Wolbachia Infection in Wild Mosquitoes (Diptera: Culicidae): Implications for Transmission Modes and Host-Endosymbiont Associations

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Research

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Abstract

Background: *Wolbachia* is an intracellular bacterial endosymbiont found in most insect lineages. In mosquitoes, the endosymbiont's influence on host reproduction and arboviral transmission has spurred numerous studies aimed at using *Wolbachia*-infection as a vector control technique. However, there exist several gaps in the literature regarding the natural *Wolbachia* infection across species, modes of transmission as well as the associations between various *Wolbachia* lineages and their hosts. This study aims to address these by exploring mosquito-*Wolbachia* associations and their evolutionary implications.

Methods: We conducted tissue-specific PCR screening of *Wolbachia* infection in wild mosquitoes from Singapore using the *wsp* molecular marker. Tissues examined include leg, gut, and reproductive tissues. We also explored mosquito-*Wolbachia* associations using three methods – a tanglegram, distance-based, and event-based method, and inferred instances of vertical transmission and host shifts.

Results: We screened 271 adult mosquitoes (41 species and 14 genera) for *Wolbachia* and found that 43.9% of all individuals harboured *Wolbachia*. Eight out of the 21 infected species were not previously reported. We detected *Wolbachia* infections predominantly in the reproductive tissues, a strong indication of vertical transmission. Despite this, *Wolbachia* infection rates vary widely within a mosquito host species. There was no clear signal of co-phylogeny between the mosquito hosts and the twelve putative *Wolbachia* strains observed in our study. Host shift events were also observed.

Conclusions: Our results suggest that the mosquito-*Wolbachia* relationship is complex and that a combination of transmission modes and multiple evolutionary events likely explain the distribution of *Wolbachia* diversity observed across mosquito hosts. This has implications towards understanding *Wolbachia*'s diversity, ecology, and utility as a biocontrol method.

Background

Wolbachia is an intracellular endosymbiotic bacterium that alters host reproduction [1]. It is widespread in arthropods, infecting a wide range of insects, crustacean, and nematode species [2, 3]. In some cases, *Wolbachia* has a mutualistic relationship with their hosts [4–6]. However, *Wolbachia* is most often recognised as a reproductive manipulator that biases the sex-ratio of the host's offspring towards infected females [7, 8]. This reproductive manipulation is commonly achieved through four phenotypes – male-killing [9], feminisation [10, 11], parthenogenesis [12, 13], and cytoplasmic incompatibility [14, 15] – increasing the endosymbionts' reproductive success [16]. Owing to their strong influence on host reproduction, an increasing amount of research is dedicated to exploring the impacts of reproductive endosymbiont on host population dynamics and evolution [17, 18], especially in medically important insects such as mosquitoes. The promise to alter both mosquito reproduction [19], and arboviral transmission [20], have prompted the deployment of novel *Wolbachia*-infected mosquitoes for population replacement and suppression [21].

Several countries, including Singapore, have started to employ *Wolbachia* as a biocontrol agent of mosquitoes [22–24], but most have not assessed the presence of naturally occurring infection in their wild mosquito populations. The release of artificially infected mosquitoes might have a profound impact on wild mosquito populations due to close interactions. The effect of endosymbiont on non-target species could potentially result in a population crash, opening a niche for other vector species [25]. Another implication of such a bio-control will be the increased likelihood of co-infections with naturally occurring *Wolbachia* strains or other endosymbionts, such as *Cardinium*, *Rickettsia*, and *Spiroplasma*, which can have a synergistic effect on host fitness and future transmission of endosymbionts [26–29]. Without a detailed characterisation of *Wolbachia* prevalence and diversity among wild mosquitoes, the ecological risk of releasing artificially infected mosquitoes might be overlooked. Therefore, with the precautionary principle in mind, it is important to investigate the natural occurrences of *Wolbachia*.

There is also a need to discern the mode of infection transmission among mosquitoes. Even though *Wolbachia* are mainly thought to be transmitted vertically [15, 30], there are accounts of horizontal transmissions into wild populations through parasitism [31, 32], or being near infected individuals [33]. Previous research also proposes that *Wolbachia* may not be strictly localised in the germline tissues and have been detected in somatic tissues such as the gastrointestinal tract and haemolymph [34–36]. Detection in the gastrointestinal tract suggests horizontal transmission through uptake from the environment or host sharing [34, 37, 38], whereas a detection in non-gastrointestinal somatic tissues, such as the jointed appendages, could be a case of horizontal bacterial genome integration into the host genome [36]. Currently, most research on the detection of *Wolbachia* in mosquitoes adopts conventional PCR methods which extract DNA from the entire individual or its abdomen [39–47]. This limits our ability to identify the exact location of endosymbiont infection within an individual. A tissue-specific screening of *Wolbachia* is necessary to provide insights into the extent of vertical and horizontal transmissions.

Given that vertical transmission is widely believed to be the main mode of *Wolbachia* transmission [15, 30], it has been proposed that host mitochondrial DNA (mtDNA) and *Wolbachia* are maternally co-transmitted within the cytoplasm [17, 48]. This results in congruency between host mtDNA and *Wolbachia* phylogenies – a consequence of cytoplasmic hitchhiking driven by endosymbiont transmission [17]. In insect systems such as bedbugs where vertical transmission has been established to be the main mode of transmission, *Wolbachia* exhibits clear patterns of co-phylogeny with its hosts, with few instances of host shifting or multiple infections within a single host species [49, 50]. In contrast, co-phylogeny is not apparent among nematodes and bees, and there are numerous acquisitions of *Wolbachia* infections through horizontal transmission as well as losses in these diversified host lineages [51, 52]. As the modes of *Wolbachia* transmission are not well-established among mosquitoes, the extent of host shifting or multiple infections within mosquito hosts are not well-explored.

Currently, there has yet been a comprehensive analysis of the evolutionary associations between *Wolbachia* and their mosquito host species. An understanding of host-endosymbiont association will not only further our ability to discern the mode of transmission which influences *Wolbachi*a diversity, but it will also allow for evaluation of *Wolbachia*'s host specificity, speciation, and its ability to establish itself

in new hosts. All of these are key to understanding *Wolbachia*'s diversity, ecology, and utility as a biocontrol method.

In this current study, we have three major research objectives: First, we examined the prevalence and diversity of *Wolbachia* among wild mosquitoes from Singapore. Second, using a tissue-specific PCR screening method, we also determined the localisation of *Wolbachia* infection within an individual. Finally, we reconstructed the evolutionary associations between *Wolbachia* and their mosquito hosts to provide a basis to understand host-endosymbiont evolution.

Methods

Adult mosquito collection and identification

We collected mosquito samples from twelve localities across Singapore between March 2018 and November 2019 (Fig. 1A). We employed three methods to collect the samples: CO_2-baited Centers for Disease Control and Prevention (CDC) traps, sweep netting using handheld fan traps, and larval sampling [53]. For the latter, we carried out the dipping method at streams and ponds and used pipettes to collect larvae from various microhabitats including tree holes, plant axils, and artificial containers. Thereafter, we reared the field-collected larvae to adults in an incubator maintained at 26 °C, 70% RH, and under a photoperiod of 12:12 (day:night) hour diurnal cycle. We fed the larvae with pulverised fish food (TetraMin Granules) daily. Mosquitoes were identified using relevant taxonomic keys and descriptions [54–59]. We randomly selected a subset of individuals from commonly sampled species and preserved them in phosphate-buffered saline solution at -80 °C for the subsequent dissection step.

Tissue-specific Dissection

We carried out dissection on each adult mosquito sample and isolated the leg, gut, and reproductive tissues (Fig. 1B – Fig. 1D). To prevent the contamination of tissues, we dissected the leg first to prevent contamination by any bacteria residing within the mosquito. Between each dissection, all dissection equipment and microscope slides were thoroughly wiped with 70% ethanol before commencing dissection for the next sample. We placed the dissected tissues individually into a 96-well plate which was placed on ice to prevent DNA degradation.

Dna Extraction, Pcr Amplification, And Sequencing

We used 7µL of QuickExtract™ DNA Extraction Solution (Lucigen) to extract DNA from each of the dissected tissues. We placed the samples in a thermocycler with the following protocol: 65 °C for 18 minutes, followed by 98^{o}C for 2 minutes, and ending with cooling on ice for at least 10 minutes. All dissected tissues were screened for *Wolbachia* infections following single-primer PCR protocols described by Martin *et al.* [26] with slight modifications. We used the *Wolbachia* surface protein (*wsp*)

general primers, wsp81F (5'- TGGTCCAATAAGTGATGAAGAAACTAGCT-3') and wsp691R (5'-AAAAATTAAACGCTACTCCAGCTTCTGCAC-3') [60]. We chose the *wsp* molecular marker as it can successfully detect *Wolbachia* infection across numerous taxa and it is also ideal for strain genotyping and evolutionary comparison between the detected *Wolbachia* strains [60]. In addition, we amplified a section of the *cytochrome c oxidase subunit I* (*COI*) gene of the mosquito hosts using primers LCO1498 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3') [61]. This serves to confirm host identity and acts as an internal control. We used DNA from known *Wolbachia*-infected *Nasonia* specimens as positive controls for this study.

We performed all PCR procedures in reaction mixtures consisting of 12.5μL of GoTaq® G2 Green Mastermix (Promega), 1μL of 1 mg mL^{-1} bovine serum albumin, 0.184μL of 25 mM magnesium chloride, 1.5μL of extracted DNA, and 1.5μL each of 5 μM *wsp* forward and reverse primers for *Wolbachia* PCR screens or 1.0μL each of 5 μM LCO1498 and HCO2198 primers for *COI* PCRs. We used double-distilled water (ddH_2O) to top up the reaction mixture to a final volume of 25μL. PCR amplification of positive and negative controls (by replacing DNA with ddH_2O) was also conducted simultaneously.

We used the following PCR conditions: 94 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min. We separated the amplicons by gel electrophoresis on 2% agarose gel stained with GelRed® (Biotium) and visualised under a UV transilluminator (GeneSys). Amplicons were purified using SureClean Plus (Bioline) following the manufacturer's protocol and sent for Sanger sequencing at First Base Laboratories (Axil Scientific Pte. Ltd., Singapore).

Using Geneious Prime (2019.2.3) (https://geneious.com), we conducted DNA sequence clean-up on all sequenced amplicons as follows: First, we trimmed the ends of the sequences using the default setting. Second, we created contiguous sequences with forward and reverse reads using de novo assemble at the highest sensitivity. Third, we visually confirmed the DNA bases. We checked the chromatogram for multiple peaks and corrected miscalled base pairs with "N". Last, if it was not possible to generate a contiguous sequence with the forward and reverse reads, we chose the read with a higher percentage quality as the final consensus sequence. All consensus sequences were queried in National Center for Biotechnology Information BLAST [62].

Statistical Analyses

To identify significant differences in the infection across the different mosquito tissues, we carried out Cochran's Q test. As a follow-up, we employed McNemar's post-hoc test to identify the tissue pairs that differed significantly in infection. Individuals which had unsuccessful amplification of the internal control (*COI* gene) for any of the three dissected tissues were excluded from this statistical analysis. We also tested for the effect of sex on host infection using binary logistics regression with sex as a categorical dependent variable and infection outcome as a binary independent variable. We conducted a logistics

regression on a subset which only includes species that have a roughly similar representation of both sexes i.e. for every species included, the number of individuals of the less common sex is at least 60% of the more common sex.

We determined the significance of the variables when the p-value is less than 0.05. We performed all statistical analyses in R version 3.6.2 [63], with packages *nonpar* [64], *rcompanion* [65], and *ISLR* [66].

Phylogenetic Analyses

We carried out multiple alignment of consensus sequences using the ClustalW algorithm with default setting ("gap penalty = 15"; "gap extension penalty = 6.66") [67], in software MEGA X [68]. We aligned mosquito *COI* sequences generated in this study with 61 reference *COI* barcodes of identified local mosquitoes from Chan *et al.* [53]. For *wsp* sequences, we aligned the sequences with 54 available *wsp* sequences of known *Wolbachia* strains which were obtained from GenBank [69]. Short sequence reads (< 500 bp) were excluded.

We conducted objective clustering on *COI* sequences to sort mosquito samples into molecular clusters [70]. We treated terminal gaps as missing and determined species using a 3% threshold [71, 72]. In a previous study which conducted *COI* barcoding of mosquitoes from Singapore, researchers supported the cut-off of 3% to discriminate mosquito species [53]. The objective clustering, thus, serves to verify the morphological sorting done previously and to correct possible cases of misidentifications in the sample.

We also constructed neighbour-joining (NJ) phylogenetic trees for mosquito hosts and *Wolbachia* using the sequenced *COI* gene fragment and the *wsp* gene, respectively. We performed NJ tree reconstruction with Kimura 2-parameters as the nucleotide substitution model in MEGA X [68]. Internal gaps were treated as indels and terminal gaps as missing for *wsp* sequences. We estimated bootstrap probabilities by generating 1000 bootstrap replicates. We designated two biting midges species, *Culicoides asiana* (KJ162955.1) and *Culicoides wadai* (KT352425.1), as outgroups for the host NJ tree construction. Due to the lack of an appropriate endosymbiont outgroup [51], we midpoint rooted *Wolbachia* NJ tree instead.

When possible, we classified *Wolbachia* strains into supergroups and putative strains using 97% bootstrap probability as a threshold [60]. *Wsp* sequences that did not have 97% bootstrap support were evaluated on a case by case basis. For example, we deemed sequences which closely cluster together and have a relatively high support value (> 90%) as the same putative strain.

We categorized putative strains which are infectious to only one host species as "specialists" and those which infect two or more hosts as "generalists". Then, we calculated the standardised phylogenetic host specificity (SPS) score of each generalist strain by adapting the method outlined by Poulin *et al.* [73] and Kembel *et al.* [74]. SPS measures the degree of phylogenetic relatedness among host species infected by the same endosymbiont strain. It also tests for significance by comparing it with null models generated with 999 replicates of random host-endosymbiont associations. A positive SPS value with high p-value

($p > 0.95$) indicates a high degree of host flexibility where *Wolbachia* infects hosts which are phylogenetically even. A negative SPS value with low p-value ($p < 0.05$) suggests a low degree of host flexibility where the infected hosts are phylogenetically clustered together. We calculated SPS score using R package *picante* [74].

Evolutionary analyses of the mosquito- Wolbachia **relationship**

Current literature adopts various approaches to explore correlation and evolution between hosts and their associated parasites or symbionts [51, 75–78]. Here, we used three distinct methods to explore the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. We carried out the analyses using pruned phylogenies where each species is represented by a single individual.

First, using the software TreeMap 3.0 [79], we created a tanglegram between host and endosymbiont NJ tree to visualise the mosquito-*Wolbachia* association. A tanglegram is useful as a pictorial representation of the interactions between two phylogenies [80]. TreeMap also seeks to minimise the entanglement between the two trees to provide a clearer visualisation of the phylogenetic relationship between host and endosymbiont [79].

Second, we employed a ParaFit Global test, a distance-based method, to quantitatively estimate congruence between the host and endosymbiont phylogenetic trees by comparing genetic distances among infected host species and the *Wolbachia* strains [81]. The null hypothesis (H_0) for this test states that the associations between host and endosymbiont trees are random, whereas the alternative hypothesis (H_1) suggests that there are strong associations with phylogenetic distances between hosts and parasites. We tested for significance by comparing the observed associations between host and endosymbiont with randomised associations generated with 5000 permutations. We also identified the respective host-endosymbiont associations which contributed significantly to the ParaFit Global statistics. We performed the ParaFit test with Caillliez correction to correct for negative eigenvalue generated [82] using R package *ape* [83].

Third, we performed an event-based analysis in Jane 4.0 [84] to map out potential evolutionary events of the endosymbiont in relation to the host phylogeny [85]. Five evolutionary events are considered: co-speciation (host and endosymbiont speciate simultaneously), duplication (intra-host speciation), duplication with host shift (endosymbiont host shifts), loss (host speciates but endosymbiont fails to establish in one of the new lineages), failure to diverge (host speciates and endosymbiont remains in both lineages). Due to the differing likelihood of each event, we attached different cost values to each of the events. Jane 4.0 determined the best reconstruction of evolutionary events by minimising the overall cost. We used the following cost-scheme regime with 100 generations and a population size of 300: co-speciation = 0, duplication = 1, duplication with host shift = 2, loss = 1, and failure to diverge = 1 [78]. As a follow-up, we carried out random tip mapping (randomisation of host-endosymbiont associations) for 50 iterations, to determine if the overall cost of reconstruction is significantly lower than expected by chance.

If 5% or fewer of the random solutions have costs lower than the reconstructed coevolution phylogeny, there is support for the coevolution of the hosts and endosymbionts through co-speciation.

Each of the three methods has its own merits: (i) a tanglegram allows for clear visualisation of host-endosymbiont association without taking into account any evolutionary relationships, but there are calls for careful interpretation as the degree of entanglement may not necessarily represent phylogeny congruency [86]. (ii) A global ParaFit test seeks to address the limitation of the tanglegram by testing for global congruency in an unbiased, statistical approach [81]. (iii) An event-based method enables evaluation of potential evolutionary events that might have occurred throughout the endosymbiont's evolutionary history such as co-speciation, duplication, and host shifting. Taken together, they allow for a well-round and comprehensive examination of the mosquito-*Wolbachia* association.

Results

Prevalence of Wolbachia in wild-caught mosquitoes

A total of 271 adult mosquitoes, representing 41 species and 14 genera, were collected from twelve localities in Singapore. Each specimen was dissected into three parts (leg, gut, and reproductive organ) and screened individually for the presence of *Wolbachia* using *wsp* primers [see Additional file 1]. Overall, infection prevalence was moderate with 119 out of 271 (43.9%) individuals screened positive for *Wolbachia*. In total, 21 (51.2%) species were positive for *Wolbachia*. Out of which, infection in eight species is reported here for the first time [Additional file 1]. All genera, except for *Aedeomyia*, *Anopheles*, and *Mimomyia* (i.e. 11 out of 14 genera; 78.6%), had positive detection of *Wolbachia*. Five out of seven *Aedes* species (71.4%) had positive detections of *Wolbachia*, while in the genus *Culex*, five out of 17 species (29.4%) are positive. Some of the positively screened species in the genera *Aedes* and *Culex*, such as *Aedes albopictus* and *Culex quinquefasciatus*, are medically important vector species which are responsible for the transmission of arboviral diseases and filariasis [87].

The infection rates vary across mosquito species. Notably, we observed variation in the percentage of infection between species that are epidemiologically related to each other. For instance, there was no *Wolbachia* infection detected in *Aedes aegypti*, the primary vector of dengue and Zika viruses [88]. However, infection was moderately high (56.8%) for *Aedes albopictus* which is considered as the secondary vector of dengue. We also observed a difference in the infection rate of two closely-related species *Culex pseudovishnui* (86.4%) and *Culex vishnui* (0%) [53], with the latter found to naturally harbour Japanese encephalitis virus in the Southeast Asian region [89].

Locality did not seem to play a part in the infection of mosquito hosts. Among species that have a wide range across Singapore, we observed that percentage infection was consistent in populations across different habitats. For example, the infection percentage was consistently high for *Culex pseudovishnui*, while consistently low for *Malaya genurostris*. Based on observation, species identity was better at predicting infection status than locality differences.

We explored the effect of sex on infection status using a binary logistics regression on a data subset containing 153 individuals (45.8% males) representing twelve species. We ran the regression on a data subset to ensure that both sexes of each species were similarly represented, preventing the introduction of bias due to unequal representation of sex. Sex was a significant explanatory variable and there was a significantly lower infection prevalence in males than females with an odds ratio of 0.434 ($p = 0.013$, $D_f = 151$).

Localisation of Wolbachia infection within a mosquito

Only individuals (n = 159) which had successful amplification of the *COI* fragment across all three tissues were included in the analyses. *Wolbachia* infection was mainly observed in the reproductive tissues. Among 159 reproductive tissues, 42.1% (n = 67) were infected. Percentage infection was lower among gut (5.7%, n = 9) and leg (3.1%, n = 5) tissues, respectively. The difference in percentage infection across the three dissected tissues was statistically significant ($p < 0.001$, $D_f = 2$). As a follow-up, percentage infection in the reproductive tissue was significantly higher than both the gut ($p < 0.001$) and leg tissue ($p < 0.001$). The difference in percentage infection between the gut and leg tissue was not significant ($p = 1.0$). Notably, the amplicon size of *wsp* in the gut and leg tend to be shorter than 400 base pairs.

Wolbachia diversity among mosquito fauna from Singapore

Following Zhou *et al.* [60], all *wsp* sequences obtained in this study can be broadly classified into A and B *Wolbachia* supergroups – out of 21 positively infected species, six were infected with supergroup A, ten with supergroup B, and one species, *Aedes albopictus*, were infected with both (Fig. 2). Infection of the remaining four species was unclassified due to either short sequences (< 400 bp) or sequence alignment issues during phylogenetic analyses. The analysed *wsp* sequences were also clustered into twelve putative strains which are labelled from “Wol 1” to “Wol 12” respectively. Four out of the twelve putative strains matched to previously typed strains [60, 90] – Wol 1, Wol 2, Wol 3, and Wol 8 were identified as wPip, wAlbB, wCra, and wRi strain respectively. Wolbachia strains from this study are also closely related to those from other insect groups (Fig. 2). For instance, Wol 9 and Wol 10 are closely related to the Wolbachia strains harboured by Drosophila spp. (bootstrap value > 99%).

Host specificity of Wolbachia strains

The degree of host specificity varied across the twelve putative strains. We considered seven out of the twelve strains (Wol 2, Wol 4, Wol 5, Wol 6, Wol 8, Wol 10, and Wol 12) as specialists. These strains were host specific and were only detected in one host species each (Fig. 3). The remaining five strains were considered as generalists as they were found in more than one host. Wol 3 was found in three host species, *Coquillettidia crassipes*, *Mansonia* sp. 1, and *Culex sitiens*, the most out of the generalists. Standardised phylogenetic host specificity (SPS) score was calculated for each generalist strain (Table 1). The SPS scores revealed that Wol 1 had the lowest degree of host flexibility ($z = -1.41$) and this was significant ($p = 0.049$). On the other hand, Wol 7 had the highest degree of host flexibility ($z = 0.07$), but this was not significant ($p = 0.779$)

Table 1
Standardised phylogenetic host specificity score of putative *Wolbachia* generalists.

Wolbachia putative strain	Number of infected hosts	Phylogenetic host specificity score	Standardised phylogenetic host specificity score	p-value
Wol 1	2	0.281	−1.41	0.049*
Wol 3	3	0.391	−0.162	0.421
Wol 7	2	0.281	0.068	0.779
Wol 9	2	0.281	−0.234	0.249
Wol 11	2	0.281	−0.817	0.157

Evolutionary relationship between mosquitoes and Wolbachia

We recorded 18 counts of mosquito-*Wolbachia* associations in wild-caught mosquitoes from Singapore. A visualisation of these associations using a tanglegram showed patterns of broad associations (Fig. 3). For instance, the clade which consists of *Aedes* species was observed to be mostly associated with *Wolbachia* supergroup A. In contrast, other species, especially the clade representing various *Culex* species, had numerous associations with *Wolbachia* supergroup B. Nevertheless, there were several one-to-many and many-to-one host-endosymbiont associations which complicated the general mosquito-*Wolbachia* association pattern.

The distance-based quantitative test showed that mosquito and *Wolbachia* phylogenies were weakly congruent at the global level (ParaFit Global = 0.006, p = 0.048). Among the numerous host-endosymbiont links, only the association between *Mansonia sp.* 1 and Wol 3 was statistically significant (p = 0.031) (Fig. 3).

The event-based analysis between mosquito and *Wolbachia* phylogenies resulted in a reconstructed output of one co-speciation event, four counts of duplication, six counts of duplication with host shift, 31 losses, and six counts of failure to diverge, amounting to a total cost of 53 (Fig. 4). Interestingly, the number of duplications with a host shift and losses was much greater than co-speciation events. Notably, multiple host shift events tend to follow after loss events occurring earlier in the evolutionary history of the endosymbiont. For example, we see instances of consecutive host shifts to new hosts that were not previously infected (red arrows in Fig. 4). Additionally, based on random tip mapping, 14% of random solutions have costs lower than the reconstructed output. Overall, there was support for multiple host shift events and losses of *Wolbachia* among the mosquitoes, and there was no clear signal for mosquito-*Wolbachia* co-phylogeny.

Discussion

Detection of Wolbachia infection and distribution in wild mosquitoes

Using PCR-based *Wolbachia* screening, we successfully detected *Wolbachia* infections in wild-caught mosquitoes. The PCR-based method has a high positive detection rate with 86.3% of all sequenced amplicons having successful BLAST matches to *Wolbachia*. This suggests that the conventional PCR method is adequate in detecting *Wolbachia* infection. Even if the study was to proceed without the additional DNA sequencing step to confirm infection, an observation of an amplicon band would likely indicate a true positive *Wolbachia* infection.

Based on our results, *Wolbachia* is highly widespread across members of the Culicidae family. Here, we report infection in eight mosquito species that have not been previously described for harbouring *Wolbachia*. Overall, the percentage infection of screened individuals was 43.9% which was largely congruent with percentages reported in past studies from the Oriental region: 31% infection in Malaysia [91], 26.4% in Sri Lanka [39], and 61.6% in Thailand [92]. At the species level, past studies reported *Wolbachia* infection in 40% of all tested species in India [93], 18.2% in Sri Lanka [39], 51.7% in Taiwan [94], and between 28.1% and 37.8% in Thailand [92, 95]. Our study showed that 51.2% of all tested species were infected with *Wolbachia* which is generally higher than previous studies. This is likely attributed to the broad range of species tested in this study, including species that are usually not included in these studies – species from the *Malaya*, *Verrallina*, and *Zeugnomyia* genera [95]. It is also possible that infection prevalence may vary across geographical regions.

Wolbachia detection in three medically important mosquito genera, *Culex*, *Anopheles*, and *Aedes*, was highly consistent with past studies. Among the *Culex* mosquitoes, *Wolbachia* infection has been reported to be variable across its member species [39, 46, 92, 94]. Similarly, we observed infection only in five out of 17 *Culex* species. We noticed moderately high *Wolbachia* infection in *Culex quinquefasciatus* which is a member of the *Culex pipiens* complex responsible for the transmission of filariasis worm disease in Singapore [87, 88]. Surprisingly, between closely-related species, *Culex pseudovishnui* and *Culex vishnui* [96], we only found high percentage infection in the former. However, previous studies in India and Thailand showed a reverse pattern – infection in *Cx. vishnui* and not in *Cx. psuedovishnui* [39, 95]. Even though the two species are morphologically similar [53], species misidentification in our study was unlikely as we had carried out DNA barcoding to verify the identification of the species. This lends further support that infection prevalence may vary between populations that are found in geographically distal regions.

We did not detect *Wolbachia* in any of the wild-caught *Anopheles* species (18 individuals representing three species) examined in this study, many of which are potential malaria vectors [87]. This is largely consistent with previous reports published globally [39, 97, 98]. The absence of *Wolbachia* in *Anopheles* mosquitoes is thought to be due to the unsuitability of *Anopheles* reproductive tissues for *Wolbachia* establishment [94, 95]. However, in recent years, there are reports of sporadic *Wolbachia* detection in field *Anopheles* mosquitoes [42, 99, 100]. Knowledge of natural *Wolbachia* infections in *Anopheles* mosquitoes has implications on malaria control strategies [100], hence more wild-caught *Anopheles* samples should be screened to determine the infection status in Singapore more accurately.

We did not detect *Wolbachi*a infection in *Aedes aegypti*, the primary vector of dengue in the Southeast Asian region [88]. Conversely, *Wolbachia* infection was moderately high in the secondary vector *Aedes albopictus*. This pattern is highly consistent with past studies that found an absence of infection in wild *Ae. aegypti* [21, 101], but stable infection in wild *Ae. albopictus* [102]. Although *Ae. aegypti* and *Ae. albopictus* belong to the same subgenus Stegomyia, and occupy similar ecological niches [103], they are rarely found in the same locality which was likewise observed in this study [43, 104, 105]. This could imply a certain degree of competitive exclusion between the two species, preventing them from occupying the same space. There is evidence showing that symbionts may influence host's resource acquisition and specificity which ultimately lead to competitive exclusion between closely related host species with differing symbiont infections [106, 107]. However, research on *Wolbachia*-induced competitive exclusion is scarce except for a few studies that have looked at heterogonic gall wasps [108], grasshoppers [109], and gall-inducing aphids [110]. Given the widespread influence of *Wolbachia*, future research can explore potential cases of *Wolbachia*-induced competitive exclusion between closely related species which have a huge implication on understanding symbiosis and speciation.

Additionally, given the frequent artificial *Wolbachia* infection into *Ae. aegypti* for bio-control purposes [111–115], our finding could suggest that *Ae. aegypti* might not be stably maintained in the wild. This can be advantageous for vector population suppression as the cytoplasmic-incompatibility effect of any artificially introduced *Wolbachia* strain will likely be fully manifested in the uninfected native population [21]. However, this also implies that such a bio-control method may have low long-term effectiveness if the infection cannot be naturally sustained in the wild population. Natural *Wolbachia* infection in wild *Ae. aegypti*, therefore, has a huge implication on vector control programmes [21]. Not only does it inform the selection of *Wolbachia* strain prior to its field-release, but it can also be used to gauge the long-term effectiveness of the programme.

Interestingly, there was an effect of sex on infection status. From our study, females had higher odds of being infected than males. This could be an artefact of the various reproductive phenotypes induced by *Wolbachia* such as parthenogenesis and male-killing. They result in offspring that are largely female or end up female only because males are killed [15]. Over multiple generations with vertical *Wolbachia* transmission, one would observe an increasing proportion of females that are infected. Hence, the phenomenon could be a consequence of *Wolbachia's* reproductive manipulation and vertical transmission.

We were unable to statistically test for the effects of locality on infection status due to uneven and small sample sizes of the respective species across different localities. Nevertheless, we documented that infection status was consistent across localities for widespread species such as *Culex pseudovishnui* and *Malaya genurostris*. This implies that mosquitoes found in localities across Singapore have roughly equal chances of having *Wolbachia*. This also suggests that underlying physiological factors and phylogenetic relatedness in mosquitoes contributed more to the *Wolbachia* infections than the habitat which they are found in.

The reproductive effect of *Wolbachia* can be masked or enhanced by other endosymbionts and there are studies which looked at infections with other reproductive endosymbionts such as *Cardinium*, *Rickettsia*, and *Spiroplasma* [7, 26–29]. Unfortunately, in our preliminary screening, we were unable to detect those endosymbionts due to a high degree of false positives using PCR-based screening methods [Additional file 2]. This is likely attributed to primers which were not optimised for mosquito screening [116–118]. As a result, we were unable to identify co-infections of various reproductive endosymbionts among wild mosquitoes which would have provided greater insights into the synergistic effects of co-infections on mosquito evolution. There is, hence, a need to develop and optimise alternative screening methods, such as multilocus sequence typing (MLST) techniques, especially for the detection of *Cardinium*, *Rickettsia*, and *Spiroplasma* in mosquitoes.

Localisation of Wolbachia infection in mosquitoes

In this study, we detected *Wolbachia* mainly in the reproductive tissues which is in line with past studies across multiple insect groups [15, 94, 119]. This suggests the vertical transmission of *Wolbachia*. Interestingly, through the course of this study, we noticed a significant variation in the size of reproductive traits (testis and ovary length) across and within species. These reproductive traits did not vary significantly with *Wolbachia* infection status, even after accounting for phylogenetic relatedness [see Additional file 3].

We also detected infection in the gut and leg tissues, albeit infrequently. This is not surprising as previous reports have detected *Wolbachia* in those tissues [34–36, 120]. Interestingly, the nucleotide sequences from gut and leg infections tend to be shorter in length. Considering that *Wolbachia* is unlikely to survive extracellularly for a long duration [35], the small amplicon size suggests potential horizontal integration of the *Wolbachia* genome into the host genome for a few species. This phenomenon has been observed in several *Wolbachia* hosts [121, 122], and mosquito species such as *Aedes aegypti* and *Culex quinquefasciatus* [123, 124]. For instance, a recent study showed that horizontal integration of *Wolbachia* genome into the host genome can have sex determination and evolution implications. This is evident in the common pillbug *Armadillidium vulgare*, resulting in the formation of a new sex chromosome [125]. Researchers have also proposed that horizontal gene transfer between endosymbiont and host can result in evolutionary innovation where new functional genes arise for both host and bacteria [123, 124].

Future research should explore the relative importance of each transmission method with relation to host-endosymbiont ecology and evolution. Such tissue-specific screening methods can be used in other arthropods especially when the mode of transmission is not clear. Currently, most *Wolbachia* screening is conducted on ground specimens or specimens in their entirety [39–41]. By doing so, researchers would be unable to localise *Wolbachia* infection within an individual which could have provided clues to its mode of transmission. In this context, adopting tissue-specific screening methods can seek to verify or refute the assumption that *Wolbachia* is transmitted vertically which is common in literature [15, 30].

Diversity and host-specificity of Wolbachia strains

Wolbachia wsp sequences generated in this study were clustered into twelve putative *Wolbachia* strains falling with the supergroup A or B which is consistent with previous studies that looked at *Wolbachia* infections in mosquitoes [39, 90, 95]. Each mosquito host species was only infected by strains belonging to A or B, with the exception of *Aedes albopictus* which harboured both. Infection of more than one strain (superinfection of wild *Ae. albopictus* with *Wolbachia* supergroup A and B) has been previously reported, and this phenomenon was commonly observed to be fixed in those examined populations due to strong cytoplasmic incompatibility effects [126, 127]. This suggests stable vertical transmission of both strains in *Ae. albopictus*. Additionally, only four out of twelve putative strains were identified to previously typed *Wolbachia* strains reported by Zhou *et al.* [60] and Ruang-Areerate *et al.* [90].

Host specificity is thought to be a characteristic of the ancestral *Wolbachia* strain, with host flexibility reported mainly in *Wolbachia* supergroup A and B [128]. In our study, we found a combination of specialists and generalists with more counts of the former. A study of mosquitoes from Taiwan showed a similar pattern [94]. In bees, which harboured supergroup A *Wolbachia*, a mixture of host-specific and host flexible strains in the population has also been reported [49]. While our estimates of specialists and generalists could vary with greater sampling effort, the higher numbers of specialists observed can be explained by the process of reciprocal selection between host and endosymbiont over evolutionary time [75]. This is also known as the “Red Queen” dynamics, where the endosymbiont constantly adapts to its host to ensure continued establishment in the same host [129]. An alternative strategy of being a generalist can also be maintained in a population. It ensures survivorship in an environment where resources (i.e. hosts) are rarely found [75]. However, there are generally more instances of host specialists than generalists across numerous parasitic and endosymbiotic taxa [130–132].

The standardised phylogenetic host specificity scores revealed that host flexibility among generalists varied greatly. Wol 1 had the lowest degree of host flexibility and was shown to infect mosquito hosts that are closely related: *Culex pseudovishnui* and *Culex quinquefasciatus*. Although Wol 3 infected the greatest number of mosquito species, Wol 7 was the most host flexible strain as it infected distally related *Uranotaenia trilineata* and *Malaya genurostris*. Understanding *Wolbachia* host specificity has huge implications especially for the optimisation of *Wolbachia* biocontrol strategy. Not only should researchers select strains that can limit pathogen replication [133], they should also select strains for their host specificity. This would not be possible without the screening of a wide variety of species or closely related species which was achieved in this study. A host-specific strain will decrease the likelihood of infection host shift to non-target species, thereby minimising the strategy’s overall ecological risk.

Evolutionary relationship between mosquito and Wolbachia

Host-*Wolbachia* relationships are often understudied and limited to a few taxa [52]. Current attempts to reconstruct host-*Wolbachia* evolutionary association have found co-phylogeny patterns in beetles and bedbugs [49, 50]; co-speciation with infrequent horizontal acquisitions patterns in filarial nematodes and bees [51, 52]; and evidence for host shifting across distantly related species in butterflies and moths [134]. Hence, patterns for *Wolbachia* transmission and diversification tend to vary across the various

taxa. In our study, the relationship between mosquito hosts and *Wolbachia* is highly complex, with neither co-speciation nor host shifting fully accounting for the evolutionary association in these lineages.

A broad association pattern between mosquitoes and *Wolbachia* strains was observed (Fig. 3). *Aedes* mosquitoes tend to be associated with supergroup A *Wolbachia*, while other clades, particularly the genus *Culex*, were largely associated with supergroup B *Wolbachia*. This showed that closely related *Wolbachia* strains were likely to establish themselves in related hosts. There might have been radiation of *Wolbachia* in these clades after their respective initial establishments. However, without information about the time of divergence, this could be an ecological event where closely related *Wolbachia* strains occupied similar niches.

The ParaFit analysis showed weak support for congruency between host and endosymbiont phylogenies. Among the 18 host-*Wolbachia* associations, only the link between *Mansonia* sp. 1 and *Wol* 3 showed a significant association (Fig. 3). This was interesting considering that Wol 3 was largely host flexible. Given that this was the only significant association, a genus-specific study on *Mansonia* spp. is worthy of further exploration to elucidate coevolutionary patterns within a group of closely related mosquito species. Perhaps, the degree to which *Wolbachia* coevolves with its mosquito host can vary across phylogenetic resolution [81]. The analyses thus far suggest that mosquito-*Wolbachia* associations are likely random at higher taxonomic levels with occasions of mosquito-*Wolbachia* co-speciation at finer phylogenetic resolution (i.e. like patterns seen in diffuse coevolution).

Referring to the least cost coevolutionary construction by Jane 4.0 (Fig. 4), co-speciation events were infrequent as compared to other evolutionary events. We noticed a greater proportion of host shifts and numerous losses. Interestingly, Jane indicated multiple consecutive host shifts occurring near the tips of the cladogram. This suggests that co-speciation does not fully explain the evolutionary association between mosquito hosts and *Wolbachia*. Instead, recent host shifting through horizontal transmission seems to promote *Wolbachia* diversification. This lends greater support that horizontal transmission between distantly related species is possible [32, 33, 135]. The consecutive host shifts are possible cases of *Wolbachia* radiation by host shift and that horizontal transmission is likely to better explain the observed *Wolbachia* diversity than vertical transmission. However, an absence of host shifts during early parts of Wolbachia's evolutionary history questions the effectiveness of horizontal transmission in maintaining infection over generations.

Furthermore, losses, which represent endosymbiont extinction events that occurred upon host speciation, seemed to dominate the evolutionary history of *Wolbachia*. Extinction events are believed to be frequent in host-endosymbiont systems [75], due to either evolution of resistance in the host or declining host population size which results in the inability for highly specialised endosymbionts to establish themselves [136, 137]. Additionally, losses could potentially influence endosymbiont evolution through the creation of vacant niches [136]. The observed losses followed by host shifts in the mosquito-*Wolbachia* relationship are possible consequences of vacant niche exploitation by generalists. Perhaps, this enabled successful endosymbiont invasion due to minimal intra-strain competition. Therefore,

horizontal *Wolbachia* transmission and losses may play a bigger role in accounting for *Wolbachia* diversity than previously expected.

Based on our study, it is difficult to determine the mechanism which explains *Wolbachia*'s diversity and evolutionary association. The presence of numerous specialists could be a sign of mosquito-*Wolbachia* coevolution since coevolution is fundamentally reciprocal selection between host and endosymbiont which gives rise to micro-evolutionary changes [138]. Being highly adapted to their hosts could imply strain level evolution. The numerous host shifts and losses might have, however, blurred the effects of vertical transmission over the long evolutionary period [52]. Thus, we propose that co-speciation might have occurred within smaller clades, but at the broader perspective, horizontal transmission and loss events are more likely the prominent force driving *Wolbachia* evolution.

The *Wolbachia wsp* gene has been shown to provide phylogenies with a good resolution [60], and our study provides an exploratory snapshot of the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. Of course, this is a potential caveat since we only used a single gene each to construct the respective phylogenetic trees. To obtain a more accurate phylogeny, future studies can adopt MLST [17, 51], or whole-genome shotgun sequencing in their methods [52]. The former could potentially characterise putative *Wolbachia* strains that cannot be distinguished with *wsp* gene primers.

A limitation of an event-based analysis (Jane 4.0) is that such a method cannot distinguish between topological congruence and an evolutionary event [75]. It is ecologically likely that there might be a co-phylogenetic coincidence in a eukaryote-bacteria relationship given that bacterial lineages often evolve faster than the hosts [139, 140], and that host shifts among closely related species are highly likely [138]. In other words, our event-based analysis does not take into account the time of divergences for both symbiont and host and is, thus, unable to accurately differentiate co-phylogeny and co-speciation.

Notwithstanding the limitations, the employment of various analytical methods allows for a comprehensive study of the evolutionary association between *Wolbachia* and mosquito hosts which are lacking in current literature. Using single genes to reconstruct evolutionary trees, this study serves as an initial exploratory study which examined mosquito-*Wolbachia* evolutionary associations across a wide range of host mosquito species. Future studies interested in the evolution of medically important vector species could narrow their scope on the Aedini tribe which will provide greater statistical power for the examination of mosquito-endosymbiont association.

Conclusion

This is the first study which examines *Wolbachia* infections in the wild mosquitoes of Singapore. We detected twelve putative strains of *Wolbachia* among 41 mosquito species and recorded new infections in eight species which had not been reported before. By employing the tissue-specific PCR screening method, we observed that *Wolbachia* infections were preferentially located in the reproductive tissues which provided strong support for vertical transmission as the main mode of infection transmission. However, if infections are mainly transmitted vertically, it is unlikely to fully explain the observed

Wolbachia diversity and how closely related *Wolbachia* lineages were found in distally related mosquito species. Hence, this study also served as an exploratory study which examined mosquito-*Wolbachia* evolutionary associations through three evolutionary analyses. Overall, we propose that the evolutionary associations between mosquito hosts and *Wolbachia* are consequences of both vertical and horizontal transmission modes and various evolutionary events.

Abbreviations

BLAST
Basic local alignment search tool; CDC:Centers for Disease Control and Prevention; *COI*:*cytochrome c oxidase subunit I*; CI:Cytoplasmic incompatibility; MLST:Multilocus sequence typing; mtDNA:Mitochondrial DNA; NJ:Neighbour-joining; SPS:Standardised phylogenetic host specificity; *Wsp*:*Wolbachia* surface protein.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the Dryad repository, http://doi.org/10.5061/dryad.zs7h44j63. Sequence data that support the findings of this study have been deposited in Genbank with the accession codes MT645167–MT645184.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HY and NP designed the research. HD and HY collected mosquitoes from the field. HY identified mosquito samples. HD performed DNA extraction and PCR. HD and HY carried out sequence and phylogenetic

analyses. HD, HY, and NP interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

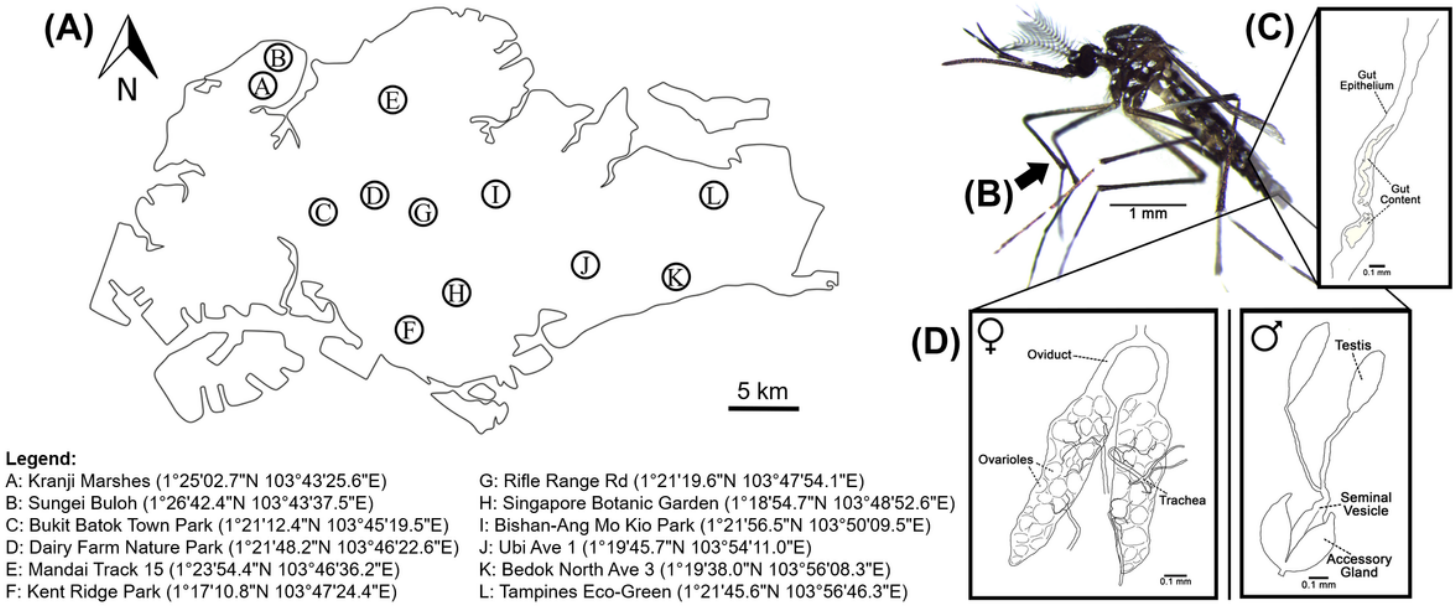

Figure 1

Map of sampling sites and diagrammatic image of Aedes aegpyti with its dissected tissues. A – various mosquito collection localities across Singapore and their respective coordinates; B – mosquito leg; C – gut; D – female reproductive tissue (left) and male reproductive tissue (right).

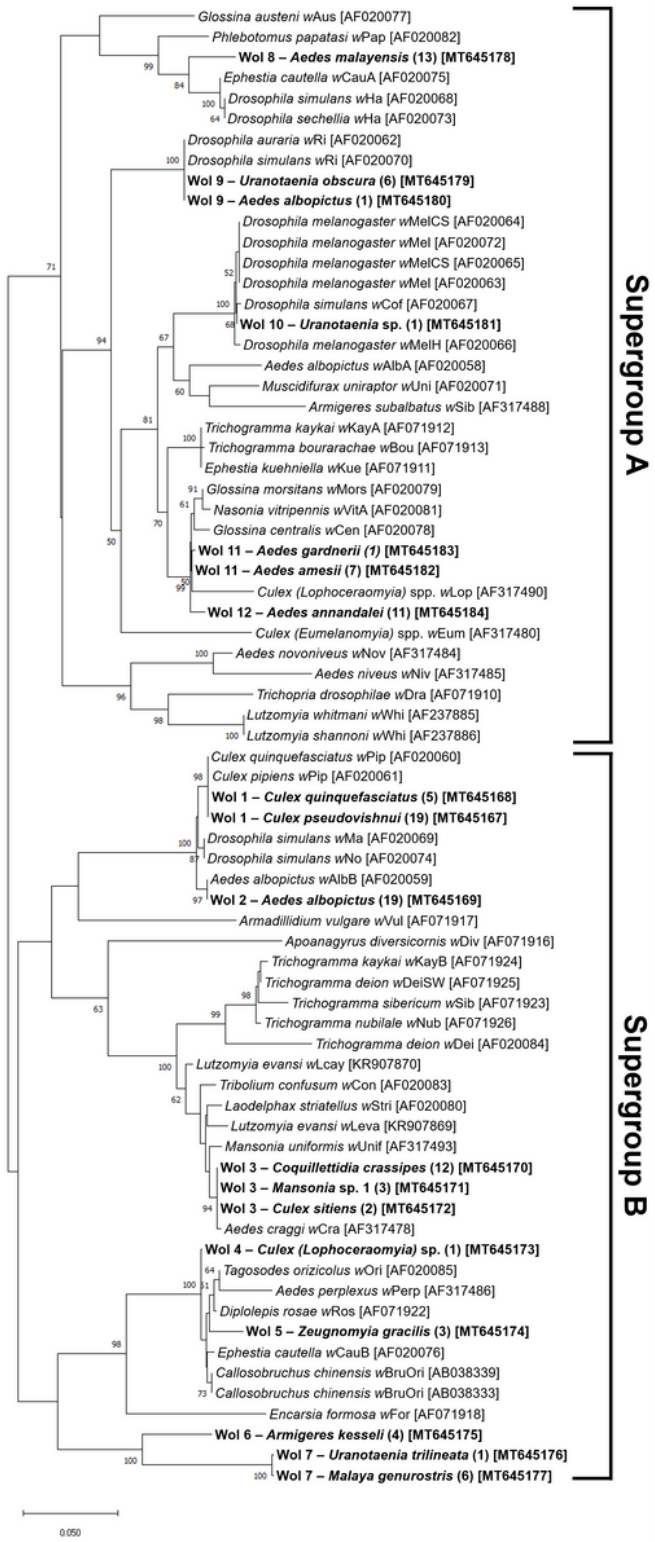

Figure 2

Wolbachia neighbour-joining tree constructed with Wolbachia wsp gene. All analysed sequences generated from this study (bold) were broadly classified into either Wolbachia supergroup A or B and clustered into 12 putative strains “Wol 1 – Wol 12”. The number of sequences of each putative strain is indicated within the brackets. Also included are 54 sequences obtained from GenBank. Taxa are labelled as the host from which the Wolbachia strain was isolated, followed by the strain name. Neighbour-joining

tree was mid rooted due to a lack of appropriate outgroup [45]. Bootstrap probability (generated with 1000 replicates) higher than 50% are indicated on the tree. Genbank accession number of each sequence is indicated within square brackets.

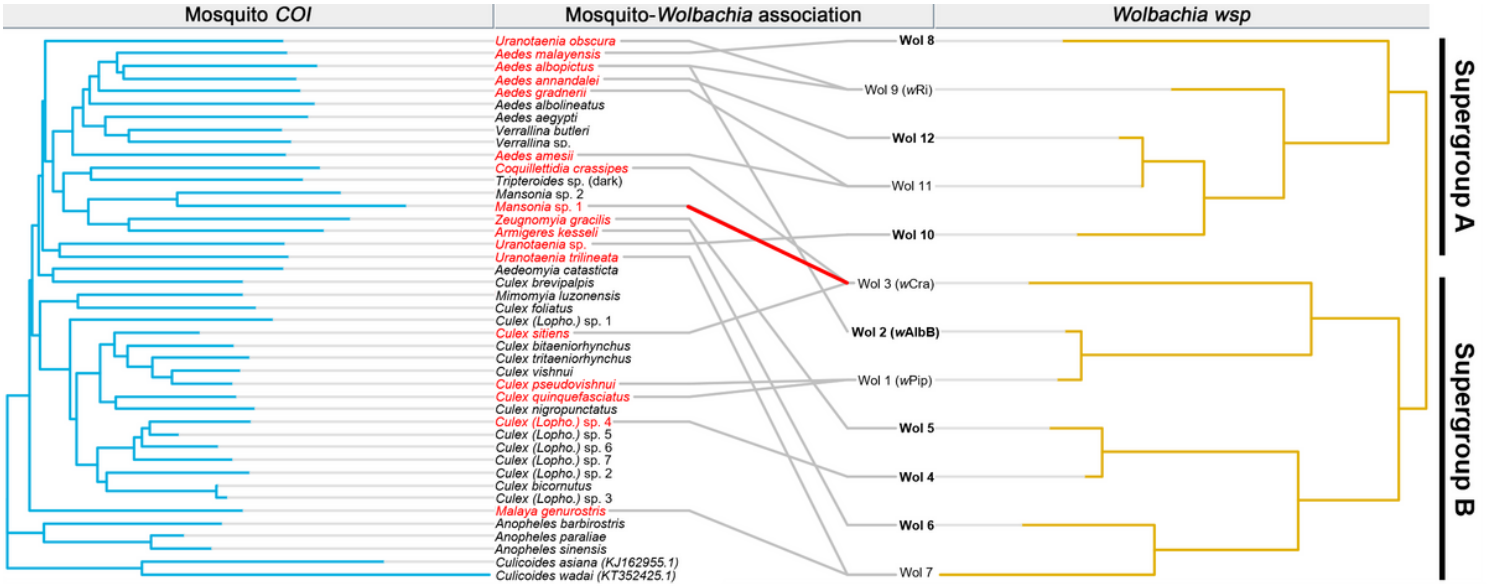

Figure 3

Tanglegram of mosquito COI neighbour-joining (NJ) tree compared to Wolbachia endosymbiont NJ tree. Mosquito host species which harboured Wolbachia infection are indicated in red. Specialist Wolbachia strains are bolded. Grey lines represent the associations between hosts and endosymbionts. A red line indicates the host-endosymbiont association that was significant in the Global ParaFit test of congruence between host and endosymbiont phylogenies (p = 0.031).

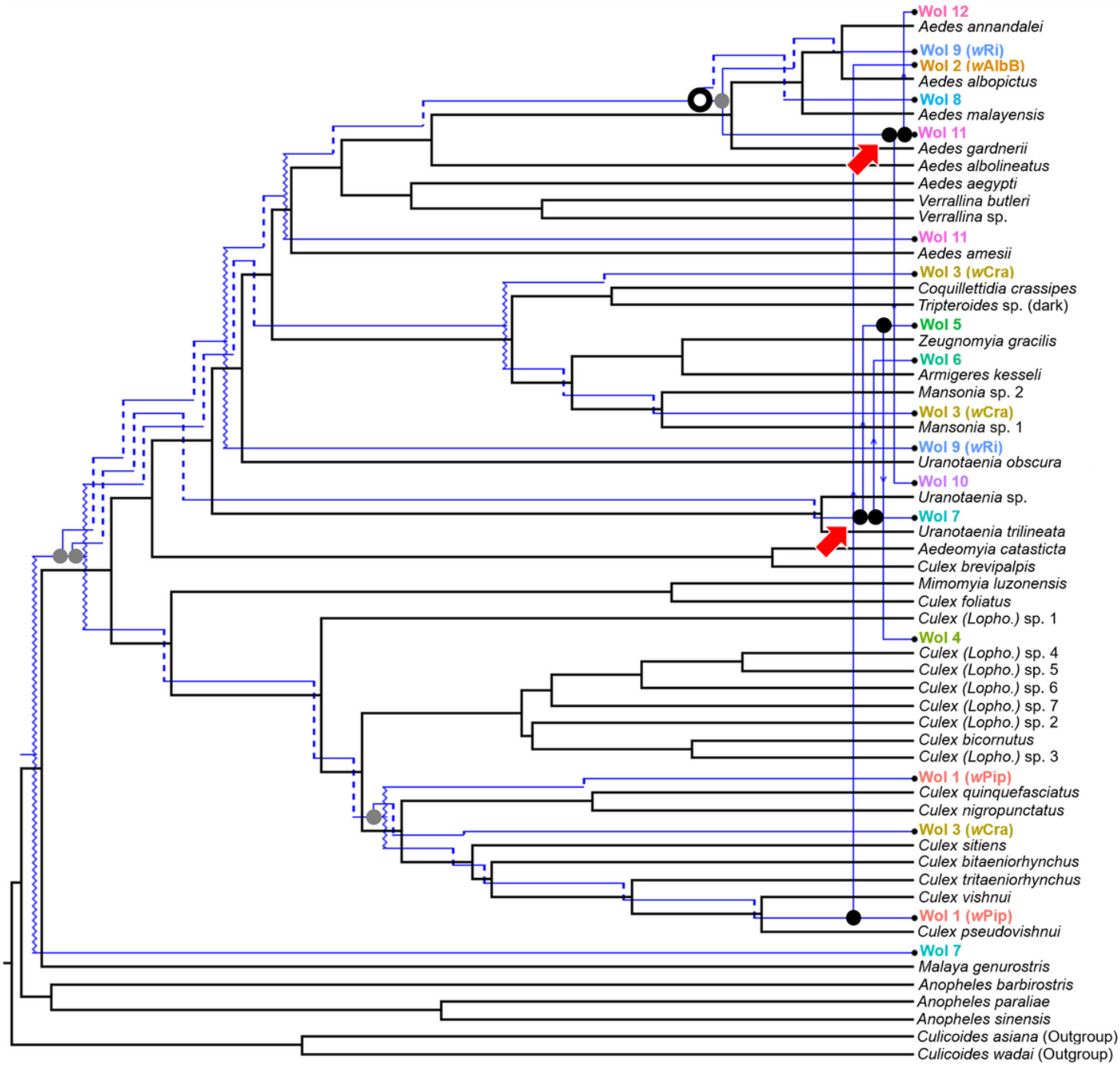

Figure 4

Least cost evolutionary reconstruction between mosquito (black) and Wolbachia (blue) phylogenies achieved using Jane 4.0. In total one co-speciation event (open circle), four counts of duplication (grey dot), six counts of duplication with host shift (black dot with an arrow pointed outwards), 31 losses (dotted line), and six counts of failure to diverge (squiggly line) were mapped out. Red arrows indicate periods where multiple host shifts occurred in succession.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DingetalWolbachiaInWildMosquitoesAdditionalfile1.docx
- DingetalWolbachiaInWildMosquitoesAdditionalfile2.docx
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- DingetalWolbachiaInWildMosquitoesGA.tif