothesis. However, some structural genomic traits may be significant and subsidiarily used to distinguish species or genera. Here, Cox1-tRNALEU-Cox2 showed shared differences and similarities in their nucleotides sizes and leaf structure that agrees with the most recent taxonomic hypothesis proposed to leafcutting ants. The species from *Atta* share an insertion of three nucleotides just before the stop-codon of Cox1 gene does not present in *Amoimyrmex* and *Acromyrmex*, whereas *Amoimyrmex* Cox2 ends is longer than *Atta* e *Acromyrmex*.

Integrative taxonomy has emerged as a multidisciplinary approach that integrates several fields of study as morphology, cytogenetic, embryology, molecular genetics, etc (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), leafcutting ants were spliced into three genera (Cristiano et al. 2013; Cristiano et al. 2020). Morphological, molecular, cytogenetical and ecological data were taken together to split three species previously placed into *Acromyrmex* into the new *Amoimyrmex* genus (Cristiano et al. 2020).

The AT-biased composition of all genes studied here is expected since it has been known that the mitochondrial genome of insects is AT-rich (Crozier & Crozier, 1993, Simon et al., 1994; Silvestre et al., 2008). Such mitochondrial trait has been largely corroborated by genomic data (Gotzek et al. 2010). Interestingly, *Atta* spp and *Acromyrmex* spp that are sister clades showed the higher values of adenine and thymine percentage compared to theit outgroup.

Likewise, the size of gene spacers is phylogenetic correlated being larger in *Atta* and *Acromyrmex* compared to *Amoimyrmex*. This is interesting because larger mitochondrial genomes have been reported to *Atta* spp. (de Melo-Rodovalho et al. 2014; Barbosa et al., 2019) and the higher number of nucleotides are supposed to the intergenic spacers. Here we found such intergenic spacer between *Cox1-tRNALeu* but no intergenic spacer was found among *tRNALeu-Cox2* suggesting that longer intergenic spacers are not arbitrary distributed across the genome.

We found slightly differences in tRNA$_{\text{LEU}}$ structure across leafcutting ants, such as in nucleotide sequence size that in turn reflect in the estimated clover-leaf structure. There is variation in the size of D-loop and TYC-loop in what species?. The acceptor stem is preserved, and no variation was found in the variable loop, as present in other ants like in *Camponotus atrox* whose bears very long V looping (Kim et al. 2016). As expected, anticodon loop is preserved but the stem in shorter in *Amoimyrmex* and some *Acromyrmex* species whereas always long in *Atta* species. Such variation seems do not affect function since are shared among some leafcutting ants. All these small differences from 1 to 8 nucleotides in stem and loops justify the variation in number of nucleotide sites across leafcutting ant genera, longer in *Acromyrmex*. Variation in cloverleaf arrangement is known (Brennan & Sudaralingam 1976), but still underreported in ants besides their apparently value in evolutionary studies (see Gotzek et al. 2010).

Taking into account the molecular structural traits observed here, all of them corroborate the recent systematic hypothesis by Cristiano et al. (2020) for leafcutting ants. Although they felt into a big clade comprising the three genera, each genera display relevant particularities and traits that justify the splitting of leafcutting ants into three genera. Molecular features of an the mitogenome segments joint to other traits as chromosome number, karyotype features, molecular phylogeny and morphological data to endorse *Amoimyrmex*, *Acromyrmex* and *Atta* (Cristiano et al., 2020).

Conclusions

The extensive use of mitochondrial DNA (mtDNA) in evolutionary and phylogenetic investigations at different taxonomic levels has demonstrated the value of this molecular marker for systematics. The results of the current study demonstrate the critical role that fine-scale structure and nucleotides sequence sizes molecular marker will play in advancing our knowledge of genomic evolutionary processes, phylogenetic relationships among leaf-cutter ants, and comparative taxonomy.

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