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Scientific Research

Analysis of the Hypervariable Regions (HVRs) of the *wsp* Gene of *Wolbachia* from *Solenopsis invicta* Ants in Southeastern Brazil

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Abstract

***Wolbachia* is a bacterium that infects many arthropods with horizontal or vertical transmission. The introduction and spreading of *Solenopsis invicta* in new areas may have influenced the acquisition of *Wolbachia* as this ant species spread from its South America origin to other parts of the globe. The *wsp* gene of *Wolbachia* was analyzed using the WSP Typing and a similarity analysis was conducted to analyse the sharing of the symbiont among nests of *S. invicta* ants. The analyses revealed the presence of two groups of *Wolbachia*: strain A belonging to *InvA S. invicta* subgroup, and the strain B belonging to *Acromyrmex insinuator*. The *wsp* gene and its hypervariable regions are shared among the *Wolbachia* present in different types of ants inhabiting in the New World. *Wolbachia* strains found in the nests of *S. invicta* are ant-specialist symbionts which may have spread by several means among the ant population.**

Keywords

Ant, Recombination, Horizontal Transmission, Symbiont

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1. Introduction

Wolbachia is an intracellular bacterium that belongs to the group of α-proteobacteria and infects a large number of hosts such as insects, crustaceans, chelicerates and nematodes. Studies suggest that infection by *Wolbachia* may affect as many as 65% of insect species [1]. Infection by *Wolbachia* can cause various effects in its hosts such as feminization, local adaption, speciation and cytoplasmic incompatibility (CI) [2]. CI has been associated with asynchrony of endomitotic divisions, resulting in nonviable embryos [3].

Wolbachia infections have been divided into eight supergroups—A, B, C, D, E, F, G and H, based on the many phylogenetic analyses using sequences from several genes (16*S rDNA*, *wsp*, *ftsZ*, *groEL*, *gltA* and *dnaA*) [4]. Transmission of *Wolbachia* can occur vertically, via the cytoplasm of the female reproductive cells, or horizontally, due to interactions between different taxa [5] [6].

Wolbachia infection of *S. invicta* populations has occurred six different times via horizontal transmission [7], but there have been multiple infections and independent loses. These researchers also noted that significant variation in the prevalence of the *Wolbachia* infection can be caused by regional limits, environment factors and loss of infection by hosts. The frequency of infection by *Wolbachia* in native populations of *S. invicta* can vary among distinct geographic populations and between nests exhibiting different social forms [8].

The *Wolbachia* variants found in New World ants are similar to each other, but differ from other variants that occur in other insect groups, suggesting that they are specialized to ants [9]. There are differences between the strains found in ants and those found in other insect groups as well as between strains that occur in New World and Old World ants [10].

Recombination between different strains of *Wolbachia* can occur [11], leading to increased variability and promoting the rise of new variants and development of novel phenotypes. These recombination regions, hypervariable regions (HVRs), of the *wsp* gene of *Wolbachia* can undergo recombination within the same strain or between different strains. The HVRs are subject to positive selection, and genetic recombination can play a major role in the host-parasite interactions and in changes in the functional characteristics of the surviving of *Wolbachia*. The WSP Typing can be used for molecular characterization of HVRs and study of recombination in *wsp* of *Wolbachia*.

In this study, we analyzed HVRs of *wsp* gene of *Wolbachia* found in *S. invicta* using WSP Typing to determine the occurrence of recombination among the variants of *Wolbachia* found in ant nests. Phylogenetic and similarity analyses were conducted using data on the *wsp* gene for verification of the horizontal transmission of *Wolbachia* variants.

2. Materials and Methods

2.1. Collection and Preservation

Solenopsis invicta nests (soil and all castes) were collected using methods describe in Banks *et al.* [12], in two different locations (P1 and P2) in each of the cities of Salesópolis, Mogi das Cruzes, São Paulo, Campinas, and Rio Claro, in São Paulo state, Brazil. The ant samples were preserved in 80% ethanol and stored in a −20˚C freezer. The geographic coordinates for each position were recorded (**Table 1**).

2.2. DNA Extraction and mtDNA Sequencing

Ant samples were homogenized in lysis buffer consisting of 100 mM Tris (pH 9.1), 100 mM NaCl, 50 mM EDTA and 0.5% SDS. The homogenized samples were incubated at 55˚C for 3 h and protein residues were precipitated with 5 M NaCl. DNA was precipitated from samples using 100% ethanol, followed by 70% ethanol. DNA was eluted with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). PCR amplification of mtDNA was conducted using methods described by Ahrens *et al.* [13] using primers C1-J-2195 and DDS-COII-4, which amplify a region of approximately 920 bp of the cytochrome oxidase I (COI) gene. Sequencing of samples was conducted using an ABI 3130 Genetic Analyzer (Applied Biosystems™).

2.3. Identification

Ant species identification was conducted based on the cytochrome oxidase I (COI) gene using the Barcode method [14]-[17]. The mtDNA sequences (approximately 920-bp region of the cytochrome oxidase I) were ana-

Table 1. Samples of *Wolbachia* from *S. invicta* from different cities in São Paulo State, Brazil.

Locations	Geographic coordinates	Similar haplotypes of *S. invicta* in GenBank*
Mogi das Cruzes P1	23˚31'07.12"S 46˚10'58.60"W	39
Mogi das Cruzes P2	23˚31'07.84"S 46˚10'58.92"W	40
Rio Claro P1	22˚23'50.18"S 47˚32'56.03"W	Lu-Chuan
Rio Claro P2	22˚23'49.00"S 47˚32'55.51"W	40
São Paulo P1	23˚33'36.69"S 46˚42'47.65"W	47
São Paulo P2	23˚33'37.18"S 46˚42'48.66"W	41
Salesópolis P1	23˚32'00.03"S 45˚50'55.46"W	47
Salesópolis P2	23˚31'46.11"S 45˚51'03.86"W	41
Campinas P2	22˚49'14.01"S 47˚03'37.47"W	47
Campinas P3	22˚49'21.43"S 47˚03'41.78"W	49

*All the haplotypes presented 99% similarity to similar haplotypes of *S. inivcta* in GenBank. The E-value was 0.0 for all sequences.

lyzed with Bioedit software [18] and compared with sequences in the National Center for Biotechnology Information (NCBI) database via BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To ensure reliability in the identification of ants, we used five ants from each nest, and their mtDNA was sequenced and analyzed separately. Ten nests positively identified as *S. invicta* were selected, and their respective values of similarity and E-values were determined (**Table 1**).

2.4. Social Form

The ten *S. invicta* nests were also analyzed to determine their social form (monogyne or polygyne). We used two sets of primers, 26BS and 16BAS; 24bS and 25bAS as describes in Valles and Porter [19], and two other sets of primers (Gp-9.for and Gp-9.rev; all_b.rev and all_b.for) as describes in Ross *et al.* [20]. Five ants from each nest were tested and the analyses were conducted in duplicate.

2.5. *Wolbachia*

Thirty ants from each nest were examined to verify infection by *Wolbachia*. PCR assays were performed using the *wsp*81F and *wsp*691R primers to amplify a region of approximately 590 bp of the *wsp* gene [21]. Sequencing of the samples was carried out with an ABI 3130 Genetic Analyzer (Applied Biosystems™).

2.6. Similarity and Phylogenetic Analyses

The similarities of the sequences were analyzed via Neighbor-joining method using Mega 4.0 software [22]. Branch distances were calculated using Kimura's 2-parameter model and the reliability of the branches was measured via bootstrap analysis (2000 replicates). Bayesian phylogenetic analysis was conducted using MrBayes software [23]. The GTR+G model was selected and the Markov chain was run for 1,000,000 generations sampled every 100 generations. The first 10% of the trees were excluded, and the subsequent probability values were calculated using the remaining trees assuming a mid point rooting [17].

For comparison purposes, we included in the analyses *Wolbachia* sequences previously detected in *S. invicta* and provided by Dr. David Dewayne Shoemaker from the Center for Medical Agricultural and Veterinary Entomology, USDA-ARS, USA. Among these sequences, two were from ants collected in Formosa, two from Roldan, two from Corrientes, and two from Rosario, all in Argentina and two sequences were from *S. invicta* from the USA. Twenty-two additional *Wolbachia* sequences were obtained from NCBI. Furthermore, five sequences were provided by researcher Ms. Cíntia Martins from Universidade Federal do Piauí, PI, Brazil, namely: Registro-SP P1, Registro-SP P2 and Ubatuba-SP P1, in *InvA* subgroup, and Picinguaba-SP P1, Registro-SP P1, in *InvB* subgroup. These sequences were deposited in GenBank together with the sequences obtained in this

study. A phylogenetic tree was generated by Mega 4.0 software and MrBayes software, but there were no significant differences between trees generated by the two softwares.

WSP Typing

For analysis of the HVRs in the *wsp* gene, the WSP Typing (online tool: http://pubmlst.org/*wolbachia*/wsp/info/wsp_typing.shtml) was used and sequences were compared to sequences deposited in the PubMLST *Wolbachia wsp* database (http://pubmlst.org/*wolbachia*/wsp). This online tool verifies the HVRs alleles of the *wsp* gene (obtained in the study) and compares them with the alleles found in the *wsp* gene from other organisms. The results of the analysis can indicate whether the *wsp* gene sequence is the result of recombination among strains. The different alleles found in the HVRs are indicated with numbers in tables.

3. Results

3.1. Mitochondrial DNA and Social Form

We observed only one lineage of mtDNA haplotypes per *S. invicta* nest (**Table 1**). The social form analyses showed that all of the investigated nests exhibited monogyny.

3.2. *Wolbachia*

Only 5 nests out of 10 sampled were infected with *Wolbachia*: 2 nests from Rio Claro, and 1 nest each from Salesópolis, Campinas, and Mogi das Cruzes. Two nests from São Paulo were not infected with the symbiont. The sequences were deposited in the NCBI database under the following accession numbers and names: *Wolbachia*-A-UbatubaSPP1 JQ425780, *Wolbachia*-A-RegistroSPAP1 JQ425781, *Wolbachia*-A-RegistroSPP2 JQ425782, *Wolbachia*-A-Rio-ClaroSPP1 JQ425783, *Wolbachia*-A-Rio-ClaroSPP2 JQ425784, *Wolbachia*-A SalesopolisSPP1 JQ425785, *Wolbachia*-A-CampinasSPP1 JQ425786, *Wolbachia*-B-RegistroSPP1 JQ425787, *Wolbachia*-B-PicinguabaSPP1 JQ425788 and *Wolbachia*-B-MogidasCruzesSPP1 JQ425789.

Wolbachia wsp sequences from ants obtained from nests from São Paulo state and provided by Dr. Shoemaker had 99% identity with samples in Gene Bank. Samples from Salesópolis, Rio Claro and Campinas were similar to group *A*, subgroup *InvA*: specifically wSinvictaA-AF243435.1 of *Wolbachia* from *S. invicta*. Sequence from Mogi das Cruzes was similar to group *B*, subgroup *B*2: specifically *A. insinuator*-AF472560, of *Wolbachia* from *Acromyrmex insinuator*. Four of the sequences provided by Dr. Shoemaker were similar to group *B*, subgroup *InvB*: specifically wSinvictaB-AF243436.1 of *Wolbachia* from *S. invicta* whereas the other four sequences were similar to group *A*, subgroup *InvA*: specifically wSinvictaA-AF243435.1 of *Wolbachia* from *S. invicta*. The three of the sequences obtained from Ms. Cintia Martins were similar to group *A*, subgroup *InvA*, while the other two were similar to group *B*, subgroup *B*2: specifically from *A. insinuator*. None of the sampled nests showed multiple *Wolbachia* infections.

3.3. Phylogenetic and Similarity Analyses

There were no differences between the results of the phylogenetic and similarity analyses of the *wsp* gene of *Wolbachia*. The phylogenetic tree resulting from the similarity analysis of the *wsp* gene of *Wolbachia* (**Figure 1**) revealed no differences among the *Wolbachia* sequences in *InvA* subgroup. Our samples in *InvA* subgroup form a polytomy with all of the sequences provided by other researchers and the sequences obtained from GenBank. The lack of resolution is reflected in the low bootstrapping value for each clade. The *Wolbachia* samples from *Linepithema humile* (from GenBank) were also included within *InvA* subgroup, and the *S. saevissima*—S1 and SS2 (from GenBank), sequences grouped with a sister clade to subgroup *InvA*. The *Wolbachia* sequences from the other *Solenopsis* species (*S. daguerrei* and *S. richteri*) from GenBank form three clades within *Wolbachia* group *A*.

3.4. WSP Typing

Some sequences of *InvA*, *InvB* subgroups and *B* group showed partial values in the analysis, these sequences were not 100% identical to the deposited sequences in the PubMLST *Wolbachia wsp* database, but when the sequences showed partial values for one allele, it was accepted. Sequences that showed partial values for several alleles were not accepted, but the alleles are shown in **Table 2**.

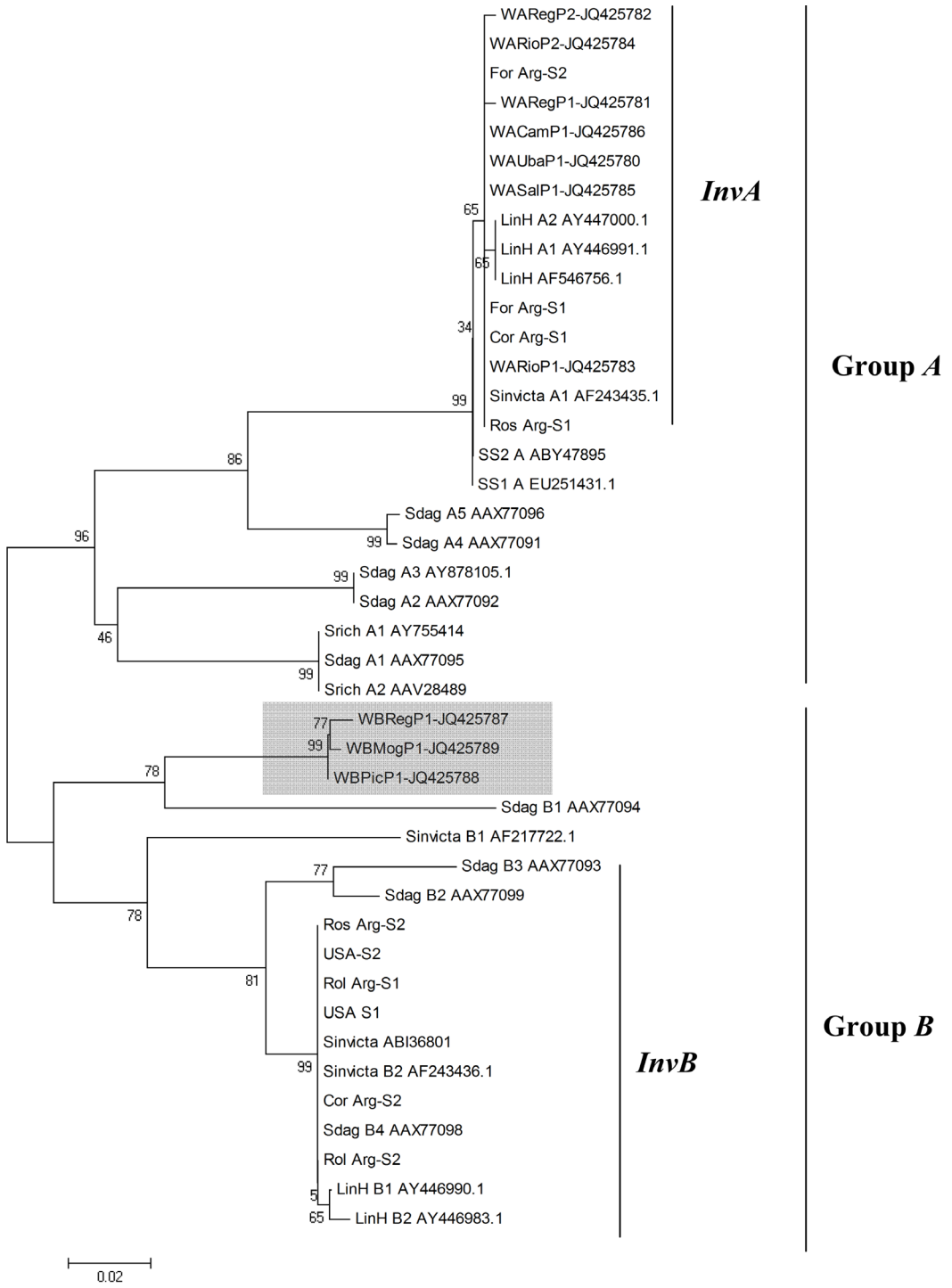

Figure 1. Phylogenetic tree of *wsp* from *Wolbachia* obtained from different populations. The gray box indicates the sequences of the *Wolbachia* of the *A. insinuator* found in nest of the *S. inivicta*. The illustrated tree was generated by software Mega 4.0. The bootstrap values are showed previously of the branches. The sequence names were shortened to the better arrangement in the figure.

The sequences of *InvA* subgroup of *Wolbachia* from *S. invicta* nest collected for this study showed the following alleles: HVR1-allele 42; HVR2-allele 43; HVR3-allele 198; and HVR4-allele 25. The sequences from Argentina shared the same alleles for the HVR1, HVR2 and HVR3 with *S. invicta* nests collected, but showed

Table 2. Alleles (numbers according to PubMLST *Wolbachia wsp* database) present as complete sequences (in orange cells), partial sequences (in gray cells) in shared HVR's of *wsp* gene in the *InvA* and *InvB* subgroup and *B* group of *Wolbachia* from *Solenopsis invicta* samples collected in São Paulo state, Brazil, and other ant samples.

Sequence source/identification*	Alleles				Group or subgroup of *Wolbachia*
	HVR1	HVR2	HVR3	HVR4	
WAUbaP1-JQ425780	42	43	198	25	*InvA*
WARegP1-JQ425781	42	43	198	25	*InvA*
WARegP2-JQ425782	42	43	198	25	*InvA*
WARioP1-JQ425783	42	43	198	25	*InvA*
WARioP2-JQ425784	42	43	198	25	*InvA*
WASalP1-JQ425785	42	43	198	25	*InvA*
WACamP1-JQ425786	42	43	198	25	*InvA*
For_Arg-S1	42	43	198	Unidentified	*InvA*
For_Arg-S2	42	43	198	Unidentified	*InvA*
Ros_Arg-S1	42	43	198	Unidentified	*InvA*
Cor_Arg-S1	42	43	198	Unidentified	*InvA*
Lhum A AY446991.1	42	43	198	Unidentified	*InvA*
Lhum A AY447000.1	42	43	198	Unidentified	*InvA*
Lhum A AF546756	42	43	198	25	*InvA*
Sdag B2 AXX77099	1	21	25	21	*InvB*
Sdag B3 AXX77093	66	21	25	21	*InvB*
Sdag B2 AXX77098	21	21	25	21	*InvB*
Sinv B2 AF243436	21	21	25	21	*InvB*
Sinv ABI36801	21	21	25	21	*InvB*
Lhum B2 AY446983.1	21	21	25	Unidentified	*InvB*
Lhum B1 AY446990.1	21	21	25	Unidentified	*InvB*
Rol_Arg-S1	21	21	25	Unidentified	*InvB*
USA_S1	21	21	25	Unidentified	*InvB*
USA-S2	21	21	25	Unidentified	*InvB*
Rol_Arg-S2	21	21	25	Unidentified	*InvB*
Cor_Arg-S2	21	21	25	Unidentified	*InvB*
Ros_Arg-S2	21	21	25	Unidentified	*InvB*
WBMogP1-JQ425789	21	40	48	Unidentified	*B*
WBRegP1-JQ425787	21	40	42	Unidentified	*B*
WBPicP1-JQ425788	21	40	42	Unidentified	*B*

*The sequence names were shortened to the better arrangement in the table.

partial results for many alleles of the HVR4. Some sequences obtained from GenBank presented partial results for just one allele in the HVRs, so these alleles were accepted. The other sequences had undefined alleles for HVR4 (**Table 2**). Some sequences from *B* group and the other sequences from *InvB* subgroup of the *wsp* gene from *Wolbachia* also showed partial values in the analysis of alleles.

The three *Wolbachia* sequences from *S. daguerrei* (obtained from GenBank) had a different allele within HVR1. The *S. dagB*2 sequence exhibited allele 1, whereas *SdagB*3 sequence had allele 66, and *SdagB*4 sequence had allele 21. All other remaining sequences of the *B* group and *InvB* subgroup of *Wolbachia* had allele 21 in HVR1 including Mogi das Cruzes, Picinguaba and Registro. The sequences of *Wolbachia* from these cities presented the allele 40 for the HVR2 whereas all sequences of the *InvB* subgroup presented allele 21, see **Table 2**. All sequences of the *InB* subgroup presented the allele 25 for the HVR3, and the sequences of the *B* group of *Wolbachia* from Picinguaba and Registro presented the allele 42. The sequence from Mogi das Cruzes presented the allele 48 of the HVR3. Some sequences from the GenBank presented the allele 21 for the HVR4, but for other sequences the alleles could not be defined (**Table 2**).

4. Discussion

Several factors influence *Wolbachia* infection of ant nests including microgeographic variation, environmental factors or intrinsic characteristics of the hosts [7]. We estimated 50% infection rate in the sampled *S. invicta* mounds which is probably due to *Wolbachia*-infected and uninfected *S. invicta* queens invading new areas, generating infected and uninfected nests [7] [8] [24] [25]. A previous study [24], found 10% to 90% prevalence of *Wolbachia* infection in native populations of *S. invicta*. The prevalence observed in this study corroborates former studies in native populations of fire ants.

Sharing of *Wolbachia* in the same subgroup (*InvA*) by different ant species may indicate a high capacity for *Wolbachia* transmission, possibly due to a loss of specialization in the symbiont, resulting in the infection a large group of insect (ants) with similar biological and ecological characteristics. Such a phenomenon would proportionally and numerically increase the local survival of the symbiont, and supports the hypothesis of a symbiont specialized in ants [9]. Because *L. humile* and different species of *Solenopsis* and *Acromyrmex* share the same *Wolbachia* subgroups *InvA* and *InvB* [6] [26], specialization to ants by the symbiont seems entirely possible. *Wolbachia* subgroups *InvA* and *InvB* (**Figure 1**) seem to be specific to New World ants [9].

The mechanism to explain the results in sharing of *Wolbachia* subgroup (*InvA*) among ant species can be horizontal transmission via social parasites [6], or parasitoids, such as the flies *Pseudacteon* genus or wasps of the Diapriidae family [26]. However, other mechanisms such as vertical transmission, loss and reintroduction of infection, populations structure, distinct behaviors and the spreading and radiation of populations [7] may also be important in the phylogenetic grouping we report here for *Wolbachia A* groups.

The high diversity of sympatric species of ants found in South America may have contributed to the generalist adaptation of *Wolbachia*. The energetic expenditure required for a symbiont to develop highly specialized relationship with a single host may be too high and may have serious implications for the survival of the symbiont. The advantage of low host-specialization and consequently a lower energy requirement may increase the local survival of a symbiont, and thus explain the sharing of the same subgroups of *Wolbachia* by different groups of New World ants.

The infection of *S. invicta* by strain *B* of *Wolbachia* from *A. insinuator* may also represent a generalist adaptation of the symbiont. However, other possible explanations such as horizontal transmission, transmission via parasitoids, close relationship between sympatric populations of *A. insinuator* and *S. invicta*, close relationship between *S. daguerrei* (social parasite of *S. invicta*) and *A. echinatior* (social host of *A. insinuator*) may also result in the acquisition and subsequent transmission of symbiont infection. The proximity of sequences for *Wolbachia* samples between *S. daguerrei* B1 and *A. insinuator* as displayed in the phylogenetic tree, may indicate an interaction between these ant species.

The partial sequences observed in the *wsp* gene may be due to punctual mutations in the sequences, or the sequences not being of an appropriate size for the analysis, both of which would interfere with the identity of the alleles. However, the great diversity of the alleles of HVR1 sequences *Wolbachia* from *S. daguerrei* may be due to social parasite behavior of this ant, which can facilitate multiple infections by different *Wolbachia* strains in only a single individual *S. daguerrei* [6], and consequent recombination of HVRs of the *wsp* gene. Baldo *et al.* [11] hypothesized that the recombination of HVRs of the *wsp* gene can produce new phenotypes for the sym-

biont and this can be selectively advantageous. Superinfection may provide genetic material for recombination in the HVRs resulting in new allele variants, which can be disseminated by the social parasitic ant. Sharing of HVR2, HVR3 and HVR4 alleles with sequences belonging to the *InvB* subgroup of *Wolbachia* is likely an indication for the occurrence of horizontal transmission via social parasite.

5. Conclusion

Although an ant-specific *Wolbachia* strain would have a greater chance for survival and establishment in new niches, the increased energetic expenditure required to infect new hosts would be disadvantageous for the bacterium and has potential consequences for its survival. This study sheds new light on the variability of *Wolbachia* HVRs, its role on the occurrence of recombined *Wolbachia* strains naturally found in ants, and potential effects of these new symbiontic relationships on the biology of both the hosts and symbionts.

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