Wolbachia infection in wild mosquitoes (Diptera: Culicidae): implications for transmission modes and host-endosymbiont associations in Singapore

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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 are several knowledge gaps in the literature and little is known about natural *Wolbachia* infection across species, transmission modes 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 for *Wolbachia* infection in the leg, gut and reproductive tissues of wild mosquitoes from Singapore using the *Wolbachia* surface protein (*wsp)* molecular marker. Mosquito-*Wolbachia* associations were explored using three methods – a tanglegram, distance-based, and event-based method, and inferred instances of vertical transmission and host shifts.

Results: Adult mosquitoes (271 specimens) representing 14 genera and 40 species were screened for *Wolbachia*. Overall, 21 species (51.2%) were found positive for *Wolbachia*, including *Aedes* (5 species) and *Culex* (5 species). Seven out of the 21 infected species were not previously reported: *Aedes* nr. *fumidus*, *Aedes annandaelei*, *Uranotaenia obscura*, *Uranotaenia trilineata*, *Verrallina butleri*, *Verrallina* sp., and *Zeugnomyia gracilis*. *Wolbachia* was predominantly detected in the reproductive tissues, an indication of vertical transmission. Despite this, *Wolbachia* infection rates vary widely within a mosquito host species. There is no clear signal of co-phylogeny between the mosquito hosts and the twelve putative *Wolbachia* strains observed in this 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* exists in 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 producing more 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 endosymbionts on host population dynamics and evolution [17,18], especially in medically important insects such as mosquitoes. The promising use of *Wolbachia* 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] by releasing infected mosquitoes. However,the presence of naturally occurring endosymbionts in the wild mosquito populations has not been adequately assessed. The release of mosquitoes artificially infected with *Wolbachia* might have a profound impact on closely interacting wild mosquito populations through various transmission modes. For instance, horizontal transmissions of theintroduced *Wolbachia* strain may result in the manipulation of the reproductive biology of non-target species, which could potentially result in an unintentional population crash, opening up niches for other vector species [25]. Another implication of such a bio-control method is the increased likelihood of co-infections with other naturally occurring *Wolbachia* strains or other endosymbionts, such as *Cardinium*, *Rickettsia*, and *Spiroplasma.* These co-infections may result in a synergistic effect on mosquito 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, keeping the precautionary principle in mind, it is important to investigate the natural occurrences of *Wolbachia*.

There is also a need to discern the main 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]. *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]. The detection of *Wolbachia* in the gastrointestinal tract suggests that it could be horizontally transmitted 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, detection of *Wolbachia* in mosquitoes mostly adopts conventional PCR methods on DNA extracted from the entire individual or its abdomen [39–47]. This limits our ability to identify the site of endosymbiont infection within an individual (tissue tropism). Tissue-specific screening of *Wolbachia* is necessary to provide insights and infer the extent of vertical and horizontal transmissions.

It has been proposed that host mitochondrial DNA (mtDNA) and *Wolbachia* are maternally co-transmitted within the cytoplasm [17,48], which predicts a 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 also not well-explored.

Currently, there has not 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 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.

This current study has three major research objectives: First, toexamine the prevalence and diversity of *Wolbachia* among wild mosquitoes from Singapore. Second, to determine the tissue tropism of *Wolbachia* infection in mosquitoes using a tissue-specific PCR screening method. Finally, to reconstruct the evolutionary associations between *Wolbachia* and their mosquito hosts to provide a basis for understanding host-endosymbiont evolution.

Methods

Adult mosquito collection and identification

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

Tissue-specific dissection

Tissue-specific dissection was carried out on each adult mosquito sample to isolate the leg, gut, and reproductive tissues (Fig. 1b – Fig. 1d). To prevent the contamination of tissues and bacteria on the external surface of the mosquito, the leg was removed first before isolating the gut and reproductive tissues.All dissection equipment and microscope slides were thoroughly wiped with 70% ethanol before commencing dissection for the next sample. Dissected tissues were individually placed into a 96-well plate on ice to prevent DNA degradation.

DNA extraction, PCR amplification, and sequencing

DNA extraction of each dissected tissue was performed using 7µL of QuickExtract™ DNA Extraction Solution (Lucigen, Madison, USA) in a thermocycler (Eppendorf, Hamburg, Germany) with the following protocol: 65°C for 18 minutes, followed by 98°C for 2 minutes, 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 to the cycle conditions. The *Wolbachia* surface protein (*wsp*) general primers, wsp81F (5'-TGGTCCAATAAGTGATGAAGAAACTAGCT-3') and wsp691R (5'- AAAAATTAAACGCTACTCCAGCTTCTGCAC-3'), were used in this study [60]. In addition, a fragment of the *cytochrome c oxidase subunit I* (*COI*) gene of the mosquito hosts using primers LCO1498 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3') was also amplified [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.

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

PCR conditions were as follow: 94°C for 5 min, followed by 35 cycles of 95°C for 30s, 55°C for 45s, and 72°C for 1 min, with a final elongation step of 72°C for 10min. Amplicons were separated by gel electrophoresis on 2% agarose gel stained with GelRed® (Biotium, Fretmont, USA) and visualised under a UV transilluminator (Syngene, Cambridge, UK). PCR products were purified using SureClean Plus (Bioline, London, UK) following the manufacturer's protocol. Samples were sequenced by the First Base Laboratories (Axil Scientific Pte. Ltd., Singapore, Singapore), using a 3730XL DNA Analyzer (Applied Biosystems, Waltham, USA). Obtained sequences were then edited and aligned using Geneious Prime (version 2019.2.3) (https://geneious.com). Similarities with publicly available sequences were assessed using the Basic Local Alignment Search Tool (BLAST) [62].

Statistical analyses

To test if there aresignificant differences in *Wolbachia* infection across the different mosquito tissues, a Cochran's Q test was carried out. As a follow-up, McNemar's post-hoc test was employed to identify the tissue pairs that differed significantly in infection. Individuals which did not amplified the internal control (*COI* gene) successfully for any of the three dissected tissues were excluded from this statistical analysis. The effect of sex on host

infection was also tested using binary logistics regression with sex as a categorical dependent variable and infection outcome as a binary independent variable. The logistics regression was conducted 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. Statistical significance was determined when the p-value is less than 0.05. All statistical analyses were performed in R version 3.6.2 [63], with packages *nonpar* [64], *rcompanion* [65], and *ISLR* [66].

Sequence analyses

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

Neighbour-joining (NJ) phylogenetic trees for mosquito hosts and *Wolbachia* were reconstructed using the sequenced *COI* gene fragment and the *wsp* gene, respectively. COI sequences from previous publications were not included considering that the aim of the research was not to compare the genetic relationship between the hosts. Instead, 54 *wsp* sequences from GenBank were included in the construction of the *Wolbachia* NJ tree. The NJ tree reconstruction was performed 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. Bootstrap probabilities were estimated by generating 1000 bootstrap replicates. We designated two biting midge 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], the *Wolbachia* NJ tree was midpoint rooted instead.

When possible, *Wolbachia* strains were classified 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, sequences which closely cluster together and have a relatively high support value (> 90%) were deemed as the same putative strain.

Putative strains which are infectious to only one host species were categorized as "specialists" and those which infect two or more hosts as "generalists". Then, the standardised phylogenetic host specificity (SPS) score of each generalist strain was calculated by adapting the method outlined by Poulin *et al.* [70] and Kembel *et al.* [71]. 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. SPS scores were calculated using R package *picante* [71].

Evolutionary analyses of the mosquito-*Wolbachia* relationship

Three distinct methods were used to explore the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. The analyses were carried out using pruned phylogenies where each species is represented by a single individual.

First, using the software TreeMap 3.0 [72], a tanglegram was created between host and endosymbiont NJ trees to visualise the mosquito-*Wolbachia* association. A tanglegram is useful as a pictorial representation of the interactions between two phylogenies [73]. TreeMap also seeks to minimise the entanglement between the two trees to provide a clearer visualisation of the phylogenetic relationship between host and endosymbiont [72].

Second, ParaFit Global test, a distance-based method, was employed to quantitatively estimate congruence between the host and endosymbiont phylogenetic trees by comparing genetic distances among infected host species and the *Wolbachia* strains [74]. 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. Significance was tested by comparing the observed associations between host and endosymbiont with randomised associations generated with 5000 permutations. The respective host-endosymbiont associations which contributed significantly to the ParaFit Global statistics were also identified. ParaFit test was performed with Caillliez correction to correct for negative eigenvalue generated [75] using R package *ape* [76].

Third, an event-based analysis was performed in Jane 4.0 [77] to map out potential evolutionary events of the endosymbiont in relation to the host phylogeny [78]. Five evolutionary events were 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). As each event is expected to have differing likelihoods, default cost values were attached to each of the events. Jane 4.0 determined the best reconstruction of evolutionary events by minimising the overall cost. The following cost-scheme regime was used 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 [79]. As a follow-up, random tip mapping (randomisation of host-endosymbiont associations) was carried out 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.

Results

Prevalence of *Wolbachia* in wild-caught mosquitoes

A total of 271 adult mosquitoes, representing 40 species and 14 genera, were collected from twelve localities in Singapore. Overall, infection prevalence was moderate with 119 out of 271 (43.9%) individuals screened positive for *Wolbachia* (Table 1). In total, 21 (51.2%) species were positive for *Wolbachia*. Out of which, infection in seven species is reported here for the first time (Table 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 16 species (31.3%) 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.

The infection rates vary across mosquito species. Notably, there was 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*. However, infection was moderately high (56.8%) for *Aedes albopictus*. There was also a difference in the infection rate of two closely-related species *Culex pseudovishnui* (86.4%) and *Culex vishnui* (0%) [53].

Locality did not seem to play a part in the infection of mosquito hosts. Among species that have a wide range across Singapore, 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.

The effect of sex on infection status was explored using a binary logistics regression on a data subset containing 153 individuals (45.8% males) representing twelve species. The regression was conducted on a subset of the data to ensure that both sexes of each species were similarly represented, preventing biased analysis on a dataset with unequal representation of each 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 ($Z = -2.48$, $P = 0.013$, $df = 151$).

Tissues tropism of ***Wolbachi***a infection in mosquitoesOnly 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 the dissected 159 reproductive tissues, 42.1% (n = 67) were infected. Percentage infection was lower in the 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 ($Q = 109.5$, $P < 0.0001$, $df = 2$). The percentage infection in the reproductive tissue was significantly higher than both the gut ($P < 0.0001$) and leg tissue ($P < 0.0001$), while 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*, was infected with both supergroups (Fig. 2). Infection of the remaining four species (*Culex tritaeniorhynchus*, *Tripteroides* sp., *Verrallina butleri*, and *Verrallina* sp.) was unclassified due to either short sequences (< 400bp) or sequence alignment issues during sequences analyses. The analysed *wsp* sequences were also clustered into twelve putative strains which are labelled from “Wol 1” to “Wol 12” respectively. Four (Wol 1, Wol 2, Wol 3, and Wol 8) out of the twelve putative strains matched to previously typed strains [60,80]. *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. Seven out of the twelve strains (Wol 2, Wol 4, Wol 5, Wol 6, Wol 8, Wol 10, and Wol 12) were considered 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 indiana*, and *Culex sitiens*, the most out of the generalists. 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 2).

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.

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 indiana* 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, three counts of duplication, seven counts of duplication with host shift, 29 losses, and six counts of failure to diverge, amounting to a total cost of 52 (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 no clear signal for mosquito-*Wolbachia* co-phylogeny.

Discussion

Detection of *Wolbachia* infection and distribution in wild mosquitoes

In this study, the PCR-based *Wolbachia* screening 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 for *Wolbachia* detection. Even if the study was to proceed without the additional DNA sequencing step, an observation of an amplicon band would likely indicate a true positive. Focusing on our results, *Wolbachia* is highly widespread across members of the Culicidae family. Here, we report infection in seven 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 [81], 26.4% in Sri Lanka [39], and 61.6% in Thailand [82]. At the species level, past studies reported *Wolbachia* infection in 40% of all tested species in India [83], 18.2% in Sri Lanka [39], 51.7% in Taiwan [84], and between 28.1% and 37.8% in Thailand [82,85]. Our study showed that 51.2% of all tested species were infected with *Wolbachia* which is generally higher than most studies. This is likely attributed to the broad range of species tested in this study, including species from the genera *Malaya*, *Verrallina*, and *Zeugnomyia* [85]. 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. These genera are responsible for the transmission of vector-borne diseases such as filariasis, malaria, and arboviral diseases [86]. Among the *Culex* mosquitoes, *Wolbachia* infection has been reported to be variable across its member species [39,46,82,84]. Similarly, infections were observed only in five out of 16 *Culex* species. We noticed moderately high *Wolbachia* infection in *Culex quinquefasciatus* (62.5%) which is a member of the *Culex pipiens* complex responsible for the transmission of filariasis worm disease in Singapore [86,87]. Surprisingly, between two closely related species, *Culex pseudovishnui* and *Culex vishnui* [88], no *Wolbachia* infection was observed in the latter which has been found to harbour Japanese encephalitis virus in the Southeast Asian region [89]. However, studies in India and Thailand showed a reverse pattern, with *Wolbachia* infection present in *Cx. vishnui* but not in *Cx. pseudovishnui* [39,85]. Although the two species are morphologically similar [53], in this study, DNA barcoding was conducted to aid morphological identification and thus, avoid any misidentification. This lends further support that infection prevalence may vary between populations which are distal geographically.

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 [86]. This is largely consistent with previous reports published globally [39,90,91]. The absence of *Wolbachia* in *Anopheles* mosquitoes is thought to be due to the unsuitability of *Anopheles* reproductive tissues for *Wolbachia* establishment [84,85]. However, in recent years, there are reports *Wolbachia* detection in field *Anopheles* mosquitoes from West Africa [42,92,93] and Malaysia [94]. Knowledge of natural *Wolbachia* infections in *Anopheles* mosquitoes has implications on malaria control strategies [93], hence more wild-caught *Anopheles* samples should be screened to determine the infection status in Singapore more accurately.

*Wolbachi*a was not detected in *Aedes aegypti*, the primary vector of dengue in the Southeast Asian region [87]. Conversely, *Wolbachia* infection was moderately high in the secondary vector *Aedes albopictus*. This pattern is highly consistent with past studies which reported an absence of infection in wild *Ae. aegypti* [21,95], but found stable infection in wild *Ae. albopictus* [96]. Although *Ae. aegypti* and *Ae. albopictus* belong to the same subgenus *Stegomyia*, and occupy similar ecological niches [97], they are rarely found in the same locality which was likewise observed in this study [43,98,99]. 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 [100,101]. However, research on *Wolbachia*-induced competitive exclusion is scarce except for a few studies on heterogonic gall wasps [102], grasshoppers [103], and gall-inducing aphids [104]. Given the widespread influence of *Wolbachia*, future research can explore potential cases of *Wolbachia*-induced competitive exclusion between closely related species which will have a huge implication on understanding symbiosis and speciation.

Additionally, given the frequent artificial *Wolbachia* infection into *Ae. aegypti* for bio-control purposes [105–109], our findings could suggest that infected *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. The detection of 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 suitable *Wolbachia* strain prior to its field-release, but it can also be used to gauge the long-term effectiveness of the vector control programme.

Interestingly, the sex of the mosquitoes had an effect on *Wolbachia* infection status. This could be an artefact of the various *Wolbachia*-induced reproductive phenotypes such as parthenogenesis and male-killingresulting in offsprings which are largely female [15]. Over multiple generations with vertical *Wolbachia* transmission, one would observe an increasing proportion of females that are infected. Hence, this phenomenon could be a consequence of *Wolbachia's* reproductive manipulation and vertical transmission.

While 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, our results suggest that mosquitoes found in localities across Singapore have roughly equal chances of harbouring *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 reproductive endosymbionts such as *Cardinium*, *Rickettsia*, and *Spiroplasma* [7,26–29]. Unfortunately, we were unable to detect those endosymbionts due to a high degree of false positives using PCR-based screening methods [Additional file 1]. This is likely attributed to primers which are not optimised for screening mosquito-specific endosymbionts [110–112]. As a result, co-infections of various reproductive endosymbionts were not identified 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.

Tissue tropism of *Wolbachia* infection in mosquitoes

Wolbachia was detected mainly in the reproductive tissues which corroborates with results from studies across multiple insect groups [15,84,113],suggesting that *Wolbachia* is mainly vertically transmitted. Interestingly, through the course of this study, there was 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 2].

Infection in the gut and leg tissues was detected, albeit infrequently. This is not surprising as previous reports have detected *Wolbachia* in those tissues [34–36,114]. 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 [115,116], and mosquito species such as *Aedes aegypti* and *Culex quinquefasciatus* [117,118]. 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 [119]. 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 [117,118].

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 grounded specimens or specimens in their entirety [39–41]. By doing so, researchers would be unable to determine tissue tropism of *Wolbachia* infection which could 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

Not only does the *wsp* molecular marker allow successful detection of *Wolbachia* infection across numerous taxa, it also enables strain genotyping and evolutionary comparison between detected *Wolbachia* strains [60]. In this study, *Wolbachia wsp* sequences were clustered into twelve putative *Wolbachia* strains falling within the supergroup A or B. This is consistent with previous studies that looked at *Wolbachia* infections in mosquitoes [39,80,85]. 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 [120,121]. 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.* [80] – Wol 1, Wol 2, Wol 3, and Wol 8 were identified as *w*Pip, *w*AlbB, *w*Cra, and *w*Ri strain respectively.

Host specificity is thought to be a characteristic of the ancestral *Wolbachia* strain, with host flexibility reported mainly in *Wolbachia* supergroup A and B [122]. 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 [84]. In bees, a mixture of *Wolbachia* supergroup A host-specific and host flexible strains in the population has also been

reported [49]. While our estimates of specialists and generalists might 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 [123]. 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 [124]. 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 [123]. However, there are generally more instances of host specialists than generalists across numerous parasitic and endosymbiotic taxa [125–127].

The standardised phylogenetic host specificity scores revealed that host flexibility among generalists varied greatly. Understanding *Wolbachia* host specificity has huge implications especially for the optimisation of *Wolbachia* biocontrol strategy. Not only should researchers select strains which can effectively limit pathogen replication [128], 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]. Studies have shown that the evolutionary associations between *Wolbachia* and their insect hosts do vary across taxa [49–52,129]. Likewise, our exploratory analyses of the mosquito hosts and their *Wolbachia* support such a complex relationship, with neither co-speciation nor host shifting fully accounting for the evolutionary association in these lineages.

Based on the tanglegram, a broad association pattern between mosquitoes and *Wolbachia* strains was observed (Fig. 3). *Aedes* mosquitoes tend to be associated with *Wolbachia* supergroup A, while other species, particularly of the genus *Culex*, were largely associated with *Wolbachia* supergroup B. This showed that closely related *Wolbachia* strains are likely to establish themselves in related hosts. There might have been radiation of *Wolbachia* in these clades after their respective initial establishments. Nevertheless, the observed variations on host-endosymbiont associations make the mosquito-*Wolbachia* association pattern still questionable.

The ParaFit analysis showed weak support for congruency between host and endosymbiont phylogenies. Among the 18 host-*Wolbachia* associations, only the link between *Mansonia indiana* 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, it is worth carrying out further genus-specific study on *Mansonia* spp. to elucidate coevolutionary patterns within a group of closely related mosquito species. Perhaps, the degree to which *Wolbachia* coevolves with its mosquito host vary across different taxa levels [74]. 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. similar to patterns seen in diffuse coevolution).

Referring to the event-based analysis performed in 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, the least cost coevolutionary reconstruction indicated multiple consecutive host shifts occurring near the tips of the cladogram. This suggests that co-speciation does not fully explain the evolutionary associations 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 [32,33,130].

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 [123], 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 [131,132]. Additionally, losses could potentially influence endosymbiont evolution through the creation of vacant niches [131]. 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.

As this was an exploratory study, we were unable to determine the exact mechanism behind *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 [133]. The numerous host shifts and losses might have, however, blurred the effects of vertical transmission over a long evolutionary period [52]. Thus, co-speciation might have occurred within smaller clades of *Wolbachia* and mosquitoes, but at higher taxa levels, horizontal transmission and loss events are more likely the prominent force driving *Wolbachia* evolution.

Strengths, limitations, and future direction

Three distinct methods were employed to explore evolutionary associations given their respective strengths and limitations: (i) The 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 [134]. (ii) The global ParaFit test seeks to address this limitation by testing for global congruency in an unbiased, statistical approach [74]. (iii) The 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. This last method, however, cannot fully differentiate a topological congruence from an evolutionary event [135]. Without the time of divergence for

both symbiont and host, a co-phylogenetic pattern may be better explained by ecological factors (as compared to co-speciation) given that bacterial lineages often evolve faster than the hosts [136,137], and the high likelihood of host shifts among closely related species [133].

The *Wolbachia wsp* gene has been shown to provide phylogenies with a good resolution [60], and this study provides an exploratory snapshot of the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. Of course, this is a potential caveat since only a single gene was used 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.

Notwithstanding the limitations, the employment of various analytical methods allows for a well-rounded and comprehensive examination of the evolutionary association between *Wolbachia* and mosquito hosts which are lacking in current literature. 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 40 mosquito species and recorded new infections in seven 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 supported 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 across a wide range of host mosquito species 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.

Declarations

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

Abbreviations

BLAST: Basic local alignment search tool; *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.

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Tables

Table 1. Percentage infection of *Wolbachia* in 40 mosquito species collected from twelve Singapore localities.

Mosquito species	Localities												Total	Infection (%)	Super-group
	BN	BA	BB	DF	KR	KJ	M	RR	SBG	SBL	T	U			
Aedeomyia catastica	-	0/1	-	-	-	-	-	-	-	-	-	-	0/1	0.0	-
Aedes aegypti	0/1	-	-	-	-	-	-	-	-	-	-	0/13	0/14	0.0	-
Aedes albolineatus	-	-	-	-	-	-	0/3	-	-	-	-	-	0/3	0.0	-
Aedes albopictus	-	-	-	6/10	6/10	3/6	6/11	-	-	-	-	-	21/37	56.8	A, B
Aedes annandalei	-	-	-	-	3/4	-	8/9	-	-	-	-	-	11/13	84.6	A
Aedes* nr. *fumidus	-	-	-	-	-	-	-	-	-	6/10	-	-	6/10	60.0	A
Aedes gardnerii	-	-	-	-	-	-	1/1	-	-	-	-	-	1/1	100.0	A
Aedes malayensis	-	-	-	1/2	13/16	0/2	-	-	-	-	-	-	14/20	70.0	A
Anopheles barbirostris complex	-	-	-	0/2	-	-	0/2	-	-	-	-	-	0/4	0.0	-
Anopheles lesteri	-	-	-	-	-	0/2	-	-	-	-	-	-	0/2	0.0	-
Anopheles sinensis	-	0/12	-	-	-	-	-	-	-	-	-	-	0/12	0.0	-
Armigeres kesseli	-	-	-	-	3/3	-	-	-	-	-	-	-	3/3	100.0	B
Coquillettidia crassipes	-	-	-	2/2	6/7	4/4	-	-	-	-	-	-	12/13	92.3	B
Culex (Lophoceramyia) spp.*	-	-	-	-	0/1	0/2	1/9	-	-	-	0/2	-	1/14	7.1	B
Culex bitaeniorhynchus	-	-	-	-	0/1	-	-	-	-	-	-	-	0/1	0.0	-
Culex brevipalpis	-	-	-	0/1	-	-	0/2	-	-	-	-	-	0/3	0.0	-
Culex nigropunctatus	-	-	-	-	-	0/1	0/2	-	-	-	-	-	0/3	0.0	-
Culex pseudovishnui	-	-	-	-	11/12	-	4/4	-	3/5	1/1	-	-	19/22	86.4	B
Culex quinquefasciatus	-	5/8	-	-	-	-	-	-	-	-	-	-	5/8	62.5	B
Culex sitiens	-	-	-	-	-	-	-	-	-	2/4	-	-	2/4	50.0	B
Culex sp.	-	-	-	-	-	-	0/2	-	-	-	-	-	0/2	0.0	-
Culex tritaeniorhynchus	-	-	-	-	-	2/5	-	-	-	0/1	0/1	-	2/7	28.6	UC
Culex vishnui	-	-	-	-	-	-	0/2	-	-	-	0/3	-	0/5	0.0	-
Malaya genurostris	-	-	2/4	-	0/1	4/13	-	-	0/1	-	-	-	6/19	31.6	B
Mansonia dives	-	-	-	-	-	-	0/2	-	-	-	-	-	0/2	0.0	-
Mansonia indiana	-	-	-	-	-	3/3	-	-	-	-	-	-	3/3	100.0	B

Mosquito species	Localities												Total	Infection (%)	Super-group
	BN	BA	BB	DF	KR	KJ	M	RR	SBG	SBL	T	U			
Mimomyia luzonensis	-	-	-	-	-	0/1	-	-	-	-	-	-	0/1	0.0	-
Tripteroides sp.	-	-	-	-	0/7	-	1/2	-	-	-	-	-	1/9	11.1	UC
Uranotaenia obscura	-	-	-	2/4	-	-	2/2	1/1	-	-	-	-	5/7	71.4	A
Uranotaenia sp.	-	-	-	1/2	-	-	-	-	-	-	-	-	1/2	50.0	A
Uranotaenia trilineata	-	-	-	-	-	-	1/1	-	-	-	-	-	1/1	100.0	B
Verrallina butleri	-	-	-	-	-	1/1	-	-	-	-	-	-	1/1	100.0	UC
***Verrallina* sp.**	-	-	-	-	-	-	-	1/5	-	-	-	-	1/5	20.0	UC
Zeugnomyia gracilis	-	-	-	1/2	-	-	1/13	1/4	-	-	-	-	3/19	15.8	B
Total	0/1	5/21	2/4	13/25	42/62	17/40	25/67	3/10	3/6	9/16	0/6	0/13	119/271	43.9	

Bolded species names represent *Wolbachia*-infected species with infection that have not been reported previously. Abbreviations: *BN* Bedok North Avenue 3, *BA* Bishan-Ang Mo Kio Park, *BB* Bukit Batok Town Park, *DF* Dairy Farm Nature Park, *KR* Kent Ridge Park, *KJ* Kranji Marshes, *M* Mandai Track 15, *RR* Rifle Range Road, *SBG* Singapore Botanic Garden, *SBL* Sungei-Buloh, *T* Tampines Eco-Green, *U* Ubi Avenue 1. UC denotes *Wolbachia* infections that were unclassified to supergroup [1]. Their DNA sequences were either too short (< 400bp) or had alignment issues during phylogenetic analyses. **Culex (Lophoceramyia)* spp. consists of 7 unique species which are not identified.

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1. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc London Ser B Biol Sci. 1998;265:509–15.

Table 2. 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

Figures

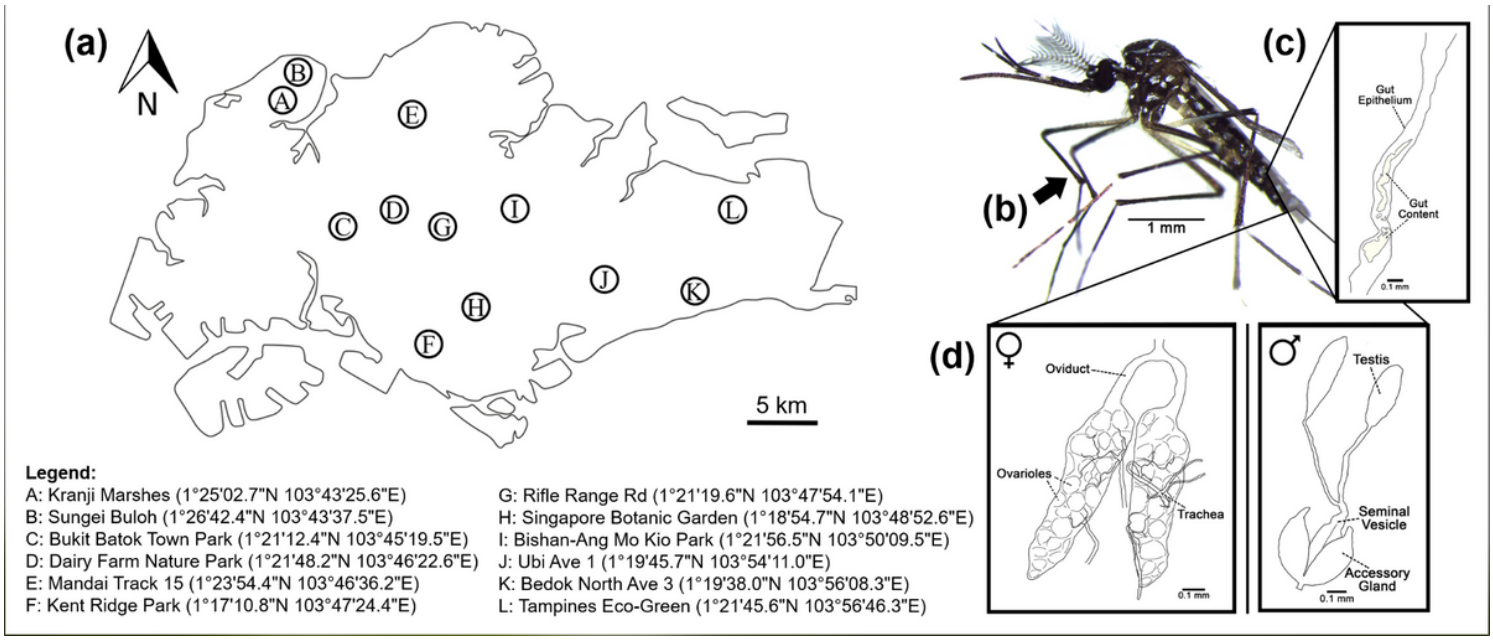

Figure 1

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).

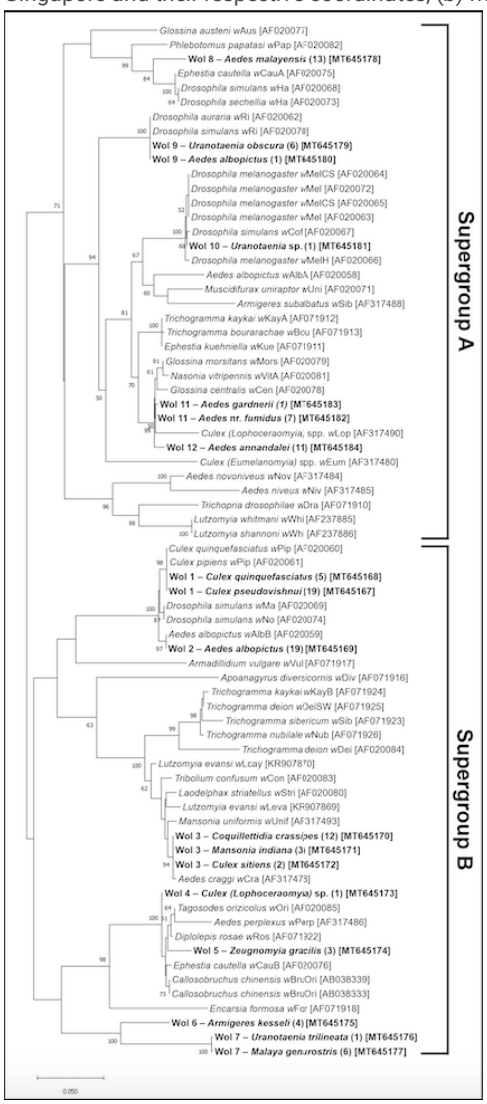

Figure 2

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.

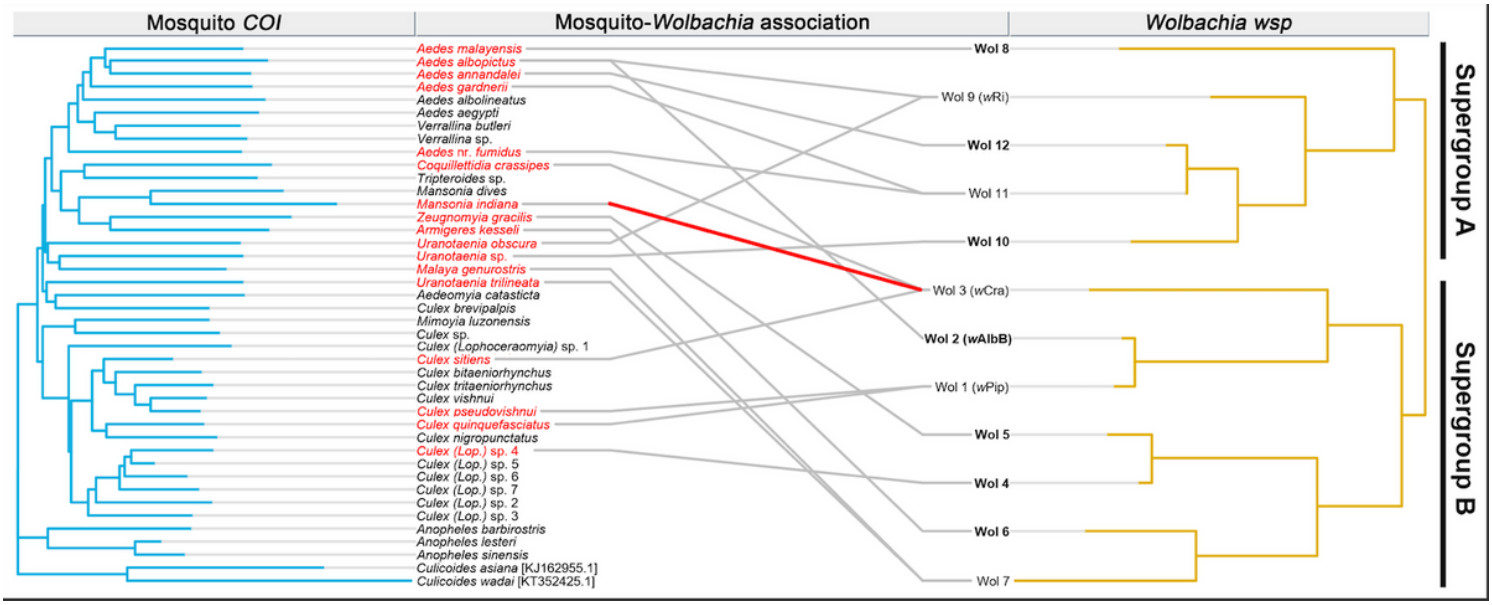

Figure 3

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).

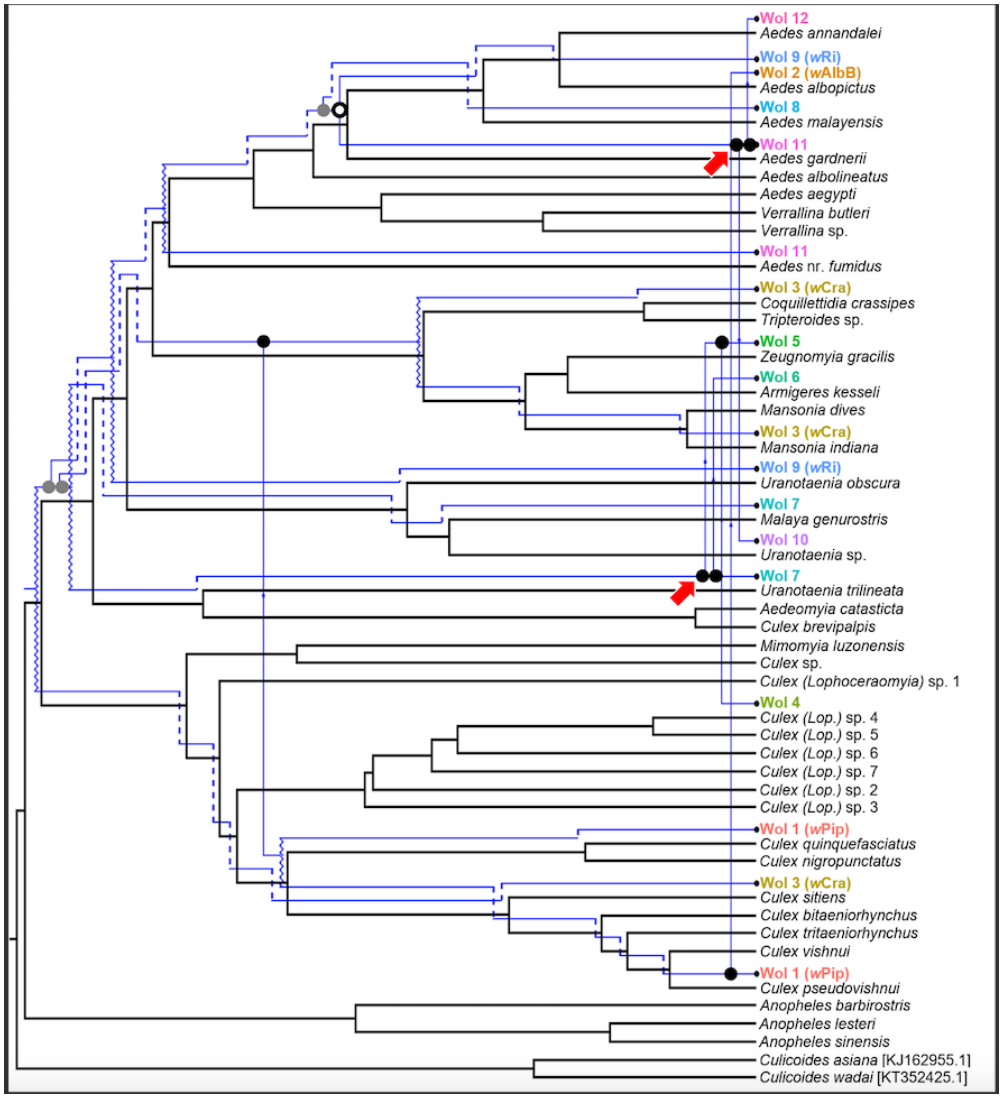

Figure 4

Least cost evolutionary reconstruction between mosquito (black) and Wolbachia (blue) phylogenies achieved using Jane 4.0. In total one co-speciation event (open circle), three counts of duplication (grey dot), seven counts of duplication with host shift (black dot with an arrow pointed outwards), 29 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

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