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DIVERSITY OF *Wasmannia auropunctata* (HYMENOPTERA: FORMICIDAE) AND THE USE OF MITOCHONDRIAL INTERGENIC SPACER AND LEUCINE tRNA FOR ITS IDENTIFICATION

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ABSTRACT

The invasiveness, reproductive system and control of *Wasmannia auropunctata* (Roger, 1863) have been the focus of many studies. However, few of these have addressed its genetic diversity or offered new techniques for species identification. In this work, we studied the diversity of *W. auropunctata* in two regions of its native range, Brazil and Colombia, and evaluated new identification methods based on a molecular approach. We sampled ants from 31 localities and sequenced a mitochondrial DNA fragment covering the cytochrome c oxidase subunit I (COI) gene, an intergenic spacer (IGS) region, a transfer RNA (tRNA)-encoding gene and the cytochrome c oxidase subunit II (COII) gene. We found high haplotype diversity, and most of the mitotypes in our samples (80%) had not been recorded before. Data analyses and comparisons with published data revealed that the tRNA between the IGS region and the COII gene was similar in all ants of this species, while the IGS region between the COI and the tRNA-encoding genes was variable in length. These findings may contribute for interspecific and intraspecific identification, respectively. Our work demonstrates the importance of more molecular studies of this kind, because they may offer new perspectives on the diversity of this species and the methods used to identify it.

Keywords: Clades; Little Fire Ant; Sequencing

RESUMO

Diversidade de *Wasmannia auropunctata* (Hymenoptera: Formicidae) e utilização do espaçador intergênico mitocondrial e do tRNA-Leucina em sua identificação. Formigas da espécie *Wasmannia auropunctata* (Roger, 1863) tem sido o foco de muitos estudos abordando o seu *status* de invasora, o seu sistema reprodutivo e o seu controle. Porém, poucos estudos têm analisado sua diversidade genética ou estudado novas técnicas para a identificação da espécie. No presente artigo foi estudada a diversidade de *W. auropunctata* em duas de suas regiões nativas, Brasil e Colômbia, testando novos métodos moleculares de identificação. Para tal, foi sequenciado o gene citocromo c oxidase subunidade I (COI), o espaçador intergênico (IGS), o RNA transportador (tRNA) e o gene citocromo c oxidase subunidade II (COII) de operárias de 31 localidades. Os resultados obtidos mostraram que 80% dos mitótipos identificados nunca haviam sido registrados anteriormente e que as amostras apresentaram uma alta diversidade haplotípica. A análise dos dados e comparação com diferentes estudos publicados indicam que o tRNA entre o IGS e o gene COII foi similar para todas as formigas dessa espécie, enquanto o IGS entre o gene COI e o tRNA variou em compri-

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mento. Sendo assim, tais padrões poderiam contribuir para a identificação inter e intraespecífica, respectivamente. O presente trabalho demonstra a importância de mais estudos moleculares na área, uma vez que podem oferecer novas perspectivas sobre a diversidade da espécie, assim como métodos de identificá-la.

Palavras-chave: Clados; Pixixica; Sequenciamento

INTRODUCTION

Wasmannia auropunctata (Roger, 1863), commonly known as little fire ant, is a generalist ant native to the Neotropics that has spread worldwide (Foucaud et al., 2010). When these ants are introduced to new areas, often as a result of human activity, they can cause environmental, economic and health problems. This species is considered invasive and has a negative impact on populations of both vertebrates and invertebrates, including other species of ants (Rabitsch, 2011). Recent studies have focused on its invasiveness (Orivel et al., 2009; Vonshak et al., 2012; Wetterer, 2013), reproductive system (Foucaud et al., 2007; Rey et al., 2013a, b; Miyakawa and Mikheyev, 2015) and control (Hara et al., 2011; Calcaterra et al., 2012; Lee et al., 2015; Montgomery et al., 2015). However, few studies have addressed the genetic diversity of *W. auropunctata* or proposed new identification techniques.

In a population genetics study of *W. auropunctata*, Fournier et al. (2005) isolated and characterized 12 polymorphic microsatellite loci of native and introduced populations of this species. Mikheyev and Mueller (2007) conducted a phylogenetic study, also based on native and introduced populations, using the so-called COI–COII region of the mitochondrial DNA (mtDNA), which includes the cytochrome c oxidase subunit I (COI) gene, the intergenic spacer (IGS) region, a transfer RNA (tRNA)-encoding gene and the cytochrome c oxidase subunit II (COII) gene. They identified two clades, which were separated by one indel in the IGS region. In 2009, Souza et al. (2009) used randomly amplified polymorphic DNA (RAPD) to distinguish between populations of *W. auropunctata* and *Wasmannia rochai* (Forel, 1912) and determine their genetic variability.

The mtDNA COI–COII region has also been used as the main or auxiliary tool in evolutionary studies of several genera of ants (Feldhaar et al., 2003; Janda et al., 2004; Shoemaker et al., 2006; Bacci Jr et al., 2009) and numerous other insects (Blum et al., 2003; Memon et al., 2006; Nakahara and Muraji, 2008; Tatarnic et al., 2013; Shankar et al., 2015; Zanini et al., 2015). According to Hwang and Kim (1999), the use of protein-coding genes at the species or population level is justified by their high rate of substitution in the third codon position. Compared to protein-coding genes, the tRNA-encoding gene is highly conserved, because it is functionally or structurally more constrained, while the *IGS*, a non-coding region, is hypervariable (Simon et al., 1994).

Recently, Chifflet et al. (2016) conducted an in-depth study of the evolutionary history of *W. auropunctata* prior to its global expansion. This study, which used the mitochondrial COI gene, also identified two major clades, A and B, that split around 3.92 million years (mY) ago. Although they used a different fragment of mtDNA, their results corroborated the results of Mikheyev and Mueller (2007), who had identified this pattern for the first time using a COI-COII fragment.

Here, we compare the data from Mikheyev and Mueller (2007) and Chifflet et al. (2016) to 31 samples of *W. auropunctata* from Brazil and Colombia (both part of its native range), using the same mtDNA region as Mikheyev and Mueller (2007). First, we assessed if our samples had the same pattern of IGS indel

and then, if this pattern correlated with the results of Chifflet et al. (2016). To achieve this, we tested if a single individual from ten random samples could be classified as belonging to clade A or B based on IGS length and compared the results to the output based on COI.

Since there is great interest in finding new ways to easily identify an individual to the level of species, we also compared our fragments against fragments from previous studies. Specifically, we examined if the mitochondrial tRNA sequence between COI and COII was the same for all individuals of this species, as observed in other species of ants (Martins et al., 2012; Ramalho et al., 2016). If this is true, sequencing this gene may help distinguish *W. auropunctata* from other species.

MATERIALS AND METHODS

Collection Sites and DNA Extraction, Amplification and Sequencing

Workers were collected between 2013 and 2014 from 31 colonies of *W. auropunctata* in south, southeast and northeast Brazil and Colombia (Table 1 and Figure 1), and the specimens were deposited in the "Coleção de Referência das Formigas do Alto Tietê" (Universidade de Mogi das Cruzes/SP). Each sample included at least two individuals per colony. Samples were stored in 80% ethanol at 2–8 ºC. Collectors identified ants based on morphology, and the identification was verified using Longino and Fernandez (2007). Each extraction consisted of one macerated individual, 250 µL of TNES solution (100 mM Tris, 100 mM NaCl, 50 mM EDTA, 0.5% SDS, pH 9.1) and 1.5 µL of proteinase K (20 mg/mL) incubated in dry bath at 55 ºC for 3 h, followed by protein precipitation with 100 µL of 5 M NaCl and DNA precipitation with two consecutive washes (100% isopropanol followed by 70% ethanol). The DNA was hydrated in 20 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

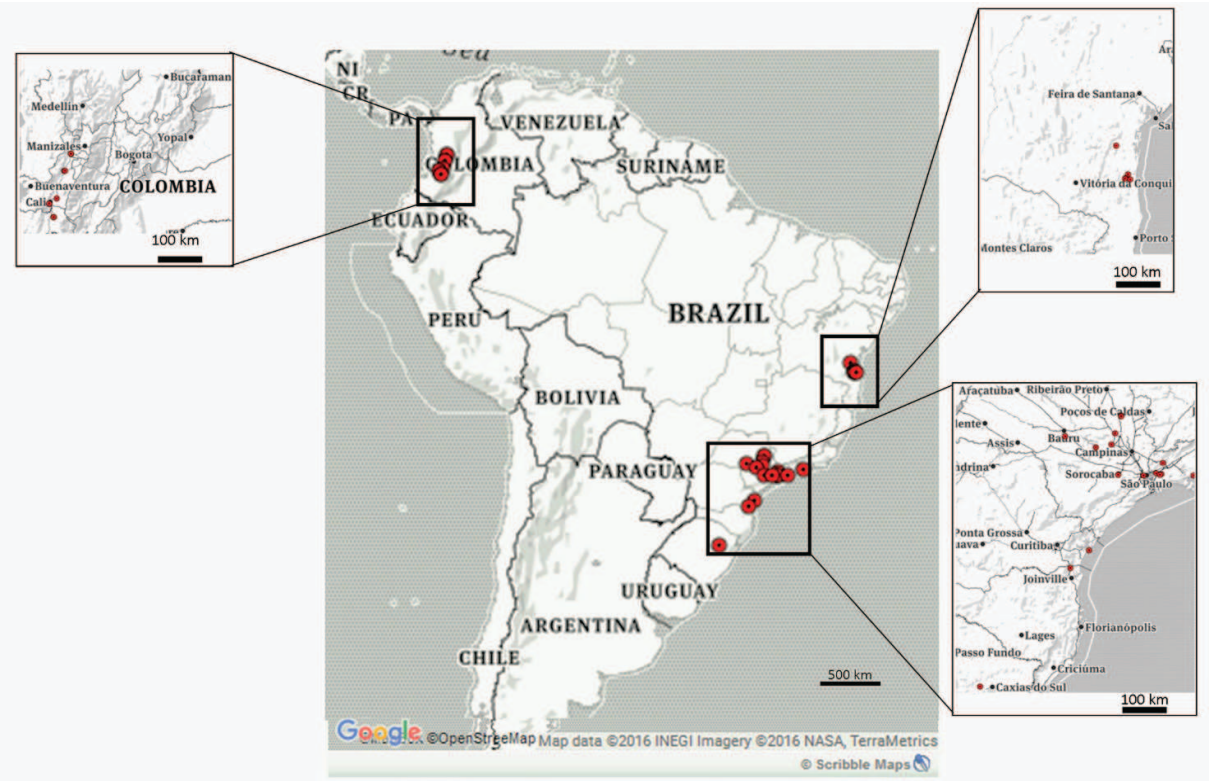

Figure 1. Samples collection sites of *W. auropunctata* used in this work.

Table 1. Coordinates and sampling localities of *W. auropunctata* specimens used in this work. *Geographic coordinates not available.

	Sample	Longitude (°)	Latitude (°)	Locality
BRAZIL	RC	-47.5429790	-22.3926460	Rio Claro, SP
	CE	-47.3718160	-21.9311280	Pirassununga, SP
	An	-48.1256130	-22.7850350	Anhembi, SP
	Ag	-49.0208310	-22.4793500	Agudos, SP
	So	-47.4543100	-23.5160620	Sorocaba, SP
	Pi	-47.6683880	-22.7012730	Piracicaba, SP
	Ub	-45.2323680	-23.5398910	Ubatuba, SP
	LF	-46.2240860	-23.5304500	Mogi das Cruzes, SP
	PEI	-46.1517270	-23.2048556	Igaratá, SP
	IMT	-46.1973470	-23.5118160	Mogi das Cruzes, SP
	PQ	-46.3497417	-23.4802500	Itaquaquecetuba, SP
	PVL	-46.7180778	-23.5476000	São Paulo, SP
	It	-39.2300500	-14.7590100	Itabuna, BA
	Ij	-39.3537900	-14.7120400	Itajuípe, BA
	Ur	-39.2786300	-14.6003000	Uruçuca, BA
	BP	-39.3857000	-14.7350200	Barro Preto, BA
	Ir	-39.6293643	-13.7768125	Itamari, BA
	IM	-43.9777600	-23.0604500	Mangaratiba, RJ
	BG	-51.5177109	-29.1500391	Bento Gonçalves, RS
	Ga	-48.8582190	-26.0347430	Garuva, SC
	Me	-48.3143200	-25.5714880	Paranaguá, PR
COLÔMBIA	Co	-76.5315830	3.3736670	Cali
	51M	-76.0840390	4.3378890	Cali
	Br6	-75.8876550	4.8602610	Pereira
	C1	-76.4016190	2.9852190	Caloto
	Pa	-76.3169190	3.5303890	Palmira
	Mi	-76.5312100	3.3739000	Cali
	A2*			Cali
	N6	-76.079061	4.33605	Cali
	4M	-75.88898	4.860711	Pereira
	28M	-76.39905	2.983319	Caloto

For molecular characterization, we amplified an mtDNA fragment covering the COI gene, IGS, tRNA-encoding gene and COII gene. Two individuals were analyzed per colony. Each PCR tube was filled with 25 µL of reaction mix containing 1.0 mM $MgCl_2$, 0.1 mM F primer, 0.1 mM R primer, 1× GoTaq® Colorless Master Mix (Promega, Madison, WI), 2.5 µL of template and nuclease-free water. The primers used for mitochondrial gene amplification were WaF (5'-TANCAATTCTTATATTCATTATCTGAGAAGC-3´) and WaR

(5'-TTANTATGNATTTCATCTGTAAGGTAAAG-3') (Mikheyev and Mueller, 2007). The thermocycler conditions started with 5 min of initial denaturation at 95 ºC, followed by 35 cycles of denaturation at 95 ºC for 1 min, annealing at 54 ºC for 1.5 min and extension at 72 ºC for 3 min, and ended with 6 min of final extension at 70 ºC.We also amplified an additional fragment of the COI gene from ten random samples for comparison with the results of Chifflet et et al. (2016). In the case of these samples, we replaced the WaF primer with the LEP-F1 primer (5´-ATTCAACCAATCATAAAGATAT-3´) (Hebert et al., 2004) and used an annealing temperature of 48 ºC.

All amplifications were visualized in 0.8% agarose gel. The PCR products were purified with GFX PCR and Gel Band Purification Kit (GE Healthcare), and sequencing was carried out in an automated Genetic Analyzer 3130 (Applied Biosystems) with BigDye Terminator chemistry (Applied Biosystem Inc), using the Sanger method.

Molecular Analyses

Sequences were manually edited in BioEdit Sequence Alignment Editor (Hall, 1999), aligned in the accessory application ClustalW Multiple Alignment (Higgins et al., 1992) and deposited in GenBank with the accession numbers KY33386-KY433416 for the 31 COI-COII sequences and KY433376–KY433385 for the ten sequences used for COI comparison.

Haplotype analyses were run in DNASP Software (Librado and Rozas, 2009), and the network was constructed using the median-joining method (Bandelt et al., 1999) in Network 4.6 (available from www.fluxus-engineering.com). To compare our samples with the results of Chifflet et al. (2016), we aligned our ten COI sequences with samples from their study, and analyzed these data using the neighbor-joining method in MEGA 6 (Tamura et al., 2013). For the tRNA analysis, we used tRNAscan-SE 1.21 (Schattner et al., 2005). Comparisons with pre-existing data were made using BLAST (Basic Local Alignment Search Tool, Altschul et al., 1990) in GenBank (Benson et al., 2013).

RESULTS AND DISCUSSION

Diversity Based on COI-COII Sequences

We obtained 31 sequences with 431 continuous base pairs covering the COI gene, IGS, tRNA-encoding gene and COII gene. The COI fragment had 82 base pairs (bp) and 11 polymorphic sites; nine of these were parsimony informative. The COII fragment was 266-bp long, had 29 polymorphic sites, including 23 parsimony-informative sites (22 with two variants and one with three variants), and a 54-bp conserved region.

Haplotype analysis identified 15 mitotypes (h) (Table 2). Of these, 12 (80%) were considered new. Haplotype diversity (Hd) was 0.9226; see Table 2 for frequency and sampling locations of each mitotype.

Table 2. Mitotypes in decreasing order of frequency. The geographic origin of each sample is shown in parentheses, and the hash symbol indicates newly-recorded mitotypes.

Mitotype (h)	Frequency	Samples
1	7	An and CE (SP), It and Ij (BA), IM (RJ), Mi and 28M (Colombia)
2	4	IMT, PQ, PVL and RC (SP)
3	3	51M, C1 and N6 (Colombia)
4#	3	Br6 and A2 (Colombia), Ir (BA)
5#	2	Ga and Me (South of Brazil)
6#	2	Pi and Ub (SP)
7#	2	Pa and 4M (Colombia)
8#	1	Ag (SP)
9#	1	BP (BA)
10#	1	BG (South of Brazil)
11#	1	LF (SP)
12#	1	PEI (SP)
13#	1	So (SP)
14#	1	Ur (BA)
15#	1	Co (Colombia)

Mitochondrial IGS and Intraspecific Relationships

The IGS region varied in both composition and size. Twenty samples had a 10-bp IGS with no variation in composition, and the remaining 11 samples had an 18-bp IGS that varied in the composition of the last ten base pairs. Figure 2 shows one representative of each IGS variant from this study, and two additional variants retrieved from GenBank.

Network analysis (Figure 3) revealed a clear separation into two groups according to *IGS* length variation. Samples with 18-bp-long *IGS* clustered into Group I, while 10-bp-long samples clustered into Group II. Even within Group I there was an obvious pattern, where samples in subgroup A and subgroup B both had the same *IGS* sequence. This subdivision was also observed when the network analysis included only *IGS* data (not shown) or excluded these data.

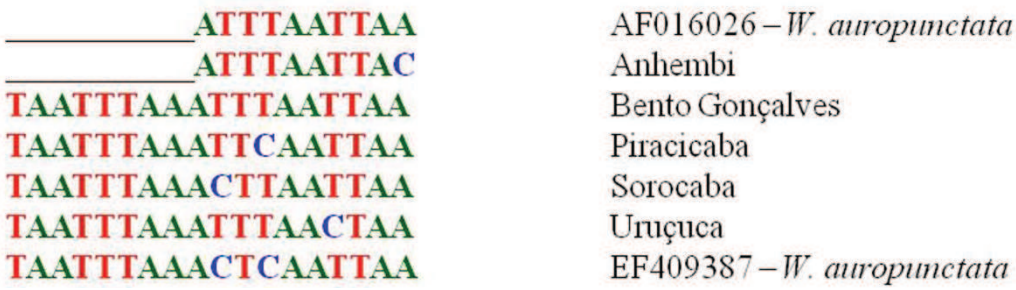

Figure 2. Five *IGS* variants found in this work and two additional variants retrieved from GenBank, listed with their respective accession numbers.

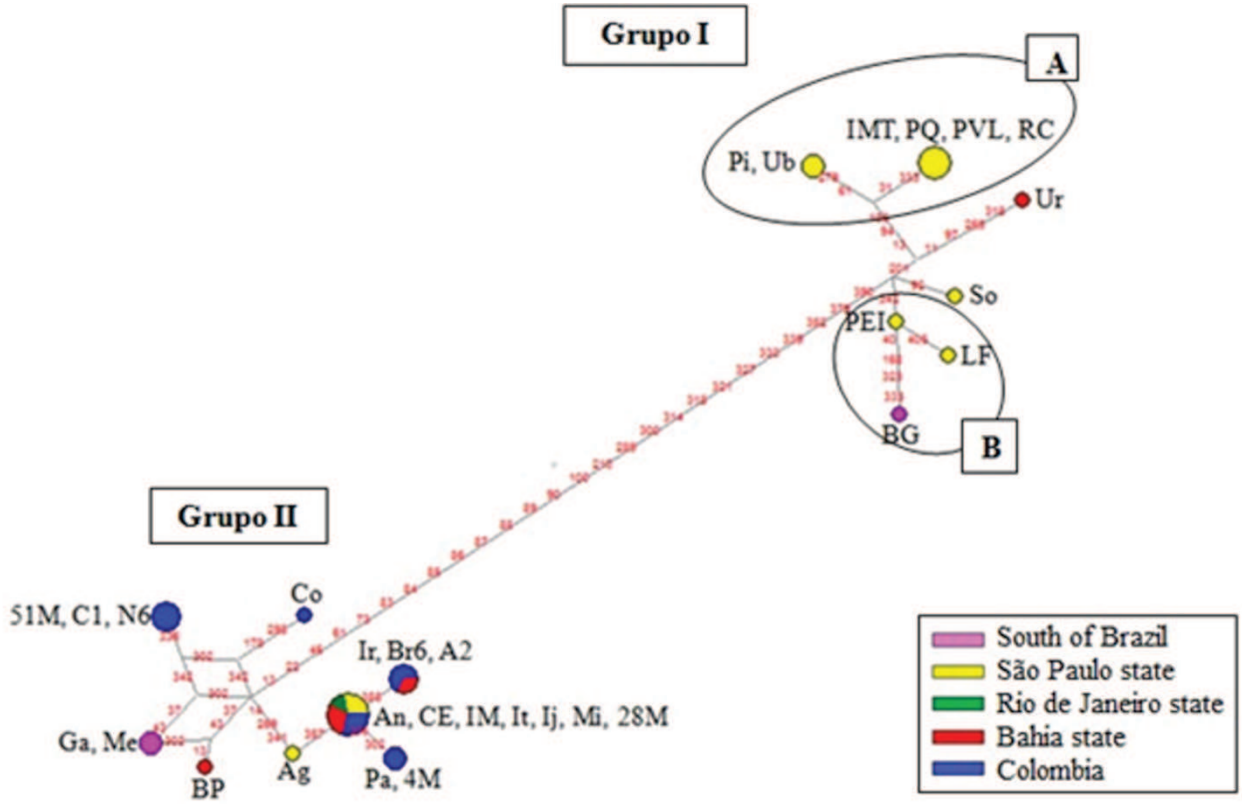

Figure 3. Mitochondrial network of *Wasmannia auropunctata* showing a separation into two clades (Group I and II) and two subclades (A and B) based on *IGS* polymorphism. Colors within circles indicate the geographic origin of each sample (n = 31).

There was no clear geographic pattern, since both groups had samples from São Paulo, Bahia, and south Brazil. However, most samples from São Paulo were in Group I, while Group II was more diverse, including samples from all regions analyzed. In addition, the most frequent mitotype belonged to Group II.

The split into two clades has been observed before. Mikheyev and Mueller (2007), who used the same mitochondrial fragment as we did to study native and introduced little fire ants, found the same pattern of distribution based on variation in IGS length. More recently, Chifflet et al. (2016) analyzed the evolutionary history of these ants based on the COI gene, among others, and also identified two clades.

Length polymorphism of the IGS between COI and COII has been documented before in Hymenoptera. In Attini ants, some authors proposed using this region for phylogenetic analysis, since basal groups seem to have shorter IGS than more derived groups (Wetterer et al., 1998; Krounauer et al., 2004). In fact, Rodovalho et al. (2014) suggested that the IGS may be useful to distinguish between sibling species of this tribe. Similarly, Garnery et al. (1993) and Franck et al. (1998) used IGS polymorphism to successfully differentiate lineages of the honeybee *Apis mellifera* (Linnaeus, 1758).

We found a similar pattern of distribution as Mikheyev and Mueller (2007), who identified two clades differentiated by one indel in the IGS of *W. auropunctata*. Thus, this IGS may be useful to distinguish between different lineages of *W. auropunctata*. The fact that Group II (shorter IGS) is more diverse, includes samples from all regions analyzed and has the most frequent mitotype suggests that populations with longer IGS are more derived. To test this hypothesis, we sequenced a fragment of the COI gene from ten random samples and compared our results with data from Chifflet et al. (2016).

COI comparison

The ten samples used for COI comparison were BP, Ij, It, CE, An, and Ag from Group II (shorter IGS), and RC, Pi, Ub, and So from Group I (longer IGS). Using a neighbor-joining analysis, we compared the COI sequences of these samples with the COI in Chifflet et al. (2016) (Figure 4). All samples from our Group II were grouped into Clade A and all samples from our Group I were grouped into Clade B. Specifically, the RC sample was grouped with haplogroup BVII, Pi and Ub with BVIII, and So with BIII, while BP, Ij, It, CE, An and Ag were grouped in haplogroup AII.

Given that Clade A and Clade B are thought to have originated 1.26 My before present (BP) and 1.22 My BP respectively (Chifflet et al., 2016), our results support the idea that individuals with shorter IGS (10 bp) belong to Clade A and that this clade appeared earlier than Clade B, as individuals with longer IGS (18 bp) belong to Clade B and are more derived.

Mitochondrial tRNA and Interspecific Relationships

The mitochondrial tRNA between the IGS and the COII gene was a 65-bp leucine tRNA with anticodon TAA, and it was the same in all *W. auropunctata* samples analyzed. The GenBank samples of *W. auropunctata,* including representatives from the Mikheyev and Mueller (2007) study in native and introduced areas, also had all the same tRNALeu gene.

A BLASTn analysis revealed no other species with the same tRNA. The closest tRNA sequence in Genbank belonged to a sample of *Cataglyphis cursor* (Fonscolombe, 1846) (Formicidae: Formicinae), which shared only 94% identity. A comparison of the two tRNAs (Figure 5) revealed that *C. cursor* also has a tRNALeu with anticodon TAA. However, the *C. cursor* tRNA is 67-bp long and has two U insertions in the D arm (blue arrows) and two U–A substitutions in the TψC arm (red arrows), for a total of four mutations compared to the *W. auropunctata* tRNALeu.

Higgs et al. (2003) do not consider a single tRNA enough to resolve phylogenies at the species level, but Chiotis et al. (2000), studying Dolichoderinae ants, argued that closer genera tend to have similar tRNAs. Additionally, Martins et al. (2012) and Ramalho et al. (2016) found that the tRNALeu between the IGS and the COII gene was conserved in the ants Linepithema micans (Forel, 1908) and Camponotus textor (Forel, 1899), but there were differences among species of the same genus.Since tRNALeu was unique for all samples of *W. auropunctata* analyzed, as well as for all samples of this species deposited in GenBank, and considering that no other species share the same tRNALeu, sequencing this gene may assist in species identification.

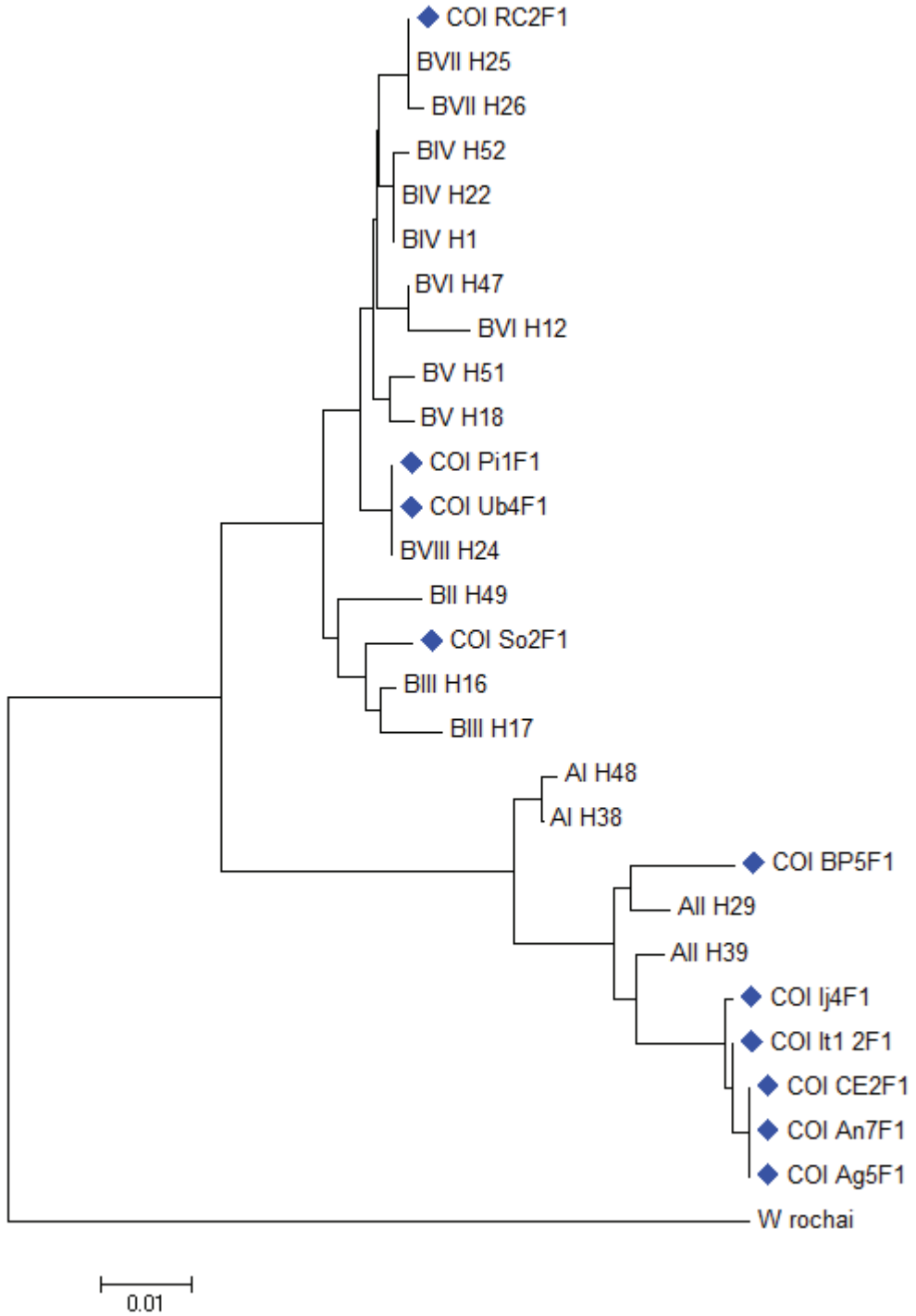

Figure 4. Neighbor-joining tree obtained from MEGA 6. Ten samples from the present work (marked in blue) were analyzed with samples representing different haplogroups from Chifflet et al. (2016). All our samples that grouped within Clade A had a shorter IGS (10 bp), and all our samples grouped in Clade B had a longer IGS (18 bp).

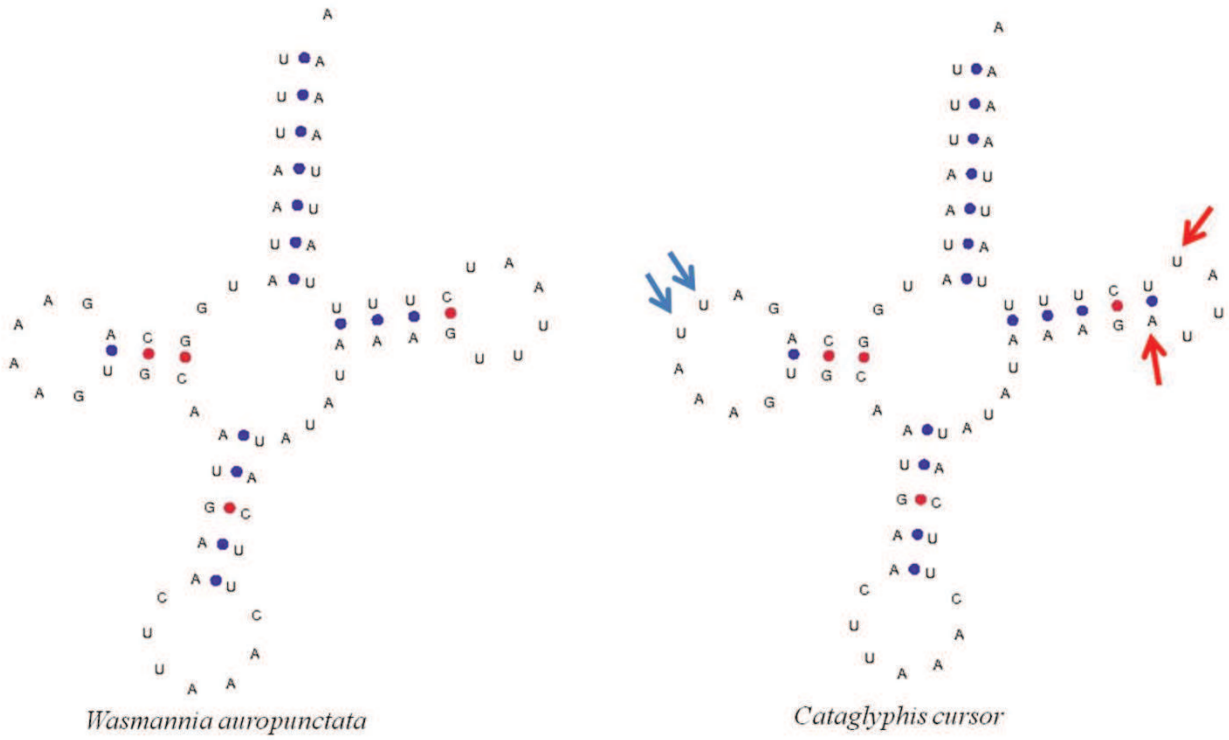

Figure 5. Secondary structure of the tRNALeu of *W. auropunctata* and *Cataglyphis cursor* (GenBank accession DQ105551.1), obtained from tRNAscan-SE1.21. All samples of *W. auropunctata* analyzed for this gene had the same tRNA, which was exclusive to this species. The closest tRNA in GenBank was from *C. cursor*, which shared only 94% identity with *W. auropunctata*. Blue arrows indicate insertions and red arrows indicate substitutions compared with the *W. auropunctata* tRNALeu gene.

CONCLUSION

Our results suggest that sequencing the COI–COII mtDNA region could be helpful both for interspecific and intraspecific identification of *Wasmannia auropunctata*. The tRNA between the IGS region and the COII gene was unique to this species and similar in all ants analyzed, indicating that this could be a good marker for interspecific identification of these ants. Additionally, the IGS region between the COI and the tRNA-encoding genes varied in length, splitting our samples into two clades. Since this distribution matches the results of previous authors, this region could also be a good marker for intraspecific identification of this species, with the shorter IGS as an indicator of earlier lineages. Our work also demonstrates the importance of more molecular studies of this kind. They may offer new perspectives on the diversity of this species, because 80% of the mitotypes identified in this work were new.

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