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New Strains of *Wolbachia* Unveiling the Complexity of This Symbiotic Interaction in *Solenopsis* (Hymenoptera: Formicidae)

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Abstract: Bacteria of the genus *Wolbachia* are widely distributed in arthropods, particularly in ants; nevertheless, it is still little explored with the Multilocus Sequence Typing (MLST) methodology, especially in the genus *Solenopsis*, which includes species native to South America. Ants from this genus have species distributed in a cosmopolitan way with some of them being native to South America. In Brazil, they are widely spread and preferentially associated with areas of human activity. This study aimed to investigate the diversity of *Wolbachia* in ants of the genus *Solenopsis* through the MLST approach and their phylogenetic relationship, including the relationship between mtDNA from the host and the related *Wolbachia* strain. We also tested the geographic correlation between the strains to infer transmission and distributional patterns. Fifteen new strains and eleven previously unknown alleles were obtained by sequencing and analyzing the five genes that make up the MLST. The phylogenetic relationship between the strains showed a polyphyletic pattern, indicative of the complexity of the evolutionary history of these bacteria in the analyzed species. We detected the correlation of host's mitochondrial DNA with *Wolbachia* diversity which imply that related strains exist in related hosts, strongly suggesting the occurrence of vertical transfer. We found no specificity of the *Wolbachia* strain for a given geographic region that could indicate either that there is no horizontal transfer of the strain from the environment for the host or that the human action could be shuffling the distribution of the *Solenopsis* ants and the endosymbiont *Wolbachia*, as well. Our study highlights the complexity and novelty of *Wolbachia* diversity with this specific group of ants and the need for further studies that focus on understanding of this intricate relationship.

Keywords: Multilocus Sequence Typing; endosymbiont; South America; ants

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1. Introduction

Wolbachia (Alphaproteobacteria: Rickettsiales) is an endosymbiont widely distributed in insects, and estimates by Zug and Hammerstein [1] indicate that around 40% of arthropods are infected. Particularly, in ants, this infection rate is around 34% [2], being indicative of the most prevalent heritable symbiont in ants [3]. Its ability to reproductively alter its hosts has made it the subject of many studies in several insect species. However, in ants, its performance in reproductive alterations is not yet widely known, especially because of obstacles in such experimental procedures [2]. Still, few studies have been successful in understanding the effects of *Wolbachia* on Formicidae, with acceleration of the colony life cycle observed in *Monomorium pharaonis* and nutritional supplementation of vitamin B detected in *Tapinoma melanocephalum* in the presence of *Wolbachia* [4,5].

Studies involving *Wolbachia* occurrence in ants are numerous (reviewed by References [2,6,7]), but most of them aimed the analysis of the *wsp* gene (*Wolbachia* Surface

Protein), which, according to Baldo et al. [8], undergoes extensive recombination. Through a more robust and rigorous approach to study the evolution of *Wolbachia*, the MLST (Multilocus Sequence Typing) developed by Baldo et al. [9] had several studies with Formicidae [7,9–14]; however, studies in specific ant genera are still restricted.

The genus *Solenopsis* Westwood, 1840, despite being highly diverse, having about 194 valid species [15], is famous mainly for the species *S. saevissima* and *S. invicta* known as fire ants, highly distributed in South America, where they are considered native [16]. Several studies have already been carried out examining the distribution and prevalence of *Wolbachia* infections using the *wsp* methodology in native populations of the species *Solenopsis invicta*, Buren, and other species of the genus, as well as the effects of *Wolbachia* in the variation of mitochondrial DNA in these species [17–23]. Previous research focusing on *wsp* genes from *Solenopsis* populations from Brazil documented high frequency of *Wolbachia* infection with 51% of the nest infected and several multi-infected nests [20]. In Russell's [2] compilation about publications of PCR screening from *Wolbachia*, he indicated that *Solenopsis* occupies the third position in prevalence of *Wolbachia* in Formicidae, with around 60% of frequency. Despite this high frequency, we still have a very limited view of the phylogeny and diversity of *Wolbachia* in *Solenopsis*, with all the studies in the genus carried out so far based on the *wsp* gene.

Baldo et al. [9] was the first study to characterize strains of *Wolbachia* in *Solenopsis* using the MLST methodology and identified the ST29 in *S. invicta*, although the focus of the study was not on the genus or species. In addition, to our knowledge there is no further investigations regarding *Wolbachia* diversity with a focus exclusively on native *Solenopsis* using MLST approach, which highlights the need to deepen studies with a more precise methodology to reconstruct the evolution and review the diversity of *Wolbachia* strains in *Solenopsis* species. Although genomic approches are more robust and suitable to undertand *Wolbachia* dynamics and the real strain diversity (see References [24–26]), the difficulty of accessing many genomes due to the high costs makes MLST a more accessible method for initial surveys of specific groups of host and larger samples. Furthermore, the number of *Wolbachia* ant genomes are still restricted [26,27], and the MLST has an extensive database for initial comparative analyses.

Given this scenario, the goals of this study were: (a) to investigate the diversity of *Wolbachia* strains associated with the *Solenopsis* ant genus from South America through Multilocus Sequence Typing (MLST) analysis; (b) to test the hypothesis of the relationship between the host's DNAmt and the associated *Wolbachia* strain; and (c) to assess whether there is geographical correlation of the host and strains of *Wolbachia* in order to infer patterns of transmission and distribution. This is the first study that took into account samples of *Solenopsis* in its native area, and the data of this symbiotic interaction are interesting from an evolutionary point of view.

2. Materials and Methods

2.1. Ant Samples and Wolbachia Isolation

One hundred and fourteen nest samples from native populations of *Solenopsis* were used, from which 33 had previously shown presence of *Wolbachia* through the sequencing of the *wsp* gene [20]. Additionally, five samples of *S. invicta* nests previously analyzed by Souza et al. [23] were added to our analysis. It is important to clarify that these samples did not show multiple infections through the previous analysis of the *wsp* gene, since the premise of single infection is necessary for the application of the MLST methodology according to Baldo et al. [9]. The ant sample used were collected directly from the nests and kept in ethanol and frozen until DNA extraction that was done using the whole ant body (samples from Reference [20] were pooled with five individuals per nest). For the *wsp* gene amplification, we used the control primer EF1α-532F (5′-AGGCAAATGTCTTATTGAAG-3′) and EF1α-610R (5′-GCGGGTGCGAAGGTAACAAC-3′) [22] and wsp81F (5′-TGGTCCATTAAGTGATGAAGAAAC-3′) and wsp691R (5′-AAA AATTAAACGCTACTCCA-3′) [28]. The first two sets amplify a fragment of 400 bp of the

nuclear gene EF1α (elongation factor), used as a control for the reaction, and the presence of its product and the absence of *wsp* fragment most likely reflects the absence of the bacteria [22]. In cases with absence of EF1α and *wsp* gene fragments, the genomic DNA was diluted, and the PCR protocol repeated.

Lastly, these 38 nest samples were analyzed for the strain of the bacterium present using the MLST methodology following the standard protocol of Baldo et al. [9], where five genes of MLST approach were amplified and sequenced (*coxA*, *gatB*, *hcpA*, *ftsZ*, and *fbpA*). Table 1 details the collection locations and species. The morphological identification of ants was based on Pitts [29] and Trager [30].

Table 1. *Wolbachia* associated with *Solenopsis* analyzed with the respective nest collection codes, geographic coordinates, and definition of the *wsp* for each sample, as well as the COI, Sequence type (ST), and the corresponding alleles. * indicates new alleles or STs found in the present study. *wsp*: *Wolbachia* surface protein gene; HVR: hypervariable region of the *wsp* gene.id: identification provided for each submission. ST: sequence type (equivalent to a strain or a haplotype). COI: cytochrome oxidase I. *gatB*: Glutamyl-tRNA(Gln) amidotransferase, subunit B. *coxA*: Cytochrome coxidase, subunit I. *hcpA*: Conserved hypothetical protein. *ftsZ*: Cell division protein. *fbpA*: Fructose-bisphosphate aldolase. na = does not apply. p = partial definition of *wsp*. For more details on location, see Figure 2.

Ant Species and Collection Code	Location	Geographic Coordinates	*wsp*	HVR1	HVR2	HVR3	HVR4	Id	ST	COI	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
S. saevissima E1714	Buritizeiro, MG, Brazil	S17°5′20″ W44°56′54″	28	21	21	25	21	516	314 *	JN808797	75	184 *	45	37	46
S. saevissima E1821	Manaus, AM, Brazil	S03°06′25″ W60°01′34″	28	21	21	25	21	518	316 *	JN808830	75	20	45	17	252 *
S. saevissima E1792	S. Cristovão do Sul, SC, Brazil	S27°15′32″ W50°26′50″	p	42	43	9		528	319 *	JN808815	19	20	207 *	43	253 *
S. saevissima E1738	Rio de Janeiro, RJ, Brazil	S22°58′51″ W43°16′75″	28	21	21	25	21	533	29	JN808818	19	20	22	17	20
S. saevissima E1740	Rio de Janeiro, RJ, Brazil	S22°58′51″ W43°16′75″	28	21	21	25	21	534	29	JN808818	19	20	22	17	20
S. saevissima E1742	São Paulo, SP, Brazil	S23°32′53″ W46°38′11″	50	42	43	9	269	540	320 *	JN808781	19	20	207 *	37	253 *
S. saevissima E1743	Ubatuba, SP, Brazil	S23°30′21″ W45°07′55″	50	42	43	9	269	541	320 *	JN808781	19	20	207 *	37	253 *
S. saevissima E1751	Ubatuba, SP, Brazil	S23°30′21″ W45°07′55″	p	42	43	9		543	321 *	JN808807	19	20	208 *	43	253 *
S. saevissima E1713	Buritizeiro, MG, Brazil	S17°25′20″ W44°56′54″	28	21	21	25	21	529	323 *	JN808797	75	20	45	17	46
S. saevissima E1746	Ubatuba, SP, Brazil	S23°30′21″ W45°07′55″	28	21	21	25	21	542	29	JN808805	19	20	22	17	20
S. saevissima E1753	Ubatuba, SP, Brazil	S23°30′21″ W45°07′55″	28	21	21	25	21	544	29	JN808808	19	20	22	17	20
S. megergates E1782	São Francisco, SC, Brazil	S26°33′53″ W48°43′10″	p	42	43	198		525	315 *	JN808826	196 *	20	45	37	251 *
S. megergates E1643	Caçador, SC, Brazil	S26°46′32″ W51°00′56″	59	21	40	42	39	538	315 *	JN808826	196 *	20	45	37	251 *
S. megergates E1644	Caçador, SC, Brazil	S26°46′32″ W51°00′56″	59	21	40	42	39	539	315 *	JN808826	196 *	20	45	37	251 *
S. invicta E1805	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	515	29	JN808817	19	20	22	17	20
S. invicta E1686	Picinguaba, SP, Brazil	S23°19′02″ W44°54′04″	59	21	40	42	39	517	315 *	JN808784	196 *	20	45	37	251 *
S. invicta E1801	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	519	29	JN808817	19	20	22	17	20
S. invicta E1802	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	520	29	JN808817	19	20	22	17	20
S. invicta E1803	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	521	29	JN808817	19	20	22	17	20
S. invicta E1807	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	522	29	JN808817	19	20	22	17	20
S. invicta E1808	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	523	317 *	JN808817	19	20	22	37	20
S. invicta E1810	Corrientes, Argentina	S27°18′39″ W58°33′44″	28	21	21	25	21	524	29	JN808817	19	20	22	17	20
S. invicta E1784	Lages, SC, Brazil	S27°48′57″ W50°22′17″	505	42	43	198	269	526	318 *	JN808819	19	20	55	37	46

Table 1. *Cont.*

Ant Species and Collection Code	Location	Geographic Coordinates	*wsp*	HVR1	HVR2	HVR3	HVR4	Id	ST	COI	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
S. invicta E1789	Pinto Bandeira, RS, Brazil	S29°07′21″ W51°26′56″	p	42	43	198		527	322 *	JN808814	19	20	55	160*	46
S. invicta E1749	Ubatuba, SP, Brazil	S23°30′21″ W45°07′55″	505	42	43	198	269	537	318 *	JN808783	19	20	55	37	46
S. invicta E1645_1	Caçador, SC, Brazil	S26°46′32″ W51°00′56″	59	21	40	42	39	535	324 *	JN808837	196 *	183 *	45	17	251 *
S. invicta E1645_2	Caçador, SC, Brazil	S26°46′32″ W51°00′56″	59	21	40	42	39	536	325 *	JN808837	196 *	183 *	45	17	254 *
S. invicta Sol128	Campinas, SP, Brazil	S22°49′21″ W47°03′42″	p	42	43	198		546	327 *	KJ690243	19	20	210 *	160 *	46
S. invicta Sol106	Rio Claro, SP, Brazil	S22°23′50″ W47°32′56″	p	42	43	198		547	328 *	KJ690242	3	20	210 *	37	46
S. invicta Sol158	Salesópolis, SP, Brazil	S23°32′00″ W45°50′55″	p	42	43	198		548	326 *	KJ690244	3	20	210 *	160 *	46
S. invicta Sol71	Cachoeira de Minas, MG, Brazil	S22°23′49″ W45°50′55″	p	42	43	198		545	326 *	KJ690241	3	20	210 *	160 *	46
S. invicta E1725	Porto Alegre, RS, Brazil	S29°59′14″ W51°09′580″	505	42	43	198	269	530	na	JN808802	19	20	55		46
S. invicta E1726	Porto Alegre, RS, Brazil	S29°59′14″ W51°09′580″	505	42	43	198	269	531	na	JN808802	19	20	55		46
S. invicta E1727	Porto Alegre, RS, Brazil	S29°59′14″ W51°09′580″	505	42	43	198	269	532	na	JN808803	19	20	55		46
S. invicta Sol48	Mogi das Cruzes, SP, Brazil	S23°25′19″ W46°05′24″	p	21	40			549	na	KJ690243	3		210 *	37	
S. invicta E1739	Rio de Janeiro, RJ, Brazil	S22°58′51″ W43°16′75″	p	21	40	42		552	na	JN808826	19	20	45		251 *
S. invicta E1646_A	Caçador, SC, Brazil	S26°46′32″ W51°00′56″	59	21	40	42	39	553	na	JN808838		20	45	37	251 *
S. invicta E1646_B	Caçador, SC, Brazil	S26°46′32″ W51°00′56″	59	21	40	42	39	554	na	JN808838		20	45	37	254 *
S. geminata E1818	Manaus, AM, Brazil	S03°06′25″ W60°01′34″	28	21	21	25	21	550	na	JN808828				37	
S. geminata E1822	Manaus, AM, Brazil	S03°06′25″ W60°01′34″	28	21	21	25	21	551	na	JN808832				37	

For MLST amplification, a final reaction volume of 25 µL was used, about 1.5 µL of genomic DNA, 1X buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 µL of each primer with dilution 1 µM, and 0.5U of Taq DNA polymerase (Invitrogen, Waltham, MA USA). The thermal cycler was programmed following the one suggested by the *Wolbachia* MLST Database (http://pubmlst.org/wolbachia/ (accessed on 28 June 2021) [31]: 94 °C for 2 min, 37 cycles of 94 °C for 30 s, specific annealing temperature for each primer combination for 45 s, 72 °C for 1.5 min, and a final cycle of 72 °C for 10 min. The confirmation of the amplifications was visualized on a 1% agarose gel. In some cases, it was necessary to use alternative primers, which are indicated for individuals with double infections (supergroups A and B) by Baldo et al. [9] and described in the *Wolbachia* MLST Database. In cases where the existing primer combinations did not produce positive PCR reactions, the nested PCR was used, with the template DNA being the dilution of the product of the first PCR in the proportion of 1:9.

In cases where the existing primer combinations for the *ftsZ* gene generated as a sequencing product a fragment with a size smaller than necessary for the application of the MLST approach, a new primer combination was designed using Gene Runner Version 3.05 (Hastings Software Inc. Hastings, NY, USA) and NCBI Primer-BLAST tool [32]. The new primer pair designed (ftsZnewF 5′- CATATGCTTTTCATTACAGCAGGAATGGGC—3′, and ftsZnewR 5′- CGCAGCTTCCGCAGCACTAA—3′) amplifies a region of the *ftsZ* gene of about 480 bp with annealing temperature of 60 °C.

2.2. DNA Sequencing

To purify the amplified products the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) was used following the manufacturer's recommendations. The result of the purification was quantified in NanoDrop® 2000 (Thermo Scientific, Waltham, MA, USA). The sequencing reactions were performed with BigDye Terminator chemistry (Applied Biosystem Inc., Waltham, MA, USA) and sequenced in a 3130 Genetic Analyzer automatic sequencer (Applied Biosystem Inc., Waltham, MA, USA).

2.3. Sequence Editing, Allele Identification and Sequence Typing

The sequences obtained from the five MLST genes were initially analyzed separately using the BioEdit program [33], aligned using the ClustalW application [34], and manually edited. Allele identification and sequence typing (the definition of a sequence type or ST by the MLST approach [9] is equivalent to a strain or a haplotype) was performed on the *Wolbachia* MLST Database. In cases where the allele was not registered in the database, the new allele found was deposited according to the rules of the *Wolbachia* MLST Database. The same was done to register new STs. The records for each allele and ST in the *Wolbachia* MLST Database, as well as the access code for each COI (from Martins et al. [35]), are shown in Table 1.

2.4. Phylogenetic Reconstruction and Mantel Test

The MLST concatenated data (2079 bp) generated in the present study, together with data from the *Wolbachia* MLST Database related to Formicidae deposited until the moment of the analysis, were used for partitioned phylogenetic reconstruction. PartitionFinder2 (2.1.1) [36] was used to choose the best model of molecular evolution that returned GTR + G. Bayesian inference was carried out by MrBayes (3.2.6) on the Cipres Portal [37] for phylogenetic reconstruction with the Markov chain Monte Carlo analysis for 1,000,000 generations with sampling every 1000 generations and discarding the first 25% of trees as burnin. ST78 was used as an outgroup because *Opistophthalmus chaperi* (supergroup F) strain shows similarity with strains associated with ants but has not been found associated with ants [38].

Genetic distance analyzes in pairs were performed with the host's COI data and the concatenated data of the five genes of the endosymbiont sequenced in this work using the R software [39] http://www.R-project.org (accessed on 16 October 2017) with APE package [40], using k80 distance method [41]. A geographic distance matrix paired between the samples was calculated with the data obtained by GPS transformed into UTM, and the distance calculated through euclidean distance, using R software with rgdal package [42]. To test the correlation, Mantel test was performed with R software with vegan package [43]. Geographic maps were reconstructed with the geographical coordinates of the collection points through the speciesMapper website [44], and the corresponding strains in each location were indicated on the maps.

3. Results

All samples of *Solenopsis* spp. infected with *Wolbachia* (n = 38) were successfully sequenced in at least one of the MLST genes. Table 1 summarizes the results obtained in addition to other data generated in previous studies (the *wsp* gene and the COI gene) that were added for analysis in the present study [20,23,35].

Of the 38 populations analyzed, 10 showed evidence of multiple infection after analyzing the sequences of MLST (samples E1713, E1725, E1726, E1727, E1739, E1746, E1789 in the *ftsZ* gene, E1645 in the *fbpA* gene, E1646 in the *gatB* gene, and Sol48 in the *coxA* and *fbpA* genes). It is important to note that the previous sequencing of the *wsp* gene [20] did not detect these multiple infections, since the electropherogram peaks were clearly unique to this gene, and these were only detected with the sequencing of the MLST genes, which indicates the ineffectiveness of using a single gene.

Several genes in the samples with multiple infections were successfully sequenced and had their alleles determined, but, in some cases, it was impossible to separate the correct combination of the genes of MLST for each sample. In a single sample, it was possible to detect the two strains because a single allele was variable, and the grouping of the five genes was possible (sample E1645, variation only in the *fbpA* gene, with 60 variable nucleotides, or 14%, corresponding to ST324 and ST325). For sample E1646, it was possible to sequence only four of the five alleles of MLST, and *fbpA* showed two different alleles that were separated using the alternative primers that are indicated for individuals with double infections (supergroups A and B) by Baldo et al. [9] and described in the *Wolbachia* MLST Database. *gatB*, however, was not determined because its sequencing resulted in several double peaks in eletropherogram, and it was not successfully separated with alternative primers. It should be noted that, in the case of sample E1646, even if success was achieved in sequencing the *gatB* genes, the combination of alleles would be undetermined, since there would be variation in more than one allele.

Among these alleles, a total of 11 new ones of MLST genes were found in the analyzed *Solenopsis* samples. An unprecedented allele was found for *gatB* (allele 196), two alleles for *coxA* (183 and 184), *hcpA* three alleles (207, 208 and 210), *fbpA* four alleles (251, 252, 253 and 254), and *ftsZ* one allele (160). Table 1 illustrates these alleles with an asterisk and in which samples they were found. These new alleles identified could be ant-specific because they were not found in groups other than ants.

Incomplete profiles were obtained for samples E1818, E1822 due to low yield in amplification reactions, and in samples E1725, E1726, E1727, Sol48, E1739, and E1646 due to the presence of multiple infection and impossibility of separation, despite the use of alternative primers for multiple infections.

The definitions of the sequence type (STs) through the *Wolbachia* MLST database were performed on 30 samples that had all five *Wolbachia* MLST genes sequenced, and the results are shown in Table 1. The characterizations of the allelic profiles revealed the presence of 16 STs in the samples of this study, of which 15 were new and registered and deposited in the *Wolbachia* MLST Database (ST314 to ST328 indicated with an asterisk in Table 1); ST 29, previously registered by Baldo et al. [9], was frequent in the samples analyzed in the present study. The "id" numbers of the samples studied here are 515 to 554, shown in Table 1.

The phylogenetic inference of the *Wolbachia* associated with *Solenopsis* show that the endosymbionts are distributed in a very diffuse way in the phylogenetic tree clustering with unrelated species and were not grouped in the same ant species and not even as to the geographic location (Figure 1 and Mantel Test results). It is important to highlight that, although most of the tips are grouped in a clade with polytomy, it is evident the *Wolbachia* grouping in New World and Old World Formicidae in the nested clades.

In a deeper analysis regarding the correlation between the host's mitochondrial DNA (COI gene) and *Wolbachia* diversity, our results indicated a significant correlation (Mantel test, $r^2 = 0.4476$, $p = 0.00005$), which suggests that related strains exist in related hosts, a strong indication of the occurrence of vertical transfer. However, there was no significant correlation (Mantel test, $r^2 = 0.02175$, $p = 0.27281$) between the geographic location of the host and the associated *Wolbachia* diversity. Figure 2 illustrates the location of each population and its respective strain.

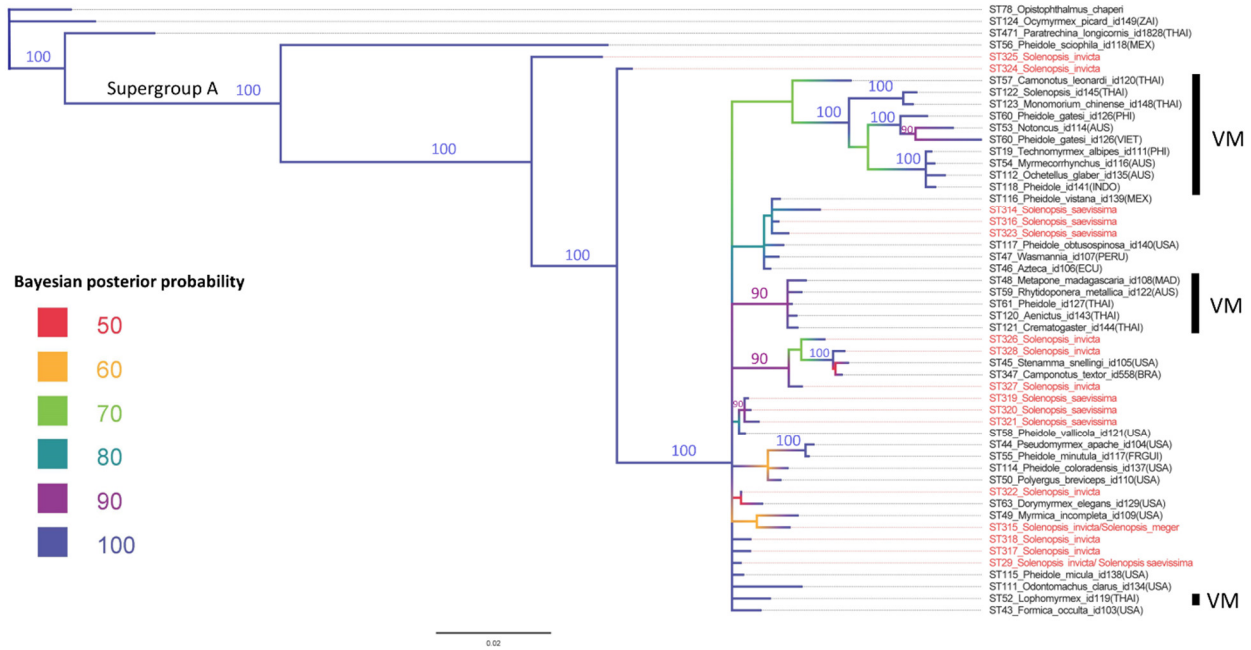

Figure 1. Bayesian inference of strains of *Wolbachia* in Formicidae. Note that most of them belong to supergroup A, with the exceptions of ST124 and ST471, which are classified in supergroup F, ST56 is are classified in supergroup B, and ST78 as outgroup (ST78). The terminals marked in red correspond to the STs detected in the present study. VM = Old World. The subsequent Bayesian posterior probability values are indicated according to the colors of the branches. However, numbers equivalent to high Bayesian posterior probability support (100 and 90) were also added to the nodes. Lower numbers have not been added for ease of viewing.

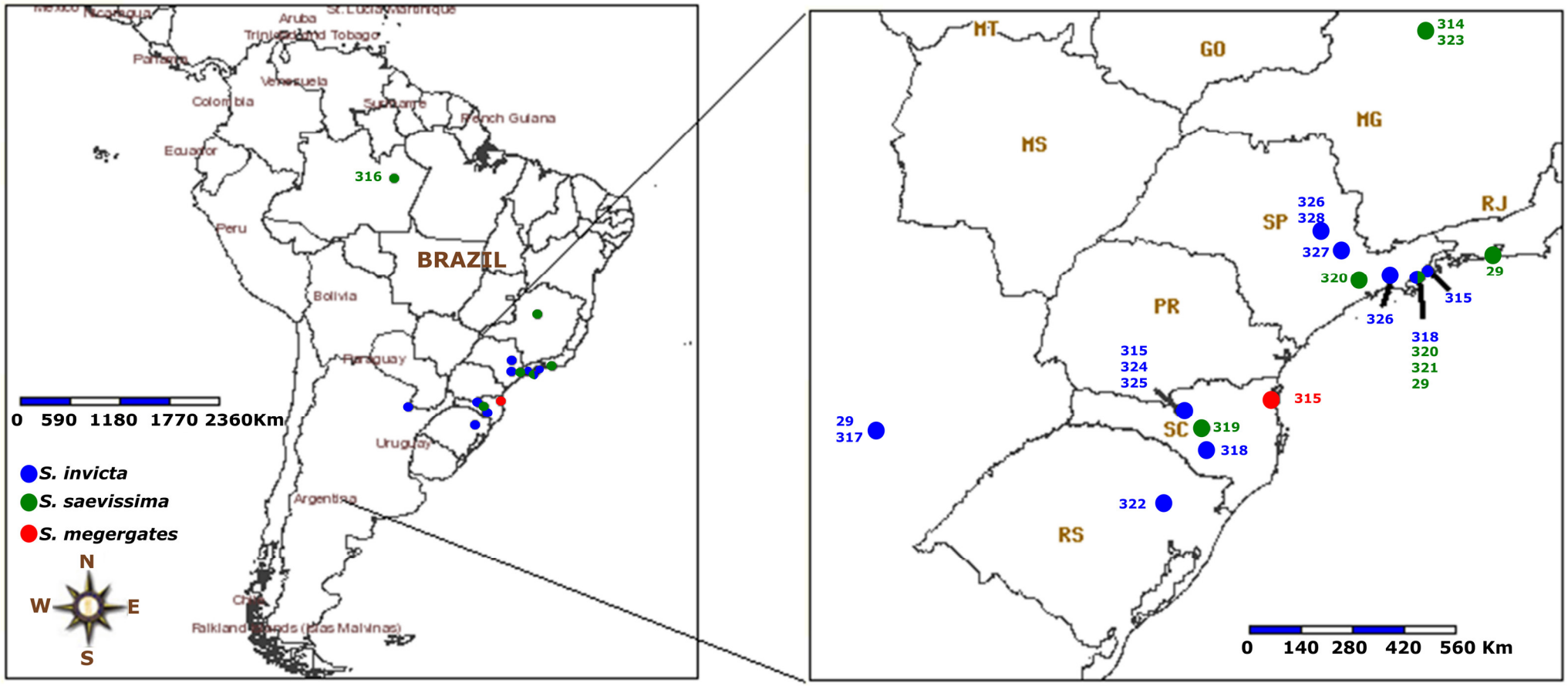

Figure 2. Map illustrating the geographical location of each strain and the associated ant species. The numbers indicate the STs, the coloring is referring to the ant species. Source: speciesMapper [44].

4. Discussion

The present study sought to understand the complexity of the symbiotic interaction between *Solenopsis* species present in their native region and the associated *Wolbachia* diversity. Our data emphasizes the complexity and novelty of these interactions and being able to identify multiple infections in some samples (n = 10), and the detection of 16 strains, 15 of which are new, in addition to 11 new alleles, highlighting the ineffectiveness of the *wsp* to define the diversity of *Wolbachia* strains. The divergence between MLST and *wsp* anlysis was also found by Baldo et al. [45], supporting the conclusion that *wsp* alone is not indicated to predict the diversity of strains or divergence.

Some samples (n = 10) analyzed were determined to be multi-infected by detecting multiple peaks on the electropherogram, making it impossible in most of these samples to define the strains present by the multilocus approach. The exception was one sample in which it was possible to identify two close strains in the same nest. Cases of multiple infections have already been described in other studies with ants, such as *Formica exsecta* [46], *Acromyrmex* [47], *Solenopsis invicta* [20,48], *Solenopsis saevissima* [19], *Solenopsis daguerrei* [18], *Formica rufa* species group [49], *Camponotus* [12,50], *Polyrhachis* [51], and in *Cephalotes atratus* [11,13], therefore being relatively frequent.

Focusing on some specific strains, we must first highlight the presence of strains ST325 and ST324, both identified in *Solenopsis invicta* (Figures 1 and 2). These strains of *Wolbachia* have ancestral characteristics of Supergroup A not only within the host *Solenopsis* but within the family Formicidae [38]. Another strain that deserves further investigation is the strain ST29 that has been observed several times in *Solenopsis* species in the native region (35% of our *S. invicta* samples and 36% of *S. saevissima*), and in several locations (n = 3) (Table 1 and Figure 2). In addition, considering that the ST29 is related to several COI haplotypes (Table 1), this may be an indication that the infection is old and already fixed in the analyzed populations, differing from what was observed by Zhang et al. [52], where two strains related to the same host COI haplotype are indicative of being a recent infection and in the process of spreading. Supporting our hypothesis, it is emphasized that most species of *Solenopsis* are native to the Neotropical region, with South America having a high diversity of species [29], and the southern portion of South America is likely the region of origin of the genus and coincidentally a region where the analyzed nests had, for the most part, infection by the ST29 strain. Therefore, if the origin of the genus is pointed to this region, the origin of the Supergroup A of *Wolbachia* present in *Solenopsis* [38] could indicate association with this region and may have diverged together with the genus. However, this hypothesis needs to be tested, especially with other genes or genome analyzes that would give more support to answer these questions and even more collecting locations and *Solenopsis* species.

Our data also recovered some strains shared by different species (Table 1, Figures 1 and 2), that is, ST29 occurring in *S. invicta* and *S. saevissima*, and ST315 occurring in *S. invicta* and *S. megergates*. This scenario shows the possibility that these strains were acquired vertically in the common ancestor of these species. Results like these highlight the complexity of the symbiotic interactions that *Solenopsis* spp. engages with *Wolbachia* being highly diverse.

The phylogenetic inference of the *Wolbachia* distribution associated with Formicidae indicates the complex evolutionary history of this symbiotic interaction, with special emphasis on the *Solenopsis* STs identified in the present study. The *Wolbachia* associated with Formicidae did not group on the ant species, not even as to the geographic location of the nest (as evidenced in the Mantel Test), and is distributed in a very diffuse way in the phylogenetic tree, forming clusters with unrelated species. This lack of codivergence was already documented in several studies [11,12,14,38].

The lack of geographic correlation between the host and the strain found shown by the Mantel test is an indicative that there was no specificity of the *Wolbachia* strain for a given geographic region, or it may mean that: (1) there is no horizontal transfer of the strain of the environment for the host, or even that (2) the human action in modifying the environment and consequently the distribution of the *Solenopsis* populations shuffles

the distribution of the endosymbiont *Wolbachia*, as well. Absences of correlation of this type have already been found in ants from Thailand [14], *Linepthema micans* from southern Brazil [53], in *Hylyphantes graminicola* spiders [54], and in spiders of the genus *Agelenopsis* and the species *A. aperta* [55]. The increased mobility of the host through human transport could be "mixing" *Solenopsis* colonies, and, consequently, *Wolbachia* strains in non-original geographical locations, that may be the cause of the lack of correlation between *Wolbachia* and the geographic location of populations analyzed. Supporting this hypothesis, there are data that suggest that *Solenopsis* populations are being moved by human activity in Brazil at a much higher frequency than previously suggested [35,56]. Another recent study that approached *Wolbachia* through the MLST methodology focused on the species of *Cephalotes atratus* and found a correlation between the diversity of strains with the geographic location of the host [11]. This could suggest that *Cephalotes atratus*, unlike *Solenopsis* spp., is not being impacted by human transport.

Despite the absence of correlation between geographic locations and strains of *Wolbachia*, in nests with sympatric occurrence, a tendency for similar or relatively close strains of *Wolbachia* to occur was observed (Table 1). Most nests in Corrientes (Argentina) have the same strain (ST29), with only one nest with a different strain (ST317), with variation only in the *ftsZ* gene compared to ST29. The same fact occurred with samples from Rio de Janeiro, RJ that also share the strains (ST29). Likewise, samples from Buritizeiro, MG have strains close to ST323 and ST314, with variation in the *coxA* and *ftsZ* genes. These data support the hypothesis of movement of propagules of the nests and, consequently, of the strains by human activity, as there are nearby nests that have related strains, being an indication that the nearby strains would occur in approximate geographical locations. In addition, the occurrence of related strains occurring in adjacent nests (which also have the same COI) constitutes evidence of vertical transfer of the strains, since related strains are occurring in related COI.

Therefore, it is assumed that the most frequent mode of transmission is the vertical, since *Wolbachia* is transmitted to offspring through the egg cytoplasm [57,58] and also because related hosts have related strains [11,14,55,59]. However, evidence obtained through the *wsp* gene and mitochondrial DNA patterns indicate that, in *S. invicta*, the evolutionary history of *Wolbachia* seems to be more complex, involving multiple invasions or horizontal transmission events [48]. Although horizontal transmission is not a common event in ants and appears to occasionally occur in related hosts [14,59], this complex history of evolutionary history, with both vertical and horizontal transmissions have recently been suggested in other species, using the multilocus approach, as, for example, in Baldo et al. [55]; Frost et al. [10]; Raychoudhury et al. [60]; Watanabe et al. [61]; and Zhang et al. [62], showing the complexity of the *Wolbachia*—host relationship and the need for further studies that lead to an understanding of this complicated relationship.

5. Conclusions

Our results highlight the complexity and novelty of *Wolbachia* diversity with one specific group of ants and unravel an unprecedent variety of 15 new strains detected and eleven previously unknown alleles using MLST approach. We found a polyphyletic pattern which we believe is an indicative of the generalized capacity of *Wolbachia* to infect this ant species. But we also found some related strains associated with related hosts which were indicative of vertical transfer and that the human activities could be shuffling the distribution pattern of the *Solenopsis* ants and the endosymbiont *Wolbachia*, as well. We believe that further studies should focus in specific groups of ants to help elucidate the complex history and evolutionary relationship of *Wolbachia* with ants. Moreover, given the recent studies on *Wolbachia* genome and the low resolution on MLST approach [24,25], new approaches, such as comparative genome studies, should change substantially our knowledge of this complex history and help understand the real strain diversity and the evolutionary dynamics of *Wolbachia* and its host.

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