Wolbachia and Mosquitoes: Exploring Transmission Modes and Coevolutionary Dynamics in Shandong Province, China

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Research Article

Keywords: Wolbachia, cytochrome oxidase subunit I, Culex pipiens pallens, genetic structures

Posted Date: August 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3284101/v1

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Abstract

Background

Mosquito-borne diseases, including outbreaks of novel mosquito-borne diseases, pose a serious threat to human health. Deployment of the intracellular symbiont *Wolbachia* has been proposed as a novel strategy to modify mosquitoes with increased resistance to pathogen infection. However, little is known about its interaction with mitochondria during maternal transmission.

Method

Here, we aimed to determine the genetic structures of 11 *Culex pipiens pallens* populations and the incidence of *Wolbachia* infections in Shandong Province, China, to gain a better understanding of the relationship between mosquitoes and *Wolbachia*. The genetic structure of the *Cx. p. pallens* population was investigated using the cytochrome oxidase subunit I (*COI*) gene. *Wolbachia* infection status assessment, molecular classification, and phylogenetic analysis were performed using molecular markers for the *Wolbachia* surface protein (WSP) gene. Mosquito–*Wolbachia* relationship was investigated using tanglegram and distance-based approaches.

Results

Sequence analysis of the *COI* gene revealed 26 different mitochondrial DNA haplotypes. The neutrality test and haplotype networks for *Cx. p. pallens* populations indicated that the species is undergoing demographic expansion in Shandong Province, with significant genetic differentiation between the populations from Qingdao and most other cities. The overall *Wolbachia* infection rate of *Cx. p. pallens* was 90.7%; 15 WSP haplotypes were detected.

Conclusion

Our study revealed the genetic structure of *Cx. p. pallens* and the prevalence of *Wolbachia* in Shandong Province, China, offering important scientific information required for developing *Wolbachia*-based vector control approaches in Shandong Province. These findings would advance current understanding of *Wolbachia*’s diversity and evolution, aiding in its application as a biocontrol agent.

Background

*Culex pipiens pallens*, the most predominant *Culex* mosquito species in northern China, is a significant vector for the transmission of Japanese encephalitis virus (JEV), Bancroftian filariasis, and potentially, West Nile virus (WNV)[1]. The incidence of *Culex*-mediated disease outbreaks is increasing, posing a significant public health challenge[2].

Historically, mosquito-borne infectious diseases, including Japanese encephalitis (JE), malaria, and filariasis, have been prevalent in Shandong Province, China. For example, in the sixties and seventies of the last century, there were two major malaria outbreaks in Shandong Province, with more than 6 million and 4.6 million malaria cases, respectively[3]. During the early years following the foundation of New China, more than 2.5 million people were infected with microfilariae, and more than 2.5 million symptomatic patients were reported in Shandong Province[4]. JE is a common viral encephalitis in Asia, with an annual incidence of 70,000 cases and 15,000 deaths; China accounts for 50% of the reported JE cases[5]. Currently, Shandong Province is the northernmost focal point of dengue fever cases diagnosed in China[6], underscoring its epidemiological significance within the country. Climate change may have increased the northward expansion of dengue outbreaks in China[7]. In 2018, a significant increase in the number of West Nile virus infections was observed in 11 European Union/European Economic Area member states, with 1,605 human cases, including 166 lethal cases[8].

Insecticides have been extensively used to reduce the spread of mosquito-borne diseases but with limited success. Meanwhile, mosquitoes are becoming increasingly resistant to insecticides because of their negligent and improper use[8]. In the last two decades, *Wolbachia* infection has been ecologically and evolutionarily important to its host species, potentially reducing the
transmission rates of mosquito-borne diseases by interfering with the lifespan of adult mosquitoes, reducing their reproductive capacity, and inhibiting the growth and replication of pathogens [9].

In recent years, Guangzhou has implemented a mosquito control strategy involving the release of hundreds of thousands of *Aedes albopictus* mosquitoes artificially infected with *Wolbachia* [10], with the aim of population modifications to decrease the population of mosquitoes and suppress the transmission of the aforementioned diseases. However, the potential effect of this intervention should be considered on the prevalence and distribution of *Wolbachia* within mosquito populations. The release of large numbers of *Wolbachia*-infected mosquitoes may lead to changes in the prevalence and distribution of *Wolbachia* within mosquito populations.

The artificially introduced *Wolbachia* strain could spread and establish itself within the local mosquito population, potentially altering the natural dynamics of *Wolbachia* infections [11]. Furthermore, the interactions between *Wolbachia* strains and the pathogens carried by mosquitoes, such as JEV or WNV, may be affected by the presence of artificially introduced *Wolbachia* strains [12]. Such mass releases have been conducted in several countries around the world, such as China [10], Singapore [13], Australia [14], Brazil [15], Thailand [16], and the USA [17].

Moreover, how *Wolbachia* infection interferes with the genetic structure of *Cx. p. pallens* remains unclear. Understanding the effects of the mass release of *Wolbachia*-infected mosquitoes on the prevalence of *Wolbachia*, host population genetic structure, and the dynamics of gene flow patterns is crucial for assessing the long-term effectiveness and sustainability of this population modification strategy. Further research and surveillance are necessary to monitor any changes in *Wolbachia* prevalence and evaluate the effect of this intervention on disease transmission dynamics in Guangzhou and other areas implementing similar strategies.

In this study, we investigated the infection frequencies and distribution patterns of *Wolbachia* in Shandong Province through an analysis of the *Wolbachia* surface protein (WSP) gene in natural populations. Further, given that *Wolbachia*’s and the host’s mitochondria are co-transmitted within the egg cytoplasm and are therefore in linkage disequilibrium, we investigated mitochondrial diversity by mitochondrial DNA marker sequencing. The standard barcode region of the cytochrome oxidase subunit I (*COI*) gene fragment was used to determine the population structure of *Cx. p. pallens*. Next, the relationship between *Wolbachia* and the mitochondria of *Cx. p. pallens* was investigated to determine the potential role of *Wolbachia* in shaping genetic diversity in *Cx. p. pallens*. These findings will allow us to evaluate their roles in disease transmission and provide a scientific basis for developing more effective management strategies for controlling mosquito-borne diseases.

**Methods**

**Sample collection and preparation**

*Cx. p. pallens* were collected between July and September 2022 from 11 locations in Shandong Province (Table 1, Fig. 1). Captured mosquitoes were immediately preserved in separate sterile tubes containing 200 µL of RNAprotect® Tissue Reagent. The samples were transported to the laboratory at room temperature for species identification and DNA extraction.
Table 1
Summary of Culex pipiens pallens specimen collection sites in Shandong Province

<table>
<thead>
<tr>
<th>Site ID</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Collection date</th>
<th>Life-stages analysed</th>
<th>Specimen genotyped</th>
<th>Wolbachia infection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northwest Shandong Plain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dezhou</td>
<td>37°45′</td>
<td>116°31′</td>
<td>18-Jul</td>
<td>adult</td>
<td>115</td>
<td>99</td>
</tr>
<tr>
<td>Jiaolai Hills</td>
<td>37°54′</td>
<td>121°39′</td>
<td>13-Jul</td>
<td>adult</td>
<td>170</td>
<td>100</td>
</tr>
<tr>
<td>Qingdao</td>
<td>36°09′</td>
<td>120°37′</td>
<td>28-Jun</td>
<td>adult</td>
<td>56</td>
<td>88.2</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liaocheng</td>
<td>36°46′</td>
<td>115°98′</td>
<td>4-Jul</td>
<td>adult</td>
<td>69</td>
<td>92.7</td>
</tr>
<tr>
<td>Heze</td>
<td>35°25′</td>
<td>115°47′</td>
<td>18-Aug</td>
<td>adult</td>
<td>137</td>
<td>98.9</td>
</tr>
<tr>
<td>Alluvial plain of the Yellow River</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dongying</td>
<td>37°46′</td>
<td>118°49′</td>
<td>22-Aug</td>
<td>adult</td>
<td>44</td>
<td>69.2</td>
</tr>
<tr>
<td>Binzhou</td>
<td>37°36′</td>
<td>118°03′</td>
<td>5-Aug</td>
<td>adult</td>
<td>93</td>
<td>97.7</td>
</tr>
<tr>
<td>Central south of Shandong Plain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zibo</td>
<td>36°81′</td>
<td>118°04′</td>
<td>12-Jun</td>
<td>adult</td>
<td>28</td>
<td>97.3</td>
</tr>
<tr>
<td>Linyi</td>
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<td>118°33′</td>
<td>11-Aug</td>
<td>adult</td>
<td>50</td>
<td>86.3</td>
</tr>
<tr>
<td>Rizhao</td>
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<td>119°46′</td>
<td>15-Jul</td>
<td>adult</td>
<td>59</td>
<td>64.1</td>
</tr>
<tr>
<td>Jining</td>
<td>36°40′</td>
<td>117°02′</td>
<td>9-Jun</td>
<td>adult</td>
<td>70</td>
<td>98.1</td>
</tr>
</tbody>
</table>

Mosquito species identification and DNA extraction

The mosquito species were identified according to their morphological characteristics (https://www.wrbu.si.edu/) using a microscope (Olympus, SZX7, Japan) and further confirmed through molecular marker analysis. DNA was extracted from individual mosquitoes using the Cador® Pathogen 96 QIAcube® HT Kit following the manufacturer’s protocols and stored at −80°C.

Polymerase chain reaction (PCR) identification of Wolbachia infections

To detect the presence of the Wolbachia endosymbiont in mosquitoes, PCR-based molecular approaches were employed, using the WSP gene as the most commonly used DNA marker, with the forward primer 81F and reverse primer 691R [18]. PCR mixtures comprised 25 µL of 2X Phanta Max Master Mix, 1 µL each of 10 µM forward and reverse primers, 2 µL of template DNA, and nuclease-free water to a final volume of 50 µL. PCR conditions were as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 54°C for 45 s, and 72°C for 1 min; and 72°C for 5 min. PCR products were separated electrophoretically and sequenced at Sangon Biotech (Shanghai, China).

Amplification and sequencing of COI

The COI gene of the mitochondrial genome was amplified using primers LCO 1490 and HCO 2198 [19]. PCR mixtures comprised 25 µL 2X Phanta Max Master Mix, 1 µL each of 10 µM forward and reverse primers, 2 µL template DNA, and nuclease-free water to a final volume of 50 µL. PCR conditions were as follows: 94°C for 1 min; 5 cycles of 94°C for 40 s, 45°C for 40 s and 72°C for 1 min; 30 cycles of 94°C for 40 s, 53°C for 40 s, and 72°C for 1 min; and 72°C for 5 min. The PCR products were sequenced by Sangon Biotech (Shanghai, China).

Data analysis
All sequences were manually aligned, checked, and edited using BioEdit version 7.0 and compared with other sequences available in the GenBank database to determine the percentage identity using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Specimens showing more than 99% nucleotide sequence identity with the available species sequences in the databases were considered. Based on the mitochondrial COI gene sequences, we used Arlequin v3.5[20] and DnaSP v6[21] to calculate the number of segregating sites, haplotypes, haplotype diversity (Hd), and nucleotide diversity (Pi) and performed neutrality tests[22], namely, Tajima's D test and Fu's Fs test, to investigate the genetic diversity of Cx. p. pallens[23]. The genetic structure of the mosquito populations was ascertained via analysis of molecular variance (AMOVA) to partition the genetic variation among groups, populations within groups, and Fst[24]. Pairwise Fst and Nm values were calculated for all the populations. In addition, haplotype networks of Cx. p. pallens were constructed using the haplotype network (TCS) method in PopArt 1.7 to visualize the relationships among populations. The Bayesian clustering method in STRUCTURE v.2.3 was utilized to evaluate the geographical structure of the population. The method identified the most probable K value and assigned individuals to corresponding clusters, providing valuable insights into population structure. To achieve a deeper understanding of the data, several experiments were conducted using diverse datasets to facilitate further clustering analysis. Subsequently, COI and WSP sequences were aligned using ClustalX and a neighbour-joining phylogenetic tree model based on genetic distance values that were created using the MEGA X software[25].

Results

Prevalence and genotyping of Wolbachia in wild-caught mosquitoes

The status of Wolbachia infection in all adult Cx. p. pallens mosquitoes collected from the 11 localities in Shandong Province was determined by checking for the presence or absence of WSP genes. Overall, the infection prevalence was high, with 777 of 856 (90.7%) individuals being positive for Wolbachia. It is notable that the prevalence of Wolbachia infection exhibited variation amongst the aforementioned urban area in Rizhao and the highest (100%) in Yantai (Fig. 2). In total, 15 WSP haplotypes were detected in Wolbachia infections and clustered into 15 putative strains, named Wol 01 to Wol 15. Zhou et al.[18] identified and categorized all WSP sequences into two groups of Wolbachia strains, designated as types A and B. In this study, all the Wolbachia infections observed in Cx. p. pallens were identified as type B infections (Fig. 3).

Polymorphisms of the mitochondrial gene COI sequence

In total, 1,780 COI sequences were generated from 11 populations. The COI sequences were aligned, yielding a total length of 603 bp and 27 variable sites. The overall Hd and Pi were measured as 0.352 and 0.98 × 10^-2, respectively (Table 2). Among the 11 populations, Cx. p. pallens from Dezhou exhibited the highest haplotype diversity, and that from Yantai exhibited the highest nucleotide diversity in Shandong Province (Table 2). In general, Cx. p. pallens from coastal cities exhibited higher COI haplotype and nucleotide diversity than those from other cities. Based on differences in the nucleotide composition of the COI gene, we identified 26 mitochondrial haplotypes in the 11 studied populations, which were denoted as H01–H26 (Fig. 4).

Table 2 Polymorphism of COI and neutrality test of Culex pipiens pallens populations
<table>
<thead>
<tr>
<th>Site</th>
<th>n</th>
<th>s</th>
<th>$P_i (10^{-2})$</th>
<th>h</th>
<th>$H_d$</th>
<th>Tajima’s $D$</th>
<th>$F_u$’s $F_s$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Northwest Shandong Plain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dezhou</td>
<td>230</td>
<td>5</td>
<td>0.097</td>
<td>6</td>
<td>0.532</td>
<td>0.55576</td>
<td>0.35800</td>
<td>1.40882</td>
</tr>
<tr>
<td>Jiaolai Hills</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yantai</td>
<td>340</td>
<td>13</td>
<td>0.187</td>
<td>10</td>
<td>0.522</td>
<td>1.03730</td>
<td>0.14500</td>
<td>1.81529</td>
</tr>
<tr>
<td>Qingdao</td>
<td>112</td>
<td>1</td>
<td>0.084</td>
<td>2</td>
<td>0.504</td>
<td>1.86793</td>
<td>0.98400</td>
<td>2.41107</td>
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<td><strong>Southwest Shandong Plain</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liaocheng</td>
<td>138</td>
<td>2</td>
<td>0.019</td>
<td>3</td>
<td>0.112</td>
<td>0.99803</td>
<td>0.09800</td>
<td>-1.98235</td>
</tr>
<tr>
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<td>3</td>
<td>0.071</td>
<td>1.01339</td>
<td>0.10200</td>
<td>2.58453</td>
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<tr>
<td><strong>Alluvial plain of the Yellow River</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dongying</td>
<td>88</td>
<td>1</td>
<td>0.007</td>
<td>2</td>
<td>0.045</td>
<td>0.91012</td>
<td>0.17300</td>
<td>1.37585</td>
</tr>
<tr>
<td>Binzhou</td>
<td>186</td>
<td>8</td>
<td>0.053</td>
<td>5</td>
<td>0.145</td>
<td>1.71164</td>
<td>0.01100</td>
<td>2.33474</td>
</tr>
<tr>
<td><strong>Central south of Shandong Plain</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zibo</td>
<td>56</td>
<td>4</td>
<td>0.052</td>
<td>5</td>
<td>0.293</td>
<td>-1.42345</td>
<td>0.05900</td>
<td>3.28950</td>
</tr>
<tr>
<td>Linyi</td>
<td>100</td>
<td>6</td>
<td>0.071</td>
<td>3</td>
<td>0.116</td>
<td>-1.41815</td>
<td>0.06200</td>
<td>0.41333</td>
</tr>
<tr>
<td>Rizhao</td>
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<td>6</td>
<td>0.102</td>
<td>3</td>
<td>0.338</td>
<td>-0.99169</td>
<td>0.16200</td>
<td>1.35630</td>
</tr>
<tr>
<td>Jining</td>
<td>140</td>
<td>16</td>
<td>0.152</td>
<td>8</td>
<td>0.298</td>
<td>-1.78621</td>
<td>0.01400</td>
<td>1.29182</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01

$n$ = number of genes (two per individual), $s$ = number of polymorphic (i.e., segregating) sites, $P_i$ = nucleotide diversity, $h$ = number of haplotypes, $H_d$ = haplotype diversity.
Table 3
Mitochondrial DNA-based population differentiation for population pairs (estimates of $F_{st}$ below the diagonal and $Nm$ above the diagonal)

<table>
<thead>
<tr>
<th></th>
<th>DY</th>
<th>BZ</th>
<th>DZ</th>
<th>HZ</th>
<th>LC</th>
<th>LY</th>
<th>QD</th>
<th>RZ</th>
<th>YT</th>
<th>JN</th>
<th>ZB</th>
</tr>
</thead>
<tbody>
<tr>
<td>DY</td>
<td>-</td>
<td>16.33</td>
<td>0.80</td>
<td>17.75</td>
<td>12.93</td>
<td>9.05</td>
<td>0.3</td>
<td>2.56</td>
<td>1.13</td>
<td>10.88</td>
<td>8.16</td>
</tr>
<tr>
<td>BZ</td>
<td>0.01568</td>
<td>-</td>
<td>1.13</td>
<td>19.90</td>
<td>14.25</td>
<td>664.76</td>
<td>0.64</td>
<td>4.31</td>
<td>1.38</td>
<td>10.52</td>
<td>12.76</td>
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<tr>
<td>DZ</td>
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<td>0.18052</td>
<td>-</td>
<td>0.83</td>
<td>1.07</td>
<td>1.21</td>
<td>5.11</td>
<td>6.23</td>
<td>26.27</td>
<td>1.73</td>
<td>1.10</td>
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<tr>
<td>HZ</td>
<td>0.01389</td>
<td>0.01241</td>
<td>0.23112</td>
<td>-</td>
<td>12.65</td>
<td>11.04</td>
<td>0.31</td>
<td>2.73</td>
<td>1.16</td>
<td>9.59</td>
<td>8.32</td>
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<tr>
<td>LC</td>
<td>0.01897</td>
<td>0.01724</td>
<td>0.16888</td>
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<td>-</td>
<td>8.98</td>
<td>0.38</td>
<td>3.90</td>
<td>1.43</td>
<td>9.47</td>
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<tr>
<td>LY</td>
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<td>0.00038</td>
<td>0.17107</td>
<td>0.02215</td>
<td>0.02710</td>
<td>-</td>
<td>0.49</td>
<td>5.30</td>
<td>1.46</td>
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<tr>
<td>QD</td>
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<td>0.36122</td>
<td>0.04664</td>
<td>0.44496</td>
<td>0.39740</td>
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<td>RZ</td>
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<td>0.36619</td>
<td>0.07417</td>
<td>0.15531</td>
<td>0.02588</td>
<td>-</td>
</tr>
</tbody>
</table>

DY: Dongying; BZ: Binzhou; DZ: Dezhou; HZ: Heze; LC: Liaocheng; LY: Linyi; QD: Qingdao; RZ: Rizhao; YT: Yantai; JN: Jining; ZB: Zibo

Haplotype network construction of Cx. p. pallens

The network was generated with TCS using 860 COI sequences of Cx. p. pallens, which identified 26 haplotypes across the sampled sites (Fig. 4). H01 and H06 were the most frequently observed haplotypes. H01 was the central haplotype that was highly connected to the haplotype lines and was the only haplotype found in all the localities in Shandong Province. Nearly all other haplotypes originated from H01 through one or more mutations, and H01 was likely the ancestral variant. The haplotype distribution showed that H01, H05, H06, H08, H11, H13, and H15 were shared in some populations, whereas the remaining haplotypes were only observed in another population.

Population genetic structure

In total, 890 individuals from 11 populations were evaluated to analyse the genetic diversity. Pairwise comparisons revealed that the genetic variation in populations among groups ($F_{st}$) values were significantly different from zero, with the lowest value (0.00109) found between Binzhou and Linyi and the highest value (0.54103) between Qingdao and Heze (Table 2). Generally, highly significant pairwise population differentiation was observed between Qingdao and most other populations ($F_{st} > 0.25$). However, frequent gene flow occurred in other populations (pairwise gene flow [$Nm$] > 1). AMOVA showed that the majority of the genetic variance occurred within populations (87.91%) (Table 4). The total $F_{st}$ was 0.13135 ($P > 0.05$), and $Nm$ was 1.65, reflecting moderate population differentiation.

Table 4
Analysis of molecular variance test of all 11 Culex pipiens pallens populations collected from different regions of Shandong province

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>10</td>
<td>59.331</td>
<td>0.03597</td>
<td>12.09</td>
</tr>
<tr>
<td>Within populations</td>
<td>1770</td>
<td>462.799</td>
<td>0.26147</td>
<td>87.91</td>
</tr>
</tbody>
</table>
Fu’s $F_s$ and Tajima’s $D$ values for the *Cx. p. pallens* populations in Shandong Province were almost entirely negative, except in Qingdao (Table 2). Tajima’s $D$ value ($-1.97$) and Fu’s $F_s$ value ($-27.58$) for the overall population were significant, indicating a high number of low-frequency mutations, and that *Cx. p. pallens* in Shandong Province is undergoing demographic expansion. Among specific populations, Jining and Binzhou had significantly negative $D$ values, whereas Dongying and Zibo exhibited significant negative $F_s$ values. Tajima’s $D$ and Fu’s $F_s$ tests revealed that Jining, Binzhou, Dongying, and Zibo were significantly negative, suggesting recent population expansion or selection.

Multilocus cluster Bayesian analysis of all 11 population samples showed the genetic structure among *Cx. p. pallens* populations (number of genetic clusters $K = 2–11$) (Fig. 5a) [26]. The results showed that when $K = 4$, a larger delta $K$ value was obtained, and when $K = 7$, the delta $K$ value was the largest. Therefore, the optimal $K$ value was determined to be $4–7$ (Fig. 5b). Based on the geographical distribution and genetic differentiation of Shandong Province, we divided the 11 populations into five groups: north-west Shandong Plain (Dezhou), Jiaolai Hills (Yantai, Qingdao), the south-west Shandong Plain (Liaocheng, Heze), alluvial plain of the Yellow River (Dongying, Binzhou), and south-central Shandong Plain (Zibo, Linyi, Rizhao, Jining).

**Migration and gene flow patterns**

LAMARC analysis revealed that historical gene flow rates varied from 0.88–96.02. High migration rates were observed among neighbouring populations within each of the five locality groups (Fig. 6). Medium migration levels were observed in the five locality groups. The coalescent analysis revealed that migration was not symmetric. (Fig. 6).

**Evolutionary relationships between mosquitoes and Wolbachia**

We documented the occurrence of associations between mosquitoes and the *Wolbachia* symbiont in wild-caught specimens from Shandong Province. Visualization of these associations using a tanglegram revealed patterns of broad associations (Fig. 7). The distance-based quantitative test showed no significant consistency between the mosquito and *Wolbachia* phylogenies at the global level (ParaFit Global test: ParaFit Global < 0.001, $P = 1$). Among the host–endosymbiont links, the associations between H07 and Wol 08, H07 and Wol 12, H07 and Wol 13, and H15 and Wol 3 were statistically significant (Table 5).

<table>
<thead>
<tr>
<th>Host</th>
<th>Wolbachia</th>
<th>PF1.statistic</th>
<th>PF1 P-value</th>
<th>PF2.statistic</th>
<th>PF2 P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H07</td>
<td>Wol08</td>
<td>3.79×10^{-9}</td>
<td>0.03</td>
<td>-1.5×10^{-3}</td>
<td>0.03</td>
</tr>
<tr>
<td>H07</td>
<td>Wol12</td>
<td>5.07×10^{-9}</td>
<td>0.04</td>
<td>-2.01×10^{-3}</td>
<td>0.04</td>
</tr>
<tr>
<td>H07</td>
<td>Wol13</td>
<td>1.92×10^{-9}</td>
<td>0.03</td>
<td>-0.76×10^{-3}</td>
<td>0.03</td>
</tr>
<tr>
<td>H15</td>
<td>Wol07</td>
<td>2.77×10^{-9}</td>
<td>0.04</td>
<td>-1.1×10^{-3}</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Discussion**

**Detection of Wolbachia infection and its distribution in wild mosquitoes**

Herein, we assessed the prevalence of *Wolbachia* in *Cx. p. pallens* collected from Shandong Province. The overall infection rate of all tested mosquitoes was 90.7%, indicating that *Wolbachia* was widespread in *Cx. p. pallens*. The WSP molecular marker has enabled the successful detection of *Wolbachia* infection across numerous taxa. Additionally, it has facilitated the genotyping of *Wolbachia* strains and the analysis of their evolutionary relationships. We found a low genetic diversity of *Wolbachia* strains in *Cx. p. pallens*; wAlbA and wAlbB coinfection was not observed in natural populations, and they were only infected with wAlbB. Studies have shown that animal habitats such as wetlands and forests tend to have relatively higher infection rates of mosquito-borne viruses and bacteria [27]. However, infection rates of *Cx. p. pallens* in the Yellow River Delta wetland of Dongying, an important habitat for migratory birds [28], were lower than those in the other places. These observations provide new insights into the application of *Cx. p. pallens* to mediate the transmission of mosquito-borne diseases via *Wolbachia*.

**Effect of the Guangzhou “mosquito factory” on mosquitoes in other areas**
The centrepiece of the “mosquito factory” is a colony of Wolbachia mosquitoes, commonly known as the brood stock, who breed all future populations of Wolbachia mosquito offspring. Owing to cytoplasmic incompatibility, the release of Wolbachia-infected male mosquitoes into the wild has become a promising strategy for suppressing wild mosquito populations[29]. However, the effects of Wolbachia on the behavioural patterns of the host need further studies to evaluate the consequences of releasing these Wolbachia-infected insects into the environment[30], for example, if the release of numerous Wolbachia-infected Aedes albopictus in Guangzhou affects the original ecological balance because of the increase or decrease in the abundance of target species. Because Wolbachia can switch from one host species to another, their use is not considered a successful approach [31]. This raises further questions regarding the effects on other species.

Because of the growing population of Cx. p. pallens in Shandong Province, in addition to assessing the geographic regions affected by climate change, the taxa of hosts, and transmission types of pathogens, future research needs to carefully consider the potential effects of Wolbachia. We believe that the results of our study can provide a basis for related future research, policies, and practices.

Herein, we found that the infection rate of Cx. p. pallens in Shandong Province was high. The phylogenetic tree analysis showed that Cx. p. pallens had low homology with Wolbachia-infected Aedes albopictus from Guangzhou, whereas its homology with Wolbachia-infected Culex mosquitoes from Guangzhou was high. The effects of Wolbachia infection on host behaviour under field conditions and the ecosystem need to be investigated. The molecular mechanisms underlying the effects of Wolbachia on host behaviour also need to be further elucidated. The effects of Wolbachia on host fitness traits may be multidimensional, and its infection may modify various host genes, micro RNAs, and proteins.

Geographical isolation of Cx. p. pallens populations from Qingdao

Herein, we analysed the population genetics of Cx. p. pallens collected from different regions of Shandong Province based on the COI gene. Most (87.91%) of the genetic variations occurred within individuals, whereas only approximately 12.09% of the total variations were detected among populations. We found considerable genetic differentiation and limited gene flow between the Qingdao population and populations from other cities. In contrast to other cities within Shandong Province, Qingdao is primarily situated on a hilly terrain that slopes from east (Laoshan Mountain Group) to west (Jiaozhou Bay), with rolling hills in the north and the Fushan Mountain range to the south along the Pacific coast. Moreover, the northern side of Qingdao is bordered by the expansive Pacific Ocean, which forms a natural geographical barrier between Qingdao and other cities within the province. In addition, there are differences in the geographical environment between other cities. Therefore, precise countermeasures need to be implemented for the prevention and control of mosquito vectors.

Demographic expansion of Cx. p. pallens in Shandong Province

The demographic expansion closely corresponded to the COI haplotype network. The haplotype profiles were star-shaped, reflecting their recent appearance and rapid population growth. The neutrality test results were markedly negative, which further supported this phenomenon. A strong link exists between demographic expansion and climatic variability. Meteorological factors, specifically including temperature, humidity, and precipitation, have a significant impact on the number, density, and distribution of disease vectors, as well as their spatial and temporal dynamics, including epidemic frequency and intensity of vector-borne diseases, with the coexistence of spatiotemporal heterogeneity. The development and survival of mosquitoes and viral replication depend on environmental conditions, particularly climatic conditions. According to a recent study, the temperature in most regions of China has risen over the past five decades, with the rates of annual temperature change varying substantially from −0.22°C/year to 0.58°C/year among the observed sites [7]. Notably, Shandong Province reported its first indigenous case of dengue fever on August 16, 2017. A total of 95 cases were subsequently reported across the province, with 79 of these occurring in Jining, thenorthernmost region where local cases were detected [32]. Therefore, upgrading surveillance systems for vectors and vector-borne diseases regarding climate change can strengthen research on risk assessments, predictions, early warnings, control strategies, and intervention measures to effectively cope with the new challenges of climate-sensitive vector-borne diseases.

Evolutionary relationships between mosquitoes and Wolbachia

The phenomenon of symbiosis is a significant driving force behind evolutionary change, having a profound influence on virtually every aspect of biology, from population ecology and evolution to genomics and molecular/biochemical mechanisms of development and reproduction. A broad association pattern was observed between mosquitoes and Wolbachia strains based on the
tanglegram (Fig. 7). A previous study reported that *Aedes* mosquitoes were significantly associated with *Wolbachia* supergroup A, whereas other species, particularly *Culex* mosquitoes, were more closely linked with *Wolbachia* supergroup B. This indicated that closely-related *Wolbachia* strains are likely to establish themselves in related hosts. Herein, we found that all infected *Wolbachia* in *Cx. p. pallens* were associated with *Wolbachia* supergroup B, which is consistent with the results of the above-mentioned study. Wol08, Wol12, Wol13, and haplotypes H07, Wol07, and H15 have shown notable co-evolution. Further investigation is necessary to determine whether these variations in mosquito-*Wolbachia* interactions are the result of ongoing evolution or are due to chance infections or specific local environments. The implications of understanding *Wolbachia* host specificity are significant for effectively implementing strategies to control these bacteria. These factors will be instrumental in optimizing such measures to effectively contain and manage the spread of these bacterial infections. Besides the selection of strains that can effectively limit pathogen replication, strains should also be selected based on their host specificity.

**Conclusions**

This study summarizes the population genetic structure of *Cx. p. pallens* in Shandong Province and predicts the risk of mosquito-borne disease transmission based on global climate changes and the specificity of a geographical environment. The release of *Wolbachia* as a vector control has been widespread in many countries, exhibiting potential reductions in host life span and the prevention of pathogens from completing their life cycle. Therefore, it is imperative to conduct extensive research on the natural infection of *Wolbachia* in mosquito vectors using advanced and precise detection methods. Further exploration on the long-term impact of *Wolbachia* on new hosts and its influence on pathogen suppression is essential to gain a comprehensive understanding. The combination of *Wolbachia* and mitochondrial markers highlights the evolutionary relationship between *Wolbachia* infections and their hosts. This study provides an experimental basis for future studies regarding scientific and accurate control of vector-borne mosquito diseases.

**Abbreviations**

WSP, *Wolbachia* surface protein; COI, cytochrome oxidase subunit I; *Cx. p. pallens*, *Culex pipiens pallens*; JEV, Japanese encephalitis virus; WNV, West Nile virus; JE, Japanese encephalitis; Hd, haplotype diversity; Pi, nucleotide diversity; Fst, genetic variation in populations among groups; Nm, pairwise gene flow; AMOVA, analysis of molecular variance; K, number of genetic clusters; and PCR, Polymerase chain reaction.

**Declarations**

**Acknowledgements**

The authors would like to thank all the reviewers who participated in the review during the preparation of this manuscript.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China [81871685 to MQG], Shandong Provincial Natural Science Foundation (ZR2020KH001 to HML), NHC Key Laboratory of Parasite and Vector Biology (National Institute of Parasitic Diseases, Chinese Center for Diseases Control and Prevention, NHCKFKT2021-02 to HML), Academic promotion programme of Shandong First Medical University (2019QL005), The Innovation Project of Shandong Academy of Medical Sciences and Qilu Health and Health Outstanding Young Talents Project Special Fund.

**Availability of data and materials**

Not applicable.

**Authors’ contributions**

CHZ, XJW, YL, HFW, QTS, PC, and YZ provided the experimental data and wrote the manuscript. MQG and HML reviewed and edited the manuscript. All authors read and approved the final manuscript.

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

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


Figure 1

The distribution of field-collected *Culex pipiens pallens* mosquitoes sampled in this study. Site abbreviations: BZ, Binzhou; DZ, Dezhou; LC, Liaocheng; HZ, Heze; JN, Jining; DY, Dongying; ZB, Zibo; LY, Linyi; YT, Yantai; QD, Qingdao and RZ, Rizhao.
Figure 2

_Wolbachia_ prevalence in 11 populations of *Culex pipiens pallens* in Shandong province. The prevalence was determined based on the polymerase chain amplification of the _Wolbachia_ surface protein marker.
Figure 3

Surface protein gene sequence-based maximum likelihood phylogenetic tree for *Wolbachia* from different hosts. The sequences were extracted from GenBank.
Figure 4

Phylogenetic network of 11 cytochrome oxidase subunit I gene haplotypes in *Culex pipiens pallens*. The haplotype network graph was constructed through the implementation of the TCS method. The diameter of each circle is indicative of its corresponding frequency. The illustration of different hues represents distinct populations of *Cx. p. pallens* in Shandong Province. The numbers above the line refer to variable asynchronous numbers.
Figure 5

Population structure of *Culex pipiens pallens* in Shandong Province. a. Stacked bar plots of STRUCTURE for K = 2 to 11 subgroups. Each vertical bar represents an individual, and the length of coloured partitions represents the proportion of the genome of that individual. b. Delta K plotted against putative K ranging from 2 to 11.
Figure 6

Bayesian analysis of historical migration asymmetry between populations of *Culex pipiens pallens*. The five localities can be identified by the dotted circles. Arrows indicate the direction of the migration rates, indicating how they are moving.
Figure 7

Tanglegrams of mosquito cytochrome oxidase subunit I and *Wolbachia* endosymbiont neighbour-joining trees. The host–endosymbiont association revealed by the Global Parafit test to be significant in terms of congruence between host and endosymbiont phylogenies is indicated by these lines.