Identification and molecular characterization of Wolbachia strains in natural populations of Aedes albopictus in China

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Abstract

Background: Aedes albopictus is naturally infected with Wolbachia spp., maternally transmitted bacteria that influence the reproduction of hosts. However, little is known regarding the prevalence of infection, multiple infection status, and the relationship between Wolbachia density and dengue outbreaks in different regions. Here, we assessed Wolbachia infection in natural populations of Ae. albopictus in China and compared Wolbachia density between regions with similar climates, without dengue and with either imported or local dengue.

Results: To explore the prevalence of Wolbachia infection, Wolbachia DNA was detected in mosquito samples via PCR amplification of the 16S rRNA gene and the surface protein gene wsp. We found that 93.36% of Ae. albopictus in China were positive for Wolbachia. After sequencing gatB, coxA, hcpA,ftsZ, fbpA and wsp genes of Wolbachia strains, we identified a new sequence type (ST) of wAlbB (464/465). Phylogenetic analysis indicated that wAlbA and wAlbB strains formed a cluster with strains from other mosquitoes in a wsp-based maximum likelihood (ML) tree. However, in a ML tree based on multilocus sequence typing (MLST), wAlbB STs (464/465) did not form a cluster with Wolbachia strains from other mosquitoes. To better understand the association between Wolbachia spp., and dengue infection, the prevalence of Wolbachia in Ae. albopictus from different regions (containing local dengue cases, imported dengue cases and no dengue cases) was determined. We found that the prevalence of Wolbachia was lower in regions with only imported dengue cases.

Conclusions: The natural prevalence of Wolbachia infections in China was much lower than in other countries or regions. The phylogenetic relationships among Wolbachia spp., isolated from field-collected Ae. albopictus reflected the presence of dominant and stable strains. However, wAlbB (464/465) and Wolbachia strains did not form a clade with Wolbachia strains from other mosquitoes. Moreover, lower densities of Wolbachia in regions with only imported dengue cases suggest a relationship between fluctuations in Wolbachia density in field-collected Ae. albopictus and the potential for dengue invasion into these regions.

Keywords: Aedes albopictus, Wolbachia, Infection, MLST genes, Phylogenetic analysis, Dengue virus

Background

Dengue is a rapidly spreading infectious disease transmitted between humans by mosquitoes of the genus Aedes. It is estimated that 400 million people are infected with dengue per year worldwide. To date, no effective vaccine or curative antiviral drug is available to prevent or treat dengue fever [1]. Thus, vector control has become the primary tool for dengue intervention. In China,
Aedes albopictus is the primary dengue vector, and was responsible for the epidemic in 2014 resulting in approximately 47,000 infections. Use of insecticides is effective in controlling dengue, but is often prohibitively expensive, unsustainable and environmentally unfriendly. Other approaches require constant interventions that are expensive and difficult to implement in urban areas [2]. In recent years, the Wolbachia-based approach has been proposed as a new vector control strategy [3].

Wolbachia is a genus of Gram-negative bacteria that infect arthropods and filarial nematodes. It has been recently estimated that ~40% of arthropod species and ~28.1% of mosquitoes are infected with Wolbachia [4, 5]. These alpha-proteobacteria endosymbionts are transmitted vertically through host eggs and alter host biology in diverse ways, including reproductive manipulations such as feminization, parthenogenesis, male killing and sperm-egg incompatibility [6–8]. Furthermore, a large number of studies have shown that Wolbachia have an effect on the host's olfactory sense, immunity and lifespan [9, 10]. After Hedges et al. [11] and Teixeira et al. [12] reported that Wolbachia can protect Drosophila flies from viral infections, a novel control strategy was proposed using Wolbachia to control or limit the spread of mosquito-transmitted diseases such as dengue and malaria. A Wolbachia strain from Drosophila could be transferred into Aedes aegypti; releasing this transinfected mosquito may result in invasion and spread of Wolbachia into wild mosquito populations [13]. Additionally, these strains also interfere with the host's reproduction, inhibit viral replication and reduce adult lifespan [14].

wMel-transinfected Aedes aegypti populations have already been established and successfully released in Australia [3, 15]. Subsequently, other countries and regions in which Aedes aegypti is the major vector of dengue, such as Vietnam, Brazil, Colombia and Indonesia, have also started to release wMel-infected mosquitoes [16, 17]. In different parts of China, especially the south (e.g. Guangdong), Aedes albopictus is the major vector of dengue. Thus, studies are currently underway to apply a Wolbachia strain, wPip, from a Culex mosquito species to control Aedes albopictus. Although the theory and technology are already established, the prevalence and characteristics of Wolbachia in natural Aedes albopictus populations are poorly understood.

Aedes albopictus carries Wolbachia superinfections with two strains, wAlba and wAlbB. In a given region Aedes albopictus harbors only single wAlbA infections, and field-collected mosquitoes with single wAlbB infections were identified in Changsha, Chenzhou and Wuhan, as has been previously reported in Guangzhou [18]. Studies of natural Wolbachia infections of Ae. albopictus in China have been much less conclusive and were mainly based on the wsp gene. In addition, multilocus sequence typing (MLST), a robust classification system that accomplishes strain typing based on variation in five conserved housekeeping genes (ftsZ, gatB, coxA, hcpA and fbpA), was applied in mosquitoes singly infected with supergroup A or B Wolbachia [19]. No studies have applied MLST to assess co-infection with supergroups A and B Wolbachia in Ae. albopictus. In previous studies, quantification of Wolbachia in mosquitoes aimed to examine the direct association between Wolbachia and virus in vivo, and several studies were carried out to understand virus-Wolbachia relationships in natural mosquito populations [20, 21].

The present study aimed to determine the natural prevalence of Wolbachia infections and to investigate differences in Wolbachia infection among five different climatic regions. MLST and wsp analyses were applied to characterize Wolbachia strains and estimate the phylogenetic relationships between Wolbachia strains in field-collected Aedes albopictus from China. Our findings illuminate the characteristics and prevalence of Wolbachia in natural populations of Ae. albopictus in China.

Methods
Mosquito sampling
According to the geographical distribution and climatic characteristics of Ae. albopictus in China, we selected 6–8 sites in each of five climate zones of Ae. albopictus distribution. Samples were collected at each site according to a five-point method. In this study, a total of 704 adult Ae. albopictus (190 males and 514 females) were collected from 34 districts between June and October 2014 (Table 1). For analysis of prevalence, sampling locations were placed into five climate groups as defined in the Chinese Climatic Regions, based on the following climate classifications: Edge of tropical; South subtropical; Mid-subtropical; North subtropical; and Warm temperate zone (Fig. 1) [22]. BG traps, human baited net traps and manual aspirators were used to catch adult mosquitoes. Pipettes and dippers were used for capturing larvae or pupae from different containers at each site. The same operation was repeated at least five times in each location to reduce sampling error. Sampling staff were well protected whilst catching adults to avoid mosquito bites. The collected larvae and pupae were reared to adults and supplemented with yeast extract. The adults collected in the field were examined morphologically to confirm whether they were Ae. albopictus [23]. Samples were stored at –80 °C in individual tubes containing 95% ethanol until DNA extraction.
DNA extraction and prevalence of Wolbachia infection

To assess the prevalence of Wolbachia infection, 2–37 Ae. albopictus were used from each population to extract total DNA. After drying the Ae. albopictus for several minutes, they were washed three times in ddH₂O. DNA was then individually extracted using a DNAeasy Tissue Kit (Qiagen, Valencia, CA, USA). Two 16S rDNA primers and four wsp-specific primers, WAF/WAR and WBF/WBR, were used to detect Wolbachia DNA by polymerase chain reaction (PCR) using the DNA of a single mosquito as a template [24, 25]. The 28S rRNA gene was used to assess the quality of DNA extraction and the cox1 mitochondrial gene was sequenced to exclude mosquitoes that were not Ae. albopictus. The full-length cox1 gene was amplified using four primers, cox1F/cox1R and cox1F/cox1R (Table 2). PCR reactions were performed in a final volume of 25 µl containing 2 µl of DNA, 11 µl of ddH₂O, 1 µM of each primer and 10 µl of SuperMix. The temperature was cycled at 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min, and then a final extension step at 72 °C for 10 min. DNA extracted from Wolbachia-infected Ae. albopictus was used as a positive control and ddH₂O was used as a negative control. PCR products were run on 1% agarose gels and the cox1 PCR products were sequenced directly.

Table 1  Sample information

| Climate zone            | District    | Coordinates         | No. of samples | ♀  | ♂  |
|-------------------------|-------------|---------------------|----------------|----|----|
| Edge of tropical        | Wenchang    | 19.57°N, 110.80°E  | 33             | 25 | 8  |
|                         | Wanning     | 18.81°N, 110.39°E  | 21             | 16 | 5  |
|                         | Haikou      | 20.02°N, 110.20°E  | 33             | 21 | 12 |
|                         | Qiongzhou   | 19.04°N, 109.83°E  | 18             | 10 | 8  |
|                         | Sanya       | 18.25°N, 109.51°E  | 37             | 22 | 15 |
|                         | Jinghong    | 22.01°N, 100.77°E  | 34             | 23 | 11 |
|                         | Dehong      | 24.43°N, 98.59°E   | 21             | 19 | 2  |
| South tropical          | Nanning     | 22.82°N, 108.36°E  | 25             | 17 | 8  |
|                         | Foshan      | 23.02°N, 113.11°E  | 12             | 10 | 2  |
|                         | Guangzhou   | 23.41°N, 113.23°E  | 33             | 19 | 14 |
|                         | Jiangmen    | 22.50°N, 113.40°E  | 5              | 2  | 3  |
|                         | Zhongshan   | 22.40°N, 112.72°E  | 20             | 12 | 8  |
|                         | Fuzhou      | 26.08°N, 119.30°E  | 24             | 18 | 6  |
|                         | Xiamen      | 24.59°N, 118.10°E  | 24             | 15 | 9  |
| Mid-subtropical         | Changsha    | 28.21°N, 112.99°E  | 25             | 11 | 14 |
|                         | Chenzhou    | 25.77°N, 113.01°E  | 18             | 13 | 5  |
|                         | Nanchang    | 28.68°N, 115.86°E  | 22             | 16 | 6  |
|                         | Chengdu     | 30.66°N, 104.07°E  | 18             | 14 | 4  |
|                         | Nanchong    | 30.49°N, 106.04°E  | 2              | 1  | 1  |
|                         | Chongqing   | 29.57°N, 106.55°E  | 24             | 24 | 24 |
| North subtropical       | Hefei       | 31.82°N, 117.23°E  | 9              | 8  | 1  |
|                         | Nanjing     | 32.05°N, 118.79°E  | 27             | 20 | 7  |
|                         | Shanghai    | 31.23°N, 121.48°E  | 31             | 24 | 7  |
|                         | Wuhan       | 30.35°N, 114.17°E  | 6              | 2  | 4  |
|                         | Wuxi        | 31.34°N, 120.18°E  | 3              | 3  | 0  |
|                         | Hangzhou    | 30.18°N, 119.5°E   | 26             | 18 | 8  |
| Warm temperate zone     | Beijing     | 39.77°N, 116.66°E  | 30             | 24 | 6  |
|                         | Shangqiu    | 34.17°N, 116.20°E  | 13             | 13 | 0  |
|                         | Taijuan     | 37.98°N, 112.32°E  | 31             | 31 | 0  |
|                         | Xian        | 34.17°N, 108.21°E  | 18             | 18 | 0  |
|                         | Tangshan    | 39.96°N, 118.81°E  | 3              | 2  | 1  |
|                         | Kaifeng     | 34.80°N, 114.27°E  | 15             | 12 | 3  |
|                         | Tianshui    | 34.71°N, 105.47°E  | 22             | 20 | 2  |
|                         | Dalian      | 38.94°N, 121.40°E  | 21             | 15 | 6  |
Cloning and sequencing of wsp and MLST genes

The WSP loci were amplified with wsp (Wolbachia surface protein gene) primers to confirm multiple infections. PCR reactions were performed in a final volume of 25 μl containing 2 μl of DNA, 11 μl of ddH₂O, 1 μM of each primer and 10 μl of SuperMix. The temperature was cycled at 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, 53 °C for 45 s and 72 °C for 1 min, and then a final extension step at 72 °C for 10 min.

The five MLST loci were amplified according to previously published protocols (http://pubmlst.org/Wolbachia/). PCR reactions were performed in a final volume of 25 μl containing 2 μl of DNA, 11 μl of ddH₂O, 1 μM of each primer and 10 μl of SuperMix. The temperature was cycled at 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, Tₘ (Tₘ values for each primer pair are shown in Table 2) for 45 s and 72 °C for 90 s, and then a final extension step at 72 °C for 10 min.

The five MLST loci were amplified according to previously published protocols (http://pubmlst.org/Wolbachia/). PCR reactions were performed in a final volume of 25 μl containing 2 μl of DNA, 11 μl of ddH₂O, 1 μM of each primer and 10 μl of SuperMix. The temperature was cycled at 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, Tₘ (Tₘ values for each primer pair are shown in Table 2) for 45 s and 72 °C for 90 s, and then a final extension step at 72 °C for 10 min. For co-infected samples, the coxA and ftsZ genes were amplified using primers coxA_F1 (5′-TTG GRG CRA TYA ACT TTA TAG-3′) and coxA_R1 (5′-CT AAA GAC TTT KAC RCC AGT-3′), and ftsZ-F (5′-TAC TGA CTT TGG GAG TTG TAA CTA AGC CGT-3′) and ftsZ-R (5′-TGC CAG TGG CAA GAA CAG AAA CTC TAA CTC-3′), respectively. For the fragment of coxA, primers for B-specific MLST protocols for AB infections were not used in our study, and ftsZ fragments were not long enough to be amplified by A-specific and B-specific primers. Fragments of coxA, ftsZ and wsp with the expected sizes were excised from the gel and purified using the Pure Yield™ Plasmid Miniprep System (Promega, Madison, USA). The purified DNA was ligated into pEASY-T5 Zero Cloning vector (Trans) and then transferred to Trans1-T1 phage resistant chemically competent cells (Trans). Putative clones of expected fragments were submitted for DNA sequencing. For all three kinds of fragments, at least eight clones were sequenced for each mosquito using both M13 forward and reverse primers, with three individuals being analyzed for each geographical population.

Nucleotide sequence accession numbers

All newly generated sequences for wsp, cox1, gatB, coxA, hcpA, ftsZ, fbpA genes were deposited in the GenBank database under accession numbers KU738304-KU738385, KU738386-KU738431, MK809569-MK809640, MK809709-MK809776, MK809845-MK809912, MK809777-MK809844, MK809641-MK809708, respectively. According to the MLST protocol, the sequences of gatB, coxA, hcpA, ftsZ, fbpA and wsp were submitted to the PubMLST database for sequence typing, generating a MLST allelic profile and a WSP hypervariable region (HVR) profile. Strain and host information were deposited in the MLST database.
**Table 2** Primers for amplification and sequencing

| Gene    | Primer | Sequence (5′–3′) | Annealing T (°C) |
|---------|--------|------------------|------------------|
| 16S rDNA | 16SF   | CGGGGAAAAATTATGCT | 55               |
|         | 16SR   | AGCTGGTAACTGAAAGTAA | 55               |
| wAlbA-wsp | WAF    | CACGCAGATCTATTGCG | 55               |
| wAlbB-wsp | WBF    | AAGGACCGAAGGCTATG  | 55               |
|         | WBR    | AGAAATTAAACCGCTATCCA | 53               |
| wsp     | 81     | TGTCCTAAATGTAGTGAAGAAC | 53               |
|         | 691    | AAAATTAACCGCTATCCA  | 53               |
| FtsZ    | ftsZ-F | TACTGACTGTTGAGTTGAATCTA | 58               |
|         | ftsZ-R | TGGCAGTGGCAAGAACAAACTCTTACCT | 58               |
| 28S rRNA | 28F    | TACCCTGAAGGAAATGTGAA | 55               |
|         | 28R    | AGACTCTCTGGTTCGGITTTT | 55               |
| cox1    | cox1F  | TTTACAATTTATCGCTTAACTTCT | 55               |
|         | cox1R  | CATGGCAGAATCTGCGATACTACTATCA | 55               |
|         | cox1F  | GGGGAGACCTTATTATTA  | 55               |
|         | cox1R  | TAAACTCTGGTGGCAACAAATCA | 55               |
| wAlbAq-wsp | qAF    | GGTTGATGTTGCAAGGAG  | 55               |
|         | qAR    | CACGCCCTTTACTTGACC  | 55               |
| wAlbBq-wsp | qBF    | ACCTTGTTGTGCAAGATTTGG | 58               |
|         | qBR    | TAAAGCCAGCACCAGATAACG | 55               |
| RPS     | RPS6-F | CTTCGTCAGGAACGTTATCG | 55               |
|         | RPS6-R | TCTTGCCAGCCTTGGACGCA | 55               |

Note: Primers cox1F/cox1R were used for sequencing
Abbreviation: T, temperature

**Sequence typing and phylogenetic analyses**

For *Wolbachia*-specific *wsp* gene sequence analysis, several reported sequences with similarities of > 97% were obtained from GenBank for comparisons. The *wsp* sequence of *Brugia malayi* was selected as the outgroup. We also analyzed co-infection with different *Wolbachia* species. Furthermore, a reference list of *Wolbachia* isolates was constructed by searching the MLST database, which was selected for having a complete set of MLST and HVR profiles. A total of 40 of known STs were from supergroup A, supergroup B, supergroup D and supergroup F *Wolbachia*, and supergroup D (Table 3) and supergroup F *Wolbachia* were selected as outgroups. Allele sequences were downloaded from the MLST database and these *Wolbachia* sequences were manually edited with Chromas2.4 by DNAMAN and their translated amino acid sequences were aligned using MUSCLE in MEGA6.0. Then, the concatenated data set of the five MLST genes was subjected to a phylogenetic analysis using MEGA 6.0. The *wsp* sequences were also subjected to a phylogenetic analysis using MEGA 6.0 using supergroup D and F *Wolbachia* strains as outgroups (for consistency with the MLST-based analysis). Maximum likelihood (ML) methods in MEGA 6.0 were used to analyze phylogenetic relationships. To select the optimal evolutionary model by critically evaluating the selected parameters, Find Best-Fit Substitution Model was conducted in MEGA 6.0 [26]. For the NCBI-*wsp* sequences, the concatenated dataset and the *wsp* sequences, the sub-models T92 (Tamura 3-parameter), GTR+I+G and T92 (Tamura 3-parameter)+G were selected, respectively. The ML trees were constructed with 1000 bootstrap replicates.

**wAlbA and wAlbB Wolbachia strain quantitation**

Twenty-eight mosquitoes from regions with local dengue cases (Guangzhou and Jinghong), with only imported cases (Xiamen and Haikou) and without dengue cases (Wenchang and Fuzhou) were amplified individually by quantitative PCR using strain-specific primers qAF/qAR [27] and qBF/qBR (Table 2) to examine the relationship between *Wolbachia* density in field-collected *Ae. albopictus* and the presence of dengue virus. The Bio-Rad CFX96 Real-Time PCR Detection System (Hercules, USA) and GoTaq® qPCR Master Mix (Promega) were used in our study. PCR reactions were performed in a final volume of 20 µl containing 10 µl of GoTaq® qPCR Master Mix, 0.5 µM of each primer, 2 µl of template DNA and 7 µl of RNase-free water. Reactions were mixed with an electronic pipette. The thermal cycling conditions were: 10 min at 95 °C, followed by 50 cycles of 94 °C for 15 s, primer Tm (wAlbA 58 °C, wAlbB 58 °C and RPS6 55 °C) for 30 s, 72 °C for 30 s, and finally 72 °C (read temperature) for 15 s. The melting curve was constructed between 49 °C and 63 °C. We used a serial dilution of *pEASY*-T5 Zero Cloning vectors containing one copy each of RPS6 [28], wAlbAq-*wsp* and wAlbBq-*wsp* genes, and used their primers set up in each PCR to plot standard curves, in case any binding efficiency difference appeared. Every mosquito DNA template was quantified three times for each of the RPS6, wAlbAq-*wsp* and wAlbBq-*wsp* genes. Assuming that each gene was present in a single copy per haploid genome, the ratio between *wsp* and RPS6 provided the number of *Wolbachia* genomes relative to the number of *Aedes* genomes [29].

**Statistical analysis**

To compare the densities of the two *Wolbachia* strains in field mosquitoes in five regions (with different adult sizes), data were normalized to the expression of the host *rps6* gene. Analyses were carried out using SPSS Statistics (17.0). Chi-square tests were performed to compare the prevalence of *Wolbachia* infections and one-way analysis of variance (ANOVA) was performed.
to compare densities of *Wolbachia* from different regions for normally distributed data using SPSS Statistics (17.0). Differences were considered statistically significant when $P < 0.05$. For better presentation of results, locA and locB were used to denote the densities of supergroup A and supergroup B, respectively, from regions with local dengue cases; impA and impB were used to denote the densities of supergroup A and supergroup B, respectively, from regions with only imported dengue cases; and noA and noB were used to denote the densities of supergroup A and supergroup B, respectively, from regions with no dengue cases.

### Table 3  MLST allelic and WSP profiles of *Wolbachia* subjected for phylogenetic analyses

| ID | Supergroup | Host species | ST | gatB | coxA | hcpA | ftsZ | fbpA | wsp | HVR1 | HVR2 | HVR3 | HVR4 |
|----|------------|--------------|----|------|------|------|------|------|-----|------|------|------|------|
| 1  | A          | Drosophila melanogaster | 1  | 1    | 1    | 1    | 1    | 31   | 1   | 12   | 21   | 24   |
| 12 | A          | Aedes albopictus     | 2  | 3    | 2    | 2    | 10   | 3    | 1    | 1    | 1    | 1    |
| 496| A          | Aedes brumeliae      | 304| 182  | 160  | 187  | 148  | 232  |
| 114| A          | Notoncus sp.         | 53 | 46   | 42   | 23   | 6    | 17   | 49   | 9    | 9    | 12   | 9    |
| 120| A          | Camponotus leonardi  | 57 | 49   | 44   | 53   | 42   | 49   | 52   | 41   | 42   | 45   | 42   |
| 167| A          | Agelenopsis aperta   | 67 | 35   | 35   | 22   | 33   | 39   | 43   | 31   | 32   | 35   | 34   |
| 294| A          | Asobara japonica     | 370| 87   | 111  | 103  | 70   | 186  | 530  | 188  | 213  | 15   | 25   |
| 399| A          | Apanteles chilonis   | 260| 172  | 150  | 7    | 137  | 8    | 592  | 209  | 15   | 17   | 14   |
| 1682| A        | Syrphophilus asperatus | 433| 234  | 84   | 227  | 300  | 120  | 689  | 11   | 9    | 267  | 302  |
| 56 | A          | Rhagotis cerasi      | 13 | 1    | 1    | 1    | 3    | 1    | 23   | 1    | 12   | 11   |
| 2  | A          | Solenopsis invicta   | 29 | 19   | 20   | 22   | 17   | 17   | 28   | 21   | 21   | 25   | 21   |
| 61 | A          | Rhagotis cerasi      | 159| 53   | 84   | 85   | 70   | 79   | 113  | 67   | 77   | 12   | 9    |
| 68 | A          | Agelenopsis aperta   | 65 | 32   | 33   | 38   | 30   | 37   | 38   | 29   | 33   | 32   |
| 88 | A          | Drosophilia testacea | 99 | 10   | 72   | 11   | 14   | 11   | 13   | 1    | 11   | 21   | 11   |
| 107| A          | Wasmannia Peru       | 47 | 43   | 40   | 46   | 38   | 46   | 28   | 21   | 21   | 25   | 21   |
| 96 | A          | Aganapsis alujai     | 164| 54   | 52   | 62   | 82   | 62   | 75   | 11   | 9    | 15   | 25   |
| 129| A          | Dorymyrmex elegans  | 63 | 19   | 21   | 55   | 46   | 53   | 51   | 42   | 43   | 47   | 25   |
| 325| A          | Ephestia kuehniella | 92 | 54   | 59   | 68   | 3    | 67   | 83   | 51   | 55   | 55   | 57   |
| 413| A          | Chelonus muniakatae | 19 | 7    | 6    | 7    | 3    | 8    | 599  | 2    | 191  | 192  | 248  |
| 29 | B          | Culex pipiens       | 9  | 4    | 3    | 3    | 22   | 4    | 10   | 8    | 10   | 8    |
| 499| B          | Mansonia africana   | 305| 9    | 38   | 189  | 36   | 4    |
| 19 | B          | Chelymorpha alternans | 7  | 9    | 14   | 15   | 12   | 14   | 8    | 7    | 7    | 8    |
| 22 | B          | Acraea encedon      | 3  | 9    | 11   | 12   | 11   | 12   | 2    | 2    | 2    | 2    |
| 27 | B          | Drosophila simulans | 16 | 5    | 4    | 4    | 4    | 5    | 15   | 10   | 8    | 11   |
| 34 | B          | Nasonia vitripennis | 26 | 9    | 8    | 9    | 7    | 9    | 25   | 18   | 16   | 23   |
| 99 | B          | Horaga oryx        | 39 | 12   | 14   | 13   | 2    | 41   | 65   | 34   | 36   | 3    |
| 118| B          | Pheidole sciphipla | 56 | 48   | 43   | 52   | 41   | 6    | 60   | 40   | 41   | 43   |
| 408| B          | Apanteles chilonis | 271| 9    | 150  | 7    | 142  | 4    | 593  | 18   | 79   | 237  |
| 269| B          | Diaphorina Diaphorina citri | 175| 109  | 86   | 88   | 126  | 27   | 160  | 2    | 17   | 3    |
| 39 | B          | Lycaides dds       | 36 | 9    | 36   | 40   | 7    | 9    | 61   | 18   | 16   |
| 73 | B          | Lycaides melissa   | 162| 108  | 73   | 40   | 80   | 9    | 294  | 125  | 141  | 127  |
| 70 | B          | Rhagoletis cerasi  | 160| 101  | 85   | 40   | 22   | 4    | 116  | 69   | 17   |
| 40 | B          | Hypolimnas bolina | 125| 4    | 14   | 40   | 73   | 4    | 10   | 10   | 8    |
| 87 | B          | Drosophila innubila | 98 | 79   | 71   | 88   | 69   | 27   | 82   | 2    |
| 97 | B          | Anthene emolus     | 37 | 9    | 9    | 6    | 8    | 10   | 63   | 19   | 17   |
| 200| B          | Eurema mandarina   | 40 | 38   | 38   | 29   | 35   | 42   | 64   | 35   | 38   |
| 311| B          | Sagatella furcifera | 213| 106  | 11   | 13   | 105  | 162  | 463  | 2    | 191  |
| 315| B          | Macrosteles fascifrons | 217| 135  | 120  | 141  | 108  | 197  | 536  | 191  | 220  |
| 37 | D          | Brugia malayi      | 35 | 28   | 29   | 33   | 26   | 30   | 34   | 24   | 27   |
| 36 | F          | Cimex lectularius  | 8  | 26   | 27   | 31   | 24   | 28   | 7    | 6    |


Results

Prevalence of Wolbachia infections

A total of 693 adult *Ae. albopictus* were obtained from five different climatic regions in China and were examined for Wolbachia infection status. Of these, 93.36% (647/693) were PCR-positive for Wolbachia using wsp and 16S rDNA primers [30]. The quality of extracted DNA was good, and the samples were all identified as *Ae. albopictus* [31]. Specific primers for *wAlbA* and *wAlbB*, derived from the rapidly evolving wsp outer-surface protein gene of Wolbachia, were used to screen for these bacteria in *Ae. albopictus* mosquitoes. The PCR results showed that 83.26% (577/693) of the mosquitoes sampled were infected with supergroup A and 91.05% (631/693) were infected with supergroup B Wolbachia strains. The prevalence of co-infection was 80.95% (561/693). Individuals singly infected with supergroup A and supergroup B Wolbachia represented 2.31% (16/693) and 10.10% (70/693) of all mosquitoes, respectively. We also found 46 uninfected individuals (Table 4).

The natural prevalence of Wolbachia infection in 34 different locations of the five climatic regions is presented in Fig. 1. Chi-square tests of Wolbachia prevalence among the five different climate regions (Fig. 2) revealed a significant difference ($\chi^2 = 15.438$, $df = 4$, $P = 0.004$). Similarly,

### Table 4 Infection status of Wolbachia based on PCR results of field-collected *Ae. albopictus* adults

| Climate region         | Total | No. of infected (%) | Single A | Single B | A and B | W+ |
|------------------------|-------|----------------------|----------|----------|---------|-----|
| Edge of tropical       | 186   | 11 (5.91)            | 26 (13.98)| 135 (72.58) | 172 (92.47) |     |
| South subtropical      | 143   | 1 (0.70)             | 15 (10.49)| 117 (81.82) | 133 (93.01) |     |
| Mid-subtropical        | 109   | 1 (0.92)             | 13 (11.93)| 80 (73.39)  | 94 (86.24)  |     |
| North subtropical      | 102   | 2 (1.96)             | 12 (11.76)| 85 (83.33)  | 99 (97.06)  |     |
| Warm temperate zone    | 153   | 1 (0.65)             | 4 (2.61)  | 144 (94.12)| 149 (97.39) |     |
| Total                  | 693   | 16 (2.31)            | 70 (10.10)| 561 (80.95)| 647 (93.36) |     |

*Note:* $W+$ represents the positive rate of Wolbachia in *Ae. albopictus*.  

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the prevalence of supergroup A and B *Wolbachia* differed significantly among the five climate regions ($\chi^2 = 24.199, df = 4, P < 0.0001$ and $\chi^2 = 17.390, df = 4, P = 0.0020$, respectively). Further analysis showed that the prevalence of *Wolbachia* infection was significantly different in four regions: Edge of tropical vs Warm temperate zone ($\chi^2 = 4.029, df = 1, P = 0.045$); Mid-subtropical vs North subtropical ($\chi^2 = 7.906, df = 1, P = 0.005$); and Mid-subtropical vs Warm temperate zone ($\chi^2 = 11.759, df = 1, P = 0.001$). However, the prevalence of *Wolbachia* infection in the South subtropical region did not show any significant differences compared with any of the other four regions. For the prevalence of supergroup A *Wolbachia*, significant differences were detected for five regions: Edge of tropical vs North subtropical ($\chi^2 = 18.298, df = 1, P < 0.0001$); South subtropical vs Warm temperate zone ($\chi^2 = 11.204, df = 1, P = 0.001$); Mid-subtropical vs North subtropical ($\chi^2 = 5.147, df = 1, P = 0.023$); and South subtropical vs Warm temperate zone ($\chi^2 = 11.242, df = 1, P = 0.001$). Similar to the overall prevalence of *Wolbachia* infections, the south subtropical region did not show any substantial difference compared with any of the other four regions (Figs. 2, 3).

**Nucleotide sequence analysis of Wolbachia from Ae. albopictus**

DNA sequencing analysis indicated that *Ae. albopictus* from different locations in China harbored two different *Wolbachia* strains: *wAlbA* and *wAlbB* (Fig. 4). The WSP profiles of *wAlbA* and *wAlbB* for wsp, HVR1, HVR2, HVR3 and HVR4 were 1, 1, 1, 1 and 1, and 169, 10, 82, 10 and 84, respectively, suggesting that these two *Wolbachia* strains were very stable.

Phylogenetic analysis based on the concatenated sequences of all MLST loci showed that ST-2 was *wAlbA*, but no closely-related STs were identified for *wAlbB*. We submitted our sequences to the MLST database, and received new ST codes (ST-464, ST-465, designated for *wAlbB1* and *wAlbB2* respectively). *wAlbB1* and *wAlbB2* only differed by a single base pair: *gatB16A* and *gatB16B*, respectively. The five MLST genes of *wAlbA* shared the same alleles as ST-2, as previously demonstrated [19]; however, three of the five MLST genes (*fbpA, gatB* and *hcpA*) of *wAlbB1* and two of the five genes (*fbpA* and *hcpA*) of *wAlbB2* shared alleles with other STs. In total, 40 known *Wolbachia* STs in the MLST database (http://pubmlst.org/Wolbachia/) were used as a dataset to infer the phylogeny of *Wolbachia* infecting field-collected *Ae. albopictus*. The MLST-based ML tree (Fig. 5) separated the isolates into three major clusters: supergroup A, supergroup B, and supergroup D + supergroup F. For the wsp-based ML tree, the isolates were separated into supergroup A, supergroup D, supergroup F and a mixture of supergroup A and supergroup B branches. According to these data, it was safe to classify ST-464 and ST-465 as strains of supergroup B. In the wsp-based ML tree (Fig. 6), *wAlbA* and *wAlbB* formed a cluster with strains from other mosquito species (*Culex quinquefasciatus* and *Culex gelidus*). Similarly, in the MLST-based tree, *wAlbA* (ST-2) formed a clade with ST-304 whose host is *Aedes bromeliae*. In supergroup B, *wAlbB* (ST-464 and ST-465) did not form a clade with ST-305 and ST-9, whose hosts were *Mansonia africana* and *Culex pipiens*, respectively (Figs. 5, 6).

**wAlbA and wAlbB Wolbachia strain quantitation**

The relative densities of the *wAlbA* and *wAlbB* strains were estimated for individual females sampled from regions with local dengue cases, with only imported dengue cases, and without dengue cases. The data were normalized using the host *rps6* gene, which also allowed the densities of the two *Wolbachia* strains to be compared between different adult sizes.

Figure 7 shows a higher density of the *wAlbB* strain relative to *wAlbA*, and this difference was significant in three different regions: *locA* vs *locB* (ANOVA, $F_{(1, 54)} = 67.143, P < 0.0001$), *impA* vs *impB* (ANOVA, $F_{(1, 54)} = 38.955$,
and noA vs noB (ANOVA, $F_{(1,54)} = 12.650, P = 0.001$). Moreover, both wAlbA and wAlbB strains showed significantly lower densities in regions with only imported dengue cases than in the other two regions [wAlbA (ANOVA, $F_{(2, 81)} = 10.203, P < 0.0001$) and wAlbB (ANOVA, $F_{(2, 81)} = 7.468, P = 0.001$)]. Neither locA vs impA, locA vs noA, locB vs impB, nor locB vs noB showed any significant difference, which may indicate a relationship between the fluctuation of Wolbachia density in field Ae. albopictus and the invasion of dengue virus.

**Discussion**

Wolbachia is a bacterial endosymbiont that infects the reproductive tissues of arthropods, mainly insects. It is spread primarily via the ova cytoplasm and alters the reproductive success of its host, thus making it a suspected driver of development and speciation. The prevalence of Wolbachia in insects has been reported as ranging from 20% to 65% [32]. Our results showed a prevalence of 93.36% for Wolbachia in natural populations of Ae. albopictus in China, slightly lower than the 100% previously reported in Guangzhou (China), Orissa (India), Chachoengsao (Thailand) [18, 33, 35] and over 99% in Korea [34]. Furthermore, single infections with both wAlbA and wAlbB were detected in our study and the prevalence of wAlbB (10.10%) strains was higher than that of wAlbA strains (2.31%). To the best of our knowledge, this is the first report of single wAlbB infections in field-collected Ae. albopictus in Changsha, Chenzhou and Wuhan, China, and our findings were similar to those reported in Guangzhou [18]. These results thus support and validate the work of O’Neill et al. [35]. In the present study, 28S rRNA was used to assess the quality of DNA extraction [30] and the cox1 gene of Ae. albopictus was sequenced to rule out samples that were not Ae. albopictus. In addition, to obtain an accurate estimate of the prevalence of wAlbA and wAlbB, qPCR was used to check negative samples and indicated an increased prevalence of 83.26% and 91.05% for supergroup A and B Wolbachia strains, respectively.

Wolbachia significantly and efficiently reduced the proportions of mosquitoes achieving infection and transmission potential across the different regions. Wolbachia density is sensitive to temperature variations [36]. A Chi-square test of Wolbachia prevalence among the five different climate regions in China revealed that geographical location and climate may have a significant effect on the prevalence of Wolbachia in natural populations of Ae. albopictus. As shown in Fig. 3, for both wAlbA and wAlbB, the prevalence of Wolbachia infection in the Mid-subtropical region was lower than in other climate regions; the difference between the North subtropical region and the Warm temperate zone was apparent in all three measures of prevalence. There was a
Fig. 5  MLST-based maximum likelihood tree for Wolbachia from different hosts. Red dots indicate reported Wolbachia strains of mosquitoes; green dots indicate Wolbachia strains of Ae. albopictus sampled in the present study.
Fig. 6 wsp-based maximum likelihood tree for Wolbachia from different hosts. Red dots indicate reported Wolbachia strains of mosquitoes; green dots indicate Wolbachia strains of Ae. albopictus sampled in the present study.
clearly lower prevalence in Chenzhou (Fig. 2), which may be the reason why rates in the Mid-subtropical region were lower than in other regions. Aside from this, the rates of *Wolbachia* infection did not show any linear relationships, which may imply that there is no absolute correlation between climate region and *Wolbachia* infection.

MLST is an important source of sequence data for comparative genetics, providing a tool for exploring molecular evolutionary methods in intracellular bacteria [19]. Our results show that in both the MLST-based and *wsp*-based ML trees, *Wolbachia* isolates included in the analyses are placed in supergroups A and B (Fig. 5). However, in the *wsp*-based ML tree (Fig. 6), a mixed cluster of supergroups A and B was identified, with ST-19, ST29, ST47, ST65 and ST67 belonging to a supergroup associated with isolates from supergroup B. This suggests that MLST-based genotyping is perhaps more accurate than the *wsp*-based method. Our results may, however, be explained by the fact that the sharing of *wsp* sequences between A and B strain supergroups indicates a strong genetic cohesiveness of *Wolbachia* strains [37]. Moreover, for supergroup B in the *wsp*-based ML tree, *Wolbachia* of *Ae. albopictus* did not show an exact match with previously identified STs. Furthermore, we identified the new ST-464 strain wAblB1 and the new ST-465 strain wAblB2. ST-464 was found in all locations, but ST-465 strains were only found in single infected mosquitoes from Changsha and Chenzhou and co-infected mosquitoes from Wuhan and Nanchang. This may reflect the various states of *Wolbachia* infection in these locations.

The density of the endosymbiont *Wolbachia* plays an important role in crossing sterility, which is known as a cytoplasmic incompatibility and limits the degree of parental spread. *Aedes albopictus* mosquitoes can be superinfected with the *Wolbachia* strains wAlbA and wAlbB [38]. In our study, the wAlbB strain was found at a higher density than wAlbA in *Ae. albopictus*, which is consistent with the results of two previous studies [38, 39]. To our knowledge, this study is the first to assess relative *Wolbachia* densities in *Ae. albopictus* mosquitoes from different natural populations, which were sampled from regions with different dengue fever load. The relative density of *Wolbachia* (wAlbA and wAlbB) in mosquitoes from regions with only imported dengue cases was lower than that in mosquitoes from regions with local dengue cases and without dengue cases. The decrease of *Wolbachia* density could lead to the loss of protection by the host immune system [40]. We hypothesize that the imported dengue cases caused a lowering of *Wolbachia* densities in natural mosquito populations and that densities of virus in these mosquitoes will increase. Sometime later, densities of virus and *Wolbachia* would come to a balance in the natural mosquito populations and thereafter could transmit virus smoothly, resulting in local dengue case emerging. This hypothesis has yet to be substantiated by other reports, but our results may reflect the alarm reaction of natural mosquito populations in response to invasion of dengue virus, which is embodied in the fluctuation of *Wolbachia* densities. Furthermore, the low prevalence in Chenzhou, which also has imported dengue cases, may be explained if our hypothesis were correct [41, 42]. Further research is needed to explore the relationship between *Wolbachia* densities in natural *Ae. albopictus* mosquitoes and the invasion of dengue virus.

In this study, we obtained adult mosquitoes at a variety of ages from different parts of China. Because adults had only recently emerged (1 or 2 days), these may have had *Wolbachia* densities that were too low to be detected. Our subsequent studies will be based on field-collected larvae, which will be brought back to the laboratory and used for further research after emergence.

**Conclusions**

This study demonstrated that the natural prevalence of *Wolbachia* infections in China was much lower than the prevalence in other countries or regions. The prevalence of *Wolbachia* was significantly different among five
different climatic regions. The phylogenetic relationships of Wolbachia in field-collected Ae. albopictus were estimated based on MLST and wsp analyses, and showed that these strains were rather stable. However, wAlbB (464/465) and Wolbachia strains did not form a clade with Wolbachia strains from other mosquitoes. Moreover, the lower densities of Wolbachia in regions with only imported dengue cases suggested a relationship between the fluctuation of Wolbachia density in natural Ae. albopictus populations and the invasion of dengue virus.

**Abbreviations**
MLST: multilocus sequence typing; ST: sequence type; PCR: polymerase chain reaction; ddH2O: double distilled water; WSP: Wolbachia surface protein gene; HVR: hypoxic ventilatory response.

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Not applicable.

**Authors’ contributions**
YH, QL, BC, ZX and XL planned the project and wrote the paper. QL, BC, YG, DR, JW and XW conducted the field survey. YH carried out the multiplex PCR assay. XL and HW contributed to data analysis. All authors read and approved the final manuscript.

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**Availability of data and materials**
The data supporting the conclusions of this article are included within the article. Representative nucleotide sequences generated in this study were deposited in the GenBank database under the accession numbers KU738304-KU738385 and KU738386-KU738431. The sequences of gatB, coxA, hspa, trzZ, fbpA and wsp were deposited in the MLST database and the allele numbers are 247/242, 229, 166, 210, 27 respectively.

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
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**Competing interests**
The authors declare that they have no competing interests.

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