Journal of Insect Science, (2023) 23(2): 13; 1–8
https://doi.org/10.1093/jisesa/iead021
Research

ENTOMOLOGICAL SOCIETY OF AMERICA
SHARING INSECT SCIENCE GLOBALLY

OXFORD

Diversity and dynamics of endosymbionts in a single population of sweet potato weevil, *Cylas formicarius* (Coleoptera: Brentidae): a preliminary study

Jin Xu[1], Jian-Bin Tan[1], Yi-Dan Li[1], Yuan-Hao Xu[1], An Tang[1], Hong-Kai Zhou[2], Pei-Qiong Shi[1,*]

[1]Department of Biotechnology, College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, China
[2]Department of agronomy, College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, China
*Corresponding author, mail: shipeiqiong@gdou.edu.cn

Subject Editor: Phyllis Weintraub

Received on 28 August 2022; revised on 8 February 2023; accepted on 31 March 2023

Endosymbionts live symbiotically with insect hosts and play important roles in the evolution, growth, development, reproduction, and environmental fitness of hosts. Weevils are one of the most abundant insect groups that can be infected by various endosymbionts, such as *Sodalis*, *Nardonella*, and *Wolbachia*. The sweet potato weevil, *Cylas formicarius* (Coleoptera: Brentidae), is a notorious pest in sweet potato (*Ipomoea batatas* L.) cultivation. Currently, little is known about the presence of endosymbionts in *C. formicarius*. Herein, we assessed the endosymbiont load of a single geographic population of *C. formicarius*. The results showed that *Nardonella* and *Rickettsia* could infect *C. formicarius* at different rates, which also varied according to the developmental stages of *C. formicarius*. The relative titer of *Nardonella* was significantly related to *C. formicarius* developmental stages. The *Nardonella*-infecting sweet potato weevils were most closely related to the *Nardonella* in *Sphenophorus levis* (Coleoptera, Curculionidae). The *Rickettsia* be identified in bellii group. These results preliminarily revealed the endosymbionts in *C. formicarius* and helped to explore the diversity of endosymbionts in weevils and uncover the physiological roles of endosymbionts in weevils.

Key words: endosymbiosis, insect, *Nardonella*, *Rickettsia*, phylogeny

Two or more different organisms forming a stable and lasting symbiotic relationship during some or the whole period of their life cycle is very common in nature (O'Neill et al. 1992, Hilgenboecker et al. 2008). Usually, the larger member of the symbiotic relationship is called "host," and the smaller one is called "symbiont." The symbiotic relationship, combined with host metabolism, can lead to the emergence of new host characteristics (Moran 2001, Oliver et al. 2010). Many insect species harbor symbiotic bacteria, which are generally regarded as nonpathogenic and include both mutualists and neutralists (Douglas 1998, Darby et al. 2005, Su et al. 2014). Many symbiotic bacteria are intracellular bacterial symbionts (endosymbionts), and some persist over generations by transovarial vertical transmission (Griffiths and Beck 1973, Moran 2001, Login et al. 2011). The endosymbionts of insects are commonly divided into primary (obligatory) endosymbionts and secondary (facultative) endosymbionts. Studies have shown that endosymbionts play important roles in the evolution, growth, development, reproduction, and environmental fitness of host insects (Oliver et al. 2009, Vanthournout et al. 2014, Xie et al. 2015, Molloy et al. 2016, Shokal et al. 2016, Zagata et al. 2016, Zhang et al. 2016, Darby et al. 2010, Landmann et al. 2009).

The superfamily Curculionoidea (weevils), as one of the most abundant insect groups, are infected with various endosymbionts. *Nardonella* is considered to be the ancestral endosymbiots, infected in many weevil species, including the red palm weevil *Rhynchophorus ferrugineus*, black vine weevil *Otiorhynchus sulcatus*, rice water weevil *Lissorhoptrus oryzophilus*, West Indian sweet potato weevil *Euscepes postfasciatus*, *Listronotus bonariensis*, and *Steriphus variabilis* (Hirsch et al. 2012, White et al. 2015, Huang et al. 2016, Morera-Margarit et al. 2019, Maire et al. 2020b). The black hard weevil, *Pachyrhynchus infernalis*, is also infected with the *Nardonella*, which play important roles in host's cuticle formation and hardening by providing tyrosine (Anbutsu et al. 2017). Although *Nardonella* is considered to be the ancestral endosymbiots, but replaced by *Curculioniphilus* and *Sodalis*-allied symbiont in the lineage of *Curculio* and grain weevils of the genus *Sitophilus*, respectively (Conord et al. 2008, Toju et al. 2013). *Curculioniphilus* is hosted by as many as 9 *Curculio* species, including *C. glandium*, *C. elephas*, *C. pellitus*, and *C. venosus*, and 27 Curculionini species are currently considered to be the primary endosymbiont of the *Curculio* lineage and allied weevils of the tribe Curculionini (Toju et al. 2010, Merville et al. 2013). The maize weevil *Sitophilus zeamais*, cereal weevil *S.*

oryzae, *S. granarius* infected with primary endosymbiont *Sodalis pierantonius*, which play important roles in providing amino acids and vitamins to its hosts (Anselme et al. 2008, Vigneron et al. 2014, Maire et al. 2020a, Vieira and Guedes 2020). *Sodalis*-allied symbionts are also hosted in some Curculionini weevils, but with low infection rates, and then referred to as secondary symbiont (Toju et al. 2013). SOPE (*Sitophilus oryzae* principal endosymbiont) is the primary endosymbiont of the rice weevils, supplies the weevil with vitamins, and interacts with mitochondrial oxidative phosphorylation and amino acid metabolism (Heddi et al. 1999, Gil et al. 2008).

In addition to these primary endosymbionts, many weevils are also infected with various secondary symbiotic bacteria, such as *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Cardinium*, and *Arsenophonus* (Weeks et al. 2003, Thao and Baumann 2004, Weinert et al. 2009, Hirsch et al. 2012, Huang et al. 2016, Marino et al. 2018, Schebeck et al. 2018, Zaidman-Remy et al. 2018, Vieira and Guedes 2020).

The sweet potato weevil, *Cylas formicarius* Fabricius (Coleoptera: Brentidae), is a notorious pest in sweet potato (*Ipomoea batatas*) cultivation in China and countries worldwide, which causes severe damage both during growth and storage, affecting the yield and quality of sweet potato (Kawamura et al. 2007, Hua et al. 2020, Baro et al. 2022, Liu et al. 2022). Although the sweet potato weevil has narrow host ranges, damage caused by the weevils is often worse. Since endosymbionts play important roles in the growth, development, and environmental fitness of host insects, so we hypothesized that sweet potato weevils may also harbor some endosymbionts. In this study, we detected the endosymbionts infection rates and titers during the different developmental stages of a single geographic population of *C. formicarius* and conducted phylogenetic analysis. The preliminary results revealed the endosymbiots in sweet potato weevils and provided the basis for further exploring the endosymbiont-insect associations.

Methods

Insects

The wild population of sweet potato weevil *C. formicarius* from South China was used in this study. The original *C. formicarius* samples were collected from sweet potatoes from Zhanjiang, Guangdong province, China, in November 2020. The eggs, larvae, pupae, and adults of *C. formicarius* were carefully separated from the damaged sweet potatoes and stored in anhydrous ethanol at –80 °C.

PCR Detection of Endosymbionts

Genomic DNA was extracted using a genomic DNA extraction kit (TianGen, Beijing, China). The mixed extraction was randomly performed for every 10 adults for detecting the species of endosymbionts infected in sweet potato weevils, and the elytra were removed during extraction. Twenty-four samples (8 samples in each replicate, 3 biological replicates) of sweet potato weevils at each development stage (egg, larva, pupa, adult) were selected for single extraction for detecting the infection rates of endosymbionts in *C. formicarius*.

PCRs were performed in a 25-µl volume containing 1 µl of the template DNA, 2.5 mM $MgCl_2$, 200 mM for each dNTP, 1 µM of each primer, and 1 unit of DNA Taq polymerase (Invitrogen, Guangzhou, China). The specific primers of endosymbionts are listed in Table 1. The PCR procedure was as follows: predenaturation at 94 °C for 3 min followed by 34 cycles of 94 °C for 30 s, annealing temperature (listed in Table 1) for 45 s and 72 °C for 45 s, and finally extension for 8 min at 72 °C. To confirm the specificity of the detection, the *ITS-1* gene of *C. formicarius* (F: 5′-TTGATTACGTCCCTGCCCTTT-3′, R: 5′-ACGAGCCGAGTGATCCACCG-3′) (Kawamura et al. 2007) was used as positive control, and ddH_2O was used as negative control. To check whether the PCRs were actually specific to the targeted symbiont, 2 DNA amplicons randomly selected per symbiont were sent to Tianyi Huiyuan Gene Technology Co., Ltd (Guangzhou, China) for sequencing after expected bands were visible on 1% agarose gels.

Quantitative Detection of *Nardonella*

Sweet potato weevil DNA was extracted from eggs, third-instar larvae, pupae, female adults, and male adults, respectively. TIANamp Genomic DNA kit (TianGen, Beijing, China) was used for single extraction, and DNA from the eggs was also extracted as a single

Table 1. Symbionts and specific primers

Symbionts	Gene	Prime sequences (5′–3′)	Fragment size (bp)	Annealing temperature (°C)
Nardonella[a]	16S rRNA	F: AAACCCTGATGCAGCTATACCGTGTGTG R: CCAT TGTAGCACGTTTGTAGCCCTACTCA	800	55
Rickettsia[b]	16S rRNA	F: AGAGTTTGATCCTGGCTCAG R: GAAAGCATCTCTGCGATCCG	900	60
Sodalis[c]	16S rRNA	F: CGRTRGCGTTAAYAGCGC R: AACAGACCGCCTGCGTACG	200	55
SOPE[d]	16S rRNA	F: TAATAGCGCCATCGATTGAC R: CCGAAGGCACCAAGGCAT	530	53
Wolbachia[e]	16S rRNA	F: GCATGAGTGAAGAAGGCC R: AGATAGACGCCTTCGCCA	400	52
Cardinium[f]	16S rRNA	F: GCGGTGTAAAATGAGCGTG R: ACCTMTTCTTAACTCAAGCCT	500	51
Arsenophonus[g]	23 S rRNA	F: CGTTTGATGAATTCATAGTCAAA R: GGTCCTCCAGTTAGTGTTACCCAAC	600	51

[a]See reference Degnan et al. (2004).
[b]See references Lane (1991) and Schulenburg et al. (2001).
[c]See reference Toju et al. (2010).
[d]See reference Heddi et al. (1999).
[e]See reference Li et al. (2007).
[f]See reference Weeks et al. (2003).
[g]See reference Thao and Baumann (2004).

pellet. The DNA samples infected with *Nardonella* detected by regular PCR were mixed in every 10 samples per instar/developmental stage as DNA templates for quantitative detection.

qPCR was performed with the CFX connect real-time system (Bio-Rad, USA) with Thunderbird 2× SYBR Green PCR mix (TOYOBO, Osaka, Japan). The primers of *Nardonella* were as follows: F: ACACGGTCCAGACTCCTT, R: ACACGCTTTACGCCCAAT (Huang et al. 2016). The β-actin gene (primer F: CGTCACAAACTGGGATGACA, R: GAGCTTCGGTCAAAAGAACG) of *C. formicarius* was used as a housekeeping gene (Hua et al. 2020). Amplifications were performed in 10-µl reactions containing 5 µl of 2× SYBR Green PCR mix, 2 µl of template DNA, 0.5 µl of each primer (10 µM each), and 2 µl of ddH_2O. The amplification program was as follows: 5 min activation at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and finally 30 s at 72 °C. A nontemplate negative control was included for each primer set to check for primer dimers and contamination. This experiment was repeated 2 more times, for a total of 30 egg sets, 30 larvae, 30 pupae, 30 female adults, and 30 male adults sampled.

Phylogenetic Analysis

The amplified products of *Nardonella* and *Rickettsia* were purified and recovered using the agarose gel DNA recovery kit (OMEGA, USA). The recovered products were used for 2-way sequencing (Tianyi Huiyuan, Guangzhou). *Nardonella* and *Rickettsia* 16S rRNA gene sequences determined in *C. formicarius* in this study have been deposited in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). The phylogenetic trees of *Nardonella* and *Rickettsia* were constructed by combining the reported *Nardonella* and *Rickettsia* gene sequences (Hosokawa and Fukatsu 2010, Toju et al. 2010, Hirsch et al. 2012, Merville et al. 2013, Hosokawa et al. 2015, Huang et al. 2016). IQ-TREE version 1.6.12-Linux (http://www.iqtree.org) was used to construct the phylogenetic trees of *Nardonella* and *Rickettsia* using the maximum likelihood method. The self-guided replication values were set to 1,000 times.

Data Analysis

A Bio-Rad instrument (CFX Connect, USA) and the accompanying software (Bio-Rad CFX Manager) were used for qPCR data normalization, and the relative quantities of endosymbionts were calculated using the $2^{-\Delta\Delta ct}$ method. The differences were evaluated in IBM SPSS Statistics v.18.0. For all ANOVA analysis, independent-samples *t*-test, data were checked for homogeneity by the Levene's test. Multiple comparisons of means were assessed by Tukey's HSD test at a significance level $\alpha = 0.05$. Figures were generated using Sigma Plot 14.0. Error bars in all graphs represent standard error.

Results

Species and Infection Rates of Endosymbionts in *Cylas formicarius*

We detected 7 endosymbiots, *Nardonella*, *Sodalis*, SOPE, *Wolbachia*, *Cardinium*, *Rickettsia*, and *Arsenophonus*, in the sweet potato weevils by regular PCR (Fig. 1). We successfully obtained partial 16S rDNA sequences amplified with *Nardonella*- and *Rickettsia*-specific primers, respectively. The sequences amplified with *Nardonella*-specific primers found in *C. formicarius* (GenBank accession no. ON955871) matched the *Nardonella* found in *Sphenophorus levis* (GenBank accession no. FJ626248) by 96.37%. Hence, we have provisionally classified the sequences as *Nardonella*. The sequences amplified with *Rickettsia*-specific primers found in *C. formicarius* (GenBank accession no. OQ398824) matched the *Rickettsia bellii* (GenBank accession no. NR074484) by 99.66%, *Rickettsia* found in *Ochlerotatus caspius* (GenBank accession no. OP007142) by 99.66%, *Rickettsia* found in *Curculio sikkimensis* (GenBank accession no. AB545027) by 99.55%, and *Rickettsia* found in *Bemisia tabaci* (GenBank accession no. MT253088) by 99.34%. Hence, we have classified the sequences as *R. bellii*. We conducted the following analysis on the 2 endosymbionts identified (*Nardonella* and *Rickettsia*).

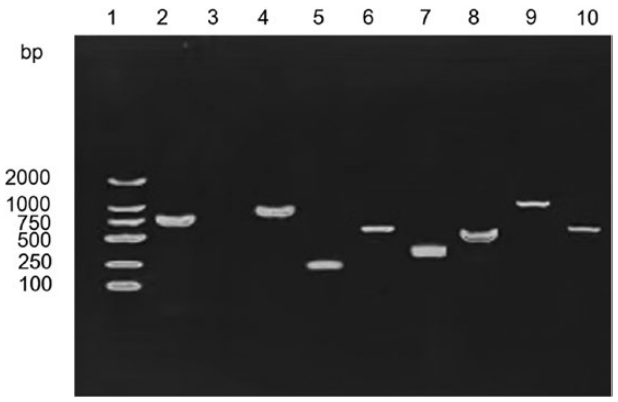

Fig. 1. Infection of endosymbionts in *Cylas formicarius* from South China. Lanes 1–9 are DNA marker, positive control (*ITS-1*), negative control (ddH_2O), *Nardonella*, *Sodalis*, SOPE, *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, respectively.

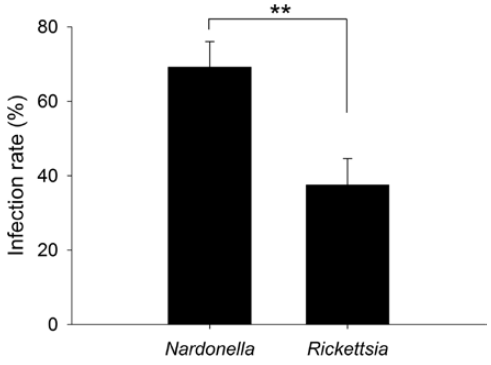

Fig. 2. The infection rates of endosymbionts in *Cylas formicarius*. **Statistically significant difference between them based on the *t*-test at a significance level $\alpha = 0.05$; bars are the mean ± SE ($n = 15$), $t = 3.191$, df = 28, $P = 0.003$.

The infection rates of the endosymbiotic bacteria were different in sweet potato weevils, and the results was shown in Fig. 2. The infection rate of *Nardonella* was 69.17% ± 6.87%, significantly higher than that of *Rickettsia* (37.50% ± 7.11%), in 120 samples of sweet potato weevils ($t = 3.191$; df = 28; $P = 0.003$; Fig. 2).

The infection rates of the endosymbionts were also different in each developmental stage of sweet potato weevil (Fig. 3). The infection rate of *Nardonella* was relatively low in weevil female (25% ± 12.50%) and at its highest in pupa (95.83% ± 4.17%; $F = 12.4375$; df = 4,10; $P = 0.000678$), with no significant difference among egg, larva, and male (Fig. 3a). The infection rate of *Rickettsia* was relatively low in weevil pupa (8.33%) and at its highest in egg and female (58.33%; $F = 6.6071$; df = 4,10; $P = 0.007213$) (Fig. 3b).

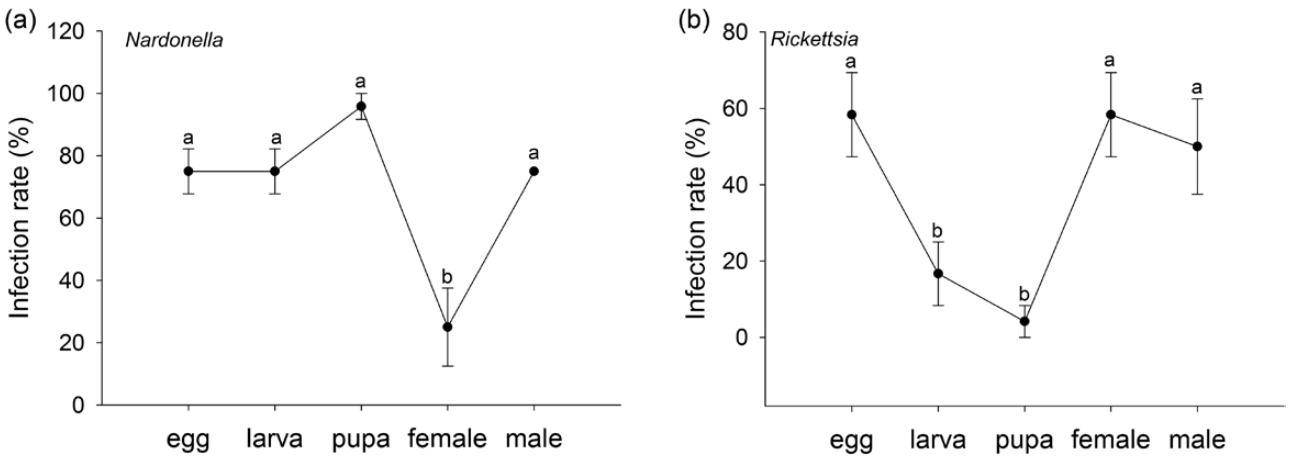

Fig. 3. The infection rates of endosymbionts in different development stages of *Cylas formicarius*. Mean ± SE ($n = 3$) marked with the different lowercase letters represent a statistically significant differences based on 1-way ANOVA with Tukey's HSD test at a significance level $\alpha = 0.05$. (a) *Nardonella*: $F = 12.4375$; df = 4, 10; $P = 0.000678$; (b) *Rickettsia*: $F = 6.6071$; df = 4, 10; $P = 0.007213$.

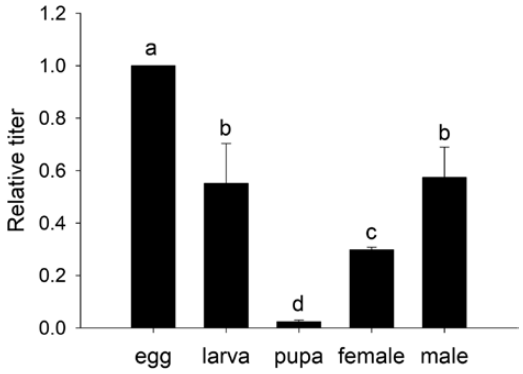

Fig. 4. The relative titers of *Nardonella* in different development stages of *Cylas formicarius*. Mean ± SE ($n = 3$) marked with the different lowercase letters represent a statistically significant differences ($F = 17.7378$; df = 4, 10; $P = 0.000155$) based on 1-way ANOVA with Tukey's HSD test at a significance level $\alpha = 0.05$.

Quantitative Detection of *Nardonella*

Nardonella is considered to be the oldest endosymbiont among weevil insects, with high infection rates in *C. formicarius*. Here, we quantitatively analyzed the relative contents of *Nardonella* in different developmental stages of sweet potato weevils. The results showed that the relative titer of *Nardonella* in the egg–pupa stages of sweet potato weevils decreased with growth and development, which were the highest in the eggs, decreased with the development and reached the lowest in the pupae, then increased in the adult stage ($F = 17.7378$; df = 4, 10; $P = 0.000155$; Fig. 4), but had no significant difference between the males and females.

Phylogenetic Analysis of Endosymbionts

Eleven 16S rDNA gene sequences of *Nardonella* were selected from the NCBI database as reference sequences to construct phylogenetic trees. The phylogenetic analyses results showed that the *Nardonella* obtained from sweet potato weevils in this study was closely related to *S. levis* (a hyacinth genus insect) (Fig. 5). The *Rickettsia* obtained from sweet potato weevils in this study was closely related to *R. bellii* (Fig. 6).

Discussion

This preliminary study describes the endosymbiotic communities hosted by a single geographic population of *C. formicarius*. The types of endosymbionts in insect populations vary with insect species, developmental stages, and geographical environments (Gottlieb et al. 2006, Himler et al. 2011, Merville et al. 2013). Previous studies showed that 4 sibling weevil species (*Curculio* spp.) were infested with one primary endosymbiont Candidatus *Curculioniphilus buchneri* and 3 secondary endosymbionts, *Rickettsia*, *Spiroplasma*, and *Wolbachia* (Merville et al. 2013). *Otiorhynchus* spp. was infested with *Rickettsia* and *Nardonella* (Hirsch et al. 2012). The West Indian sweet potato weevil, *Euscepes postfasciatus*, was infected with *Nardonella* (Hosokawa and Fukatsu 2010). Cereal weevils, maize weevils *S. zeamais*, house an obligatory nutritional endosymbiont *Sodalis pierantonius* and occasionally *Wolbachia* (Zaidman-Remy et al. 2018, Vieira and Guedes 2020). *Wolbachia* was also detected in rice weevil *S. oryzae* (Heddi et al. 1999) and the spruce bark beetle *Pityogenes chalcographus* (Schebeck et al. 2018). In this study, Candidatus *Curculioniphilus buchneri* and *Spiroplasma* were not detected (results not shown), and *Nardonella*, *Sodalis*, SOPE, *Wolbachia*, *Cardinium*, *Rickettsia*, and *Arsenophonus* were detected in *C. formicarius* by regular PCR. Only *Nardonella* and *Rickettsia* 16S rDNA sequences were successfully obtained, and whether the sweet potato weevil harbored other endosymbionts needs further confirmation.

The primary symbionts are generally localized in specialized cells called bacteriocytes, grouped together in a bacteriome, undergo vertical transmission from mother to offspring with high fidelity (Thao and Baumann 2004, Gottlieb et al. 2008, Wilson et al. 2010, Ghosh et al. 2020, Maire et al. 2020a). The secondary endosymbionts inhabit various body parts, such as bacteriocytes, hemolymph, gut tissues, and so on, and can be transmitted vertically, and undergo some horizontal transmission (Oliver et al. 2010, Carvalho et al. 2014, Li et al. 2017, Shi et al. 2018). No matter what kinds of endosymbionts, vertical transmission, from the mother to offspring, is the main transmission mode. The infection rate of *Nardonella* and *Rickettsia* were relatively higher in egg stage of *C. formicarius*. The egg is the first stage of progeny, so more endosymbiots may be retained in the eggs. The infection rate of *Nardonella* was lowest in the female adult, which was a bit unexpected. We speculated that the endosymbiot *Nardonella* failed to transfer to the reproductive

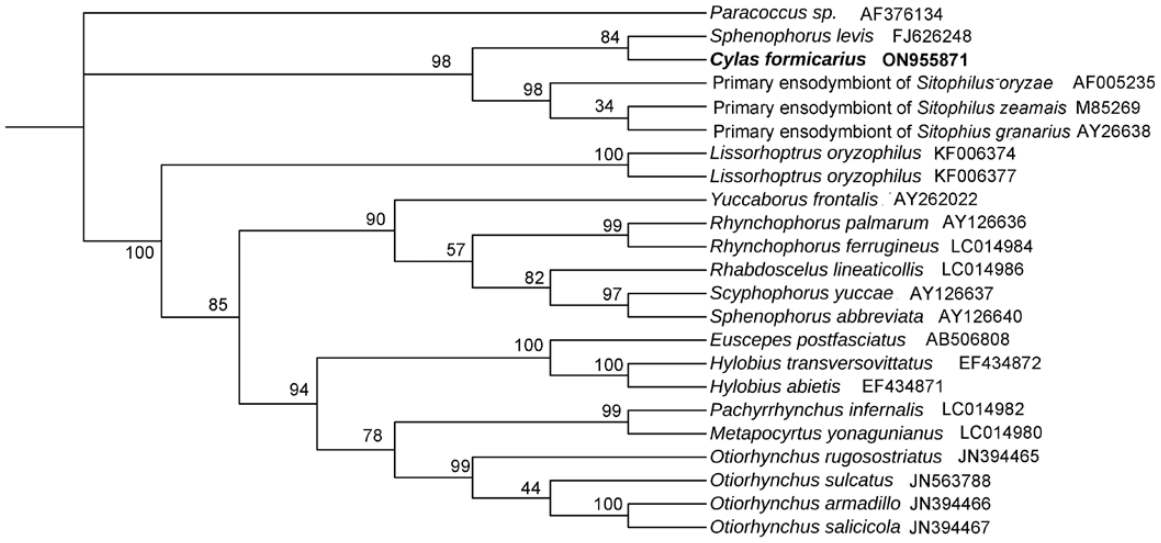

Fig. 5. The phylogenetic analysis of *Nardonella* based on 16S rRNA gene. Sequences obtained from this study shown in bold.

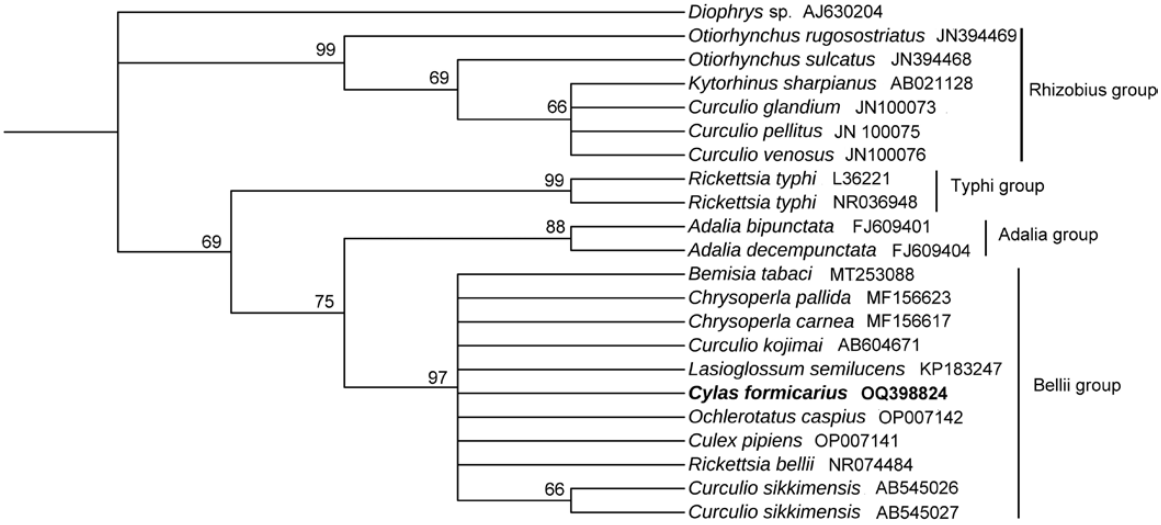

Fig. 6. The phylogenetic analysis of *Rickettsia* based on 16S rRNA gene. Sequences obtained from this study shown in bold.

system of the female sweet potato weevil and then were metabolized by the host insects. Previous studies showed that the mechanisms of symbiont translocation during development and from somatic tissues into the germline differ widely across insect groups. The endosymbiont *Buchnera* translocates from bacteriomes to the ovaries in aphids through a coordinated series of exocytosis and endocytosis event (Koga et al. 2012). The transmission of whitefly's endosymbionts was achieved by the transport of entire bacteriocytes (Kaltenpoth 2020). The transmission of *S. pierantonius* from larva to adult cereal weevil *S. oryzae* was achieved by bacteriocytes turning into spindle cells and migrating along the midgut epithelium, thereby transferring endosymbionts to midgutsites where future mesenteric caeca will develop (Maire et al. 2020a). *Nardonella* was also localized in bacteriome of weevils (Maire et al. 2020a). However, how the endosymbiots are maintained and occasionally translocated during metamorphosis in sweet potato weevils remains poorly understood. Whether the endosymbionts infection rates are related to the localization patterns, physiological state, and immune activity of different developmental stages of *C. formicarius* needs further study.

Nardonella is considered to be the most ancient, primary endosymbionts in weevils (Hosokawa and Fukatsu 2010, Kuriwada et al. 2010, Hosokawa et al. 2015, Huang et al. 2016) and infected many types of weevils, such as *Odoiporus longicollis*, *Yuccaborus frontalis*, *Rhynchophorus palmarum*, *L. oryzophilus*, etc. (Lefevre et al. 2004, Hosokawa et al. 2015, Huang et al. 2016, Maire et al. 2020a, b). Previous studies showed that *Nardonella* plays important roles in host's cuticle formation and hardening by proving tyrosine (Anbutsu et al. 2017). *Nardonella* was reported to be involved in normal growth and development of the West Indian sweet potato weevil *E. fasciatus*, and deletion of these bacteria significantly suppressed the growth rate of immature stages, and the adults were smaller in size and paler in color (Kuriwada et al. 2010). In this study, the density of *Nardonella* was significantly related to *C. formicarius* developmental stages. The relative titer of *Nardonella* decreased with the growth of *C. formicarius* from egg to pupa, and increased in adults, which was consistent with the changes in rice water weevil (Huang et al. 2016). If *Nardonella* also plays a nutritional role in the sweet potato weevil, it may be because, unlike the

egg stage, the sweet potato weevil in larval stage can eat independently the supplement nutrients, thus reducing the endosymbiont titers. The increase of endosymbiont titer in adult stage may be a preparation for vertical transmission to the progeny of host insects. However, the symbiotic mechanism of *Nardonella* and sweet potato weevil and the function of *Nardonella* in sweet potato weevil need further study. Rinke et al. (2011) detected *Nardonella* in *S. levis*, and the results of *Nardonella* phylogenetic analysis showed that the *Nardonella* infection in sweet potato weevils was most closely related to *Nardonella* in sugarcane weevil *S. levis*. Both species of weevils could be considered together when studying the function of *Nardonella*.

In recent years, molecular surveys showed that *Rickettsia* are associated with a diverse range of hosts (vertebrates, arthropods, plants, etc.) (Weinert et al. 2009, Caspi-Fluger et al. 2012). Some *Rickettsia* are symbionts, with an intimate relationship with hosts, and can be considered facultative (secondary) endosymbionts of arthropods (Weinert et al. 2009). Some *Rickettsia* can alter the reproductive behavior, such as male killing in coleopteran or parthenogenesis in hymenopteran hosts (Lawson et al. 2001, Hagimori et al. 2006, Weinert et al. 2009, Schebeck et al. 2018). In this study, there was no significant difference in the infection rate of *Rickettsia* between females and males. Therefore, we speculated that the *Rickettsia* detected in *C. formicarius* may not have the function of male killing. The *Rickettsia* detected in *C. formicarius* was most closely related to the *Rickettsia bellii* in *B. tabaci*, *C. sikkimensis*, *C. kojimai*, etc. Infection with *Rickettsia* increases susceptibility to insecticide (Kontsedalov et al. 2008), increases the thermotolerance (Brumin et al. 2011), influences the reproduction by changing the sex ratio, and influences offspring fitness of whitefly *B. tabaci* (Himler et al. 2011, Shi et al. 2021). Chestnut weevil *C. sikkimensis* also harbored *Rickettsia*, which closely related to the *Rickettsia* in *B. tabaci* and higher *Rickettsia* infections at localities of higher temperature (Toju and Fukatsu 2011). Thus it could be speculated that *Rickettsia* endosymbionts may also manipulate host thermotolerance and/or reproduction in *C. formicarius*. *Rickettsia* also be detected in 4 sibling weevil species (*C. glandium*, *C. elephas*, *C. pellitus*, and *C. venosus*) (Merville et al. 2013), and *Otiorhynchus* species be identified in rhizobius group (Hirsch et al. 2012), but little is known about their function. Molecular surveys coupled with biological experiments can help to explore the biological function of endosymbionts.

In summary, *Nardonella* and *Rickettsia* were found to infect *C. formicarius*, with different infection rates, which also varied according to the developmental states of *C. formicarius*. The relative titer of *Nardonella* in the egg–pupa stages of sweet potato weevil decreased with growth and development and subsequently increased in the adults. *Nardonella* infection in sweet potato weevils was most closely related to the *Nardonella* in *S. levis*, and the *Rickettsia* be identified in bellii group. These findings preliminarily indicated the information on endosymbionts of sweet potato weevil, provided basis to further explore the diversity of endosymbionts in weevils, and uncovered the physiological roles of endosymbionts in weevils. In the future, we can conduct microbiome studies on multiple geographic populations of sweet potato weevil to better reveal the diversity of insect symbionts.

Acknowledgments

This work was supported by the Guangdong Basic and Applied Basic Research Foundation (2019A1515110745) and the Special Fund for Science and Technology of Guangdong Province (2020A06013), Zhanjiang Science and Technology Plan Competitive Project (2019A03005). We thank Hongbo Zhu for providing insect source.

Author Contributions

Jin Xu (Conceptualization-Equal, Methodology-Equal, Writing – original draft-Equal), Jian-Bin Tan (Investigation-Equal, Methodology-Equal, Software-Equal), Yi-Dan Li (Data curation-Equal, Methodology-Equal), Yuan-Hao Xu (Data curation-Equal, Methodology-Equal), An Tang (Data curation-Equal, Methodology-Equal), Hong-Kai Zhou (Project administration-Equal, Resources-Equal), Pei-Qiong Shi (Conceptualization-Equal, Funding acquisition-Lead, Writing – original draft-Equal, Writing – review & editing-Lead)

Supplementary Material

Supplementary material is available at *Journal of Insect Science* online.

References

Anbutsu H, Moriyama M, Nikoh N, Hosokawa T, Futahashi R, Tanahashi M, Meng XY, Kuriwada T, Mori N, Oshima K, *et al*. Small genome symbiont underlies cuticle hardness in beetles. *Proc Natl Acad Sci USA*. **2017**:114(40):E8382–E8391. https://doi.org/10.1073/pnas.1712857114

Anselme C, Perez-Brocal V, Vallier A, Vincent-Monegat C, Charif D, Latorre A, Moya A, Heddi A. Identification of the weevil immune genes and their expression in the bacteriome tissue. *BMC Biol*. **2008**:6:43.

Baro Y, Schuster C, Gato Y, Marquez ME, Leclerque A. Characterization, identification and virulence of *Metarhizium* species from Cuba to control the sweet potato weevil, *Cylas formicarius* Fabricius (Coleoptera: Brentidae). *J Appl Microbiol*. **2022**:132(5):3705–3716. https://doi.org/10.1111/jam.15460

Brumin M, Kontsedalov S, Ghanim M. *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. *Insect Sci*. **2011**:18(1):57–66. https://doi.org/10.1111/j.1744-7917.2010.01396.x

Carvalho GA, Correa AS, de Oliveira LO, Guedes RNC. Evidence of horizontal transmission of primary and secondary endosymbionts between maize and rice weevils (*Sitophilus zeamais* and *Sitophilus oryzae*) and the parasitoid *Theocolax elegans*. *J Stored Prod Res*. **2014**:59:61–65.

Caspi-Fluger A, Inbar M, Mozes-Daube N, Katzir N, Portnoy V, Belausov E, Hunter MS, Zchori-Fein E. Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proc R Soc B*. **2012**:279:1791–1796.

Conord C, Despres L, Vallier A, Balmand S, Miquel C, Zundel S, Lemperiere G, Heddi A. Long-term evolutionary stability of bacterial endosymbiosis in curculionoidea: additional evidence of symbiont replacement in the dryophthoridae family. *Mol Biol Evol*. **2008**:25(5):859–868. https://doi.org/10.1093/molbev/msn027

Darby AC, Chandler SM, Welburn SC, Douglas AE. Aphid-symbiotic bacteria cultured in insect cell lines. *Appl Environ Microbiol*. **2005**:71(8):4833–4839. https://doi.org/10.1128/AEM.71.8.4833-4839.2005

Darby AC, Choi JH, Wilkes T, Hughes MA, Werren JH, Hurst GDD, Golbourne JK. Characteristics of the genome of *Arsenophonus nasoniae*, son-killer bacterium of the wasp *Nasonia*. *Insect Mol Biol*. **2010**:19:75–89.

Degnan PH, Lazarus AB, Wernegreen B. Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: cospeciation of *Camponotus* species and their endosymbionts, *Candidatus* Blochmannia. *Syst Biol*. **2004**:53(1):95–110.

Douglas AE. Nutritional interaction in insect-microbial symbiosis: aphid and their symbiotic bacteria *Buchnera*. *Annu Rev Entomol*. **1998**:43:17–37. https://doi.org/10.1146/annurev.ento.43.1.17

Ghosh S, Sela N, Kontsedalov S, Lebedev G, Haines LR, Ghanim M. An intranuclear sodalis-like symbiont and *Spiroplasma* coinfect the carrot psyllid, *Bactericera trigonica* (Hemiptera, Psylloidea). *Microorganisms*. **2020**:8(5):692. https://doi.org/10.3390/microorganisms8050692

Gil R, Belda E, Gosalbes MJ, Delaye L, Vallier A, Vincent-Monegat C, Heddi A, Silva FJ, Moya A, Latorre A. Massive presence of insertion sequences in the genome of SOPE, the primary endosymbiont of the rice weevil *Sitophilus oryzae*. *Int Microbiol*. **2008**:11(1):41–48.

Gottlieb Y, Ghanim M, Chiel E, Gerling D, Portnoy V, Steinberg S, Tzuri G, Horowitz AR, Belausov E, Mozes-Daube N, *et al.* Identification and localization of a *Rickettsia* sp. in *Bemisia tabaci* (Homoptera: Aleyrodidae). *Appl Environ Microbiol*. **2006**:72(5):3646–3652. https://doi.org/10.1128/AEM.72.5.3646-3652.2006

Gottlieb Y, Ghanim M, Gueguen G, Kontsedalov S, Vavre F, Fleury F, Zchori-Fein E. Inherited intracellular ecosystem: symbiotic bacteria share bacteriocytes in whiteflies. *FASEB J*. **2008**:22(7):2591–2599. https://doi.org/10.1096/fj.07-101162

Griffiths GW, Beck SD. Intracellular symbiotes of the pea aphid, *Acyrthosiphon pisum*. *J Insect Physiol*. **1973**:19(1):75–84. https://doi.org/10.1016/0022-1910(73)90223-0

Hagimori T, Abe Y, Date S, Miura K. The first finding of a *Rickettsia* bacterium associated with parthenogenesis induction among insects. *Curr Microbiol*. **2006**:52(2):97–101. https://doi.org/10.1007/s00284-005-0092-0

Heddi A, Grenier A, Khatchadourian C, Charles H, Nardon P. Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proc Natl Acad Sci USA*. **1999**:96(12):6814–6819. https://doi.org/10.1073/pnas.96.12.6814

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia?* A statistical analysis of current data. *FEMS Microbiol Lett*. **2008**:218:215–220.

Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, Chiel E, Duckworth VE, Dennehy TJ, Zchori-Fein E, *et al.* Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science*. **2011**:332(6026):254–256. https://doi.org/10.1126/science.1199410

Hirsch J, Strohmeier S, Pfannkuchen M, Reineke A. Assessment of bacterial endosymbiont diversity in *Otiorhynchus* spp. (Coleoptera: Curculionidae) larvae using a multitag 454 pyrosequencing approach. *BMC Microbiol*. **2012**:12(Suppl 1):S6. https://doi.org/10.1186/1471-2180-12-S1-S6

Hosokawa T, Fukatsu T. *Nardonella* endosymbiont in the West Indian sweet potato weevil *Euscepes postfasciatus* (Coleoptera: Curculionidae). *Appl Entomol Zool*. **2010**:45(1):115–120. https://doi.org/10.1303/aez.2010.115

Hosokawa T, Koga R, Tanaka K, Moriyama M, Anbutsu H, Fukatsu T. *Nardonella* endosymbionts of Japanese pest and non-pest weevils (Coleoptera: Curculionidae). *Appl Entomol Zool*. **2015**:50(2):223–229. https://doi.org/10.1007/s13355-015-0326-y

Hua J, Pan C, Huang Y, Li Y, Li H, Wu C, Chen T, Ma D, Li Z. Functional characteristic analysis of three odorant-binding proteins from the sweet potato weevil (*Cylas formicarius*) in the perception of sex pheromones and host plant volatiles. *Pest Manag Sci*. **2020**:77(1):300–312. https://doi.org/10.1002/ps.6019

Huang X, Huang Y, Zhang J, Lu F, Wei J, Jiang M. The symbiotic bacteria *Nardonella* in rice water weevil (Coleoptera: Curculionidae): diversity, density, and associations with host reproduction. *Ann Entomol Soc Am*. **2016**:109(3):415–423. https://doi.org/10.1093/aesa/saw015

Kaltenpoth M. An endosymbiont's journey through metamorphosis of its insect host. *Proc Natl Acad Sci USA*. **2020**:117(35):20994–20996. https://doi.org/10.1073/pnas.2014598117

Kawamura K, Sugimoto T, Kakutani K, Matsuda Y, Toyoda H. Genetic variation of sweet potato weevils, *Cylas formicarius* (Fabricius) (Coleoptera: Brentidae), in main infested areas in the world based upon the internal transcribed spacer-1 (*ITS*-1) region. *Appl Entomol Zool*. **2007**:42(1):89–96. https://doi.org/10.1303/aez.2007.89

Koga R, Meng XY, Tsuchida T, Fukatsu T. Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proc Natl Acad Sci USA*. **2012**:109(20):E1230–E1237. https://doi.org/10.1073/pnas.1119212109

Kontsedalov S, Zchori-Fein E, Chiel E, Gottlieb Y, Inbar M, Ghanim M. The presence of *Rickettsia* is associated with increased susceptibility of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides. *Pest Manag Sci*. **2008**:64(8):789–792. https://doi.org/10.1002/ps.1595

Kuriwada T, Hosokawa T, Kumano N, Shiromoto K, Haraguchi D, Fukatsu T. Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS One*. **2010**:5(10):e13101. https://doi.org/10.1371/journal.pone.0013101

Landmann F, Orsi GA, Loppin B, Sullivan W. *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathog*. **2009**:5:e10003433. https://doi.org/10.1371/journal.ppat.1000343

Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. *Nucleic acid techniques in bacterial systematics*. Chichester (UK): Wiley; **1991**. p. 115–175.

Lawson ET, Mousseau TA, Klaper R, Hunter MD, Werren JH. *Rickettsia* associated with male-killing in a buprestid beetle. *Heredity*. **2001**:86(Pt 4):497–505. https://doi.org/10.1046/j.1365-2540.2001.00848.x

Lefevre C, Charles H, Vallier A, Delobel B, Farrell B, Heddi A. Endosymbiont phylogenesis in the Dryophthoridae weevils: evidence for bacterial replacement. *Mol Biol Evol*. **2004**:21(6):965–973. https://doi.org/10.1093/molbev/msh063

Li SJ, Ahmed MZ, Lv N, Shi PQ, Wang XM, Huang JL, Qiu BL. Plant-mediated horizontal transmission of *Wolbachia* between whiteflies. *ISME J*. **2017**:11(4):1019–1028. https://doi.org/10.1038/ismej.2016.164

Li ZX, Lin HZ, Guo XP. Prevalence of *Wolbachia* infection in *Bemisia tabaci*. *Curr Microbiol*. **2007**:54(6):467–471. https://doi.org/10.1007/s00284-007-0011-7

Liu X, Wang Y, Zhu H, Mei G, Liao Y, Rao S, Li S, Chen A, Liu H, Zeng L, *et al.* Natural allelic variation confers high resistance to sweet potato weevils in sweet potato. *Nat Plants*. **2022**:8:1233–1244.

Login FH, Balmand S, Vallier A, Vincent-Monegat C, Vigneron A, Weiss-Gayet M, Rochat D, Heddi A. Antimicrobial peptides keep insect endosymbionts under control. *Science*. **2011**:334(6054):362–365. https://doi.org/10.1126/science.1209728

Maire J, Chouaia B, Zaidman-Remy A, Heddi A. Endosymbiosis morphological reorganization during metamorphosis diverges in weevils. *Commun Integr Biol*. **2020b**:13(1):184–188. https://doi.org/10.1080/19420889.2020.1840707

Maire J, Parisot N, Ferrarini MG, Vallier A, Gillet B, Hughes S, Balmand S, Vincent-Monegat C, Zaidman-Remy A, Heddi A. Spatial and morphological reorganization of endosymbiosis during metamorphosis accommodates adult metabolic requirements in a weevil. *Proc Natl Acad Sci USA*. **2020a**:117:19347–19358.

Marino YA, Ospina OE, Verle Rodrigues JC, Bayman P. High diversity and variability in the bacterial microbiota of the coffee berry borer (Coleoptera: Curculionidae), with emphasis on *Wolbachia*. *J Appl Microbiol*. **2018**:125(2):528–543. https://doi.org/10.1111/jam.13768

Merville A, Venner S, Henri H, Vallier A, Menu F, Vavre F, Heddi A, Bel-Venner M-C. Endosymbiont diversity among sibling weevil species competing for the same resource. *BMC Evol Biol*. **2013**:13:28. https://doi.org/10.1186/1471-2148-13-28

Molloy JC, Sommer U, Viant MR, Sinkins SP. *Wolbachia* modulates lipid metabolism in *Aedes albopictus*mosquito cells. *Appl Environ Microbiol*. **2016**:82(10):3109–3120. https://doi.org/10.1128/AEM.00275-16

Moran NA. The coevolution of bacterial endosymbionts and phloem-feeding insects. *Ann Mo Bot Gard*. **2001**:88(1):35–44. https://doi.org/10.2307/2666130

Morera-Margarit P, Bulgarelli D, Pope TW, Graham RI, Mitchell C, Karley AJ. The bacterial community associated with adult vine weevil (*Otiorhynchus sulcatus*) in UK populations growing on strawberry is dominated by *Candidatus* Nardonella. *Entomol Exp Appl*. **2019**:167:186–196.

O'Neill SL, Giordano R, Colbert AME, Karr TL, Robertson HM. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA*. **1992**:89:2699–2702.

Oliver KM, Degnan PH, Burke GR, Moran NA. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol*. **2010**:55:247–266.

Oliver KM, Degnan PH, Hunter MS, Moran NA. Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science*. **2009**:325(5943):992–994. https://doi.org/10.1126/science.1174463

Rinke R, Costa AS, Fonseca FPP, Almeida LC, Delalibera I, Henrique-Silva F. Microbial diversity in the larval gut of field and laboratory populations of the sugarcane weevil *Sphenophorus levis* (Coleoptera, Curculionidae). *Genet Mol Res*. **2011**:10:2679–2691.

Schebeck M, Feldkirchner L, Marin B, Krumbock S, Schuler H, Stauffer C. Reproductive manipulators in the bark beetle pityogenes chalcographus (Coleoptera: Curculionidae)—the role of *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*. *J Insect Sci*. **2018**:18(3):4.

Schulenburg J, Habig M, Sloggett JJ, Webberley KM, Bertrand D, Hurst G, Majerus M. Incidence of male-killing *Rickettsia* spp. (alpha-proteobacteria) in the ten-spot ladybird beetle *Adalia decempunctata* L. (Coleoptera: Coccinellidae). *Appl Environ Microbiol*. **2001**:67(1):270–277.

Shi PQ, Chen XY, chen XS, Lv N, Liu Y, Qiu BL. *Rickettsia* increases its infection and spread in whitefly populations by manipulating the defense patterns of the host plant. *FEMS Microbiol Ecol*. **2021**:97(4):fiab032. https://doi.org/10.1093/femsec/fiab032

Shi PQ, Wang L, Liu Y, An X, Chen XS, Ahmed MZ, Qiu BL, Sang W. Infection dynamics of endosymbionts reveal three novel localization patterns of *Rickettsia* during the development of whitefly *Bemisia tabaci*. *FEMS Microbiol Ecol*. **2018**:94:fiy165.

Shokal U, Yadav S, Atri J, Accetta J, Kenney E, Banks K, Katakam A, Jaenike J, Eleftherianos I. Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. *BMC Microbiol*. **2016**:16:16. https://doi.org/10.1186/s12866-016-0634-6

Su Q, Wei X, Wang SL, Wu QJ, Liu BM, Fang Y, Xu BY, Zhang YJ. The endosymbiont *Hamiltonella* increases the growth rate of its host *Bemisia tabaci* during periods of nutritional stress. *PLoS One*. **2014**:9(2):e89002.

Thao ML, Baumann P. Evidence for multiple acquisition of *Arsenophonus* by whitefly species (Sternorrhyncha: Aleyrodidae). *Curr Microbiol*. **2004**:48(2):140–144. https://doi.org/10.1007/s00284-003-4157-7

Toju H, Fukatsu T. Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Mol Ecol*. **2011**:20(4):853–868. https://doi.org/10.1111/j.1365-294X.2010.04980.x

Toju H, Hosokawa T, Koga R, Nikoh N, Meng XY, Kimura N, Fukatsu T. 'Candidatus *Curculioniphilus buchneri*', a novel clade of bacterial endocellular symbionts from weevils of the genus *Curculio*. *Appl Environ Microbiol*. **2010**:76(1):275–282. https://doi.org/10.1128/AEM.02154-09

Toju H, Tanabe AS, Notsu Y, Sota T, Fukatsu T. Diversification of endosymbiosis: replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. *ISME J*. **2013**:7(7):1378–1390. https://doi.org/10.1038/ismej.2013.27

Vanthournout B, Vandomme V, Hendrickx F. Sex ratio bias caused by endosymbiont infection in the dwarf spider *Oedothorax retusus*. *J Arachn*. **2014**:42(1):24–33. https://doi.org/10.1636/hi12-37.1

Vieira JL, Guedes RNC. Endosymbiont load, personality and reproductive output of maize weevils (*Sitophilus zeamais*). *J Pest Sci*. **2020**:94(3):691–701. https://doi.org/10.1007/s10340-020-01287-7

Vigneron A, Masson F, Vallier A, Balmand S, Rey M, Vincent-Monegat C, Aksoy E, Aubailly-Giraud E, Zaidman-Remy A, Heddi A. Insects recycle endosymbionts when the benefit is over. *Curr Biol*. **2014**:24(19):2267–2273. https://doi.org/10.1016/j.cub.2014.07.065

Weeks AR, Velten R, Stouthamer R. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc R Soc B Biol Sci*. **2003**:270:1857–1865.

Weinert LA, Werren JH, Aebi A, Stone GN, Jiggins FM. Evolution and diversity of *Rickettsia* bacteria. *BMC Biol*. **2009**:7:6. https://doi.org/10.1186/1741-7007-7-6

White JA, Richards NK, Laugraud A, Saeed A, Curry MM, McNeill MR. Endosymbiotic candidates for parasitoid defense in exotic and native New Zealand weevils. *Microb Ecol*. **2015**:70(1):274–286. https://doi.org/10.1007/s00248-014-0561-8

Wilson ACC, Ashton PD, Calevro F, Charles H, Colella S, Febvay G, Jander G, Kushlan PF, Macdonald SJ, Schwartz JF, *et al*. Genomic insight into the amino acid relations of thepea aphid, *Acyrthosiphon pisum*, with its symbioticbacterium *Buchnera aphidicola*. *Insect Mol Biol*. **2010**:19(Suppl 2):249–258. https://doi.org/10.1111/j.1365-2583.2009.00942.x

Xie JL, Winter C, Winter L, Mateos M. Rapid spread of the defensive endosymbiont *Spiroplasma* in *Drosophila hydei* under high parasitoid wasp pressure. *FEMS Microbiol Ecol*. **2015**:91:1–11.

Zagata P, Greczek-Stachura M, Tarcz S, Rautian M. The evolutionary relationships between endosymbiotic green algae of *Paramecium bursaria* syngens originating from different geographical locations. *Folia Biologica-Krakow*. **2016**:64:47–54.

Zaidman-Remy A, Vigneron A, Weiss BL, Heddi A. What can a weevil teach a fly, and reciprocally? Interaction of host immune systems with endosymbionts in *Glossina* and *Sitophilus*. *BMC Microbiol*. **2018**:18(Suppl 1):150. https://doi.org/10.1186/s12866-018-1278-5

Zhang YC, Cao WJ, Zhong LR, Godfray HCJ, Liu XD. Host plant determines the population size of an obligate symbiont (*Buchnera aphidicola*) in aphids. *Appl Environ Microbiol*. **2016**:82(8):2336–2346. https://doi.org/10.1128/AEM.04131-15