Horizontal transmission and recombination of *Wolbachia* in the butterfly tribe Aeromachini Tutt, 1906 (Lepidoptera: Hesperidae)

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**Abstract**

*Wolbachia* is arguably one of the most ubiquitous heritable symbionts among insects and understanding its transmission dynamics is crucial for understanding why it is so common. While previous research has studied the transmission pathways of *Wolbachia* in several insect lineages including Lepidoptera, this study takes advantage of data collected from the lepidopteran tribe Aeromachini in an effort to assess patterns of transmission. Twenty-one of the 46 species of Aeromachini species were infected with *Wolbachia*. Overall, 25% (31/125) of Aeromachini specimens tested were *Wolbachia* positive. All *Wolbachia* strains were species-specific except for the wJho strain which appeared to be shared by three host species with a sympatric distribution based on a cophylogenetic comparison between *Wolbachia* and the Aeromachini species. Two tests of phylogenetic congruence did not find any evidence for cospeciation between *Wolbachia* strains and their butterfly hosts. The cophylogenetic comparison, divergence time estimation, and *Wolbachia* recombination analysis revealed that *Wolbachia* acquisition in Aeromachini appears to have mainly occurred mainly through horizontal transmission rather than codivergence.

**Keywords:** Aeromachini; *Wolbachia*; divergence time; cophylogeny; recombination; horizontal transmission

**Introduction**

*Wolbachia* is the most widespread endosymbiotic bacterium that infects a large variety of arthropods and filarial nematodes (Bandi et al. 1998; Weinert et al. 2015). In butterflies, *Wolbachia* infections have been reported in five families (Papilionidae, Hesperidae, Nymphalidae, Pieridae, and Lycaenidae) so far (Jiggins et al. 2000; Dyson et al. 2002; Hiroki et al. 2004; Tagami and Miura 2004; Russell et al. 2009; Bipinchandra et al. 2012; Jiang et al. 2018). The transmission pattern of *Wolbachia* is predominantly vertical and secondarily horizontal (Raychoudhury et al. 2009). It induces various reproductive alterations to alter host biology, like cytoplasmic incompatibility (CI), male killing (MK), feminization induction (FI), and thelytokous parthenogenesis (Yen and Barr 1971, Rousset et al. 1992, Stouthamer et al. 1993; Hurst and Jiggins 2000). In butterflies, some of these effects are well established, especially MK in Hypolimnas bolina and Acraea encedon (Jiggins et al. 2001; Dyson and Hurst 2000). CI in H. bolina and Polygonia calhouni (Hornett et al. 2008; Kodandaramaiah et al. 2011) and FI in Eurema hecabe (Kageyama et al. 2008).

Based on phylogenetic reconstructions with a set of loci (MLST) used to type *Wolbachia* strains, *Wolbachia* fall into 17 supergroups designated by the letters A–R, with supergroup G being the most widespread endosymbiotic bacterium that infects a large variety of arthropods and filarial nematodes (Bandi et al. 1998; Weinert et al. 2015). In butterflies, *Wolbachia* infections have been reported in five families (Papilionidae, Hesperidae, Nymphalidae, Pieridae, and Lycaenidae) so far (Jiggins et al. 2000; Dyson et al. 2002; Hiroki et al. 2004; Tagami and Miura 2004; Russell et al. 2009; Bipinchandra et al. 2012; Jiang et al. 2018). The transmission pattern of *Wolbachia* is predominantly vertical and secondarily horizontal (Raychoudhury et al. 2009). It induces various reproductive alterations to alter host biology, like cytoplasmic incompatibility (CI), male killing (MK), feminization induction (FI), and thelytokous parthenogenesis (Yen and Barr 1971, Rousset et al. 1992, Stouthamer et al. 1993; Hurst and Jiggins 2000). In butterflies, some of these effects are well established, especially MK in Hypolimnas bolina and Acraea encedon (Jiggins et al. 2001; Dyson and Hurst 2000). CI in H. bolina and Polygonia calhouni (Hornett et al. 2008; Kodandaramaiah et al. 2011) and FI in Eurema hecabe (Kageyama et al. 2008).

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Most species are restricted geographically to the Oriental Region, and a few species are found in Afrotropical Region and Palearctic Region (Eliot 1969). The common ancestor of this tribe was inferred to originate in Southeast Asia (Huang et al. 2019). We have reported the external features and the molecular phylogeny of the tribe in a preliminary study (Li et al. 2019). In prior screening, we found Wolbachia in Aeromachus inachus, A. virgata, and Halpe dizangpusa which prompted our further study of all Aeromachini species in China.

In this study, we characterized the Wolbachia in tribe Aeromachini by MLST genotyping. Furthermore, we conducted a cophylogenetic analysis between Wolbachia and their Aeromachini hosts, compared the age of Wolbachia divergence with that of host species, and analyzed the actual and potential recombination of Wolbachia in Aeromachini to provide information on the patterns of Wolbachia transmission across this tribe.

Materials and methods

Samples collection, DNA extraction, and Wolbachia MLST typing

We collected a total of 125 Aeromachini butterflies representing 10 genera and 46 species from 42 local regions in China across the last 12 years (Figure 1 and Supplementary Table S1). All specimens were caught with sweep nets and saved in small envelopes. The species were identified with morphological characteristics and molecular techniques (Jiang et al. 2019; Li et al. 2019). The DNA was isolated from whole abdomens of specimens using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany).

To screen for Wolbachia infection status, the wsp locus was amplified followed the published protocols described by Zhou et al. (1998; Supplementary Table S2). The characterization of Wolbachia strains was performed to sequence multiple loci suggested by Wolbachia MLST database (http://pubmlst.org/wolbachia) (Zhou et al. 1998; Supplementary Table S2). The MLST typing consisted of five Wolbachia gene fragments (gatB, coxA, hcpA, ftsZ, and fbpA). The PCR product was purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). The purified product was ligated with the pGEM-T easy vector (Promega, Madison, WI, USA) using a ligation mix (TaKaRa). Competent cells (Escherichia coli JM109, TaKaRa) were then transformed with the plasmid. Plasmid DNA was extracted using the Pure Yield Plasmid Miniprep System (Promega, Madison, WI, USA). The sequencing was performed using an ABI 377 automated DNA sequencer.

A Mantel test was used to compare Wolbachia frequency (pooled across species) and geographical distribution of their corresponding Aeromachini hosts with the software Isolation by Distance (IBD; Bohonak 2002). It was performed on the pairwise node distance matrix of Wolbachia frequency and host Aeromachini species to test for an association between matrices (Maddison 2015).

Cophylogenetic analysis

The MLST sequences were aligned with outgroups retrieved from the MLST database (host: Brugia malayi, Cordylochernes scorpioides, and Opistophthalmus capensis; Supplementary Table S1) using Bioedit v. 7.0 (Hall 1999). The HKY + I model was selected as the best-fit substitution model with PartitionFinder v2.1.1 (Lanfear et al. 2012) using the Bayesian Information Criterion (BIC). Maximum likelihood (ML) tree was constructed with the concatenated data using IQtree 1.4.2 (Nguyen et al. 2015). To assess nodal support, we performed 1000 ultrafast bootstrap replicates with UFBoot and an SH-aLRT test with 1000 replicates (Hoang et al. 2018). For the molecular phylogenetic constructions of Aeromachini species (the concatenated mitochondrial and nuclear genes), we retrieved the mitochondrial genes COI, COII, and three variable domains of the nuclear DNA (D3 region of 28S rDNA, V4 and V7 regions of 18S rDNA) from GenBank (MK344780–MK345418). The method of ML tree construction follows that used for the hosts as

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Figure 1 The distribution of specimens collected in China infected or uninfected with Wolbachia. The sizes of the circles are directly proportional to the number of individuals analyzed (black: infected with Wolbachia, white: uninfected). The triangles refer to the location of collection sites and the letters are the Abbreviation of place names. For full site names and other details, please see Supplementary Table S1.
described above. The GTR + G model was selected as the best-fit substitution model for this dataset.

A Mantel test was used to compare genetic and Wolbachia distance matrices with IBD (Bohonak 2002). It was performed on the pairwise node distance matrix of Wolbachia strains and host Aeromachini species to test for an association between matrices (Hall 1999). Another test of phylogenetic congruence between butterflies and endosymbiont partners was undertaken with the Procrustean Approach to Cophylogeny (PACo; Balbuena et al. 2013). The analysis was performed in R with 100,000 permutations using packages VEGAN v.2.4.6 (Oksanen et al. 2018) and APE v.4.1 (Paradis et al. 2004).

**Estimation of divergence time**

We referred to a molecular dating analysis of Wolbachia supergroups A and B to compare the divergence times of Wolbachia (Gerth and Bleidorn 2016) with the age of Aeromachini species divergence. The divergence times of all Wolbachia-infected Aeromachini species were inferred with the relaxed-clock molecular dating estimation by BEAST 1.5.2 (Excoffier et al. 2005). The HKY model of nucleotide substitution with gamma distributed rate variation among sites was used to analyze and the Yule specification method was assumed. We used the age ranges estimated from Chazot et al. (2019) to calibrate the split between Hesperiidae and Hedyliidae (81–114 Mya) and the age ranges between Hesperini and Heteropterini (35–55 Mya). We also used a recently described fossil hesperid, Pamphilites abdita Scudder, 1875 to constrain the minimum stem age of subfamily Hesperini to 25 Mya (de Jong 2016). Chains were run for 50 million generations, with the first 20% discarded as burn-in. The results were summarized with TRACER 1.5 (Fu and Li 1993).

**Recombination analysis**

Gene recombination can interfere with and mislead phylogenetic relationships of species. We detected recombination events within each MLST gene and wsp gene, to clarify whether horizontal transmission had occurred among these Wolbachia strains. To examine recombination among Wolbachia strains from Aeromachini species, each MLST gene and wsp gene were detected using RDP3 (Martin et al. 2010), a program for detecting recombination. The potential recombination events can be detected by any of the methods listed above. As recommended for this procedure, the breakpoint positions and recombinant sequences inferred from every potential recombination event were manually checked and adjusted following the phylogenetic and recombination signal analysis features available in RDP3.

To visualize potential recombination events, ML trees for each MLST gene and wsp gene were constructed with 10 reference STs and 3 outgroups retrieved from the MLST database (Supplementary Table S1) using IQtree 1.4.2 (Nguyen et al. 2015). They were checked for their supergroup clustering in ML trees. A potential recombination event could be found from inconsistencies between gene trees (Werren and Bartos 2001; Baldo et al. 2006).

**Results**

**Infection rates and diversity of Wolbachia**

In the examined butterflies, 25% (31/125) of samples were Wolbachia positive and 46% (21/46) of Aeromachini species in this study were considered infected with Wolbachia, with some of these shown to be polymorphic for the infection despite limited sampling. The infection status and geographical distribution of each sample and species is shown in Figure 1, Supplementary Tables S1 and S3. The Mantel test analysis indicated a nonsignificant correlation between Wolbachia frequency and geographic location of their corresponding Aeromachini hosts when pooled across species and samples ($r = 0.1714, P = 0.060$), suggesting a weak spatial structure in the incidence of Wolbachia. However, there is no obvious association between Wolbachia frequency overall and latitude (Figure 1), a pattern previously noted for moths (Ahmed et al. 2015). We amplified five MLST loci to characterize Wolbachia strains. Each of the five MLST genes and the wsp gene detected from each Aeromachini species had the same sequence. The strains are denoted based on the MLST loci as $w$Vir, $w$Mag, $w$Mag, $w$Kyn, $w$Jho, $w$Yin, $w$Lua, $w$Dio, $w$Hy, $w$Bai, $w$Lin, $w$Vir, $w$Pes, $w$Dol, $w$Lat, $w$Sub, $w$Kua, $w$Diz, and $w$Str (GenBank accession numbers: MT935975–MT936085).

**Comparison of Wolbachia and Lepidoptera phylogenies**

All Wolbachia strains were species specific except for $w$Jho shared by three host species (Aeromachus jhora, Aeromachus propinquus, and Pedesta bivitta) sympatric in Yunnan Province, southwest China (Figure 2). Although the concatenated sequences of hosts and Wolbachia strain types matched well, the topologies of Aeromachini hosts and corresponding Wolbachia strains (which fell into supergroups A and B) were not congruent (Figure 2). It is possible that coevolution could have occurred between hosts and their Wolbachia in the Aeromachus clade, although the Mantel test indicated no significant correlation between the genetic distances of the Wolbachia strains and their host Aeromachini species ($r = -0.094, P = 0.719$). This points to the horizontal transmission being an important mode of transmission. Similarly, PACo provided no evidence for congruence between the phylogeny of Aeromachini and that of their endosymbionts (PACo $r^2 = 0.033, P = 0.402$).

**Divergence time estimation**

Divergence time of the Aeromachini was estimated with the relaxed clock molecular dating implemented in BEAST. We compared the divergence between Wolbachia supergroups based on genomic data (Gerth and Bleidorn 2016) with divergence times of Aeromachini and found the youngest divergence between species at 6.69 Mya (8.82–4.03, 95% HPD) and the oldest gap between Parasius perbelia and the other species at 43.30 Mya (47.93–39.61, 95% HPD) (Figure 3).

**Recombination of MLST and wsp genes**

The recombination analysis within each MLST gene and wsp gene showed that the polymorphic sites of the alignment of the FtsZ alleles are not randomly distributed, but a mosaic pattern consistent with recombination in a coinfecting host. To estimate the approximate recombination events, all events were confirmed with five of seven RDP3 algorithms (Table 1). The FtsZ sequence of four Wolbachia strains ($w$Yin from A. inachus; $w$Jho from A. jhora, A. propinquus, and P. biivitta; $w$Yin from Pedesta yingqii; and $w$Dol from Sebstonyma dolopia) are the same recombinant between Wolbachia strain $w$Lat detected from Pedesta latris and Wolbachia strain $w$Dio from Ampitia discordiae (Supplementary Figure S1).

We also reconstructed ML trees for each MLST gene and the wsp gene separately (Figure 4). Eleven of the nineteen Wolbachia strains ($w$Jho, $w$Pic, $w$Mag, $w$Lin, $w$Vir, $w$Pes, $w$Dol, $w$Lat, $w$Sub, $w$Diz, and $w$Str) were found to have inconsistent supergroup
allocation among the five MLST gene trees. For example, the localization of \( wJho \) on the ML tree was with the B-supergroup (Figure 2). This was associated with a \( coxA \) allele that belonged to supergroup A, in contrast to alleles at other loci belonging to supergroup B (Figure 4). Therefore, there was substantial incongruence between the \( \text{Wolbachia} \) phylogenies based on the MLST genes and the \( \text{wsp} \) gene sequences (Figure 4) and highlights limitations of supergroup assignment.

**Discussion**

Two reports have predicted the incidence of \( \text{Wolbachia} \) in lepidopteran insects and arthropods more generally (Weinert et al. 2015; Ahmed et al. 2016). The estimated infection incidence in species was predicted to be 80% in Lepidoptera, which is much higher than the 52% incidence predicted in arthropods. However, the mean prevalence of \( \text{Wolbachia} \) in Lepidoptera (27%) is similar to that that in arthropods (24%). The high incidence and low prevalence of \( \text{Wolbachia} \) in Lepidoptera was interpreted as indicating substantial horizontal transmission of \( \text{Wolbachia} \) (Ahmed et al. 2016). For the Aeromachini butterflies considered in this study, the mean prevalence in samples (25%) was like the value in other Lepidoptera (27%) and arthropods more generally (24%). On the other hand, the presence of the infection at the species level (46%) was similar to that in arthropods (52%) but considerably lower than reported previously in Lepidoptera (80%). However, the 21 uninfected species in this study are often represented by only 1 or 2 individuals, such as \( \text{Ampittia trimacula} \), \( \text{A. jhora} \), \( \text{Pedesta xiaozingae} \), and \( \text{Pedesta zinnia} \). The proportion of species infected should therefore be considered as an underestimate of the actual incidence of \( \text{Wolbachia} \) infection across Aeromachini species until larger sample sizes across the geographic range of species are considered.

Two cophylogenetic analyses revealed no correlation of genetic distances between \( \text{Wolbachia} \) strains and their butterfly hosts, which further supports horizontal transmission of \( \text{Wolbachia} \) in the tribe. The divergence time of \( \text{Wolbachia} \) supergroups was compared with that of Aeromachini species (Figure 3). Gerth and Bleidorn (2016) estimated the divergence time between \( \text{Wolbachia} \) supergroups A and B was 216.61 Mya. This implies that transfers of \( \text{Wolbachia} \) from different supergroups between Aeromachini species cannot due to divergence coinciding with speciation events which are dated between 6.69 and 43.30 Mya. Instead, these analyses point to clear cases of horizontal transmission. The \( \text{Wolbachia} \) strain \( wJho \) provides a particularly strong argument for horizontal transmission, given that it was present in three species in the tribe (Figure 2). The individuals of \( \text{A. jhora} \), \( \text{A. propinquus} \), and \( \text{P. biutta} \), infected with \( wJho \), co-occur in Yunnan Province, southwest of China, presumably reflecting an opportunity for horizontal transmission.

Pathways of horizontal transmission for \( \text{Wolbachia} \) could occur through hybridization (e.g., Jiang et al. 2018), feeding on common plants (e.g., Sintupachee et al. 2006; Li et al. 2017), ectoparasitic mites (e.g., Jaenike et al. 2007; Gehrer and Vorburger 2012), or parasitoids (e.g., Vavre et al. 1999; Ahmed et al. 2015). To our knowledge, there is no report of hybridization in the tribe Aeromachini so far. Although sympatric species \( \text{A. jhora} \) and \( \text{A. propinquus} \) harbor the same \( \text{Wolbachia} \) strains based on MLST typing, we cannot confirm \( \text{Wolbachia} \) spread through introgressive hybridization based on the ML trees constructed with mt+nDNA, mtDNA, and nDNA using IQtree (Supplementary Figure S2). We also found the topological structure based on mtDNA sequence was consistent with mt+nDNA, but different from nDNA. The discordance between these patterns may have several reasons including inaccurate species taxonomy, paralogous pseudogenes, incomplete lineage sorting (ILS), and introgressive hybridization.
We can exclude the possibility of inaccurate species taxonomy and paralogous pseudogenes in our case, as all specimens were identified carefully by experts and all sequences were checked for paralogous pseudogenes prior to analysis. However, we cannot really distinguish ILS from introgressive hybridization on the evidence we have so far. Also, the few substitutions detected in the nuclear markers tested here make it difficult to use these data to reconstruct fine-scale phylogenies. However, since most butterfly larvae feed on plant tissue, and adults obtain nectar from flowers or tree sap, the close relationship between

![Figure 3](image)

**Figure 3** (A) Estimated divergence times of Wolbachia Supergroups A and B based on Gerth and Bleidorn (2016), and (B) Bayesian Inference (BI) tree of mtDNA datasets for Aeromachini species using uncorrelated lognormal relaxed clock in BEAST v1.5.2. Posterior probabilities of nodes are shown to the right of the node branch when higher than 0.95. The violet bars (B) indicate 95% highest posterior density interval (HPD) of the node ages.

**Table 1** Average P-values of recombinations estimated using the RDP3 program

| Recombination strains | RDP      | GENECONV | BootScan | MaxChi   | Chimaera | SiScan  | 3Seq     |
|-----------------------|----------|----------|----------|----------|----------|---------|----------|
| wIna                  | 5.306 × 10^-09 | 2.475 × 10^-08 | 5.032 × 10^-10 | 8.266 × 10^-11 | 7.207 × 10^-12 | —       | 1.395 × 10^-18 |
| wJho                  | —        | 1.585 × 10^-06 | 1.516 × 10^-08 | 5.784 × 10^-11 | 5.719 × 10^-11 | —       | 8.129 × 10^-18 |
| wYin                  | —        | 1.308 × 10^-10 | 1.904 × 10^-12 | 2.522 × 10^-11 | 7.657 × 10^-12 | —       | 1.177 × 10^-18 |
| wDol                  | —        | 1.585 × 10^-16 | 2.550 × 10^-09 | 5.784 × 10^-11 | 5.784 × 10^-11 | —       | 5.360 × 10^-18 |
butterflies and host plants might lead to infection transmission through plant mediation (Sintupachee et al. 2006). There are many known hymenopteran parasitoids found on both lepidopteran and dipteran hosts, and generalist parasitoids may also have mediated horizontal transmission (Apiwathnasorn 2012). This could be further tested by examining Wolbachia strains in parasitoids particularly in those from Yunnan province.

The recombination analysis of each MLST allele and wsp using RDP3 found intragenic recombination in the FtsZ gene in four Wolbachia strains. This result also argues for horizontal transmission between Wolbachia strains in the tribe Aeromachini; the very similar recombined FtsZ sequence in four species-specific Wolbachia strains may reflect a second horizontal transmission in these closely related species (Supplementary Figure S1). In our reconstructed ML trees for each MLST allele and wsp gene (Figure 4), we found potential recombination events by checking every allele for supergroup localization among the gene trees. Eleven Wolbachia strains from Aeromachini species showed inconsistent supergroup localization for the five MLST allele trees. The substantial incongruence between the Wolbachia phylogenies based on the MLST concatenated sequences and the wsp gene (Figure 4) suggests that the different Wolbachia genes have undergone independent evolutionary trajectories. This has also been observed in rice planthoppers, butterflies, and moths (Zhang et al. 2013; Ilinsky and Kosterin 2017) and highlights the limitations of the MLST system for classifying Wolbachia strains, whereas full genome sequencing may be required to further establish relationships among Wolbachia strains (Conner et al. 2017; Cooper et al. 2019; Meany et al. 2019).

Taken together, this study provides a conservative estimate of Wolbachia prevalence (25%) of the butterfly tribe Aeromachini with a species incidence of >46%. The cophylogenetic
comparison, divergence time estimation, and Wolbachia recombination analysis revealed that Wolbachia acquisition in Aeromachinini is often through horizontal transmission as also found for other groups such as fruit flies (Turelli et al. 2018), spiders (Baldo et al. 2008), wasps (Hugens et al. 2004), trypetids (Schuler et al. 2013), leaf beetles (Jactel et al. 2013), moths (Ahmed et al. 2016), rice planthoppers (Zhang et al. 2013), and mosquitoes (Shaikevich et al. 2019).

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**Data availability**

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All original raw sequence data files are available via the GenBank (accession number MT935975–MT936085 and MK344780–MK345418). Supplementary material is available at G3 online.

**Conflicts of interest**

None declared.

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