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

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Research Paper

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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 microbiotal involvement in host reproductive biology. In this study, we first identified and compared the compositions of microbiota between these sibling species, revealing higher microbiotal 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 microbiotal 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.*, 2016*a*). *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.*, 2018*a*). 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

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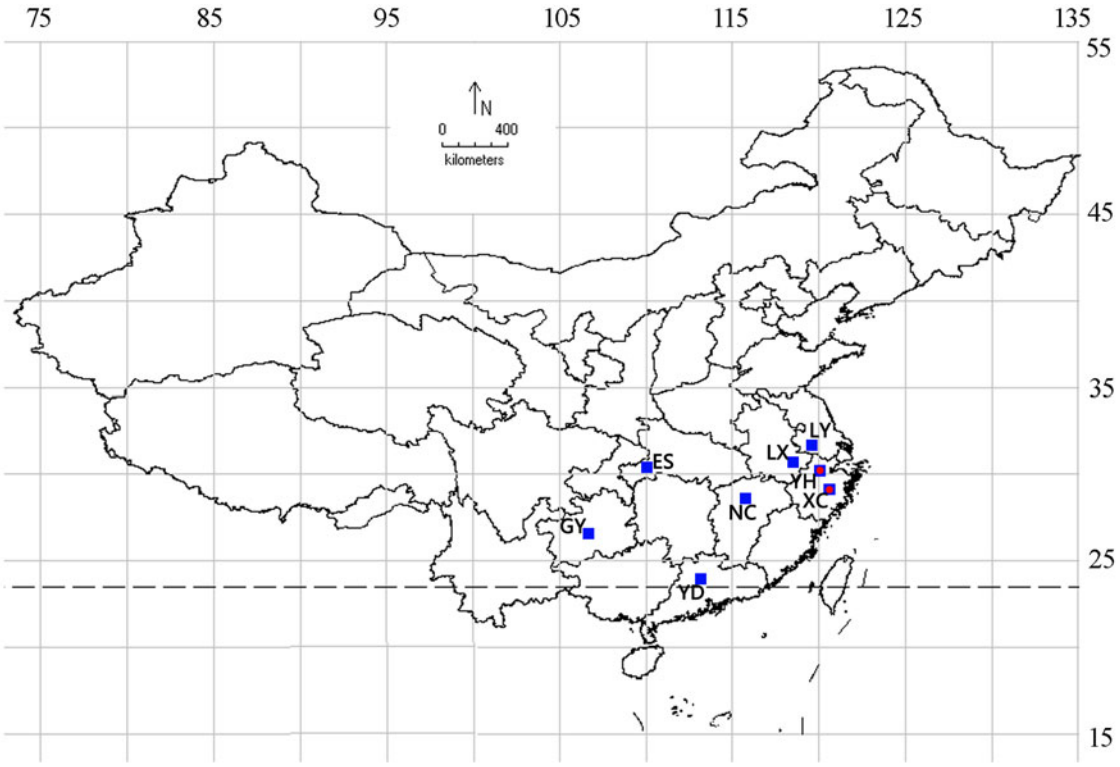

Figure 1. Sampling localities of tea geometrid. ■represent samples used for the detection of *Wolbachia* infection rate,●represent samples used for analysing bacterial communities. The abbreviation of different geographical population as follows: XC, Xinchang; YH, Yuhang; LY, Liyang; LX, Langxi; NC, Nanchang; ES, Enshi; GY, Guiyang; GY, Guiyang.

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.*, 2018*b*). 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.*, 2016*a*). 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.*, 2016*a*, 2016*b*). 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

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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′-ATTCAACCAATCATAAAGATATTGG-3′ and the reverse primer Enh_LepR1 5′-CTCCWCCAGCAGGATCAAAA-3′ (Jiang *et al.*, 2014). PCR using 2x Master Mix (TSINGKE Bio Inc., Hang zhou City, Zhe jiang, 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. Sequences were assembled and edited with SeqMan 7.1.0. and aligned with CLUSTAL 1.83. The sequence of *E. obliqua COI* gene (accession number KJ704358), which was obtained from GenBank, was used as a control to calculate genetic distance based on Kimura-2-parameter model (100 bootstrap replicates) and build a cluster analysis tree using a maximum likelihood approach implemented in MEGA 5.05. Compared to the control sample (KJ704358), genetic distances of ∼0–2.5 and 3.2–4.0%, respectively, were identified for *E. obliqua* and *E. grisescens* (Jiang *et al.*, 2014).

PCR amplification of microbial 16S rDNA genes

The V3-V4 region of 16S rDNA was amplified using the forward primer 341F 5′-CCTACGGGNGGCWGCAG-3′ and the reverse primer 805R 5′-GACTACHVGGGTATCTAATCC-3′ (Sinclair *et al.*, 2015). 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 TruSeq® DNA PCR-free Sample Preparation Kit (Illumina) according to the manufacturer's instruction. 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′-GTCCAATARSTGATGARGAAAC-3′ and the reverse primer *wsp*_R1 5′-CYGCACCAAYAGYRCTRTAAA-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 pheatmap 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

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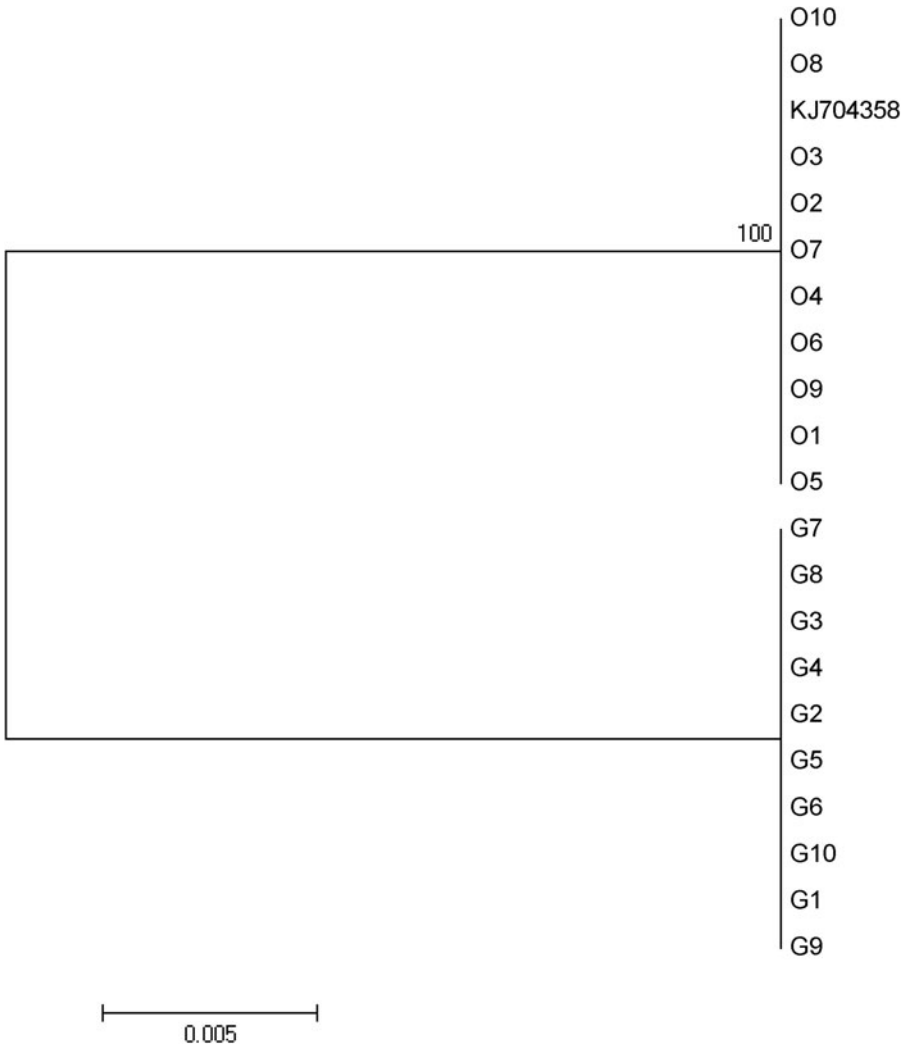

Figure 2. The maximum likelihood (ML) tree of samples used in bacterial community analysis based on 516 bp gene segment of cytochrome oxidase I (*COI*) sequences. Kimura-2-parameter model was used with bootstrap percentages shown on the clades. KJ704358 (*E. obliqua*) was the control sample downloaded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

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[a]	52.17 ± 4.98[a]	47.96 ± 4.38[a]	0.99

[a]Significantly different compared to group O by Tukey test ($P < 0.05$); means ± SE for α diversity indices.

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%),

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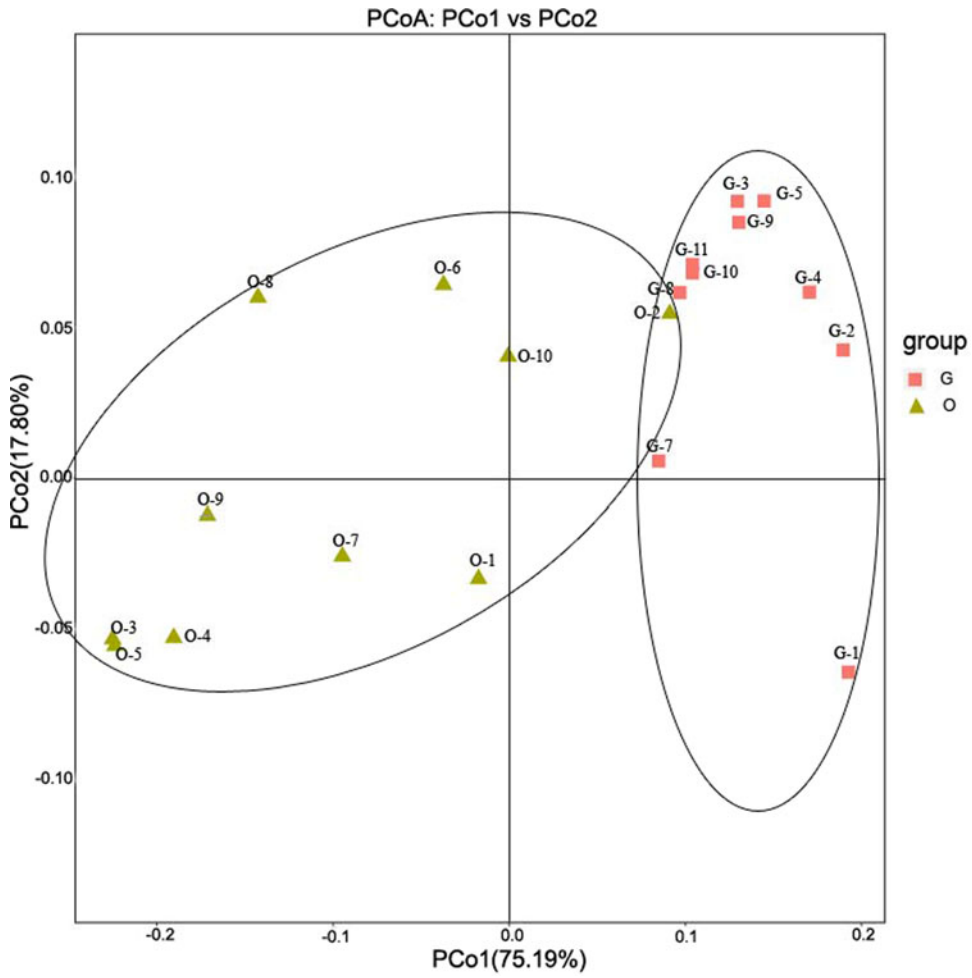

Figure 3. Principal coordinate analysis of microbiota in 20 samples based on OTUs. Each point corresponds to a sample. Red represents *E. grisescens*, and yellow represents *E. obliqua*. PCo1 and PCo2 are shown with the percentage variation explained for each axis.

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.*, 2016*b*). 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

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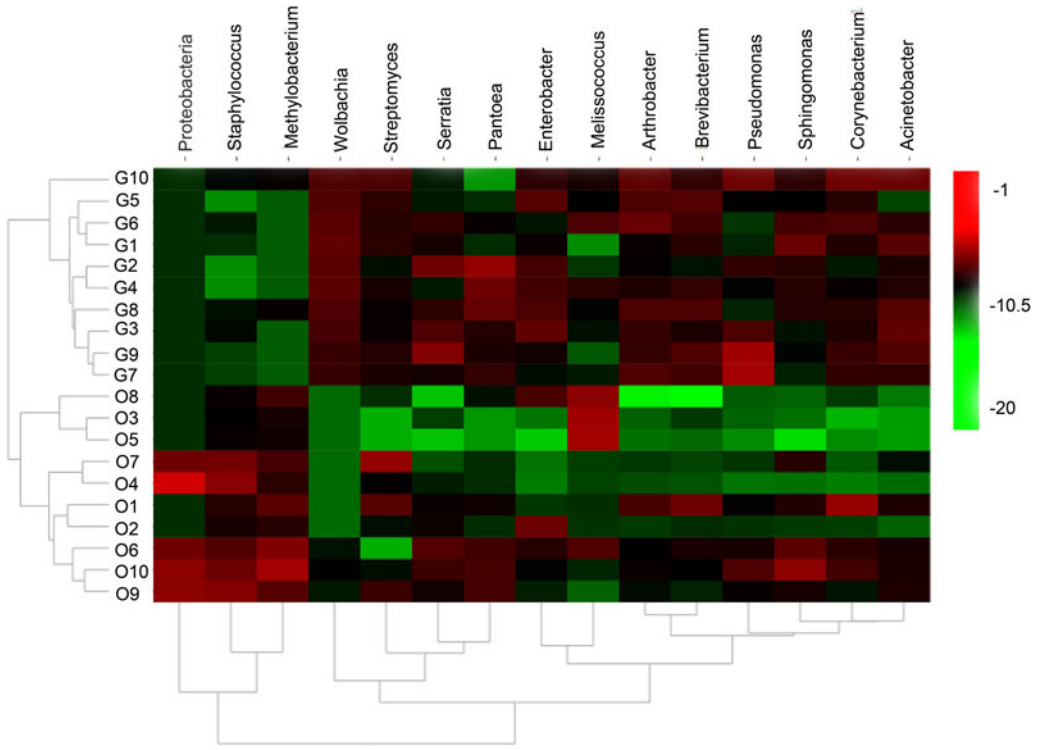

Figure 4. Hierarchical clustering analysis of 20 samples based on the relative abundance of the top 15 microbiota genera identified in sibling species of tea geometrid moths.

significant difference between female and male moths was found (table 2).

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

Discussion

The sibling species of tea geometrid moths *E. obliqua* and *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 microbiotal diversity for *E. grisescens* than *E. obliqua*, which may offer clues for understanding why *E. grisescens* has a wider distribution and greater adaptability than does *E. obliqua*. In general, predominant microbiota differed at the genus level between these sibling species. The predominant microbiota were *Melissococcus*, *Staphylococcus* and *Enterobacter* in *E. obliqua* but *Wolbachia*, *Enterobacter* and *Pseudomonas* in *E. grisescens*. Regarding microbiota species related to reproduction, *Wolbachia* abundance was significantly different between these sibling species.

Wolbachia is Gram-negative bacterium first found in the oophoron of *Culex pipiens* (Hedges *et al.*, 2008). *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 *Wolbachia* in nematode species and many arthropods, with widespread infection in insecta (Hilgenboecker *et al.*, 2010). Indeed, *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). *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 *Wolbachia* as a known cause

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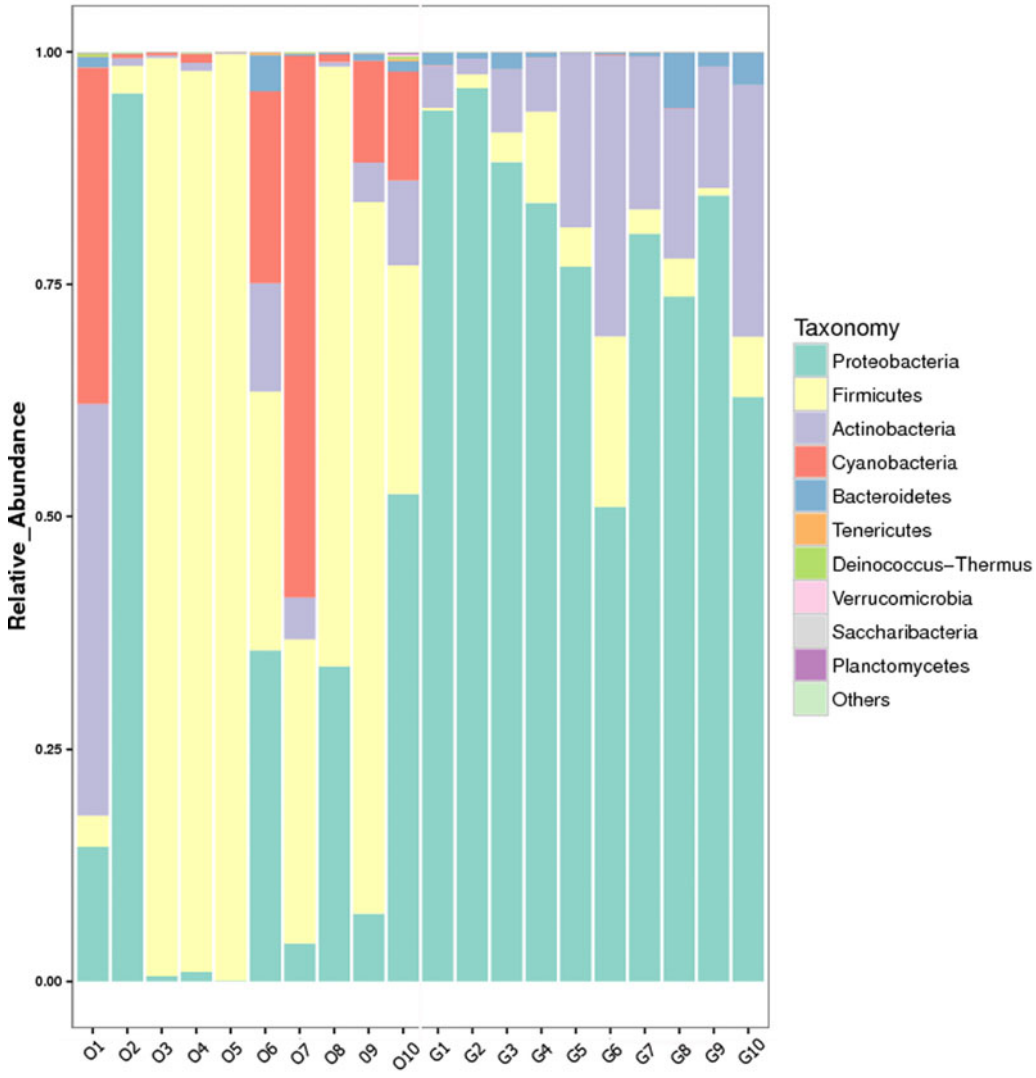

Figure 5. Relative abundances of microbiota phyla in sibling species of tea geometrid moths. Columns of different colour represent the abundance of the top ten microbiota. Others represent the sum abundance of those microbiota not among the top ten.

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

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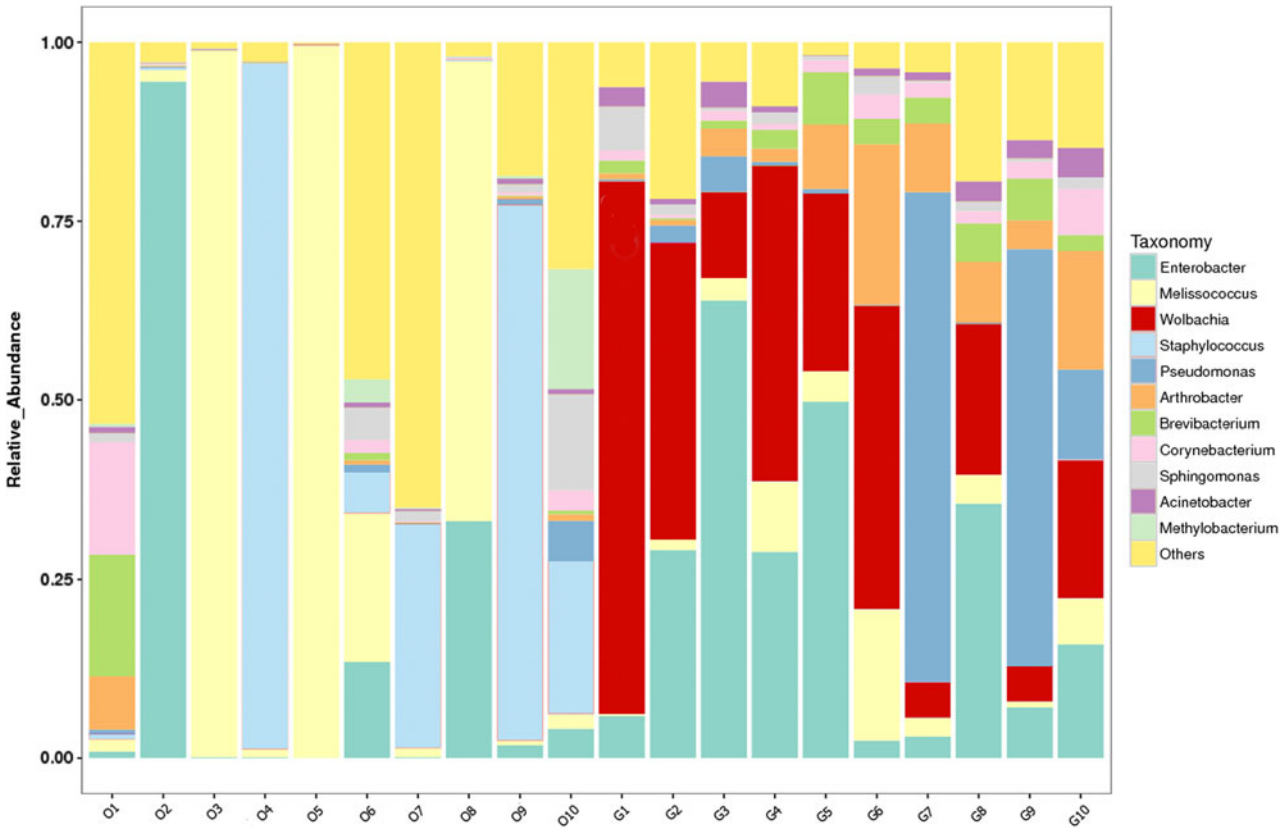

Figure 6. Relative abundances of dominant microbiota genera in sibling species of tea geometrid moths. Columns of different colour represent dominant genera (relative abundance >1%).

Table 2. *Wolbachia* richness of 20 samples was tested using16S 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 O	0.02	Group G	28.97

Spodoptera genus (Dumas *et al.*, 2015*a*, 2015*b*). 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.*, 2015*a*). 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 *Eo*NPV. Nonetheless, further research is required to explore the mechanism by which *Wolbachia* is involved in reproductive isolation between sibling species of tea geometrid moths.

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

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