The Evolution and Biogeography of *Wolbachia* in Ants (Hymenoptera: Formicidae)

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**Abstract:** *Wolbachia* bacteria are widely distributed across invertebrate taxa, including ants, but several aspects of this host-associated interaction are still poorly explored, especially with regard to the ancestral state association, origin, and dispersion patterns of this bacterium. Therefore, in this study, we explored the association of *Wolbachia* with Formicidae in an evolutionary context. Our data suggest that supergroup F is the ancestral character state for *Wolbachia* infection in ants, and there is only one transition to supergroup A, and once ants acquired infection with supergroup A, there have been no other strains introduced. Our data also reveal that the origin of *Wolbachia* in ants likely originated in Asia and spread to the Americas, and then back to Asia. Understanding the processes and mechanisms of dispersion of these bacteria in Formicidae is a crucial step to advance the knowledge of this symbiosis and their implications in an evolutionary context.

**Keywords:** endosymbiont; ant; vertical transmission; biogeography; ancestral state reconstruction; phylogeny

1. Introduction

There are many examples of insect–microbe interactions providing benefits to all players involved [1–5], and one of the most well-documented bacteria associated with insects is *Wolbachia* [6,7]. This bacterium is known for modifying the host’s reproduction for its own benefit, including the induction of cytoplasmic incompatibility, parthenogenesis, and male-killing or feminization [8]. However, it is not known whether these functions are related to a particular *Wolbachia* strain or host [9]. As this bacterium has been found in association with hundreds of hosts and encompasses an immense diversity, the classification of strains into supergroups has been proposed and about 17 supergroups, to date, have been reported through genotyping a single gene, several genes, or even genomic approaches [10]. These supergroups are called “A to S”, with G and R no longer considered separate supergroups [11,12]. Of these, supergroups found exclusively in arthropods belong to the A, B, E, H, I, K, M, N, O, P, Q, and S supergroups [13–17], with supergroup F being common for nematodes and arthropods [18,19]. Although we know that *Wolbachia* is associated with several arthropods hosts, we focused on ants, a highly diverse group with more than 13,000 described species and a global distribution [20]. In addition, it is one of the groups of insects best studied in terms of association with *Wolbachia*. This offers us an excellent opportunity to explore the evolution and biogeography of this association.

*Wolbachia* has been identified from several ant genera, but few studies have succeeded in identifying the implications for these ant-associated interactions. *Monomorium pharaonis* appears to have an accelerated colony life cycle [21] and *Tapinoma melanocephalum* has a nutritional upgrade of vitamin B [22] in the presence of *Wolbachia*. However, whether these are specific cases within all ant
diversity is still unknown. Despite the fact that these bacteria have been identified in several ant genera, little is known about the diversity and evolutionary history of this symbiosis; see [23–35].

Historically, studying these interactions was conducted by targeted PCR amplification and sequencing of the *Wolbachia* surface protein (*wsp*) gene [36]. However, the use of this gene alone has been shown to not be appropriate for diversity studies since it has been reported to have a high rate of recombination [36]. Therefore, the Multilocus Sequence Typing (MLST) approach [37] has been shown to be a more reliable alternative, targeting five different *Wolbachia* genes, *coxA*, *fipA*, *ftsZ*, *gatB*, and *hcpA*, instead of just one [38]. These five genes are spread across the *Wolbachia* genome and evolve under purifying selection [38], making them more appropriate for phylogenetic analysis. Once a unique sequence is found with the combination of all of these genes, a sequence type (ST) is assigned and is stored in the *Wolbachia* MLST database (https://pubmlst.org/wolbachia/).

Many studies have reliably used the MLST approach [39–42], including some studies of ants [9,24,28,32,33]. However, no studies, to date, in ants have leveraged ancestral state reconstruction and biogeographic range evolution to understand the origin and biogeography of this symbiosis. As a robust phylogenetic tree is necessary to investigate lifestyle transitions in *Wolbachia*’s evolutionary history, this study aimed to (1) infer the evolutionary history of *Wolbachia* associated with Formicidae through the MLST approach (five genes), controlling for their different evolutionary models; (2) reconstruct the ancestral state of the different *Wolbachia* superfroups; (3) investigate the dispersion patterns of these bacteria associated with Formicidae through biogeographic range evolution analyses.

2. Material and Methods

2.1. Phylogenetic Reconstruction

*Wolbachia* sequences isolated from 70 Formicidae hosts (2079 bp from the five genes) were downloaded from the *Wolbachia* MLST database (https://pubmlst.org/wolbachia/), as well as metadata information. ST78 from *Opistophthalmus chaperi* (supergroup F) was added as an outgroup because it has not been found to be associated with ants but shows high similarity with strains associated with ants (Table 1). The sequences were aligned using ClustalW [43] of the BioEdit software [44] and later were used for partitioned phylogenetic reconstruction. PartitionFinder2 (2.1.1) [45] was used to choose the best model of molecular evolution and returned four partitions: charset Subset1 = 1–402 pb; charset Subset2 = 403–831 pb; charset Subset3 = 832–1266 pb and 1636–2079 pb; charset Subset4 = 1267–1635 pb, with GTR + G for Subset1, GTR + I + G for Subset2, GTR + I + G for Subset3, and TIM + I + G for Subset4. Bayesian inference was implemented using MrBayes (3.2.6) [46] on the Cipres Science Gateway [47,48] 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. We used the chronos function with the correlated model available in the Ape package [49] with R software [50] to reconstruct a chronogram with a relative time scale [51–53].

| id | Supergroup | Host Genus | Host Species | Locality | Submitter | Sequence Type (ST) |
|----|------------|------------|--------------|----------|-----------|-------------------|
| 2  | A          | *Solenopsis* | *invicta*    | Argentina | Laura Baldo | 29                |
| 4  | A          | *Camponotus* | *pennsylvanicus* | USA      | Laura Baldo | 33                |
| 103| A          | *Formica*   | *occulta*    | USA      | Jacob Russell | 43               |
| 104| A          | *Pseudomyrmex* | *apache*    | USA      | Jacob Russell | 44               |
| 105| A          | *Stenamma*  | *snellingi*  | USA      | Jacob Russell | 45               |
| 106| A          | *Azteca*    | spp.         | Ecuador  | Jacob Russell | 46               |
| 107| A          | *Wasmannia* | spp.         | Peru     | Jacob Russell | 47               |
Table 1. Cont.

| id | Supergroup | Host Genus | Host Species | Locality | Submitter   | Sequence Type (ST) |
|----|------------|------------|--------------|----------|-------------|-------------------|
| 108| A          | Metapone   | madagascaria | Madagascar | Jacob Russell | 48                |
| 109| A          | Myrmica    | incompleta   | USA       | Jacob Russell | 49                |
| 110| A          | Polyergus  | breviceps    | USA       | Jacob Russell | 50                |
| 111| A          | Technomyrmex | albipes    | Philippines | Jacob Russell | 19                |
| 112| A          | Polyrhachis | vindex       | Philippines | Jacob Russell | 51                |
| 113| A          | Anoplolepis | gracillipes | Philippines | Jacob Russell | 52                |
| 114| A          | Notoncus   | spp.         | Australia  | Jacob Russell | 53                |
| 115| A          | Leptomyrmex | spp.        | Australia  | Jacob Russell | 19                |
| 116| A          | Myrmecorhynchus | spp.     | Australia  | Jacob Russell | 54                |
| 117| A          | Pheidole   | minutula     | French Guinea | Jacob Russell | 55                |
| 119| A          | Lophomyrmex | spp.        | Thailand   | Jacob Russell | 52                |
| 120| A          | Camponotus | leonardi     | Thailand   | Jacob Russell | 57                |
| 121| A          | Pheidole   | vallicola    | USA        | Jacob Russell | 58                |
| 122| A          | Rhytidoponera | metallica | Australia  | Jacob Russell | 59                |
| 124| A          | Pheidole   | plagiara     | Thailand   | Jacob Russell | 19                |
| 125| A          | Pheidole   | sauberi      | Thailand   | Jacob Russell | 19                |
| 126| A          | Pheidole   | gatesi       | Vietnam    | Jacob Russell | 60                |
| 127| A          | Pheidole   | spp.         | Thailand   | Jacob Russell | 61                |
| 129| A          | Dorymyrmex | elegans      | USA        | Jacob Russell | 63                |
| 134| A          | Odontomachus | clarus   | USA        | Jacob Russell | 111               |
| 135| A          | Ochettellus | glaber       | Australia  | Jacob Russell | 112               |
| 137| A          | Pheidole   | coloradensis | USA        | Jacob Russell | 114               |
| 138| A          | Pheidole   | micula       | USA        | Jacob Russell | 115               |
| 139| A          | Pheidole   | vistana      | Mexico     | Jacob Russell | 116               |
| 140| A          | Pheidole   | obtusaspinosa | USA     | Jacob Russell | 117               |
| 141| A          | Pheidole   | spp.         | Indonesia  | Jacob Russell | 118               |
| 143| A          | Aenictus   | spp.         | Thailand   | Jacob Russell | 120               |
| 144| A          | Crematogaster | spp.      | Thailand   | Jacob Russell | 121               |
| 145| A          | Solenopsis | spp.         | Thailand   | Jacob Russell | 122               |
| 146| A          | Leptogenys | spp.         | Thailand   | Jacob Russell | 19                |
| 147| A          | Pheidole   | plantifrons  | Thailand   | Jacob Russell | 19                |
| 148| A          | Monomorium | chinense     | Thailand   | Jacob Russell | 123               |
| 149| F          | Ocymyrmex  | picardi      | Congo (DRC) | Jacob Russell | 124               |
| 558| A          | Camponotus | textor       | Brazil     | Manuela Ramalho | 347           |
| 1827| A         | Paratrechina | longicorns | Taiwan | Tseng ShuPing | 19                |
| 1828| F         | Paratrechina | longicorns | Taiwan | Tseng ShuPing | 471               |
| 1868| A         | Cephalotes | atratus     | Brazil     | Madeleine Kelly | 494              |
| 1869| A         | Cephalotes | atratus     | French Guiana | Madeleine Kelly | 495              |
| 1870| A         | Cephalotes | atratus     | Brazil     | Madeleine Kelly | 496              |
Table 1. Cont.

| id   | Supergroup | Host Genus | Host Species | Locality | Submitter          | Sequence Type (ST) |
|------|------------|------------|--------------|----------|--------------------|--------------------|
| 1871 | A          | Cephalotes | atratus      | Guyana   | Madeleine Kelly    | 497                |
| 1872 | A          | Cephalotes | atratus      | Brazil   | Madeleine Kelly    | 498                |
| 1873 | A          | Cephalotes | atratus      | Peru     | Madeleine Kelly    | 499                |
| 1989 | A          | Cardiocondyla | spp.    | India    | Manisha Gupta     | 550                |
| 1996 | F          | Paratrechina | spp.   | India    | Manisha Gupta     | 557                |
| 2010 | A          | Pheidole   | spp.         | India    | Manisha Gupta     | 571                |
| 2017 | A          | Anoplolepis | gracillipes | Malaysia | Tseng ShuPing     | 52                 |
| 2022 | A          | Camponotus | spp.         | Malaysia | Tseng ShuPing     | 576                |
| 2023 | A          | Camponotus | spp.         | Malaysia | Tseng ShuPing     | 577                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 314                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 315                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 316                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 317                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 318                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 319                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 320                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 321                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 322                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 323                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 324                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 325                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 326                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 327                |
| Not defined | A | Solenopsis | spp. | Brazil | Cintia Martins | 328                |
| 59   | F          | Opistophthalmus | chaperi | South Africa | Laura Baldo | 78                 |

These data were retrieved from the Wolbachia Multilocus Sequence Typing (MLST) database (https://pubmlst.org/wolbachia). Included are identification (id), provided for each submission, Wolbachia supergroup, host genus, host species, locality, submitter (researcher responsible for submitting the sequences), and the sequence type (ST) found.

2.2. Reconstruction of the Ancestral State of Wolbachia

To reconstruct the ancestral state of Wolbachia diversity associated with Formicidae, we assigned each supergroup A or F to each tip in our topology. Although there are only a few (three) observations from the supergroup F associated with ants, we included them in the analysis. However, several other studies have already reported that supergroup A is more common in ants [9,28,33], which gives us support that our data are a real representation of what we find in nature. As we only have one observation of Wolbachia belonging to the supergroup B associated with Formicidae (see [9]), we decided to remove this sample from the subsequent analyses. The ‘equal rates’ (ER) model and the ‘all rates different’ (ARD) model were compared to determine which model best explains our data using the likelihood ratio test (LRT) [54,55]. The Ape and Phytools packages [49,56] from the R software [50] were used to reconstruct ancestral character states. We used the MCMC approach to sample character histories from the probability distribution using stochastic character mapping [57]. This method samples character histories in direct proportion to their posterior probability under a model. Using the SIMMAP function [58], we generated 100 stochastic character maps from our dataset. To summarize
the set of stochastic maps in a more meaningful way, we estimated the number of changes of each type, the proportion of time spent in each state, and the later probabilities that each internal node is in each state, under the best model.

2.3. Biogeographic Range Evolution Analyses

We used the inferred phylogeny and noted where, geographically, the *Wolbachia* ST was recovered (Asia, Africa, North America, South America, or Oceania) to implement biogeographic range evolution analyses using the R package BioGeoBEARS v1.1.1 [59] and estimated the ancestral range of *Wolbachia* associated with Formicidae. We followed the recommendations and parameters available on BioGeoBEARS PhyloWiki (http://phylo.wikidot.com/biogeobears) to test whether the observed biogeographic distribution of *Wolbachia* associated with Formicidae is best explained with a model that allows for vicariance and long-distance dispersal (DEC + J model) [60] versus a model that allows for only vicariance (DEC model) [61]. In addition, as *Wolbachia* is widely spread across these five biogeographic areas, we set max_range_size = 5. We used the likelihood ratio test (LRT) and the Akaike information criterion (AIC) to see which model best fits the data.

3. Results

Our *Wolbachia* phylogeny appears to be fairly robust when examining the results of posterior probability (PP); however, it grouped taxonomically unrelated ants, indicating that there is a lack of specificity of the host. Samples from 35 ant genera were included in this analysis, and as an example, 11 strains of *Camponotus* recovered from different locations around the world (Brazil, USA, Malaysia, India, and Thailand) were distributed across the phylogeny. This suggests the lack of codivergence of the bacteria and ant host. However, there was a clear and robust distinction between *Wolbachia* supergroups A and F associated with Formicidae (Figure 1).

Based on the likelihood ratio test, the ARD (all rates different) model (LTR = −6.975) was the best fit referring to the transition rates between each state to estimate the ancestral states of the *Wolbachia* supergroup associated with Formicidae when compared with ER (equal rates) (LTR = −8.530). Our ancestral state reconstruction (ASR) results with all 70 strains of *Wolbachia* associated with Formicidae show that the ancestor’s supergroup was F and that was consistent across all 100 replicates, which suggests that supergroup F was the ancestor character state (Figure 2A). In addition, the transition from supergroup ancestor F to supergroup A occurred only once within the Formicidae family (Figure 2B). Therefore, once *Wolbachia* supergroup A is acquired, there is no transition to another type of *Wolbachia*.

For the biogeographic analysis, the DEC + J model was returned as the best fit to our data (AIC weight ratio model = 1.28 × 10^{13}), which allows for vicariance and long-distance dispersal of *Wolbachia* in Formicidae (see Table 2). According to this scenario, the ancestral origin of *Wolbachia* associated with Formicidae remains ambiguous; however, it is most probable that it originated in Asia. Then, this supergroup F, rarely found among ants, seems to have expanded to Africa. More information about *Wolbachia* associated with Formicidae in the African region could confirm this trend. Another lineage of *Wolbachia*, supergroup A, is the most common among Formicidae and appears to have likely expanded to South America. One clade then expanded into North America. In the other major clade, there was a second expansion of *Wolbachia* back to Asia from South America, introducing supergroup A into this region. Our survey revealed only a few representatives of *Wolbachia* associated with Formicidae in Oceania and Africa (Madagascar), but our results show that infections in these regions are more recent (Figure 3). Our survey did not recover any data from Europe or Central America.
For peer review, we set max_range_size = 5. We used the likelihood ratio test (LRT) and the Akaike information criterion (AIC) to see which model best fits the data.

### Results

Our Wolbachia phylogeny appears to be fairly robust when examining the results of posterior probability (PP); however, it grouped taxonomically unrelated ants, indicating that there is a lack of specificity of the host. Samples from 35 ant genera were included in this analysis, and as an example, 11 strains of Camponotus recovered from different locations around the world (Brazil, USA, Malaysia, India, and Thailand) were distributed across the phylogeny. This suggests the lack of codivergence of the bacteria and ant host. However, there was a clear and robust distinction between Wolbachia supergroups A and F associated with Formicidae (Figure 1).

**Figure 1.** Bayesian majority-rule consensus tree (MrBayes) from the concatenated dataset (coxA, fbpA, ftsZ, gatB, and hcpA genes) of Wolbachia strains in Formicidae. Note that most strains belong to supergroup A, highlighted by a dotted box red, with the exceptions of three sequence types (STs), which were classified in supergroup F, highlighted by a dotted box black, and ST78 as outgroup.

| Model          | Likelihood Ratio Test (LRT) | Akaike Information Criterion (AIC) |
|----------------|-----------------------------|-------------------------------------|
| DEC + J Model  | -77.355                     | $1.28 \times 10^{13}$               |
| DEC Model      | -108.541                    | $7.77 \times 10^{-14}$              |

Table 2. Likelihood ratio test (LRT) and the Akaike information criterion (AIC) of the DEC model and the DEC + J model to select the optimal model of ancestral areas of Wolbachia associated with Formicidae. Note that the DEC + J model is highlighted in bold because vicariance and long-distance dispersal better explained our data.
Figure 2. Ancestral state reconstruction of Wolbachia associated with Formicidae. (A) One hundred stochastic character maps from our dataset. (B) Summary of all stochastic character maps for ancestral state reconstruction. Model = all rates different (ARD).

Figure 3. Ancestral range estimation of Wolbachia associated with Formicidae (MLST approach) with the chronogram using BIOGEOBEARS (DEC + J model). The pies are color-coded with the highest maximum likelihood probability of locations on the continents, and the boxes in the corner are colored according to the geographic locations and Wolbachia supergroup of the tips. The arrow indicates when Wolbachia supergroup A was acquired.
4. Discussion

This is the first study that has sought to investigate the ancestral state of supergroups, as well as to understand the ancestral range of origin in the evolutionary context of *Wolbachia* associated with ants. In the past, identifying *Wolbachia* infection was done by genotyping a single gene, *wsp* only. In general, with this approach, the *Wolbachia* strains associated with Formicidae belonged to supergroups A and B. With the MLST approach, now with five genes, it was found that the vast majority of Formicidae strains belong to supergroup A, followed by some representatives from supergroup F. There is only a single observation of supergroup B associated with *Pheidole sciophila* found in Mexico [9] and, therefore, it was not included in the present study. In order to understand if supergroup B, in nature, is atypical as a symbiont of Formicidae or if this is mainly a sampling bias, further studies are needed. In an extensive study, Russell and colleagues [9] used the MLST approach to understand the evolution of this interaction between *Wolbachia* and its hosts. For this, the authors focused their studies on two insect groups: ants and lycaenid butterflies. In addition to showing the presence of these bacteria in these hosts, the authors also concluded that the *Wolbachia* bacteria found in each of these groups of insects are different and highly specialized. Furthermore, they concluded that phylogenetic and geographic barriers can influence the evolutionary divergence of these bacteria. The authors found about 41 STs (sequence types) of *Wolbachia* associated with Formicidae, with few sample sequences included from South America, a hotspot for biodiversity [62,63]. Thus, in the present study, we were able to expand and include samples from this region and add more STs included in the *Wolbachia* MLST database since 2009 (Table 1).

Including *Wolbachia* associated with 35 different ant genera, our results indicate a lack of codivergence of *Wolbachia* with their ant hosts, corroborating what has been found by other studies [9,28,33,64]. The evidence for this conclusion is supported since taxonomically unrelated ants were grouped together in our *Wolbachia* phylogeny. Surprisingly, supergroup F was the ancestral character state for *Wolbachia* present in Formicidae, despite being less frequent than supergroup A. As already mentioned above, this supergroup was originally described in nematodes, but with the MLST approach, it has also been found in ants, although less frequently than supergroup A [9,32]. In addition, our results agree with the findings of Comandatore et al. [65] which suggest that supergroup F is a basal branching lineage for all *Wolbachia*.

This symbiont has already been reported to be transmitted vertically from queens to eggs in several host insects [40,66–68], including ants [29,69], and horizontal transmission of *Wolbachia* in ants is not common but may occasionally occur in related hosts [9,32,70]. In addition, Tseng et al. [32] showed that not all strains of ant *Wolbachia* have the same potential for being horizontally transferred. Another interesting aspect about these two supergroups being present in ants is that the evolutionary histories seem to be different, with supergroup A being transmitted vertically (maternal) and supergroup F being acquired horizontally [32].

Regarding the potential origin of the ancestral range of *Wolbachia* in Formicidae, our results seem to explain the dispersions by across continents. Other studies also show that geography can impact *Wolbachia* diversity on different ant host [9,28,33] and also in other insects, such as butterflies and moths [71]; however, few studies have focused on the origin and pattern of dispersion of these bacteria in different hosts until recently [72]. Our data suggest that the origin of *Wolbachia* in Formicidae happened in Asia. The supergroup F, although with few samples in ants, seems to have expanded to Africa and Asia. With the evolution of supergroup A associated with ants, *Wolbachia* then appeared in South America, with one major clade remaining in South America with a single introduction into North America and other South American clades resulting in a jump back to Asia with introductions from Asia into Oceania and Africa (Madagascar). Russell et al. [9] found evidence that *Wolbachia* strains were grouped according to samples from the Old and New Worlds, and our results also found this grouping. Our work highlights that to fully understand the evolutionary history and mechanisms of *Wolbachia* dispersion in ants or any other host group, we need much broader sampling of host species
and geographic locations. However, our work is shedding light on the evolution and biogeographic history of this symbiotic interaction.

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