Comparative characterization of microbiota between the sibling species of tea geometrid moth *Ectropis obliqua* Prout and *E. grisescens* Warren

Zhibo Wang¹, Hong Li¹, Xiaogui Zhou¹, Meijun Tang¹, Liang Sun¹, Shuai Zhan² and Qiang Xiao¹

¹Key Laboratory of Tea Quality and Safety Control, Ministry of Agriculture, Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China and ²Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

Abstract

For a wide range of insect species, the microbiota has potential roles in determining host developmental programme, immunity and reproductive biology. The tea geometrid moths *Ectropis obliqua* and *E. grisescens* are two closely related species that mainly feed on tea leaves. Although they can mate, infertile hybrids are produced. Therefore, these species provide a pair of model species for studying the molecular mechanisms of microbiota involvement in host reproductive biology. In this study, we first identified and compared the compositions of microbiota between these sibling species, revealing higher microbiota diversity for *E. grisescens*. The microbiota of *E. obliqua* mainly comprised the phyla Firmicutes, Proteobacteria and Cyanobacteria, whereas that of *E. grisescens* was dominated by Proteobacteria, Actinobacteria and Firmicutes. At the genus level, the dominant microbiota of *E. grisescens* included *Wolbachia*, *Enterobacter* and *Pseudomonas* and that of *E. obliqua* included *Melissococcus*, *Staphylococcus* and *Enterobacter*. Furthermore, we verified the rate of *Wolbachia* to infect 80 samples from eight different geographical populations, and the results supported that only *E. grisescens* harboured *Wolbachia*. Taken together, our findings indicate significantly different microbiota compositions for *E. obliqua* and *E. grisescens*, with *Wolbachia* possibly being a curial factor influencing the reproductive isolation of these species. This study provides new insight into the mechanisms by which endosymbiotic bacteria, particularly *Wolbachia*, interact with sibling species.

Introduction

Important interactions of microbiota organisms with insects are very common in nature (Mao et al., 2018). Some of the microbiota harboured in host cells are considered endosymbionts, constituting a symbiotic bacteriome. Other microbiota species opportunistically colonize different tissues of insects and the gut lumen, which can be affected by many factors, such as the host’s diet and living environment (Colman et al., 2012; Engel and Moran, 2013; Huang and Zhang, 2013). Overall, the microbiota plays an important role in an insect’s life activity, providing the host with essential nutrients and protection from predators, parasites and pathogens (Tsuchida et al., 2010) and affecting reproduction (Zhang et al., 2015). Indeed, an increasing number of studies are focusing on the use of sequencing technology to analyse the functions and development of microbial communities associated with termites, silkworms and other insects (Su et al., 2016; Sun et al., 2016; Chen et al., 2017; Wang et al., 2017). However, the effect of the microbiota on host insects remains incompletely understood.

*Ectropis obliqua* and *E. grisescens* are primary defoliators in tea plantations due to their wide distribution and destructive nature (Jiang et al., 2014; Zhang et al., 2014). These moths infest thousands of hectares of tea per year, severely reducing the growth and impacting tea production in the following year (Jiang et al., 2014; Zhang et al., 2016a). *Ectropis obliqua* and *E. grisescens* were named in 1894 and 1930, respectively, but they have always been treated as the same species in tea garden management in China because they have similar morphology and are difficult to be distinguished (Jiang et al., 2014). After the two species were reacquainted in 2014, differences in morphology, reproductive capacity and virus susceptibility have been reported (Jiang et al., 2014; Mao et al., 2017; Bai et al., 2018a). In particular, the technique of DNA barcoding can accurately distinguish them according to the genetic distance of approximately 3.7% based on COI sequences (Jiang et al., 2014). Correspondingly, the distribution of the two species has become clearer, with *E. obliqua* being distributed in China, Japan
and the Korean Peninsula and *E. grisescens* only in China (Wehrli, 1945; Sato, 1984; Kim et al., 2001). In Zhejiang Province, in eastern China, *E. grisescens* is more widespread than *E. obliqua*, though they are both also found in some areas (Bai et al., 2018b). Moreover, morphological and phylogenetic evidence supports that *E. obliqua* is closely related to *E. grisescens*. Intriguingly, these sibling species can mate but produce infertile hybrids (Xi et al., 2014). Indeed, the hybrid F1 generation showed hatching, survival to adult stage and per cent of normal adult rates that were much lower than those with intra-species mating. Furthermore, a self-cross of F1 generation adults produced either infertile eggs or no eggs (Xi et al., 2014; Zhang et al., 2014). Reproductive interference also exists between these sibling species (Zhang et al., 2016a). As these phenomena differ from those resulting from common reproductive isolation, whereby different species cannot mate and breed, we suggest that these sibling species constitute a suitable model pair for exploring reproductive isolation.

Previous studies have shown some microbiota organisms can manipulate host reproduction and even cause reproductive isolation (Philipp and Nancy, 2013; Zhang et al., 2016a, 2016b). Moreover, we previously found that F1 hybrids of *E. obliqua* and *E. grisescens* showed the characteristics including unbalanced sex ratio, lower hatchability of eggs, desynchronized development of larvae and infertility that resembled cytoplasmic incompatibility which was caused by some microbiota (Bourtzis et al., 1996; Zhang et al., 2014; Wang et al., 2019). Thus, we sought to ascertain the microbiota involved in the reproductive isolation of these sibling species. In this study, we analysed and compared differences in *E. grisescens* and *E. obliqua* microbiotal composition and found that an obvious difference in the presence of *Wolbachia* may be a factor influencing their reproductive isolation.

**Materials and methods**

**Collection and preparation of samples**

*Ectropis obliqua* larvae were collected from Yuhang (Hangzhou City, Zhejiang Province) and *E. grisescens* from Xinchang (Shaoxin City, Zhejiang Province). At least 200 larvae were collected in each location. The collected larvae were reared in a phytotron (temperature 24–26°C, humidity 50–70%, photoperiod L14:D10). The larvae were fed fresh leaves of the tea cultivar Yingshuang for successive three generations. Male and female individuals were separated at the pupa stage. Two days after eclosion, adult moths were randomly collected for the analysis of bacterial communities. In addition, samples from different geographical populations were collected to evaluate the rate of *Wolbachia* infection without rearing in the phytotron. Sampling localities of tea geometrid can be seen in fig. 1.

**DNA extraction**

Wings were removed, and other tissues were washed with sterilized water and ground for 2 min. The tissue homogenate was used for metagenomic DNA extraction using the DNeasy Blood and Tissue kit (Qiagen Co. Inc., Germany) according to the manufacturer’s instruction. The quality of the extracted DNA was assessed by electrophoresis on a 1% (w/v) agarose gel. The concentration of DNA extracted was measured using a Nanodrop.
2000, and the DNA samples were stored at −20°C for experiments.

**Sample identification**

Identification of *E. obliqua* and *E. grisescens* was confirmed by sequence analysis of the mitochondrial cytochrome oxidase I gene (COI) (Jiang et al., 2014). A fragment of the COI gene was amplified using the forward primer LepF1 5′-ATTCACCAAT-CATAAGATATTGG-3′ and the reverse primer Enh_LepR1 5′-CTCCWCGAGGATCAAA-3′ (Jiang et al., 2014). PCR using 2x Master Mix (TSINGKE Bio Inc., Hangzhou City, Zhejiang, China) in a total reaction volume of 50 μl was performed using a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA), as follows: an initial denaturation step of 95°C for 2 min, 5 cycles of 95°C for 30 s, 46°C for 1 min, and 72°C for 30 s, 35 cycles of 95°C for 30 s, 51°C for 1 min, and 72°C for 30 s and a final extension at 72°C for 10 min. Sequencing of PCR products was performed using an ABI377 genetic analyser.

**PCR amplification of microbial 16S rDNA genes**

The V3-V4 region of 16S rDNA was amplified using the forward primer 805F 5′-GACTACHVGGGTATCTAATCC-3′ and the reverse primer Enh_LepR1 5′-CYGCACAYAGYRCTRTAA-3′ (Jiang et al., 2014). PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, Waltham City, MA, USA), and the conditions of PCR amplification are as follows: pre-amplification was 94°C for 3 min, 5 cycles of 94°C for 30 s, 45°C for 20 s, and 65°C for 30 s, followed by 20 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s and a final extension at 72°C for 5 min. PCR products were examined on a 2% agarose gel. Only samples with a clear band between 400 and 450 bp were chosen for further experiments.

**Sequencing of 16S rDNA gene amplicons**

Sequence libraries that included 20 individuals were generated using TrueSeq® DNA PCR-free Sample Preparation Kit (Illumina) according to the manufacturer’s instructions. Library quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system (Agilent). The libraries were sequenced using the Illumina Miseq platform by Zhejiang Tianke High Technology Development Co. Ltd. (Zhejiang, China), and 300 bp paired-end (PE) reads were generated.

**Detection of the Wolbachia infection rate**

To determine whether the samples were infected with Wolbachia, the wsp gene was amplified from genomic DNA (Gong and Shen, 2002; Laura et al., 2006). The forward primer wsp_F1 5′-GTCC-AATARSTGATGARGAAAC-3′ and the reverse primer wsp_R1 5′-CYGCACAYAGYRCTRTAA-3′ were used (Laura et al., 2006), and amplification was as follows: denaturation at 95°C for 3 min, 35 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 90 s and a final extension at 72°C for 10 min. PCR products were detected by 1% agarose gel electrophoresis.

**Data analysis**

PE reads were merged using FLASH, and quality filtering of spliced sequences (raw tags) was performed under specific conditions to obtain high-quality clean tags according to QIIME. To detect and remove chimeric sequences, the tags were compared with reference sequences obtained from the Gold database using the UCHIME algorithm. Sequence analysis was performed using UPARSE software. Sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). Representative sequence for each OTU was screened for further annotation.

Taxonomic assignment was achieved using the SILVA reference database (http://www.arb-silva.de/) with a threshold of 90%. The α diversity was applied when evaluating the complexity of species diversity for a sample using six indices: observed species, Chao1, Shannon, Simpson, ACE, Good’s coverage. All indices were calculated using QIIME (Version 1.9.1). The β diversity analysis was applied to evaluate the differences in species complexity among the different samples, and principal coordinate analysis (PCoA) was performed based on the matrices of pairwise distances among all the microbiota. Heatmap and hierarchical cluster were built based on the relative abundance of the top 15 genera identified in the bacterial communities of samples by using heatmap package in R program. The highly relative abundances of microbiota phyla and genera were visualized using ggplot2 package (version 3.0.0) in R program. Statistical analyses were performed using SPSS 17.0 (IBM). One-way ANOVA was followed by Tukey test for means comparison of α diversity. The level of significance was set at *P* < 0.05.

**Result**

**Species verification**

A total of 20 individuals were chosen for bacterial community analysis. All samples from the Yuhang population were identified as *E. obliqua* and numbered O1–10 (group O); all samples from the Xinchang population were identified as *E. grisescens* and numbered G1–10 (group G) (fig. 2; table S1).

**Sequencing data and sequence read diversity analysis**

A total of 752,452 raw PE 16S rDNA reads were generated from these 20 samples. After removal of low-quality reads, 658,249 (∼75.5%) valid reads were obtained. The length of each read was between 404 and 426 bp, with an average of 417.2 bp. Q20 values for all samples were >98%. All reads clustered into 1492 OTUs (3% distance, average neighbour clustering), covering ten phyla, 40 classes, 79 orders, 118 families, 198 genera and 125 species.

Shannon and Simpson indices were used to evaluate bacterial diversity, and Chao1 and Ace indices were employed to estimate the total number of species in the samples (Sun et al., 2016). Good’s coverage was applied for sequencing results. Significantly higher values of the Simpson index were found for *E. grisescens* compared to *E. obliqua*, whereas *E. obliqua* displayed
significantly higher Ace and Chao1 index values (table 1). Despite a lack of a significant difference for the Shannon index between *E. grisescens* and *E. obliqua*, higher bacterial community diversity was found for *E. grisescens*. Overall, the high Good’s coverage (both >99%) values suggest that the OTUs covered most of the bacterial communities present and that our metagenomic data were reliable.

To compare similarity and dissimilarity between all samples, PCoA and hierarchical clustering analysis were performed. The points in fig. 3 represent samples in the PCoA plot, and the distance of each point indicates the similarity of different samples. PCoA separated the samples into two clusters, with 75.19 and 17.80% of the total variation being explained by the PCo1 and PCo2 axes, respectively. With the exception of sample O2, which was closer to group G, samples of the same species clustered together. In addition, hierarchical clustering analysis separated the samples into two clades according to species (fig. 4). Overall, the results suggest that the bacterial communities in these two species differed and that the major contribution of the difference was PCo1.

The 16S rDNA V3-V4 region was amplified to compare the differences of the microbiota of these sibling species of tea geometrid moths. A total of 286,700 valid reads and 1043 OTUs were obtained from ten of *E. obliqua* samples, comprising ten phyla, 38 classes, 75 orders, 111 families, 179 genera and 118 species. Among these ten phyla (fig. 5), Firmicutes (52.80%), Proteobacteria (24.52%) and Cyanobacteria (14.05%) were highly abundant. At the genus level, 14.48% reads were not identifiable, and the remaining reads belong to the bacteria of 179 genera. The predominant genera (>1%) were Melissococcus (29.14%),

![Figure 2. The maximum likelihood (ML) tree of samples used in bacterial community analysis based on 516 bp gene segment of cytochrome oxidase I (COI) sequences. Kimura-2-parameter model was used with bootstrap percentages shown on the clades. KJ704358 (E. obliqua) was the control sample downloaded from GenBank.](https://doi.org/10.1017/S0007485320000164)

**Table 1.** Estimated richness and diversity indices for the bacterial communities

| Group | Effective tags | OTUs | Shannon | Simpson | Ace        | Chao1       | Good’s coverage |
|-------|----------------|------|---------|---------|------------|-------------|----------------|
| O     | 28,670         | 1043 | 1.87 ± 0.54 | 0.43 ± 0.11 | 124.77 ± 16.72 | 128.78 ± 14.69 | 0.99           |
| G     | 371,549        | 449  | 2.56 ± 0.16 | 0.70 ± 0.04 | 52.17 ± 4.98 | 47.96 ± 4.38 | 0.99           |

*S*Significantly different compared to group O by Tukey test (*P* < 0.05); means ± SE for *α* diversity indices.
Staphylococcus (23.05%), Enterobacter (14.48%), Sphingomonas (2.19%), Corynebacterium (2.30%), Methylobacterium (2.13%), Brevibacterium (1.90%) and Paracoccus (1.08%).

In total, 371,549 valid reads and 449 OTUs were obtained from the sequencing data for *E. grisescens*, covering seven phyla, 19 classes, 34 orders, 58 families, 75 genera and 40 species. Compared to *E. obliqua*, fewer microbiota organisms were identified in *E. grisescens* at all classification levels, and the composition of abundant taxa varied in these two species. The bacteria found in *E. grisescens* belong to seven phyla (fig. 5), with Proteobacteria (79.13%), Actinobacteria (14.06%) and Firmicutes (5.13%) being the most abundant. Comparatively, the abundances of Firmicutes and Cyanobacteria were significantly greater in *E. obliqua* than in *E. grisescens*, whereas the abundances of Proteobacteria and Actinobacteria were higher in *E. grisescens* (fig. 5).

At the genus level, 99.60% of the reads were identified and classified into 75 taxa. Most bacteria were found belonging to 14 genera (>1%): Wolbachia (28.97%), Enterobacter (24.16%), Pseudomonas (14.82%), Arthrobacter (7.74%), Melissococcus (5.10%), Brevibacterium (3.38%), Corynebacterium (2.19%), Acinetobacter (1.97%), Raoultella (1.94%), Sphingomonas (1.66%), Ochrobactrum (1.53%), Stenotrophomonas (1.36%), Serratia (1.34%) and Sphingobacterium (1.29%). When comparing the number of genera, 56 were shared by *E. grisescens* and *E. obliqua*, and 123 and 19 genera were unique to *E. obliqua* and *E. grisescens*, respectively. Wolbachia, Enterobacter and Pseudomonas were predominant genera in *E. grisescens*, the most predominant microbiota genera of *E. obliqua* were Melissococcus, Staphylococcus and Enterobacter, while Wolbachia (0.02%) were rare in *E. obliqua* (fig. 6; table 2).

Detection of Wolbachia in sibling species of tea geometrid moths

We screened for four microbiota (Wolbachia, Cardinium, Spiroplasma and Rickettsia), which have been shown to influence the reproduction (Zhang et al., 2016b). The results showed that only Wolbachia was found in both species. Thus, we evaluated the richness of Wolbachia in all samples used for bacterial community analysis and found it to be significantly different between the sibling species of tea geometrid moths. Specifically, the average richness of Wolbachia was 28.97% in *E. grisescens*, whereas it was dramatically lower in *E. obliqua* (0.02%). Wolbachia was not detected in female *E. obliqua* samples (O1–O5), but were detected in 60% of male samples (O6–O10). The richness of Wolbachia ranged from 0.03 to 0.15% in three male *E. obliqua* samples. In *E. grisescens*, infection rates varied among samples, and no
significant difference between female and male moths was found (table 2).

Furthermore, we detected the infection rate of \textit{Wolbachia} for samples from eight different geographical populations by amplifying \textit{wsp} gene. A total of 86 samples were randomly selected for the identification of species using the \textit{CO1} gene, which were deposited in GenBank (Supplementary material table S2). The samples from Yuhang (Zhejiang) and Liyang (Jiangsu) were identified as \textit{E. obliqua} and those from Xinchang (Zhejiang), Guiyang (Guizhou), Nanchang (Jiangxi), Yingde (Guangdong) and Enshi (Hubei) as \textit{E. grisescens}. Additionally, samples from Langxi (Anhui) were identified as the two species (ten samples of \textit{E. obliqua} and six samples of \textit{E. grisescens}). The intra-specific genetic distances between those individuals were 0–0.2%, while the inter-specific genetic distances were 3.4–3.8% (Supplementary material table S3). The results of \textit{Wolbachia} detection showed a \textit{Wolbachia} infection rate of 0 and 100% for \textit{E. obliqua} and \textit{E. grisescens}, respectively (figs S1–S5). In addition, we also detected the infection rate of \textit{Wolbachia} for 20 samples used for bacterial community analysis by \textit{wsp} gene marker. The result showed \textit{Wolbachia} were not detected in the samples with low \textit{Wolbachia} infection rates (such as O6, O9 and O10) (fig. S6).

**Discussion**

The sibling species of tea geometrid moths \textit{E. obliqua} and \textit{E. grisescens} both feed on tea leaves. In our study, we controlled the rearing conditions to eliminate the influence of food and environment to explore microbiotal differences in these species. The results showed higher microbiota diversity for \textit{E. grisescens} than \textit{E. obliqua}, which may offer clues for understanding why \textit{E. grisescens} has a wider distribution and greater adaptability than does \textit{E. obliqua}. In general, predominant microbiota differed at the genus level between these sibling species. The predominant microbiota were \textit{Melissococcus}, \textit{Staphylococcus} and \textit{Enterobacter} in \textit{E. obliqua} but \textit{Wolbachia}, \textit{Enterobacter} and \textit{Pseudomonas} in \textit{E. grisescens}. Regarding microbiota species related to reproduction, \textit{Wolbachia} abundance was significantly different between these sibling species.

\textit{Wolbachia} is Gram-negative bacterium first found in the oophoron of \textit{Culex pipiens} (Hedges et al., 2008). \textit{Wolbachia} is mainly harboured in the cytoplasm of a host germ cell, with two transmission routes: common maternal vertical transfer to progeny (Hoffmann et al., 1990) and less common horizontal transfer among different hosts, widely broadening the host range (Huigens et al., 2000; Ahmed et al., 2016). Previous studies have reported the presence of \textit{Wolbachia} in nematode species and many arthropods, with widespread infection in insecta (Hilgenboecker et al., 2010). Indeed, \textit{Wolbachia} has garnered intense interest because of its ability to alter the biology of its host, especially with regard to reproduction. This bacterium can manipulate insect-host reproduction in various ways including parthenogenesis (PI), feminization, male killing (MK) and CI (Lus, 1947; Terry et al., 1997; Zhang et al., 2015; Lindsey et al., 2016). To date, PI has been found in mites, hymenopterans and thrips (Arakaki et al., 2001; Werren et al., 2008). Generally, they have a specific sex-determination system where unfertilized eggs develop into haploid males while fertilized eggs develop into diploid females (Weeks and Breeuwer, 2001). \textit{Wolbachia} can induce PI, whereby females develop without fertilization (Lindsey et al., 2016). In MK, male individuals are killed during embryonic development, which is induced by multiple factors, and this process has been reported in more than 20 insects (Hurst et al., 1997), though with \textit{Wolbachia} as a known cause.
in only *Adalia bipunctata* and *Acraea encedon* (Jiggins et al., 2010). Among all reproductive impacts, CI is the most typical and conspicuous (Zhang et al., 2015). In the simplest form, CI can be described as embryonic mortality that occurs when uninfected females mate with *Wolbachia*-infected males (Bourtzis et al., 1996). Most CI embryos exhibit defects in paternal chromosome condensation, resulting in paternal ‘diffused chromatin’ that cannot be normally distributed to the zygote during metaphase I (Uyen et al., 2006). Previous studies have reported CI in Acari, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera and Orthoptera (Bourtzis et al., 1996; Werren et al., 2008; Chevalier, 2012; Pinto et al., 2013; Zhang et al., 2015).

In our study, *E. grisescens* was found to be infected with *Wolbachia*, but *E. obliqua* showed little infection by this genus, which is in line with the CI requirement of mating between uninfected and infected individuals. Recent research has indicated that the hatching rate of the filial generation was notably decreased when female *E. grisescens* mated with male *E. obliqua* and that it was even lower when female *E. obliqua* mated with male *E. grisescens* (Xi et al., 2014; Zhang et al., 2014). Overall, our results are in accord with CI and suggest that *Wolbachia* might be an important factor causing reproductive isolation between these species.

Generally, the fundamental rule of distinguishing species is reproductive isolation which may result from prezygotic or postzygotic barriers (Dobzhansky, 1970). In our study, the kind of phenomenon, can mate but produce sterile offspring between the two tea loopers, belongs to postzygotic isolation (Haldane, 1922). The postzygotic isolation is a rare phenomenon relative to prezygotic in nature and exists in those species which have a close genetic relationship such as *Spodoptera frugiperda* (fall armyworm). *Spodoptera frugiperda* is polyphagous and a major agricultural pest in the North and South American continent and Caribbean (Gouin et al., 2017). It consists of two sympatric host-plant strains, C strain feeding mostly on maize cotton and sorghum and R strain mostly associated with rice and various pasture grasses (Gouin et al., 2017). These two strains are morphologically indistinguishable but estimated to be 2.09% on average in the COI gene (Kergoat et al., 2012). Compared to genetic distances of other species, Dumas et al. proposed that C and R strains were pairs of differentiated species (sister-species) in the
Spodoptera genus (Dumas et al., 2015a, 2015b). More important, C and R strains also showed the phenomenon of postzygotic isolation (Groot et al., 2016). Though bacterial endosymbionts can cause genetic incompatibilities in hybrids, Dumas et al. investigated the presence of Wolbachia and several other bacteria in both C and R strains of S. frugiperda, but did not detect any of them, accordingly, eliminated the factor that bacteria manipulate their host reproduction (Dumas et al., 2015a). However, in our study, E. obliqua and E. grisescens have identical dietary habits and differential bacterial endosymbiont in Wolbachia. Therefore, this pair of insects constitute suitable material for research that Wolbachia mediating host reproduction isolation.

The results of this study reveal the diversity of microbiota taxa between sibling species of tea geometrid moths. In particular, the notable difference in Wolbachia may be the major factor influencing the reproductive isolation of these sibling species. Overall, Wolbachia is an important microbiota genus manipulating insect-host reproduction in various ways. In addition, more functions of Wolbachia can be explored in these model sibling species of tea geometrid moths, such as Wolbachia interaction with pheromones and EoNPV. Nonetheless, further research is required to explore the mechanism by which Wolbachia is involved in reproductive isolation between sibling species of tea geometrid moths.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485320000164.

Acknowledgments. We thank Dr Zhishuo Wang (University of Edinburgh, Edinburgh), Dr Liang Sun and Dr Xin Li (Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou) for providing language and writing assistance.

Financial support. This work was supported by the National Key R&D Program of China (2017YFE0107300), the National Natural Science Foundation of China (31700613), Special Project on Basic Scientific Research (2013FY113200) and the Science and Technology Innovation Project of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2016-TRICAAS).

Table 2. Wolbachia richness of 20 samples was tested using 16S rDNA by Illumina Miseq platform

| Sex  | Sample | Wolbachia (%) | Sample | Wolbachia (%) |
|------|--------|---------------|--------|---------------|
| Female | O1     | 0             | G1     | 74.51         |
|       | O2     | 0             | G2     | 41.43         |
|       | O3     | 0             | G3     | 11.99         |
|       | O4     | 0             | G4     | 44.13         |
|       | O5     | 0             | G5     | 24.87         |
| Male  | O6     | 0.03          | G6     | 42.40         |
|       | O7     | 0             | G7     | 5.02          |
|       | O8     | 0             | G8     | 20.95         |
|       | O9     | 0.03          | G9     | 4.97          |
|       | O10    | 0.15          | G10    | 19.42         |
| Average | Group  | 0.02          | Group  | 28.97         |
Conflict of interest. The authors declare that they have no conflict of interest.

Ethical standards. This article does not contain any studies with human participants or animals performed by any of the authors.

References

Ahmed MZ, Breinholt JW and Kawahara AY (2016) Evidence for common horizontal transmission of Wolbachia among butterflies and moths. BMC Evolutionary Biology 16, 118.

Arakaki N, Miyoshi T and Noda H (2001) Wolbachia-mediated parthenogenesis in the predatory thrips Frankliniella vesparium (Thysanoptera: Insecta). Proceedings of the Royal Society London B 268, 1011–1016.

Bai JH, Wang ZB and Xiao Q (2018a) Genetic differentiation and distribution of two sibling species of tea geometrids in tea-growing areas in Zhejiang, eastern China. Acta Entomologica Sinica 61, 741–748.

Bai JH, Tang MJ, Yin KS, Wang ZB and Xiao Q (2018b) Differential biological characteristics between closely related tea geometrid species, Ectropis obliqua and Ectrops gresescens. Acta Agriculturae Zhejiangensis 30, 797–803.

Bourtzis K, Nirgianaki A, Markakis G and Savakis C (2002) Molecular diagnostic techniques of Spodoptera frugiperda (Lepidoptera: Geometridae) with different host-plant ranges. Parasitology Research 88, 651–657.

Bourjorfis K, Nirgianaki A, Markakis G and Savakis C (1996) Wolbachia infection and cytoplasmic incompatibility in Drosophila species. Genetics 144, 1063–1073.

Chen BS, Lu XM and Shao YQ (2017) Diversity of the gut microbiota in lepidopteran insects and their interaction with hosts. Acta Entomologica Sinica 60, 710–722.

Chevalier F (2012) Feminizing Wolbachia: a transcriptomics approach with insights on the immune response genes in Armadillidium vulgare. BMC Microbiology 12, S1.

Colman DR, Toolson EC and Takacsveschbad CD (2012) Do diet and taxonomic influence insect gut bacterial communities? Molecular Ecology 21, 5124–5137.

Dobzhansky T (1970) Genetics of the Evolutionary Process. New York: Columbia University Press, p. 354.

Dumas P, Legrai F, Lemaitre C, Scaon E, Orsucci M, Labadie K, Gimenez S, Clamens AL, Henri H, Vavre F, Aury JM, Fournier P, Kergoat GJ and d’Alencón E (2015a) Spodoptera frugiperda (Lepidoptera: Noctuidae) host-plant variants: two host strains or two distinct species? Genetica 143, 305–316.

Dumas P, Barbut J, Le Ru B, Silvain JF, Clamens AL, Henri H, Vavre F, Aury JM, Fournier P, Kergoat GJ and d’Alencón E (2015b) Phylogenetic molecular species delimitations unravel non-male strains in populations. Acta Entomologica Zhejiangensis 27, 1016–1020.

Engel P and Moran NA (2013) The gut microbiota of insects – diversity in structure and function. FEMS Microbiology Reviews 37, 699–735.

Gong P and Shen ZR (2002) Molecular diagnostic techniques of Wolbachia. Hereditas 24, 207–210.

Gouin A, Breutaud A, Nam K, Gimenez S, Aury JM, Fournier P, Kergoat GJ, d’Alencón E and Bobet F (2016) Genetic differentiation and distribution of Wolbachia among butterflies and moths. BMC Evolutionary Biology 16, 118.

Haldane JBS (1922) Sex ratio and unisexual sterility in hybrid animals. Journal of Genetics 12, 101–109.

Hedges LM, Brownlie JC, O’Neill SL and Johnson KJ (2008) Wolbachia and virus protection in insects. Science (New York, N.Y.) 322, 702.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telchow A and Werren JH (2010) How many species are infected with Wolbachia? A statistical analysis of current data. FEMS Microbiology Letters 281, 215–220.

Hoffmann AA, Turelli M and Harshman LG (1990) Factors affecting the distribution of cytoplasmic incompatibility in Drosophila simulans. Genetics 126, 933–948.

Huang S and Zhang H (2013) The impact of environmental heterogeneity and life stage on the hindgut microbiota of Holotrichia parallela larvae (Coleoptera: Scarabaeidae). PLoS ONE 8, e57169.

Huigens ME, Luck RF, Klaassen RGH, Maas M and Stouthamer R (2000) Infectious parthenogenesis. Nature 405, 178–179.

Hurst GDD, Hammarton TC, Bandi C, Majerus TMO, Bertrand D and Majerus MEN (1997) The diversity of inherited parasites of insects: the male-killing killing of the ladybird beetle Coleomegilla maculata is a member of the Flavobacteria. Genetical Research 70, 1–6.

Jiang N, Liu SX, Xue DY, Tang MJ, Xiao Q and HanHX (2014) External morphology and molecular identification of two tea Geometrid moth from southern China. Chinese Journal of Applied Entomology 51, 987–1002.

Jiggins FM, Hurst GDD and Majerus MEN (2010) Sex ratio distortion in Acraea encedon (Lepidoptera: Nymphalidae) is caused by a male-killing bacterium. Heredity 81, 87–91.

Kergoat GJ, Provell DP, Le Ru BP, Mitchell A, Dumas P, Clamens AL, Condamine FL and Silvain JF (2012) Disentangling dispersal, vicariance and adaptive radiation patterns: a case study using armypawns in the pest genus Spodoptera (Lepidoptera: Noctuidae). Molecular Phylogenetics and Evolution 65, 855–870.

Kim SS, Beljaev EA and Oh SH (2001) Illustrated catalogue of Geometridae in Korea (Lepidoptera: Geometrinae, Ennominae). Illustration: In Park KT (ed.), Korea Research Institute of Bioscience and Biotechnology & Center for Insect Systematics, Daejeon, Korea, pp. 1–278.

Laura B, Julie CDH, Keith AJ, Seth RB, Sarah AB, Choudhury RR, Hayashi C, Maidjen MCJ, Tettel H and Werren JH (2006) Multilocus sequence typing system for the endosymbiont Wolbachia pipientis. Applied and Environmental Microbiology 72, 7098–7110.

Lindsey ARI, Werren JH, Richards S and Stouthamer R (2016) Comparative genomics of a parthenogenesis-inducing Wolbachia symbiont. G3 Genes, Genomes, Genomes 6, 2113–2123.

Lus YY (1947) Some rules of reproduction in populations of Adalia bipunctata. L. Non-male strains in populations. Doklady Akademii Nauk SSSR 57, 951–954.

Mao TF, Fu JY, Liang S, Gui ZX, Bai JH and Xiao Q (2017) The expression of the antibacterial peptide genes from two sibling species of tea geometrid was different in resistance to Esenovirus infection. Chinese Journal of Biological Control 33, 472–480.

Mao XY, Tan RR, Wang YP, Chen X, Wang HJ, Huang DJ and Gong ZM (2018) Analysis of the bacterial diversity in adults of Empoasca (Matsumurasca) orukhi based on 165 r DNA sequences. Plant Protection 44, 17–23.

Philipp E and Nancy A M (2013) The gut microbiota of insects – diversity in structure and function. FEMS Microbiology Reviews 37, 699–735.

Pinto SB, Kirsty S, Simon H, Zakaria K, Sutton ER, Bonsall MB, Julian P and Sinkins SP (2013) Transcriptional regulation of Calux pipiens mosquitoes by Wolbachia influences cytoplasmic incompatibility. PLoS Pathogens 9, e1003647.

Sato R, Sato K, Kikuchi T, Akita K, Ono Y and Makino Y (2003) Wolbachia infection influences cytoplasmic inheritance and fitness in some natural populations. Journal of Genetics 82, 153–157.

Su LJ, Yang LL, Huang S, Su XQ, Li Y, Wang FQ, Wang ET, Kang N, Xu J and Song AD (2016) Comparative gut microbiomes of four species representing the higher and the lower termites. Journal of Insect Science 16, 1–9.

Sun Z, Lu Y, Zhang H, Kumar D, Liu B, Gong Y, Zhu M, Zhu L, Liang Z and Kuang S (2016) Effects of BmCPV infection on silkworm Bombyx mori intestinal bacteria. PLoS ONE 11, e0146313.

Terry R, Dunn A and Smith J (1997) Cellular distribution of a feminizing microsporidian parasite: a strategy for transovarial transmission. Parasitology 113, 157–163.
Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, Simon JC and Fukatsu T (2010) Symbiotic bacterium modifies aphid body color. *Science (New York, N.Y.)* 330, 1102–1104.

Uyen T, Kurt F, Werren JH and William S (2006) Paternal chromosome segregation during the first mitotic division determines *Wolbachia*-induced cytoplasmic incompatibility phenotype. *Journal of Cell Science* 119, 3655–3663.

Wang XM, Wu K, Chen XG and Yan GY (2017) Research advances on diversity and function of mosquito-bacteria symbiosis. *Chinese Journal of Parasitology and Parasitic Diseases* 35, 305–312.

Wang ZB, Bai JH, Liu YJ, Li H, Zhan S and Xiao Q (2019) Transcriptomic analysis reveals insect hormone biosynthesis pathway involved in desynchronized development phenomenon in hybridized sibling species of tea Geometrids (*Ectropis grisescens* and *Ectropis obliqua*). *Insects* 10, 381.

Weeks AR and Breeuwer JAJ (2001) *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. *Proceedings of the Royal Society London B* 268, 2245–2251.

Wehrli E (1945) Subfamilie: Geometrinae. Die Grossschmetterlinge der Erde: In Seitz A (ed.), Stuttgart: Verlag A. Kernen, pp. 254–766.

Werren J, Baldo L and Clark M (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 6, 741–751.

Xi Y, Yin KS, Tang MJ and Xiao Q (2014) Geographic populations of the tea geometrid, *Ectropis obliqua* (Lepidoptera: Geometridae) in Zhejiang, eastern China have differentiated into different species. *Acta Entomologica Sinica* 57, 1117–1122.

Zhang GH, Yuan ZJ, Zhang CX, Yin KS, Tang MJ, Guo HW, Fu JY and Xiao Q (2014) Detecting deep divergence in seventeen populations of tea geometrid (*Ectropis obliqua* Prout) in China by COI mtDNA and cross-breeding. *PLoS ONE* 9, e99373.

Zhang YK, Zhang KJ, Xie RR, Zhao XD and Hong XY (2015) Research progress in cytoplasmic incompatibility induced by endosymbiont *Wolbachia*. *Acta Entomologica Sinica* 58, 1344–1355.

Zhang GH, Yuan ZJ, Yin KS, Fu JY, Tang MJ and Xiao Q (2016a) Asymmetrical reproductive interference between two sibling species of tea looper: *Ectropis grisescens* and *Ectropis obliqua*. *Bulletin of Entomological Research* 11, 1–8.

Zhang YK, Chen YT, Yang K, Qiao GX and Hong XY (2016b) Screening of spider mites (Acari: Tetranychidae) for reproductive endosymbionts reveals links between co-infection and evolutionary history. *Scientific Reports* 6, 27900.