Interaction of Arsenophonus with Wolbachia in Nilaparvata lugens

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Abstract

Background: Co-infection of endosymbionts in the same host is ubiquitous, and the interactions of the most common symbiont Wolbachia with other symbionts, including Spiroplasma, in invertebrate organisms have received increasing attention. However, the interactions between Wolbachia and Arsenophonus, another widely distributed symbiont in nature, are poorly understood. We tested the co-infection of Wolbachia and Arsenophonus in different populations of Nilaparvata lugens and investigated whether co-infection affected the population size of the symbionts in their host.

Results: A significant difference was observed in the co-infection incidence of Wolbachia and Arsenophonus among 5 populations of N. lugens from China, with nearly half of the individuals in the Zhenjiang population harbouring the two symbionts simultaneously, and the rate of occurrence was significantly higher than that of the other 4 populations. The Arsenophonus density in the superinfection line was significantly higher only in the Maanshan population compared with that of the single-infection line. Differences in the density of Wolbachia and Arsenophonus were found in all the tested double-infection lines, and the dominant symbiont species varied with the population only in the Nanjing population, with Arsenophonus the overall dominant symbiont.

Conclusions: Wolbachia and Arsenophonus could coexist in N. lugens, and the co-infection incidence varied with the geographic populations. Antagonistic interactions were not observed between Arsenophonus and Wolbachia, and the latter was the dominant symbiont in most populations.

Keywords: Nilaparvata lugens, Wolbachia, Arsenophonus, Co-infection

Background

Symbiotic associations between prokaryotic and eukaryotic organisms are ubiquitous in natural communities, and bacterial symbiosis has played a fundamental role in the evolution of eukaryotes, which range from parasitism to mutualism [2, 20, 32]. Many invertebrate hosts have been found to harbour multiple inherited symbionts within a single host [21, 27, 30, 34, 40]. Other than co-infection of different symbiont species, co-infections with multiple strains of the same symbiont species have also been found [7, 28].

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Wolbachia is an intracellular symbiont that infects between 20 and 76% of arthropod species [14, 38]. Wolbachia has been known to coinfect and interact with various symbionts in the same host, and the superinfections vary with the species of symbionts and are also affected by many other factors, including the species of insect host, environmental conditions, etc. [7, 8, 21, 28]. In Bemisia tabaci, Wolbachia was found to be present with Hamiltonella or Cardinium or both genera [8]. Co-infection of Wolbachia and Cardinium was also found in Encarsia inaron [39], and superinfection with combination of Wolbachia and Spiroplasma occurs in Drosophila melanogaster, whereas an asymmetrical interaction occurs between Wolbachia and Spiroplasma in which the population of Wolbachia organisms is negatively affected.
by *Spiroplasma* organisms while the population of *Spiroplasma* organisms is not influenced by *Wolbachia* organisms [7]. The genus *Arsenophonus* is an emerging clade of symbiotic bacteria with a vast host distribution that includes parasitic wasps, triatomine bugs, psyllids, whiteflies, aphids, ticks, planthoppers, etc. [6, 8, 10, 24, 26, 35]. However, interactions among *Arsenophonus* and *Wolbachia* are poorly understood.

Brown planthopper *Nilaparvata lugens* Stål (Homoptera: Delphacidae) is a monophagous insect herbivore of rice that causes serious damage to rice crops. *N. lugens* has been known to harbour symbionts, including *Wolbachia* and *Arsenophonus*, and previous detection has shown that although *Wolbachia* and *Arsenophonus* were present in all 15 brown planthopper populations collected from China and Southeast Asian countries, coexistence was not observed in the same individuals from Laos [26]. In this study, we investigated the co-infection of *Wolbachia* and *Arsenophonus* in different populations of *N. lugens* collected from 5 sites in China, and then we established a single-infected line (infected with only *Wolbachia*) and a double-infected line (infected with both *Wolbachia* and *Arsenophonus*). Subsequently, we examined *Wolbachia* and *Arsenophonus* titres in the double- and single-infected *N. lugens* to assess whether these two symbionts interacted mutually or competitively.

**Methods**

**Field collection of *Nilaparvata lugens***

All geographic populations of brown planthopper were collected from rice paddies in different locations of China. The details of each population were listed in Table 1. The planthoppers were maintained on rice seedlings at a constant temperature of 27 (±1) °C and a light period of 14:10 h light:dark.

**Investigation of *Wolbachia* and *Arsenophonus* infection**

To compare the co-infection of *Wolbachia* and *Arsenophonus* among different geographic populations of *N. lugens*, approximately 80 (64–88) adults were randomly collected from each population for a diagnostic PCR analysis. Extraction of DNA was the same as previously described, and only DNA samples with a ratio of OD260/OD280 ranging from 1.6 to 1.9 were used for the PCR detection [18]. The presence of *Wolbachia* and *Arsenophonus* was checked as previously described (*Wolbachia*: [41], *Arsenophonus* [31]).

**Preparation of single-infected (*Wolbachia*) line and double-infected line (**Wolbachia** and **Arsenophonus**)**

Geographic populations were set up as mass bred lines. The single-infected (*Wolbachia*) line and double-infected lines (*Wolbachia* and *Arsenophonus*) were developed from each geographic population of *N. lugens*. To minimize variation in the genetic background within populations, a pair of newly emerged female and male adults was randomly selected from the same population.

To ensure that only the single infection or the double infection was being considered, at first, newly emerged brown planthoppers from each line were screened for the presence of all the known symbionts in planthoppers, which consisted of *Wolbachia*, *Arsenophonus*, *Cardinium hertigi*, *Acinetobacter*, *Chryseobacterium*, *Serratia* and *Arthrobacter* as previously described (*Wolbachia*: [41], *Arsenophonus* [31]; *Cardinium*: [23]; *Acinetobacter*: [33]; *Chryseobacterium*: [1]; *Serratia*: [43]; *Arthrobacter*: [15]). Then female and male parents and their offspring that were only infected with *Wolbachia* or only infected with *Wolbachia* and *Arsenophonus* were kept for subsequent experiments.

**Analysis of *Wolbachia* and *Arsenophonus* density**

In order to measure the density of *Wolbachia* and *Arsenophonus*, the real-time quantitative PCR was performed with an ABI StepOne Real-Time PCR System (Applied Biosystems Inc, Foster City, CA, USA). For each line, a total of 10 female and male adults was collected as one sample, and the DNA was extracted with a Wizard® Genomic DNA Purification Kit (Promega, USA). The primers of *Wolbachia* and *Arsenophonus* for the reaction were as follows: (*Wsp*-F) 5’-ATGTAACCTCCAG AAATCAAACCT-3’, (*Wsp*-R) 5’-GATACCCAGCATC ATCCTTAGC-3’; (*ARS16S*-F) 5’-TTCCGTCTCGGAAC TCAAAGG-3’ (*ARS16S*-R) 5’-TCTGAGTTCGGCTTC CCATC-3’. The 20 µL quantitative PCR (qPCR) reaction system included 10 µL SYBR® Premix Ex Taq (Tli RNaseH Plus) (2X) (Takara, Japan), 0.4 µL forward and 0.4 µL reverse primers, 0.4 µL ROX Reference Dye, 2 µL DNA and 6.8 µL ddH2O. The RT-PCR program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s, and then 95 °C for 15 s, 60 °C for 1 min, and a final step at 95 °C for 15 s. A standard curve using real-time fluorescent quantitative PCR of the *Wolbachia* wsp gene or the *Arsenophonus* ARS16S rDNA

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**Table 1: Information for the different geographic populations of brown planthopper**

| Abbreviations | Collection site | Longitude (°’) | Latitude (°’) | Collection time |
|---------------|----------------|----------------|---------------|----------------|
| NN            | Nanning        | 108° 33’      | 22° 84’       | 2014.6         |
| MS            | Maanshan       | 118° 37’      | 31° 70’       | 2012.8         |
| NJ            | Nanjing        | 118° 46’      | 32° 03’       | 2005.8         |
| ZJ            | Zhenjiang      | 119° 55’      | 32° 00’       | 2012.8         |
| NT            | Nantong        | 120° 86’      | 32° 01’       | 2013.8         |
gene was performed to determine accurate *Wolbachia* or *Arsenophonus* gene copy numbers as described previously [42]. For each sample, there was three technical replicates, and for each line, there was three biological replicates.

**Statistics**
The infection incidence of *Arsenophonus* and *Wolbachia* among different populations were compared using the Chi-square test, and the density of *Arsenophonus* between the double-infected line and single-infected line were compared using Student’s t test, the density of *Wolbachia* among different populations were tested by ANOVAs. IBM Statistics (SPSS 19.0) software was used for these statistical analyses.

**Results**
**Co-infection of *Wolbachia* and *Arsenophonus* varies with the population of *Nilaparvata lugens***

The symbionts *Wolbachia* and *Arsenophonus* were detected in all the 5 populations of *N. lugens* from China (Fig. 1). Compared to *Wolbachia* infection, *Arsenophonus* infection was more common in *N. lugens*, with the infection incidence of *Arsenophonus* ranging from 88.9 to 100%. In the MS and NJ populations, all the tested individuals were infected with *Arsenophonus*, and the infection incidence was significantly higher than that in the NT population (88.9%) ($\chi^2 = 17.196, P = 0.002$, Fig. 1).

In all 5 tested populations, *Wolbachia* infection always coexisted with *Arsenophonus* infection, and the co-infection incidence of *Wolbachia* and *Arsenophonus* was the equivalent to the incidence of *Wolbachia* infection. The co-infection incidence in the ZJ population was 50%, which was the highest value among the 5 populations, whereas the co-infection incidence in the MS and NJ populations was rare at only 2.3% and 1.5%, respectively, while this value in the NN and NT populations was 15.5% and 25%, respectively, and a significant difference was observed among populations ($\chi^2 = 75.457, P < 0.001$, Fig. 1).

**Coexistence of *Wolbachia* does not negatively affect the density of *Arsenophonus* in most populations of *Nilaparvata lugens***

When *Arsenophonus* coexisted with *Wolbachia* in *N. lugens*, *Arsenophonus* density between the double-infected line and single-infected line varied based on the population (Fig. 2). In double-infected lines established from the NN, NJ, ZJ and NT populations, the *Arsenophonus* density was not significantly different from that in the single-infected lines (NN: $t = 0.813, df = 4, P = 0.462$; NJ: $t = 0.661, df = 4, P = 0.545$; ZJ: $t = 1.61, df = 4, P = 0.183$; NT: $t = 0.803, df = 4, P = 0.467$), whereas in double-infected lines established from the MS population, the *Arsenophonus* density was significantly higher than that in single-infected line (MS: $t = 5.66, df = 4, P = 0.005$).

**Dominance of *Wolbachia* and *Arsenophonus* varies with the population of *Nilaparvata lugens***

The *Wolbachia* density in the double-infection lines varied with the population (Fig. 3). In the line established from the ZJ population, the *Wolbachia* density was significantly higher than that in the lines from the MS, NJ, and NT populations ($F_{4,14} = 8.832, P = 0.003$).

The relative ratio of *Wolbachia* and *Arsenophonus* quantity in the double-infected lines of *N. lugens* also
Interactions between coexisting symbionts may affect infection densities because the symbionts may compete for available resources and space in the host body or they may share the resources and habitats by regulating their own exploitation to avoid damaging the whole symbiotic system [3, 12, 16, 29]. In pea aphids, the density of the primary symbiont Buchnera aphidicola is depressed when the insect is co-infected with Serratia symbiotica [16] or Rickettsia [29]. An antagonistic interaction between Hamiltonella and Cardinium has also been found in Bemisia tabaci, and the density of Cardinium increased across time and led to a decrease of Hamiltonella density [40]. Asymmetrical interactions have been found between the reproductive parasites Spiroplasma and Wolbachia in Drosophila melanogaster in which the population of Wolbachia organisms was affected by Spiroplasma while the population of Spiroplasma was not affected by Wolbachia [7]. Other than the interaction between different species of symbionts, interactions are also observed between different strains of the same symbiont. When multiple Wolbachia strains were observed in the same host, the density of each strain was specifically regulated [13, 17], which limited the segregation of symbionts through inefficient transmission by maintaining a sufficiently high density of each symbiont [4].

Discussion

When different symbionts are simultaneously present within the same host, interactions between them will take place, which might affect the dynamics of the microbial population. The interaction of the common endosymbiont Wolbachia with other symbionts has received increasing attention. An asymmetrical interaction has been found between Wolbachia and Spiroplasma [7]. Our aim in this study was to test whether interactions between Wolbachia and another popular symbiont, Arsenophonus, in the same host could affect the titre of the symbionts. We established 5 double-infected lines from different natural populations of N. lugens, and they were stable co-infections.

Previous studies have shown that the brown planthopper population from Laos was extensively infected by Wolbachia or Arsenophonus and the two bacteria may be exclusive in each host individual [26]. We found that Arsenophonus and Wolbachia could coexist in the same individual of brown planthopper in all the tested populations from China and differences among populations might result from differences in population resources.

The double-infection incidence of Wolbachia and Arsenophonus in brown planthopper varied with the geographical populations in China. In the ZJ population, the double-infection incidence was the highest, with half of the individuals simultaneously harbouring Wolbachia and Arsenophonus, whereas in the NJ and MS populations, less than 3% were infected with the two symbionts. The variance in double infection has been found in small brown planthopper, with a significantly higher co-infection incidence of Wolbachia and Serratia observed in the buprofezin-resistant strain compared with that of the buprofezin-susceptible strain [18].

Interaction between coexisting symbionts may affect infection densities because the symbionts may compete for available resources and space in the host body or they may share the resources and habitats by regulating their own exploitation to avoid damaging the whole symbiotic system [3, 12, 16, 29]. In pea aphids, the density of the primary symbiont Buchnera aphidicola is depressed when the insect is co-infected with Serratia symbiotica [16] or Rickettsia [29]. An antagonistic interaction between Hamiltonella and Cardinium has also been found in Bemisia tabaci, and the density of Cardinium increased across time and led to a decrease of Hamiltonella density [40]. Asymmetrical interactions have been found between the reproductive parasites Spiroplasma and Wolbachia in Drosophila melanogaster in which the population of Wolbachia organisms was affected by Spiroplasma while the population of Spiroplasma was not affected by Wolbachia [7]. Other than the interaction between different species of symbionts, interactions are also observed between different strains of the same symbiont. When multiple Wolbachia strains were observed in the same host, the density of each strain was specifically regulated [13, 17], which limited the segregation of symbionts through inefficient transmission by maintaining a sufficiently high density of each symbiont [4].
In our study, we found that in brown planthopper, co-infection with Wolbachia did not negatively affect eh Arsenophonus population and did not lead to lower net bacterial densities. In addition, the relative ratio of Wolbachia and Arsenophonus quantity in the double-infected lines of N. lugens varied with the geographic population. In the double-infected lines from the NN, ZJ, NT and MS populations, Wolbachia was the dominant symbiont, whereas in the double-infected line from the NJ population, Arsenophonus was the dominant symbiont and had a significantly higher density than that of Wolbachia. The difference in Arsenophonus density among lines might be related to the period of maintenance in the lab because the NJ population has been maintained for more than 14 years before investigation, which is at least 7 years longer than the other populations. This longer period of maintenance may possibly benefit the accumulation of Arsenophonus.

Wolbachia can provide protection against environmental stress, including RNA viruses and insecticides [11, 18, 19, 36], and this genus also confers certain fitness benefits to their hosts [22, 37]; however, Wolbachia can also have deleterious effects on the life history of their hosts [5, 9]. Arsenophonus was also found to provide protection against environmental stress, such as protection against the entomopathogenic fungi Metarhizium anisopliae [44], although it also induced negative effects on their hosts, such as decreasing the chemical insecticide (imidacloprid) resistance of rice brown planthopper [25]. Co-infection of Wolbachia and Arsenophonus is stable in brown planthopper, which raises the question of how these genera evolve and the effect that they have on the phenotype of their host.

Conclusions
Interactions of Wolbachia, the most common symbiont, with Arsenophonus, another widely distributed symbiont in nature has not been reported previously. Present study indicated that Wolbachia and Arsenophonus could coexist in N. lugens, and the co-infection incidence varied with the geographic populations. Antagonistic interactions were not observed between Arsenophonus and Wolbachia, and Wolbachia was the dominant symbiont in most populations.

Authors’ contributions
HG analyzed the infection incidence of symbionts, and was a major contributor in writing the manuscript. NA established most of the lines and analyzed the quantity of symbionts. HN collected part of the samples from fields. DZ established part of the lines. ZZ collected part of the samples from fields. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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