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# High prevalence of *Wolbachia* infection does not explain unidirectional cytoplasmic incompatibility of *Altica* flea beetles

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**Abstract.** 1. *Wolbachia* are intracellular bacteria found in a wide range of arthropods that can impact reproductive isolation of their hosts. Previous studies showed unidirectional cytoplasmic incompatibility (CI) and high infection rates by *Wolbachia* in the closely related leaf beetles *Altica fragariae* and *Altica viridicyanea*; however, whether this reproductive isolation was induced by *Wolbachia* remains unclear.

2. This study estimated the prevalence of *Wolbachia* in *Altica* beetles, assessed genetic diversity of *Wolbachia* strains infecting these beetles, and tested whether *Wolbachia*-induced CI explains reproductive isolation of *A. fragariae* and *A. viridicyanea*.

3. The results show that all of the 11 tested *Altica* species were infected by *Wolbachia*, and the infection rate was as high as 97.0% ($n = 235$). Multi-strain infections were common, being found in 10 of the 11 species tested and accounting for 23.0% of all screened specimens. In total, 35 *Wolbachia* strains were identified based on 208 *wsp* sequences obtained. Although a majority of *A. fragariae* and *A. viridicyanea* individuals from the Beijing population were infected with only one strain each, multi-strain infections did occur in both species. Antibiotic curing experiments did not change hatching success in either inter- or intraspecific crosses of *A. fragariae* and *A. viridicyanea*, indicating that these *Wolbachia* strains do not induce CI. These results were further corroborated by the lack of the *Wolbachia cifB* gene, which is responsible for causing CI.

4. These findings suggest that high prevalence of *Wolbachia* infection is unlikely to contribute to reproductive isolation and speciation in this system.

**Key words.** Cytoplasmic incompatibility, hatching success, MLST, speciation, *Wolbachia*, *wsp*.

## Introduction

The alpha-Proteobacterium *Wolbachia* are intracellular bacteria commonly found in a wide range of insects. Estimations based on meta-analysis indicate that about half of all insect species are infected by *Wolbachia* symbionts (Werren & Windsor, 2000; Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012). A systematic review determined that the infection rate of coleopterans is *c.* 38%, varying from 0% to 88% across families and genera (Kajtoch & Kotásková, 2018). In a recent experimental screening across 297 beetle taxa, *c.* 27% of them were found to be infected (Kajtoch *et al.*, 2019). High infection rates by *Wolbachia* can have significant effects on their hosts through changes in reproduction. Indeed, *Wolbachia* is well known to manipulate reproduction of its hosts via multiple mechanisms, including induction of parthenogenesis, feminisation of males, selective killing of male offspring, and

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cytoplasmic incompatibility (CI; Werren, 1997; Stouthamer *et al.*, 1999; Werren *et al.*, 2008; Correa & Ballard, 2016). In particular, *Wolbachia*-induced CI has been suggested as an important factor promoting rapid speciation in a diversity of insects (e.g. Bordenstein *et al.*, 2001; Telschow *et al.*, 2005a; Jaenike *et al.*, 2006; Bordenstein & Werren, 2007; Engelstädter & Hurst, 2009), and is a common phenotype associated with this endosymbiont. Cytoplasmic incompatibilities result in high levels of mortality at the embryonic stage and can be either bidirectional or unidirectional (Dobson *et al.*, 2002; Telschow *et al.*, 2005b, 2007; Perlman *et al.*, 2008; Machtelinckx *et al.*, 2009; Narita *et al.*, 2009; Alam *et al.*, 2011; Sicard *et al.*, 2014; Zhong & Li, 2014; Joanne *et al.*, 2016; Richardson *et al.*, 2016, 2018; Altinli *et al.*, 2018). Bidirectional CI arises when infected females mate with infected males containing a different *Wolbachia* strain, and unidirectional incompatibility occurs when uninfected females mate with infected males. Both forms of CI can lead to reproductive isolation and speciation; however, the presence of *Wolbachia* does not always imply reproductive incompatibility and *Wolbachia* may not be the causal agent for incompatibility in some cases (Weeks *et al.*, 2002). Consequently, studies of insect speciation should directly test for *Wolbachia*-induced mechanisms.

Beetles in the genus *Altica* (Insecta: Coleoptera: Chrysomelidae) are a good example of an insect group in which CI is a potential mechanism of speciation. *Altica* represents a highly diverse, cosmopolitan genus of *c*. 235 species (Reid & Beatson, 2015). To date, this group has offered an interesting model system for studies of ecological speciation. For instance, speciation has been shown to be strongly connected to differential host plant use, microhabitat isolation, and chemically mediated sexual isolation (Xue *et al.*, 2011, 2014, 2016, 2018). However, we also know that *Altica*, like many other coleopterans, house *Wolbachia* (Xue *et al.*, 2011; Jäkel *et al.*, 2013); thus, our understanding of speciation in this group lacks information on whether these bacteria play an important role in reproductive isolation.

There is some evidence suggesting the possibility of *Wolbachia*-induced unidirectional CI in *Altica*. Previous studies have shown that *Altica fragariae* Nakane, *Altica viridicyanea* (Baly), and *Altica cirsicola* Ohno are closely related species, and that interspecific hybrids could be generated in the laboratory (Xue *et al.*, 2009a,b, 2011). Viable, fertile hybrid $F_1$ offspring are produced when *A. fragariae* is the male parent (hatching rate of *A. cirsicola* ♀ × *A. fragariae* ♂ = 49.1%; hatching rate of *A. viridicyanea* ♀ × *A. fragariae* ♂ = 65.8%) whereas hatching rates are extremely low when *A. fragariae* is the female parent (hatching rate of *A. fragariae* ♀ × *A. cirsicola* ♂ = 6.4%; hatching rate of *A. fragariae* ♀ × *A. viridicyanea* ♂ = 1.2%), exhibiting a clear pattern of unidirectional incompatibility in hybrid crosses (Xue *et al.*, 2011). Although the ratio of *Wolbachia* infection is high in these *Altica* species (Xue *et al.*, 2011), the mechanism of incompatibility remains unclear in this system. Appreciating the causes of this one-way incompatibility is key to understanding the evolution of barriers to gene exchange and the speciation process in this group.

We had four major aims in the present study. First, we screened for infection in 11 *Altica* species from natural populations to broaden the diversity of species estimates on the prevalence of *Wolbachia* in this species-rich flea beetle genus. Second, we examined *Wolbachia* diversity infecting those *Altica* species based on the *Wolbachia* surface protein (*wsp*) gene and multilocus sequence typing (MLST) markers. Third, we conducted antibiotic curing experiments and crossing studies to test whether *Wolbachia*-induced CI explains patterns of embryonic inviability in the *Altica fragariae*–*A. viridicyanea* system. Last, we assayed the Beijing populations of *A. fragariae* and *A. viridicyanea* for the presence of two genes required for *Wolbachia*-induced CI, *cifA* and *cifB*. Together, these aims provide new insights into speciation of this specious group.

## Materials and methods

### Wolbachia *surveys in* Altica *species*

In total, 235 *Altica* individuals were collected from China, Mongolia, and England and were screened for *Wolbachia* infection. The specimens were identified to species based on genitalic morphology and the host plant from which they were collected, an important supplement for this taxonomically challenging genus (Gressitt & Kimoto, 1963; Yu *et al.*, 1996; Wang *et al.*, 2005; Bukejs, 2011). Our sampling efforts across these geographically distant sites yielded samples from 11 *Altica* species (Table S1). Beetles were stored in 100% ethanol at −30 °C until DNA extraction.

We extracted total genomic DNA from whole beetles by puncturing the thorax and abdomen with a sterile insect pin and then soaking the specimens in TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) extraction buffer overnight. Extraction followed the protocols of the kit. This allowed us to retain the specimens as vouchers after DNA extraction. PCR of the COX1 region was used to confirm the success of DNA extraction (cf. Xue *et al.*, 2014).

Once we confirmed the presence of DNA, the *Wolbachia* surface protein (*wsp*) locus was amplified using *Wolbachia*-specific primers (*wsp*81F, 5′-TGGTCCAATAAGTGATGAAGAAAC-3′, *wsp*691R, 5′-AAAAATTAAACGCTACTCCA-3′) (Braig *et al.*, 1998) to determine if *Wolbachia* were present. The PCR was carried out using standard methods and the following thermal profile: a pre-cycle denaturation at 94 °C for 5 min, a post-cycle extension at 72 °C for 10 min, and 35 cycles of a standard three-step PCR (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min). DNA from confirmed *Wolbachia*-infected *Altica* beetles and sterilised water were used as positive and negative controls in each set of reactions. Samples that showed bands for *wsp* were scored as infected with *Wolbachia*. Samples that were negative for *Wolbachia* were screened twice more. In samples that were deemed uninfected via *wsp* screening, we conducted a second confirmatory screen for the *ftsZ* and *gatB* regions that are common housekeeping genes often used in sequence typing of *Wolbachia* (Baldo *et al.*, 2006).

### *Identification of* Wolbachia *diversity*

The genus *Wolbachia* is genetically diverse and can be classified into 17 supergroups (A–R, excluding G which is a

combination of A and B) (Wang *et al.*, 2016), among which supergroups A and B are most commonly found among insects. We sequenced a fragment of the fast-evolving and highly variable *wsp* gene to examine *Wolbachia* diversity within our samples. All of the fragments were sequenced in both directions. In instances in which the direct sequencing showed multiple peaks, we assessed the presence of more than one strain of *Wolbachia* using cloning. Cloning followed standard protocols included in the pGM-T Fast Cloning Kit (Tiangen Biotech, Beijing, China). The plasmids were directly sequenced or sequenced after PCR amplification using the bacterial primers *M13f* (5′-GTAAAACGACGGCCAG-3′) and *M13r* (5′-CAGGAAACAGCTATGAC-3′) that are included in the kit.

Some studies have suggested that recombination between different *Wolbachia* strains is common at the *wsp* locus; therefore, this gene may be misleading for strain characterisation when used alone. To resolve this issue, an MLST system of five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) was established by Baldo *et al.* (2006). To further characterise the *Wolbachia* strains of the three closely related *Altica* species (i.e. *A. fragariae*, *A. viridicyanea*, and *A. cirsicola*), the MLST gene sequences of seven singly infected individuals (three *A. fragariae*, two *A. viridicyanea* and two *A. cirsicola*) were amplified using the primers and protocols listed on the PubMLST website (http://pubmlst.org/wolbachia/) (Baldo *et al.*, 2006). To characterise the *Wolbachia* strains, allelic sequences were compared with the PubMLST database using the 'sequence query' function. MLST and *wsp* (including the hypervariable region (HVR)) sequences that had no matches were submitted to the MLST database (Table S2). All *wsp* haplotypes and MLST sequences were also deposited into GenBank (*wsp*, MK395170-MK395182, Mk395188-MK395209; *coxA*, MK395210-MK395211, MK395213-MK395216, MK395218; *fbpA*, MK395219- MK395220, MK395222-MK395225, MK395227; *ftsZ*, MK395228-MK395229, MK395231-MK395233, MK395235; *gatB*, MK395236-MK395237, MK395239-MK395242, MK395244; *hcpA*, MK395245-MK395246, MK395248-MK395252). To analyse the diversity of *Wolbachia* identified in *Altica*, the distribution of *wsp* haplotypes was summarised using statistical parsimony networks inferred using TCS 1.2.1 (Clement *et al.*, 2000) with a probability cut-off set to 95%.

### *Phylogenetic analyses*

The phylogenetic relationships were reconstructed using the *wsp* and the concatenated MLST sequences. For the phylogenetic tree based on the *wsp* gene, 48 representative *wsp* sequences were retrieved from Genbank following previous studies (i.e. Zhou *et al.*, 1998; Zhong & Li, 2014; Karimian *et al.*, 2018). These sequences represent samples from across the phylogenetic diversity of *Wolbachia* strains. Two datasets were assembled. One contained all 208 *wsp* sequences from *Altica* plus the representative sequences from GenBank, and the second was a simplified dataset that contained the representative sequences along with the 35 haplotypes identified in *Altica*. To reconstruct a tree based on the MLST sequences, two new sequence types (STs) were combined with 56 concatenated reference MLST sequences (following Baldo *et al.*, 2006; and all the MLST loci from Coleoptera hosts) that were retrieved from PubMLST. These reference sequences were aligned with corresponding *Wolbachia* sequences from *Altica* hosts using CLUSTALW in MEGA 7.0 (Kumar *et al.*, 2016). Neighbour-joining trees were inferred using MEGA 7.0, and maximum likelihood phylogenetic trees were inferred using RAXML 8.2.10 (Stamatakis, 2014) on the CIPRES Science Gateway portal (Miller *et al.*, 2015). For both tree types, 1000 non-parametric bootstrap replicates were calculated. To account for repeat mutations and possible recombination events, phylogenetic analyses were also performed using CLONALFRAME v.1.1) (Didelot & Falush, 2007). We used 100 000 Markov chain Monte Carlo iterations to estimate the trees after a burn-in period of 20 000 iterations. These values were selected based on convergence of the summary statistics and four independent runs were conducted to ensure that convergence was achieved. The graphical user interface of the program was used to generate 50% consensus trees.

### *Antibiotic treatment and crossing experiments*

We conducted antibiotic curing experiments and crossing studies to test whether *Wolbachia*-induced CI occurs between *A. fragariae* and *A. viridicyanea*. To estimate the potential role of *Wolbachia* in CI, we used both intraspecific and interspecific crosses with and without antibiotic curing to explore how the presence of *Wolbachia* impacted hatching success. In addition, because $F_1$ progeny were available for the *A. viridicyanea* female × *A. fragariae* male cross, we also conducted trials with the $F_1$ generation. The reciprocal cross (*A. fragariae* female × *A. viridicyanea* male) is inviable; consequently, all crosses involving $F_1$ individuals originate from the *A. viridicyanea* female × *A. fragariae* male cross. For this additional set of experiments with $F_1$ individuals, we crossed *A. fragariae* females and $F_1$ males. Because three of the backcrosses are viable in the presence of *Wolbachia* (*A. viridicyanea* female × $F_1$ male, $F_1$ female × *A. viridicyanea* male, $F_1$ female × *A. fragariae* male), we did not include these combinations in the experiment. Instead, we chose to focus on the backcross which has previously been shown to be inviable (*A. fragariae* female × $F_1$ male) (Xue *et al.*, 2011).

Singly infected over-wintered adults of both *A. fragariae* and *A. viridicyanea* were collected from field populations living on their host plants at Yanqing, Beijing (40.54°N, 116.43°E), and maintained in the laboratory. The insects were fed their normal host plants (*Duchesnea indica* and *Geranium nepalense*, respectively) in growth chambers maintained at LD 16:8 h and 25 °C. The virgin adults of second-generation beetles were used in experimental crosses and to generate the $F_1$ generation. Upon hatching, the hybrid $F_1$ larvae were provided with both host plants. We cured beetles of their *Wolbachia* infections using tetracycline applied to host plant tissues. In brief, beetles were fed fresh leaf material soaked in a 0.3% tetracycline-HCl (Santa Cruz Biotechnology, Santa Cruz, California) solution once larvae reached the second instar, whereas the untreated beetles received untreated fresh leaf tissue. Two weeks before the crossing experiments, we increased the dose and fed adults

0.6% tetracycline-HCl-treated leaves daily. Five individuals from each treatment were randomly selected to confirm the presence of *Wolbachia* in control beetles and their absence in the antibiotic treatment. These samples were confirmed using PCR as described earlier.

To conduct the crosses, virgin beetles were paired and placed into glass jars (height 11.5 cm, diameter 12 cm), with one mating pair per jar. Glass jars also contained cuttings of fresh host plant tissue to allow the adults to feed freely. We waited 5 days before collecting eggs to ensure that mating occurred and the eggs would be fertilised. Once egg collection began, we gently scraped eggs from the leaf tissue every 48 h and placed them into 9-cm Petri dishes lined with moistened filter paper. At this time, host plant tissue was also replaced with fresh cuttings. Eggs were collected five times over the course of 10 days. Hatching success was determined by checking the eggs daily. One we had collected the final set of eggs, beetles were assessed with PCR to confirm the success of the antibiotic treatment at the end of the experiment. We had five to 10 replicates per mating combination. Because the hatching success data tended to deviate from normality, we used non-parametric Kruskal–Wallis tests to examine the effect of the antibiotic treatment on hatching success in the different crosses. These tests were conducted using SPSS 22.0 (IBM, Armonk, New York).

### *Detection of* Wolbachia *genes responsible for causing cytoplasmic incompatibility*

Recent studies have determined that the *cifA* and *cifB* (or *cidA/B*) are the *Wolbachia* genes responsible for causing CI in insects (Beckmann *et al.*, 2017; LePage *et al.,* 2017; Bonneau *et al.*, 2018; Lindsey *et al.*, 2018; Shropshire *et al.*, 2018). Because both of these genes are required for CI (LePage *et al.*, 2017), we used a PCR assay to test for the presence of these genes in the Beijing populations of *A. fragariae* and *A. viridicyanea*. We tested six representative individuals from each species using PCR amplification protocols that followed Asselin *et al.* (2019). We used the primers *CifA* (5′-AAGTG GAGCGAAGGGGTAGA-3′/5′-CAGGAAAGCAACCTTTGG CA-3′) and *CifB* (5′-GTGCAAGTGCCTAATGCTG-3′/5′-TAC CTTGCCTCGTCTTGC-3′). The thermal profile for the PCR included a pre-cycle denaturation at 94 °C for 3 min, then 35 cycles of 94 °C of denaturation for 30 s, 52 °C annealing for 1 min, and 68 °C extension for 1 min. We also included positive controls using DNA extracted from *Drosophila melanogaster* infected with the *w*Mel *Wolbachia* strain, which is known for CI induction.

## Results

### Wolbachia *prevalence*

All of the 11 *Altica* species tested in the present study were infected by *Wolbachia* (Table 1). Overall, the infection rate of the *Altica* specimens that we screened was high, at 97.0% ($n = 235$) infected. On a per-species basis, infection rates ranged from 25% to 100%. There were seven species with 100% infection rates (*A. fragariae, A. cirsicola*, *A. birmanensis*, *A. caerulescens*, *A. carinthiaca*, *A. deserticola*, and *A. engstroemi*), although some of the sample sizes were small. Additionally, infection rates were also high in other species. For example, *A. viridicyanea* had an infection rate of 98.0% ($n = 99$), and *A. koreana* had an infection rate of 90.9% ($n = 11$). Relatively lower infection rates were found in *A. oleracea* (25%, $n = 4$) and *A. opacifrons* (50%, $n = 2$), species for which we had small sample sizes (Table S1).

### *Diversity of* Wolbachia

Of the 11 species tested, 10 had infections by more than one *Wolbachia* strain (Table S1). Multiple infection was only absent among the samples of *A. engstroemi* ($n = 3$). The multiple infection rate was high in *A. caerulescens* (86.7%, $n = 15$) and *A. birmanensis* (91.7%, $n = 12$). Multiple infected individuals accounted for 23.0% of all screened specimens.

In terms of genetic diversity of *Wolbachia*, we identified 35 *wsp* haplotypes among the 208 sequences obtained from 10 *Altica* species (*wsp* clones in *A. oleracea* failed to sequence). Six of the haplotypes were shared by more than one species (Fig. 1; Table S2). In our focal species, *A. fragariae* and *A. viridicyanea,* nine and 10 *wsp* haplotypes were detected, respectively. In the Beijing population of these species, we found seven *wsp* haplotypes in *A. fragariae* and eight in *A. viridicyanea*. Interestingly, a majority of the *A. fragariae* and *A. viridicyanea* individuals from Beijing (83.3% and 75%) were each singly infected with common *wsp* haplotypes that differed by one nucleotide substitution. In addition, in the Beijing population, there were relatively high multiple infection rates, with *A. fragariae* having 4.2% of individuals with multiple infections and *A. viridicyanea* having 15.3%. Furthermore, some individuals had a complex pattern of infection, being infected with three or four *wsp* strains (BJ1811, BJ1831, BJ1845, BJ1851, BJ1863). We found this interesting because our estimates of infection are conservative, as weakly amplifying PCR products failed to sequence in some individuals.

Using the conserved MLST *Wolbachia* genes, we identified three sequence types among seven individuals of *A. cirsicola*, *A. fragariae*, and *A. viridicyanea*, and at least two of these were new to the MLST database (assigned as ST505 and ST506). Because sequencing of *ftsZ* failed in one of the samples (HNN1508), it remains unclear whether there was a third unique sequence type. Among the MLST genes, four of the five alleles were 100% identical to previously reported alleles (*gatB* 32, *coxA* 33, *ftsZ* 32, and *fbpA* 122 in *A. fragariae* individual BJ1803) and the *hcpA* closely matched *hcpA* 186, but differed by a single nucleotide substitution. We observed the same allelic profile (100% identity) in four additional Beijing individuals (*A. fragariae*, BJ1815; *A. viridicyanea*, BJ1846; *A. cirsicola*, BJ1002 and BJ1003). Furthermore, the dominant singly infected *A. cirsicola*, *A. fragariae*, and *A. viridicyanea* all shared the same strain type, ST505. For the *A. viridicyanea* individual HNN1505, there was only one allele with 100% identity to the previously reported *hcpA* 297. For the *A. fragariae* individual HNN1508, four alleles had 100% identity to previously reported

**Table 1.** Summary of *Wolbachia* infections in 11 *Altica* species.

| Host species | Population (geographic coordinates) | Individuals screened/ uninfected | Infection rate/multiple infection rate | *Wolbachia* infection rate reported previously |
|---|---|---|---|---|
| *A. fragariae* | BJ1 (40.28°N, 116.05°E) | 4/0 | 100%/10.3% ($n = 58$) | 97.9% ($n = 47$) (Xue *et al.*, 2011) |
| | BJ2 (40.54°N, 116.43°E) | 44/0 | | |
| | HNN (28.08°N, 112.67°E) | 3/0 | | |
| | SX1(33.62°N, 106.92°E) | 2/0 | | |
| | SX2 (33.44°N, 108.46°E) | 1/0 | | |
| | FJ (27.63°N, 117.98°E) | 2/0 | | |
| | GX (25.71°N, 110.80°E) | 1 / 0 | | |
| | YN (26.99°N, 98.87°E) | 1/0 | | |
| *A. viridicyanea* | BJ1 (40.35°N, 116.16°E) | 4/1 | 98.0%/12.1% ($n = 99$) | 100% ($n = 57$) (Xue *et al.*, 2011) |
| | BJ2 (40.11°N, 116.00°E) | 68/1 | | |
| | SSX (35.47°N, 112.44°E) | 4/0 | | |
| | HBB (29.89°N, 109.57°E) | 6/0 | | |
| | HNN (27.96°N, 109.60°E) | 2/0 | | |
| | SX1 (33.43°N, 108.45°E) | 3/0 | | |
| | SX2 (33.22°N, 107.55°E) | 1/0 | | |
| | SX3 (33.81°N, 109.00°E) | 1/0 | | |
| | SX4 (34.09°N, 107.71°E) | 3/0 | | |
| | SX5 (33.62°N, 106.92°E) | 1/0 | | |
| | SX6 (33.86°N, 107.82°E) | 2/0 | | |
| | SX7 (34.09°N, 110.15°E) | 1/0 | | |
| | SX8 (33.42°N, 109.15°E) | 3/0 | | |
| *A. cirsicola* | GZ (28.24°N, 107.21°E) | 3/0 | 100%/16.7% ($n = 18$) | 100% ($n = 23$) (Xue *et al.*, 2011) |
| | HB (38.42°N, 115.83°E) | 3/0 | | |
| | HN (34.62°N, 112.45°E) | 3/0 | | |
| | BJ (40.17°N, 116.22°E) | 2/0 | | |
| | SD (35.09°N, 116.39°E) | 3/0 | | |
| | SX (32.83°N, 109.37°E) | 2/0 | | |
| | SX (32.96°N, 109.44°E) | 2/0 | | |
| *A. birmanensis* | GX (22.82°N, 108.38°E) | 2/0 | 100%/91.7% ($n = 12$) | 100% ($n = 1$) (Jäkel *et al.*, 2013) |
| | YN (21.62°N, 101.59°E) | 4/0 | | |
| | JX (24.87°N, 114.61°E) | 6/0 | | |
| *A. caerulescens* | HLJ (44.09°N, 131.12°E) | 1/0 | 100%/86.7% ($n = 15$) | - |
| | HNN (27.96°N, 109.60°E) | 3/0 | | |
| | BJ1 (40.54°N, 116.42°E) | 2/0 | | |
| | BJ2 (40.18°N, 116.06°E) | 2/0 | | |
| | BJ3 (40.28°N, 116.05°E) | 5/0 | | |
| | SX1 (33.53°N, 107.99°E) | 1/0 | | |
| | SX2 (34.09°N,110.15°E) | 1/0 | | |
| *A. carinthiaca* | UK1 (51.62°N, 0.22°W) | 4/0 | 100%/50% ($n = 8$) | 100% infected ($n = 3$) (Jäkel *et al.*, 2013) |
| | UK2 (51.44°N, 0.02°E) | 4/0 | | |
| *A. deserticola* | XJ (44.30°N, 86.17°E) | 5/0 | 100%/40% ($n = 5$) | - |
| *A. engstroemi* | MG (unknown) | 2/0 | 100%/0% ($n = 3$) | 100% infected ($n = 2$) (Jäkel *et al.*, 2013) |
| | NX (36.01°N, 116.24°E) | 1/0 | | |
| *A. koreana* | MG (unknown) | 6/0 | 90.9%/9.1% ($n = 11$) | - |
| | BJ (40.35°N, 116.16°E) | 2/0 | | |
| | SX (34.09°N, 110.15°E) | 1/1 | | |
| | NX (36.01'N, 116.24°E) | 2/0 | | |
| *A. oleracea* | SC (31.04°N, 103.19°E) | 1/1 | 25%/25% ($n = 4$) | 41.7% infected ($n = 24$) (Jäkel *et al.*, 2013) |
| | SX (33.57°N, 107.56°E) | 1/1 | | |
| | YN (27.33°N, 99.22°E) | 2/1 | | |
| *A. opacifrons* | SX1 (33.91°N, 106.52°E) | 1/0 | 50%/50% ($n = 2$) | - |
| | SX2 (33.86°N, 107.82°E) | 1/1 | | |
| Total | | 235/7 | 97.0%/23.0% | |

BJ, Beijing; HNN, Hunan; SX, Shaanxi; SXX, Shanxi; FJ, Fujian; GX, Guangxi; SD, Shandong; HB, Hebei; HBB, Hubei; HN, Henan; NX, Ningxia; YN, Yunnan; SC, Sichuan; XJ, Xinjiang; GZ, Guizhou; JX, Jiangxi; HLJ, Heilongjiang; MG, Mongolia; UK, United Kingdom.

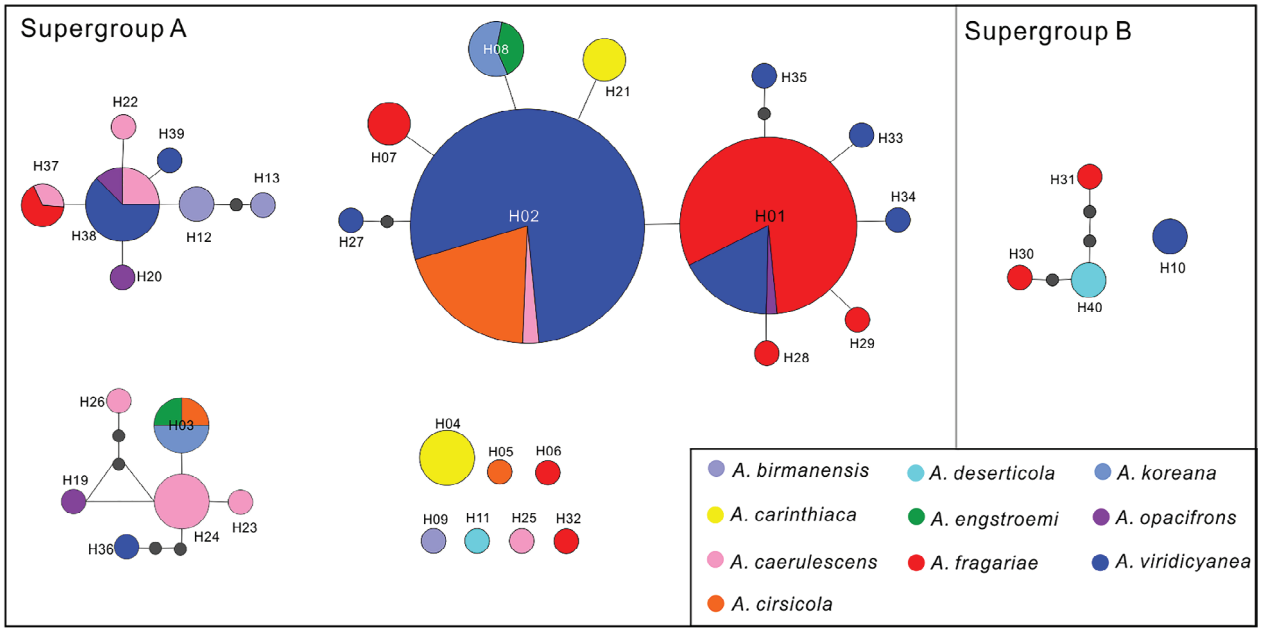


**Fig. 1.** Maximum parsimony networks for *Wolbachia* surface protein (*wsp*) sequences of *Wolbachia*. Each circle represents a haplotype and circles are sized proportionally to the number of samples with that haplotype. Each colour indicates a host species of *Altica*. Straight lines and small black dots reflect mutations and median vectors, respectively. [Colour figure can be viewed at wileyonlinelibrary.com].

**Table 2.** Multilocus sequence typing (MLST) 'alleles' amplified from *Altica* species in our study.

| | | MLST locus | | | | | |
|---|---|---|---|---|---|---|---|
| Host species | Individual | *gatB* | *coxA* | *hcpA* | *ftsZ* | *fbpA* | Sequence type |
| *A. cirsicola* | BJ1002 | 32 | 33 | ***312*** | 32 | 122 | 505 |
| | BJ1003 | 32 | 33 | ***312*** | 32 | 122 | 505 |
| *A. fragariae* | BJ1803 | 32 | 33 | ***312*** | 32 | 122 | 505 |
| | BJ1815 | 32 | 33 | ***312*** | 32 | 122 | 505 |
| | HNN1508 | 49 | 44 | 297 | – | 122 | |
| *A. viridicyanea* | BJ1846 | 32 | 33 | ***312*** | 32 | 122 | 505 |
| | HNN1505 | ***288*** | ***285*** | 297 | ***247*** | ***455*** | 506 |

The numbers correspond to the allele serial numbers in the MLST database. Sequences that were identified for the first time are bold and in italicis. PCR products that failed to sequence are indicated with a '–'.

sequences (*gatB* 49, *coxA* 44, *hcpA* 297 and *fbpA* 122), while *ftsZ* failed to sequence (Table 2).

### *Phylogenetic analysis*

Phylogenetic analysis using different methods of inference showed similar results. Based on 208 *wsp* sequences from *Altica* species and 48 representative sequences from other insects, these analyses showed that seven of the *Wolbachia* sequences from *Altica* fell into supergroup B, whereas the others were in supergroup A (Figs S1–S3). Phylogenetic analysis of *wsp* gene sequences showed that the 35 *wsp* haplotypes in *Altica* clustered into eight clades. Three clades containing seven haplotypes fell into supergroup B, whereas the others were in supergroup A (Figs 2, S4, S5). Phylogenetic analysis of the MLST sequences suggested that the dominant strain from *A. cirsicola*, *A. fragariae*, and *A. viridicyanea* (ST505) is closely related to ST348, a strain type of supergroup A from the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Floate *et al.*, 2011). ST506 also belongs to supergroup A (Figs 3, S6, S7).

### *Crossing experiments*

Intraspecific crosses of infected males and cured females showed that there was no change in hatching success from crosses with infected beetles (Table 3). Similarly, interspecific crosses showed that antibiotic curing had no effect on hatching success, and that hatching rates were not increased by antibiotic curing of individuals involved in backcrosses with $F_1$ individuals (Table 3). Thus, the results show that antibiotic curing does not decrease or rescue hatching success.

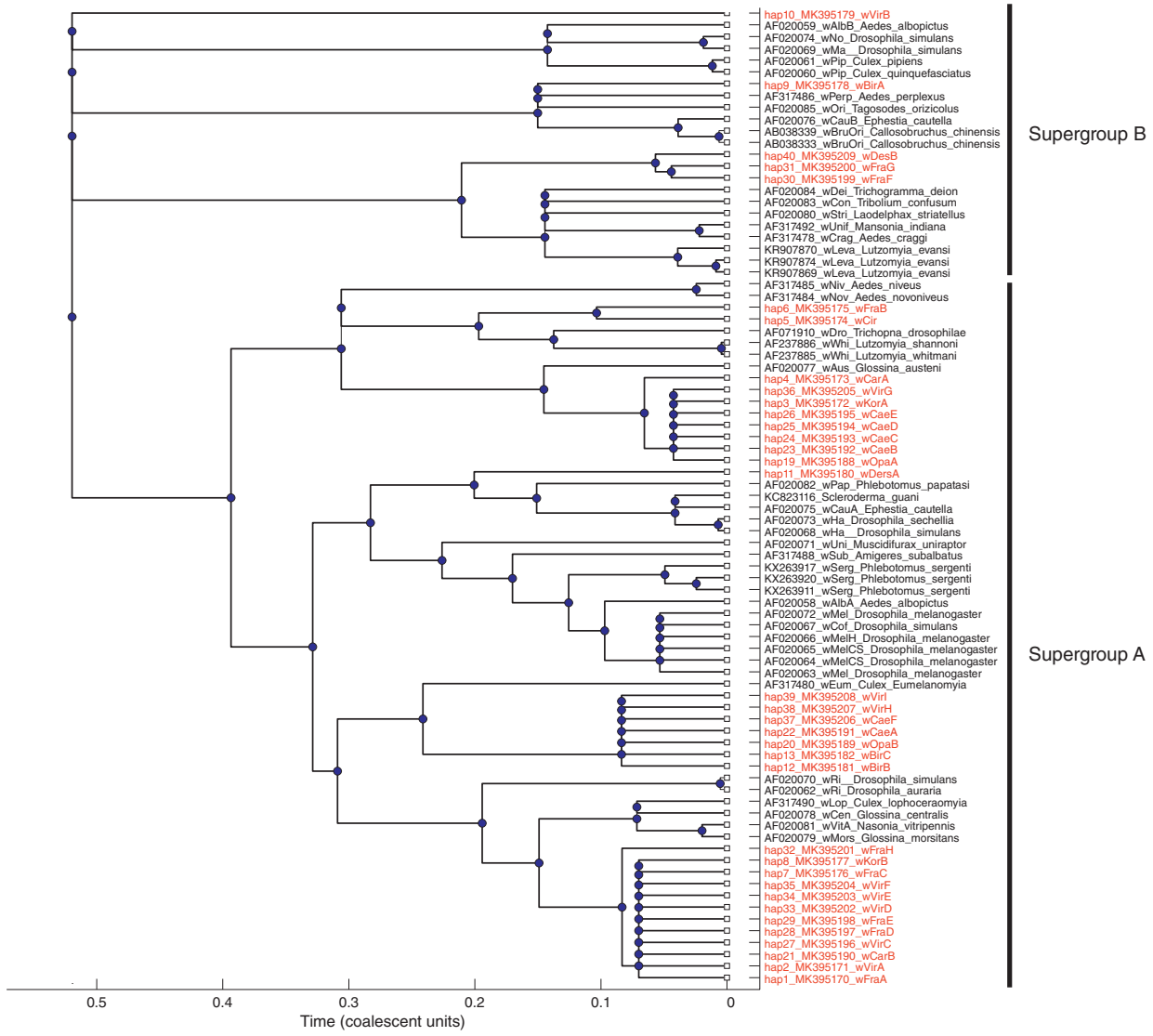


**Fig. 2.** Fifty per cent consensus tree inferred with CLONALFRAME v.1.1 based on 35 *Wolbachia* surface protein (*wsp*) haplotypes from 10 *Altica* species (present study) and 48 representative sequences from different host species. [Colour figure can be viewed at wileyonlinelibrary.com].

*Cytoplasmic incompatibility gene detection*

We found that *cifA* was readily amplified in all of the 12 representative samples, whereas *cifB* was only amplified in the positive controls (Fig. 4). Because both *cifA* and *cifB* are required for the induction of CI (LePage *et al.*, 2017; Shropshire *et al.*, 2018), our results are consistent with the outcome of the crossing experiment.

## Discussion

*Wolbachia*-induced CI has been identified as a key mechanism that can lead to the formation of new insect species, and these infections are also important in manipulating host phenotype (e.g. Werren, 1997; Jaenike *et al.*, 2006; Brucker & Bordenstein, 2012). *Wolbachia* infections have been identified previously in *Altica* beetles, but whether these contribute to reproductive isolation in this species-rich group has remained untested. Here we examined infection frequency, *Wolbachia* diversity, and tested whether *Wolbachia*-induced CI plays a role in the evolutionary ecology of *Altica*.

The results indicate that *Wolbachia* infection is a common feature of *Altica* species. All 11 species and 97.0% of individuals that we screened were infected with *Wolbachia*, clearly demonstrating that *Wolbachia* infection is highly prevalent in these species. The infection rates reported here are somewhat higher than those previously found in *Altica*. Jäkel *et al.* (2013) surveyed 18 species and found that 83.3% of species and 61.2% of individuals were infected ($n = 116$). Furthermore, multiple infections were also more common than previously recorded.

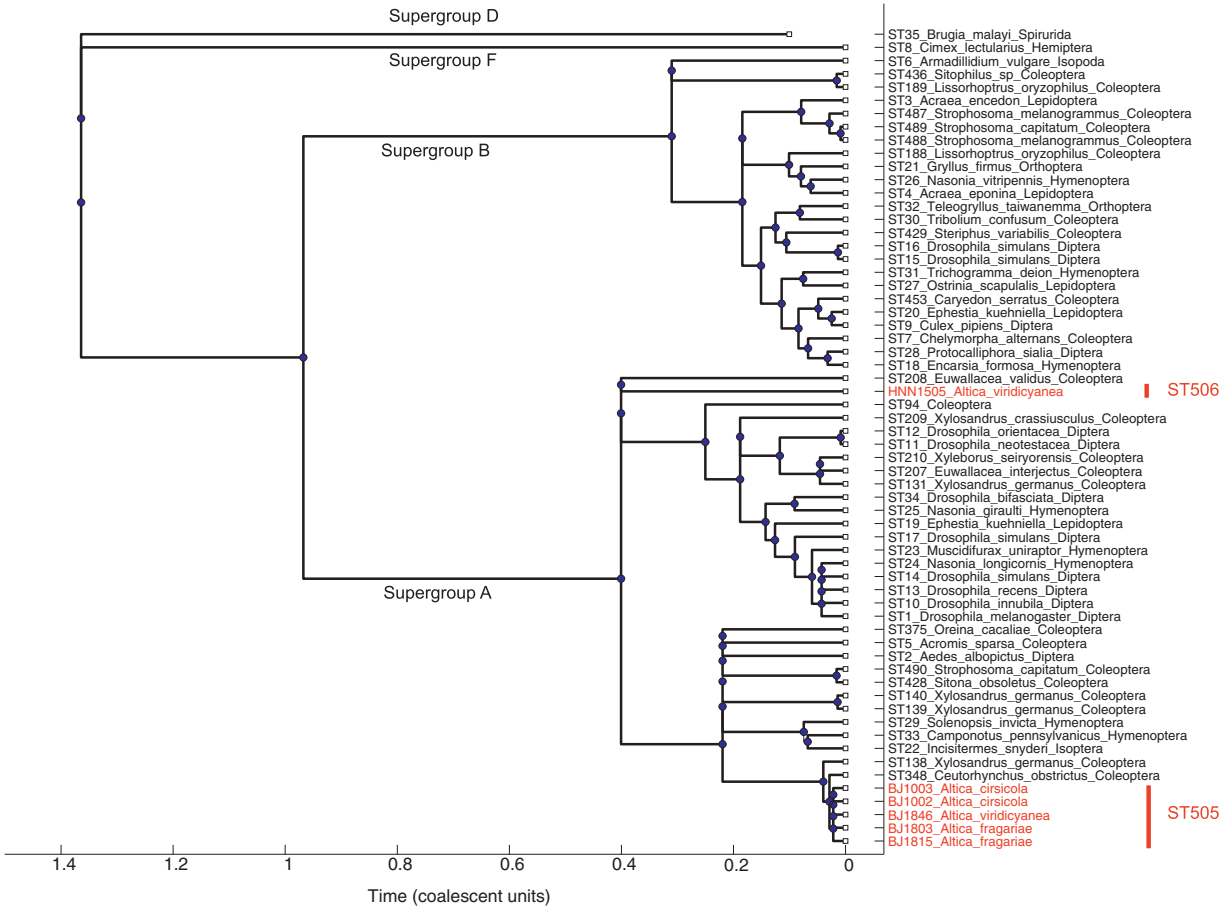


**Fig. 3.** Fifty per cent consensus tree inferred with CLONALFRAME v.1.1 based on six *Wolbachia* multilocus sequence typing (MLST) marker sequences from *Altica fragariae*, *Altica viridicyanea*, and *Altica cirsicola* and 56 representative MLST sequences. [Colour figure can be viewed at wileyonlinelibrary.com].

**Table 3.** Hatching success of experimental crosses (AF, *Altica fragariae*; AFc, cured *Altica fragariae*; AV, *Altica viridicyanea*; AVc, cured *Altica viridicyanea*; $F_1$, offspring of AV♀ × AF♂ cross; $F_1$c, cured $F_1$).

| Cross | Hatching rate (mean ± SD) | *P*-value |
|---|---|---|
| AF♀ × AF♂ ($n = 10$) | 88.34 ± 4.17 | 0.423 |
| AFc♀ × AF♂ ($n = 5$) | 90.21 ± 9.66 | |
| AV♀ × AV♂ ($n = 6$) | 89.19 ± 3.92 | 0.144 |
| AVc♀ × AV♂ ($n = 5$) | 83.47 ± 7.45 | |
| AF♀ × AV♂ ($n = 10$) | 0.60 ± 2.66 | 0.607 |
| AF♀ × AVc♂ ($n = 5$) | 0 | |
| AFc♀ × AV♂ ($n = 5$) | 0 | |
| AFc♀ × AVc♂ ($n = 4$) | 0 | |
| AF♀ × $F_1$♂ ($n = 6$) | 0 | 0.287 |
| AF♀ × $F_1$c♂ ($n = 4$) | 2.13 ± 4.26 | |
| AFc♀ × $F_1$c♂ ($n = 4$) | 0 | |

We showed multiple infections in 10 out of 11 species as compared with previous work that identified only one in 18 species (Jäkel *et al.*, 2013). Multiple infections have been commonly recorded in a number of insect hosts (Kondo *et al.*, 2002; Reuter & Keller, 2003; Keller *et al.* 2004; Malloch & Fenton, 2005; Watanabe *et al.*, 2011; Arai *et al.*, 2018), although single infections do occur in some groups (e.g. Duplouy & Brattström, 2018; Jiang *et al.*, 2018). With few exceptions, most of the *Altica* species examined in our survey had some proportion of multiply infected individuals, and multiple infection was quite common in the Beijing population of *A. fragariae* and *A. viridicyanea*. Only one species (*A. engstroemi*) lacked individuals with multiple infections, for which we only had three samples, suggesting the possibility of multiple infection in this species as well. Moreover, *Altica* is also characterised by complex infections where three or four *wsp* sequence types were detected in a single host individual (Table S2). Because cloning and sequencing of the *wsp* gene from the multiply infected individuals was not always successful, the diversity of *Wolbachia* strains was underestimated in the present study. Consequently, the rate of multiple infections is probably higher than we report here.

*Wolbachia* infection frequencies have been shown to vary spatially and temporally in a number of species (e.g. Jansen *et al.*, 2008; Hu *et al.*, 2015; Ahmed *et al.*, 2015a; Cooper *et al.*, 2017), potentially explaining the differences between the present study and previous work on *Altica*. Not only were

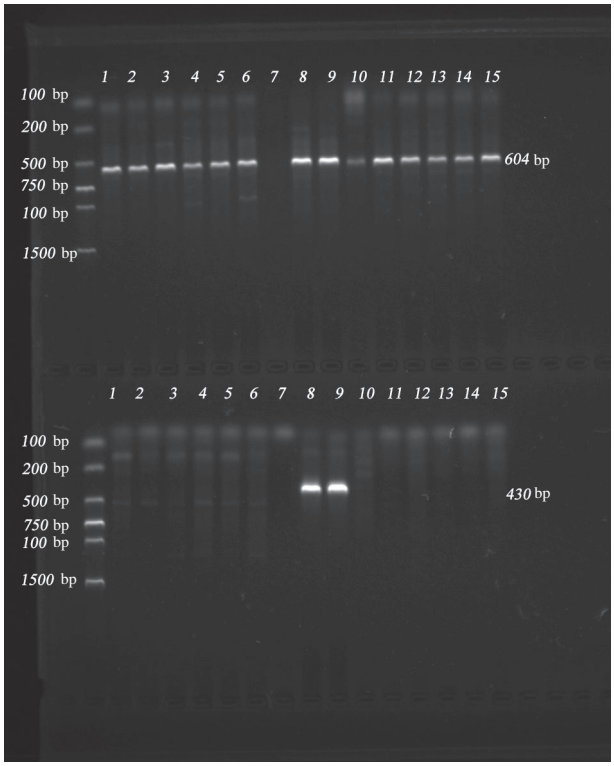


**Fig. 4.** Polymerase chain reaction amplification of the *cifA* and *cifB* *Wolbachia* genes responsible for causing cytoplasmic incompatibility. Top, *cifA* (604 bp); bottom, *cifB* (430 bp). Lanes 1–6, *Altica fragariae*; lane 7, negative control (DNase/RNase-Free water); lanes 8, 9, positive controls (*Drosophila melanogaster* infected with *w*Mel *Wolbachia* strain); lanes 10–15, *Altica viridicyanea*.

*Wolbachia* infections high in *Altica*, but we also observed that the high infection rates in *A. fragariae* and *A. viridicyanea* were maintained through time. For example, we surveyed infection in 2009–2010 in the Beijing populations of these species and determined that infection was present in 97.9% ($n = 47$) of *A. fragariae* and 100% ($n = 57$) of *A. viridicyanea* (Xue *et al.*, 2011). In the present study, 2018 infection rates in the Beijing populations were comparable, at 100% ($n = 48$) and 97.2% ($n = 72$). In our previous study, multiple infections occurred in 10.6% of *A. fragariae* and 1.8% of *A. viridicyanea*, whereas the present study showed multiple infections in 4.2% and 15.3%. Together, the data suggest that for *Altica*, *Wolbachia* infections are common and infection rates are stable while the pattern of multiple infections varied temporally in the species we examined here.

By comparison, the genetic diversity of *Wolbachia* in the singly infected *A. fragariae* and *A. viridicyanea* in the Beijing populations was low, with only one unique *wsp* haplotype detected in each host species. The one exception was in an *A. viridicyanea* individual (BJ1402) that shared the *Wolbachia* haplotype with that from *A. fragariae*. A similar pattern was found in a previous study of these species where four *A. viridicyanea* individuals shared the *Wolbachia* haplotype found in *A. fragariae* (Xue et al. 2011). One explanation for this pattern is interspecific horizontal transmission of *Wolbachia*. We know that horizontal transmission of *Wolbachia* can occur among insects through shared food sources (Sintupachee *et al.*, 2006; Kolasa *et al.*, 2017; Li *et al.*, 2017), hosts (Huigens *et al.*, 2004), and parasitoids (Heath *et al.*, 1999; Ahmed *et al.*, 2015b); however, this is doubtful in *Altica*. Although these species live sympatrically, they are specialised to feed on different host plants and so are unlikely to exchange endosymbionts through this pathway. Instead, the most likely mechanism is exchange via hybridisation. Interspecific gene flow occurs between these beetle species (Xue *et al.*, 2014) and would offer an opportunity for bacterial exchange.

Another major aim of this study was to assess the role of *Wolbachia* infection in the recent speciation event between *A. fragariae* and *A. viridicyanea*. Previous work has emphasised the role of host-plant use in causing reproductive isolation between these species, as the species do not feed on one another's hosts and mating is closely tied to the host plant (Xue *et al.*, 2011, 2014). However, the presence of *Wolbachia* suggests that CI is also a possible mechanism of isolation. Using antibiotic curing coupled with crossing experiments, we show that hatching success is unaffected by the curing treatment (Table 3), suggesting that the dominant strains of *Wolbachia* tested here cannot account for the incompatibility observed between *A. fragariae* and *A. viridicyanea*. This was further supported by the apparent lack of the required CI *cif*B gene in these *Wolbachia* strains. These results are perhaps not surprising given that the presence of *Wolbachia* does not always lead to incompatibilities (Weeks *et al.*, 2002), and nuclear incompatibilities can instead be the cause (e.g. Mandel *et al.*, 2001; Maroja *et al.*, 2008; Hamm *et al.*, 2014). Some studies have also suggested that CIs are induced by both nuclear incompatibilities and *Wolbachia* infection (e.g. Breeuwer & Werren, 1995). The outcome of crosses between individuals with more complex patterns of infection (multiple infection) remains unknown; however, it seems unlikely that *Wolbachia* infection has influenced the pattern of reproductive isolation between *A. fragariae* and *A. viridicyanea*.

Although *Wolbachia* infection may not have contributed to CI of the *Altica* species investigated here, the question remains whether there are additional effects of these bacteria. For example, *Wolbachia* infection can also impact host fitness via the selective killing of males (Richardson *et al.*, 2016; Sullivan & Jaenike, 2006), and past reports of sex ratio bias in *Altica* suggest this as a possibility. Some *Altica* species exhibit female bias in natural populations (Kangas & Rutanen, 1993, Jäkel *et al.*, 2013); for instance, males only account for 4.1–18.8% of European populations of *A. lythri*, *A. oleracea*, and *A. palustris* (Jäkel *et al.*, 2013). To examine this, we tested for sex ratio bias in samples of *A. fragariae* and *A. viridicyanea* collected in Beijing over 2 years (July–September 2012 and 2013). There was no evidence of sex ratio distortion in these beetle species (*A. fragariae*, $\chi^2 = 3.776$, $P = 0.052$, $n = 1119$; *A. viridicyanea*, $\chi^2 = 0.026$, $P = 0.871$, $n = 1851$; H.J. Xue, pers. obs.), indicating that *Wolbachia* infections did not cause male-killing in either of these species.

In addition to affecting sex ratios in natural populations, *Wolbachia* can also alter host fitness in other ways, such as contributing to iron metabolism (Brownlie *et al.*, 2009),

protecting the host from plant defensive compounds (Barr *et al.*, 2010), increasing host resistance to viruses (Hedges *et al.*, 2008; Osborne *et al.*, 2012), and having positive effects on life history and reproductive traits (Okayama *et al.* 2016; Arai *et al.*, 2018). As a consequence, the phenotypic effects of *Wolbachia* infection in *Altica* are worth further investigation.

## Acknowledgements

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## Author contributions

HJX, XKY and JW conceived the project. JW, BHX and WZL collected the data. JW and HJX analysed the data. JW and HJX wrote the first draft of the manuscript, and KAS and HJX revised the manuscript.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Phylogenetic relationships inferred from neighbour-joining analyses based on 208 *Wolbachia wsp* sequences isolated from 10 *Altica* species and 48 representative sequences from other insects.

**Fig. S2.** Maximum likelihood tree based on 208 *wsp* sequences identified from *Altica* species and 48 representative sequences from different hosts. Bootstrap values represent 1000 replicates, and only values > 60 are shown.

**Fig. S3.** Fifty per cent consensus tree inferred with CLONALFRAME (v.1.1) based on 208 *wsp* sequences from 10 *Altica* species and 48 representative sequences from different host species.

**Fig. S4.** Neighbour-joining tree based on 35 *wsp* haplotypes from 10 *Altica* species and 48 representative sequences from different host species. Bootstrap values represent 1000 replicates, and only values > 60 are shown.

**Fig. S5.** Maximum likelihood tree based on 35 *wsp* haplotypes from 10 *Altica* species (present study; clones in one species failed to sequence) and 49 representative sequences from different hosts. Bootstrap values represent 1000 replicates, and only values > 60 are shown.

**Fig. S6.** Neighbour-joining tree of six *Wolbachia* MLST sequences from *Altica fragariae*, *A. viridicyanea* and *A. cirsicola* and 56 representative MLST sequences. Bootstrap values represent 1000 replicates, and only values > 60 are shown.

**Fig. S7.** Maximum likelihood phylogenetic tree of six *Wolbachia* MLST sequences from *Altica fragariae*, *A. viridicyanea* and *A. cirsicola* and 56 representative MLST sequences. Bootstrap values represent 1000 replicates, and only values > 60 are shown.

**Table S1.** Infection status of 235 *Altica* specimens screened in present study. S, single infected; M, multiple infected; U, *Wolbachia* undetected; N, infection detected but sequence unsuccessful.

**Table S2.** *Wsp* haplotypes detected among 208 *wsp* sequences from *Altica* host species inferred with TCS 1.2.1.

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