Annals of the Entomological Society of America, 114(6), 2021, 738–749
doi: 10.1093/aesa/saab025
Advance Access Publication Date: 22 July 2021
Research

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Parthenogenesis and Sex-Ratio Distorting Bacteria in *Empoasca* (Hemiptera: Cicadellidae) Leafhoppers

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Subject Editor: Dana Nayduch

Received 16 January 2021; Editorial decision 16 May 2021

Abstract

Endosymbiotic bacteria can induce parthenogenesis in many haplo-diploid species, but little is known about their role in asexual reproduction in diplodiploids. Bacteria are essential for reproduction in the asexual lineages of springtails and booklice, and possibly some weevils, but whether they are the causative agent of parthenogenesis remains to be proven. This would require comparing unisexual forms and their closely related bisexual species, but such model species are very rare. *Empoasca* leafhoppers from the Madeira Archipelago provide an excellent case to test whether bacterial infections can cause parthenogenesis. Here we examined the diversity of the sex-distorting bacteria, *Wolbachia*, *Cardinium*, *Rickettsia,* and *Arsenophonus*, in three apomictic morphotypes (A, B, C) and three bisexual relatives of *Empoasca*. *Wolbachia* of group B was present in all unisexual and bisexual species. *Rickettsia* were the only bacteria restricted to the three unisexual forms, suggesting an association between infection and asexual reproduction. In addition, we found *Asaia* for the third time in the Cicadellidae, although this may have little to do with the reproductive mode of its host. Adult females of morphotype C reared on plants watered with tetracycline solution did not result in the production of male offspring. To our knowledge, this is the first study to suggest a possible association between bacterial infection and parthenogenesis in apomictic XO/XX diplodiploid bisexual species based on a range of sex ratio-distorting bacteria.

Key words: asexual reproduction, apomictic, endosymbiont, *Wolbachia, Rickettsia*

Many arthropods harbor endosymbiotic bacteria that are maternally inherited. Some of these bacteria can manipulate the reproduction of their hosts to increase the proportion of infected females. They can skew sex ratios by inducing parthenogenesis in haplodiploid species, feminizing genetic males, killing males during embryogenesis, or causing cytoplasmic incompatibility between gametes (Stouthamer et al. 1999, Werren et al. 2008). *Wolbachia* Hertig 1936 (Rickettsiales: Anaplasmataceae) is the best-known example of manipulative bacteria of host reproduction in arthropods (Werren and Windsor 2000, Hilgenboecker et al. 2008, Werren et al. 2008). Other manipulative reproductive parasites include *Cardinium* Zchori-Fein et al. 2004 (Sphingobacteriales: Flexibacteraceae), *Rickettsia* da Rocha-Lima, 1916 (Rickettsiales: Rickettsiaceae), *Arsenophonus* Gherna et al. 1991 (Enterobacterales: Morganellaceae), and *Spiroplasma* Saglio et al. 1973 (Entomoplasmatales: Spiroplasmataceae) (Saglio et al. 1973, Gherna et al. 1991, Duron et al. 2008, Engelstädter and Hurst 2009, Zchori-Fein et al. 2004, Ma et al. 2014). Bacteria-induced parthenogenesis has only been demonstrated in haplodiploid species of the orders Hymenoptera, Thysanoptera, and some Acari (Weeks et al. 2001, Kageyama et al. 2012). In these species, unfertilized eggs become haploid males and fertilized eggs develop into diploid females. Infected females do not produce male offspring, but convert all haploid eggs into parthenogenetic females (Stouthamer and Kazmer 1994, van der Kooi et al. 2017). When the bacteria are killed with antibiotics or heat, the cured females produce haploid males again, and the haplodiploid cycle is restored (Stouthamer and Mak 2002, Li et al. 2014). The mechanism by which bacteria induce parthenogenesis is not fully understood, but is often due to modification of meiosis (Suomalainen et al. 1987,

Adachi-Hagimori et al. 2008, Ma et al. 2015) and less commonly due to postmeiotic changes (Stouthamer 1997). In contrast to haplodiploid species, there is no conclusive case of endosymbiont-induced parthenogenesis in diplodiploid species (Huigens and Stouthamer 2003).

The relationship between bacteria and parthenogenesis in diplodiploid species has been studied in the automictic springtail, *Folsomia candida* Willem, 1902 (Collembola: Isotomidae), and the booklice, *Liposcelis bostrichophyla* Badonnel, 1931 (Psocoptera: Liposcelididae). In this springtail, *Wolbachia* are necessary for egg viability (Pike and Kingcombe 2009, Timmermans and Ellers 2009), whereas in the booklice, *Rickettsia* are necessary for egg production (Yusuf and Turner 2004, Perotti et al. 2006). Despite this strong correlation, further studies are needed to understand whether bacteria are the cause of parthenogenesis. Unlike automictics, there are no studies specifically addressing the role of bacteria in asexual reproduction in apomictic lineages (Son et al. 2008, Mazur et al. 2016, Elias-Costa et al. 2019). A growing body of data on triploid and apomictic tetraploid weevils shows a significant correlation between endosymbionts and the reproductive mode of their host (Son et al. 2008, Lachowska et al. 2010, Rodriguero et al. 2010, Chen et al. 2012). Studies on diplodiploid species have so far focused almost exclusively on the effects of *Wolbachia* (Son et al. 2008, Chen et al. 2012, Elias-Costa et al. 2019), but searches should include other manipulative bacterial species as well (Hurst et al. 1999, Hurst 2000, Koivisto and Braig 2003, Duron et al. 2008).

Here we examine sexual and apomictic asexual *Empoasca* Walsh, 1862 leafhopper species from the Madeira Archipelago for endosymbiotic bacteria. Parthenogenesis is rare within Hemiptera species of the suborder Auchenorrhyncha (Gokhman and Kuznetsova 2017). On the Madeira Archipelago, three unisexual morphotypes of *Empoasca* leafhoppers coexist with three bisexual, two species of *Empoasca* and the closely related *Asymmetrasca decedens* (Paoli, 1932) (Hemiptera: Cicadellidae) (Aguin-Pombo et al. 2006, Aguin-Pombo and Freitas, 2020). Unisexual forms are known only in Madeira, whereas bisexual species are widespread in Africa, Europe, and Central and North America, with the exception of *E. alsiosa* Ribaut, 1933, which is native to Madeira Archipelago, Canary Islands, and the western Mediterranean (Nast 1972, 1987). The other two bisexual species, *E. fabalis* DeLong, 1930 and *A. decedens*, are probably introductions from America (Aguin-Pombo and Freitas 2020) and Mediterranean region, respectively (Freitas and Aguin-Pombo 2005, Aguin-Pombo and Freitas 2008). *Empoasca* is a worldwide distributed genus with about 800 species; many of them are known to be pests of crops and, except in Madeira, only one putative case of parthenogenesis is known throughout their range (Akingbohungbe, 1983). The bisexual species in Madeira are diploid with XO/XX sex determination system, whereas the unisexual females reproduce by apomictic parthenogeneses. These morphotypes are morphologically very similar (Aguin-Pombo and Freitas, 2006, 2020) and have different chromosome numbers, morphotype A and B are triploids, and morphotype C is diploid (Aguin-Pombo et al. 2006, Kuznetsova and Aguin-Pombo 2015).

Previous studies have shown that endosymbiotic bacteria can manipulate reproduction in other leafhoppers and planthoppers. In the planthoppers *Laodelphax striatellus* (Fallen, 1826) and *Sogatella furcifera* (Horvath, 1899) (Hemiptera: Delphacidae), *Wolbachia* and *Cardinium* induce cytoplasmic incompatibility between infected and uninfected hosts (Noda 1984, Hoshizaki and Shimada 1995, Noda et al. 2001, Nakamura et al. 2012, Zhang et al. 2012, Bing et al. 2019). In leafhoppers of the genus *Zyginidia* Haupt, 1929 (Hemiptera: Cicadellidae), *Wolbachia* can skew sex ratio toward females by feminizing genetically determined males (Negri et al. 2006) and may be responsible for the production of intersex individuals in *Eupteryx* Curtis 1829 leafhoppers (Hemiptera: Cicadellidae) (Henke et al. 2013).

In this work, we screen unisexual lineages and diplodiploid bisexual species of *Empoasca* for a full range of endosymbiotic bacteria known to cause parthenogenesis (Duron et al. 2008). We examined the effect of tetracycline on the reproductive biology of morphotype C to determine whether occasional males occurring in nature (D.A.-P., personal observation) are due to incomplete transmission of the bacteria by infected females.

Material and Methods

Asymmetrasca decedens has been regarded either a species of the genus *Empoasca* (Nast, 1987) or another species of the closely related genus *Asymmetrasca*. For simplicity, we will refer to all bisexuals as *Empoasca* species. Between November 2002 and March 2006, *Empoasca* samples were collected from native populations on Madeira Island by sweeping the vegetation with a net. Specimens were identified using informative morphological characters such as forewing veins, body size, and head shape (Aguin-Pombo and Freitas 2020).

Bacterial Diversity Screening

Bacterial diversity in leafhoppers was determined in two ways: by PCR using bacteria-specific primer pairs or by sequencing cloned bacterial 16S ribosomal PCR amplicons. Ten to 20 females of the bisexual species *E. fabalis*, *E. alsiosa*, and *E. decedens* and the unisexual morphotypes A, B, and C were collected on Madeira Island and stored in 96% ethanol. Prior to DNA extraction, individual leafhoppers were surface sterilized by rinsing once with 70% ethanol and then washed twice in sterile water. The abdomen was then dissected in 95% ethanol and ground in 5 µl proteinase K (20 mg/ml) and 100 µl CTAB buffer (2% CTAB w/v in 100 mM Tris–HCl (pH8), 20 mM EDTA, and 1.42 M NaCl). After vortexing, samples were incubated at 55°C for 1 h. Next, 100 µl chloroform:isoamyl alcohol (24:1) was added, and the contents were mixed gently for 2 min. The tubes were centrifuged at 15,800 g for 10 min. After centrifugation, 80 µl of the supernatant was transferred to a clean tube and the DNA was precipitated by adding 200 µl ice-cold 96% ethanol. The tubes were incubated at 20°C for at least 1 h before centrifugation at 15,800 g for 15 min at 4°C. The supernatant was removed, and the DNA pellet was washed with 70% ethanol. The DNA was then air dried for at least 15 min, eluted in 30 µl sterile water, and stored at 20°C. DNA quality was systematically tested by polymerase chain reaction (PCR) amplification of a conserved region of arthropod mitochondrial COI with universal primers (Ma et al. 2014), which are listed in Table 1.

Bacteria-Specific Screening

Screening for *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, and *Asaia* Yamada et al. 2000 (Rhodospirillales: Acetobacteraceae) was performed using species-specific primer pairs (Table 1). Leafhoppers yielding amplicons of the expected size were scored as infected with this bacterium. Negative samples for species-specific primer pairs but yielding a product of the expected size of cytochrome COI were retested. If the second test was also negative, these samples were noted as not infected with that bacterial species.

Amplifications were performed in 25 µl reaction mixtures containing 2.5 µl DNA extraction volume, 2.5 µl 10× PCR buffer, 5 µl

Table 1. Genes and primers used in polymerase chain reaction (PCR) assays for bacterial detection and CO1 primers and DNA quality control. Tm is the annealing temperature.

Organism	Gene	Name	Primer (5′ – 3′)	Size	Tm	Reference
Wolbachia	*ftsZ*	494F	GTATGCCGATTGCAGAGCTTG	760 bp	58°C	Himler et al. 2011
		1262R	GCCATGAGTATTCACTTGGCT			
	wsp	81F	TGGTCCAATAAGTGATGAAGAAAC	610 bp	58°C	Schulenburg et al. 2001, Zhang et al. 2012
		691R	AAAAATTAAACGCTACTCCA			
Cardinium	16s	CLOf	GCGGTGTAAAATGAGCGTG	450 bp	57°C	Weeks et al. 2007
		CLOr1	ACCTMTTCTTAACTCAAGCCT			
Rickettsia	16s	RSSUF	CGGCTTTCAAAACTACTAATCTA	380 bp	50°C	Schulenburg et al. 2001,
		RSSUF	GAAAGCATCTCTGCGATCCG			
Asaia	16s	Asafor	GCGCGTAGGCGGTTTTACA	180bp	50°C	Favia et al. 2007
		Asarev	AGCGTCAGTAATGAGCCAGGTT			
Eubacterial	16s	27F	AGAGTTTGATCMTGGCTCAG	1450 bp	54°C	Lane 1991
		1492R	TACGGYTACCTTGTTACGACTT			
Arthropods	CO1	HCO	TAAACTTCAGGGTGACCAAAAAATCA	658 bp	50°C	Folmer et al. 1994
		LCO	GGTCAACAAATCATAAAGATATTGG			

dNTPs mix, 0.2 µl of each primer (20 uM), 0.2-µl Taq DNA polymerase (5µ/µl), and ultrapure water. For *wsp* and COI, the PCR mixture also contained 1.25 µl $MgCl_2$ (25 mM). PCRs were performed under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of denaturation (94°C, 30 s), annealing (50–55°C, depending on the primer, 30 s), extension (72°C, 1 min to 1 min 30 s), and a final extension at 72°C for 5 min. PCR products were electrophoresed in a 1.5% agarose gel. When a PCR product was obtained, it was sequenced from two randomly selected individuals per infected species to ensure that the data set represented a true positive and not a PCR artifact or a related bacterium. Samples were purified using the NucleoSpin Extract ll kit and sequenced using the forward primers and Big Dye 3.1 on an ABI PRISM 3730 automated DNA sequencer.

Bacterial Diversity Identification

We used general bacterial primers to amplify a 1,400 bp portion of bacterial 16S rDNA from a pool of five leafhoppers per taxon to determine whether bacteria for which we had specific primer pairs were present in leafhoppers. The 16S rDNA PCR mixture was identical to the mixture used for the bacteria-specific screen. The PCR conditions were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 54°C, and 1 min extension at 72°C, concluded with a final extension at 72°C for 5 min. The resulting PCR product was ligated into a plasmid vector, and this construct was used to transform *E. coli*. Ten clones per taxon were sequenced using standard sequencing protocols with M13 primers.

Bacterial Species Identification

After removal of PCR primers and vector sequences, bacterial sequences were compared with GenBank accessions using the Basic Local Alignment Search Tool (BLAST) to identify species. For general bacterial screening, we included 16S rDNA sequences from representatives of the bacterial groups indicated in Moya et al. (2008). We also sequenced leafhopper *Wolbachia ftsZ* and *wsp* amplicons to determine to which *Wolbachia* supergroup they belonged. In all cases, relatedness between bacterial sequences was estimated using MEGA X (Kumar et al. 2018). Sequences were aligned using the default settings of CLUSTAL as implemented in MEGA. Relationships and bacterial identities of leafhoppers were inferred by constructing neighbor-joining phylograms with 500 bootstrap replicates based on unambiguously aligned sites using the substitution model proposed by ModelTest (Posada 2008). Sequences were deposited in GenBank (Accession numbers: 16S rRNA: MW970329–MW970343; wsp: MW926320–MW926324; and ftsZ: MW926325–MW926329).

Tetracycline Treatments

Antibiotics successfully remove *Wolbachia* from infected individuals of various insect orders (Li et al. 2014). If *Wolbachia* is the causative agent of parthenogenesis, its removal restores normal sexual reproduction. Similarly, we studied the effects of *Wolbachia* on the reproductive biology of morphotype C to determine whether the occasional males in nature (D.A.-P., personal observation) may be due to incomplete transmission of the bacteria from infected females to their offspring. In similar studies on sap-feeding leafhoppers and planthoppers, the antibiotic is administered dissolved in water used to pot food plants (e.g., Zhang et al. 2020). The antibiotic is ingested while the insects suck the sap from the plant. We also exposed adults and nymphs of morphotype C to plants irrigated with a tetracycline

solution. The progeny of P and F1 was examined for the presence of males to see whether sexual reproduction was restored.

Parental lineages were started with adult females collected in the field on *Ricinus communis* L., 1753 (Magnoliopsida: Euphorbiaceae) in Ribera Brava at a site 47 m above sea level. Three experimental cages were set up. In each cage, a bean plant about 25 cm high was placed with 20 adult females. Plants in two cages were watered daily with a tetracycline solution (1 mg/l). The food plants were watered only with this solution. The plant watered with water in the third cage served as a control. It cannot be excluded that some of these adult females produced males in the field. In order not to overlook any males, we examined all the offspring of these females. Thus, the effect of the antibiotic was studied in the F1 nymphs over a period of eight days. For this purpose, 80 nymphs less than 10 d old were distributed among four plastic cages, each containing one plant and 20 nymphs. The nymphs from adults reared on untreated plants served as control, whereas the nymphs of P females reared on plants exposed to tetracycline solutions were also treated with 1, 3, and 5 mg/L solution, respectively. Experiments were conducted at a photoperiod and temperature of 26 ± 1. 9°C (23.5–29) and humidity of 69.5 ± 1.3% (67.1–71) according to the procedure described above.

Results

Bacterial Diversity in Leafhoppers

Ten clones with 16S rDNA amplicons of each taxon were sequenced and compared against the GenBank 16S rDNA database. Clades of the major bacteria known to be symbionts of insects, in particular Hemiptera, were obtained from GenBank. The final aligned data set contained 63 entries with a sequence length of 1,656 positions. The bacteria of Madeira leafhoppers can be broadly divided into four groups: *Wolbachia*, *Rickettsia* and Asaia (alpha-proteobacteria) and gammaproteobacteria (Fig. 1). The bacterium of *E. alsiosa*, which is close to *Lactococcus lactis* (Lactobacillales, Streptococcaceae), was considered a human contamination. The presence of *Wolbachia* and the absence of *Cardinium* and *Arsenophonus* in this general bacterial screening (Table 2) agreed with the specific PCRs for these species. Most interestingly, *Rickettsia* were present in all three unisexual morphotypes but were absent in the three sympatric bisexual species. The resulting neighbor-joining tree is shown in Fig. 2. Some internal branches are not well supported, and the phylogram does not necessarily represent the real phylogenetic relationships between clades. However, the phylogram does indicate the closest bacteria to the leafhopper symbionts.

The *Wolbachia* of *Empoasca* belong to strain B based on *wsp* and *ftsZ* sequences (Figs. 3 and 4). Strains A and B of *Wolbachia* are widely distributed among insects (Werren and Windsor 2000, Prakash and Puttaraju 2007) and are found in leafhoppers, including *Empoasca* (Delay, 2013), planthoppers (Noda et al. 2001), and some parthenogenetic weevils (Son et al. 2008).

Cardinium and *Arsenophonus* bacteria were not detected in any of our leafhopper samples using specific primer pairs nor with general bacterial 16S rDNA primer pair. We did not detect either any of the known Hemipteran nutritional symbionts, such as *Baumannia* Moran et al. 2003 (Proteobacteria: Gammaproteobacteria), "Candidatus Sulcia" Moran et al. 2005 (Flavobacteriia: Flavobacteriales), *Zinderia* McCutcheon and Moran 2010 (Burkholderiales: Oxalobacteraceae), or *Nasuia* Noda et al. 2012 (Proteobacteria, Betaproteobacteria) (Fig. 1). However, we did find that all bisexual and unisexual *Empoasca* were infected with *Asaia* bacteria. In addition, we found some other gamma-proteobacteria in morphotype C and *E. decedens* that grouped with *Serratia* Bizio 1823 (Enterobacterales: Yersiniaceae) based on our phylogram analysis of the 16S rRNA gene (Fig. 2). Similarly, closely related bacteria were also found in the brown planthopper, *Nilaparvata lugens* (Stal, 1854) (Hemiptera: Delphacidae), and bed bug, *Cimex lecturalius* Latreille, 1802 (Hemiptera: Cimicidae).

Antibiotic Treatments

Adult females of morphotype C reared on plants watered with tetracycline solution did not produce male offspring. Watering food plants with 1-mg tetracycline solution had no significant effect on adult survival (Fig. 5). All adult females from the control and from one of the two antibiotic trials survived, and in the third trial, 90% were still alive after 1 wk of treatment. Females from the control and antibiotic trials did not differ in the number of offspring produced; control: 63 nymphs; trial 1: 58; trial 2: 96. First nymphs (F1) also appeared at similar times in the control and treatment trials; control: 7 d; trials 1 and 2: 8 d each (Fig. 5). As with the adults, F1 nymphs from the control survived (75%) but those reared on plants watered with tetracycline solution died regardless of antibiotic concentration. At 1, 3, and 5 mg/l, nymph mortality reached 100% after 6, 8, and 8 d, respectively (Fig. 5).

Discussion

The symbionts of *Empoasca* species are largely unknown, but results from Madeira suggest that, like other leafhopper species (Takiya et al. 2006) and other Hemiptera (Thao et al. 2000, Baumann 2005, Downie and Gullan 2005, Iasur-Kruh et al. 2017, Gonella et al. 2019), *Empoasca* harbor a diverse bacterial community.

In our survey of the bacterial community of *Empoasca*, three groups of bacteria frequently showed up: *Wolbachia*, *Rickettsia*, and *Asaia*.

A first step to determine whether these bacteria may play a role in the induction of parthenogenesis is to look for associations between the mode of reproduction of the host and bacterial species. *Wolbachia* was not detected in individual specimens of *E. alsiosa*, the only native *Empoasca* species in Madeira, but a pooled control sample of five specimens tested positive, suggesting that the absence of this bacterium in *E. alsiosa* may be due to a lower bacterial load or very low prevalence. As in weevils, *Wolbachia* was more

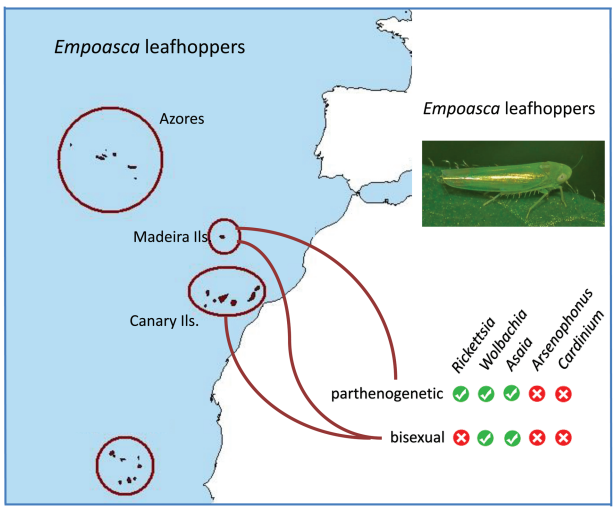

Fig. 1. Bacteria found in unisexual and bisexual leafhoppers *Empoasca* from Madeira, and distribution in Macaronesia, which includes the archipelagos of the Azores, Canary Islands, Cape Verde, and Madeira.

Table 2. Summary of the bacteria present (+) in bisexual and unisexual leafhoppers of *Empoasca*

Species	Reproduction	Distribution	*Wolbachia*	*Cardinium*	*Rickettsia*	*Asaia*	*Arsenophonus*	Other groups
Morphotype A	Unisexual	Madeira Isl	+	–	+	+	–	
Morphotype B	Unisexual	Madeira Isl	+	–	+	+	–	Gamma Proteobacteria
Morphotype C	Unisexual	Madeira Isl	+	–	+	+	–	Gamma Proteobacteria
Empoasca decedens	Bisexual	Palearctic including the Mediterranean region	+	–	–	+	–	Gamma Proteobacteria
Empoasca alsiosa	Bisexual	Mediterranean region (including Canary Isl, Madeira)	+[a]	–	–	+	–	Firmicutes

The presence of bacteria is based on the combined results of positive PCR with bacteria-specific primers (Table 1) and sequences cloned 16S rDNA amplicons. If positive in at least one of the methods, the host was listed as infected with that bacterium.

[a]Positive results of *E. alsiosa* were obtained only in a pooled sample of five individuals.

common in unisexual than in bisexual species (Lachowska et al. 2010, Rodriguero et al. 2010). However, its presence in both bisexual and unisexual *Empoasca* species suggests that *Wolbachia* is not directly linked to parthenogenesis.

Rickettsia were found in all three unisexual morphotypes but not in the bisexual species (Table 2, Fig. 1), indicating a possible link between infection and asexual reproduction. *Rickettsia* can be obligate endosymbionts (Perotti et al. 2006), reproductive parasites, or act simultaneously as nutritional mutualists and reproductive manipulators (Himler et al. 2011). As reproductive parasites, *Rickettsia* can cause male-killing (Lawson et al. 2001) and is invoked in parthenogenesis (Werren et al. 1994, Giorgini et al. 2010). For example, parthenogenetic populations of the booklice, *Liposcelis bostrychophila*, were also fixed for *Rickettsia* (Perotti et al. 2006, Perlman et al. 2015), whereas related bisexuals were not infected (Feng et al. 2018). When *Rickettsia* were removed, egg production stopped, suggesting that the bacteria may be involved in parthenogenesis of booklice (Yusuf and Turner 2004, Perotti et al. 2006). *Rickettsia* have been reported in *Empoasca papaya* Oman, 1937 (Davis et al. 1998), as well as other leafhoppers (Weinert et al. 2009, Noda et al. 2012, Ishii et al. 2013, Lian et al. 2016) and planthoppers (Gonella et al. 2011, Michalik et al. 2018), but the effects on host biology are still unknown.

Asaia bacteria were present in unisexual and bisexual *Empoasca* species. It is a newly discovered secondary symbiont of insects that belongs to the acetic acid bacteria family Acetobacteraceae. *Asaia* can be intracellular and can be both vertically and horizontally transmitted (Favia et al. 2007, Crotti et al. 2010, Gonella et al. 2018). This bacterium was recently found in the planthoppers, *Nilaparvata lugens* (Stal, 1854) and *Sogatella furcifera*, and in the leafhoppers, *Scaphoideus titanus* Ball, 1932 (Hemiptera: Cicadellidae) (Sacchi et al. 2008; Gonella et al. 2012, 2018) and *Euscelidius variegatus* (Kirschbaum, 1858) (Hemiptera: Cicadellidae) (Gonella et al. 2012). Although *Asaia* can have various functions in host biology (Roh et al. 2008) and fitness (Chouaia et al. 2012, Mitraka et al. 2013), it has not been shown to cause parthenogenesis. The fact that they are not specifically associated with unisexuals but were found in both unisexual and bisexual *Empoasca* species, makes it unlikely that they induce parthenogenesis in *Empoasca*.

We did not detect *Cardinium* and *Arsenophonus* in unisexual and bisexual species of *Empoasca* of Madeira. Although they have been reported in other leafhoppers (Bigliardi et al. 2006; Sacchi et al. 2008; Kobiałka et al. 2016, 2018a,b) and planthoppers (Gonella et al. 2011; Zhang et al. 2012, 2013; Qu et al. 2013; Bressan 2014; Li et al. 2018), so far in none of these cases they have been reported to be involved in reproductive manipulation of these hosts.

A second step to determine whether bacteria are involved in the reproduction mode of their host is to treat hosts with antibiotics to eliminate bacteria and observe changes in offspring number and sex ratio. If the bacteria of *Empoasca* cause parthenogenesis in diplodiploids, their elimination is expected to produce sterile females or females requiring sperm to fertilize eggs. However, the effects of these bacteria on the host are not all or nothing, as one would expect if parthenogenesis was induced by bacteria (Timmermans and Ellers, 2009). Antibiotic treatment of females of unisexual morphotype C did not provide a clear answer because the F1 offspring did not survive long enough to determine their sex. Mortality due to antibiotics could be due to several reasons. First, male development may simply no longer be possible due to the mutational erosion of genes involved in the process because they are not under selection. Second, antibiotics may kill bacteria that

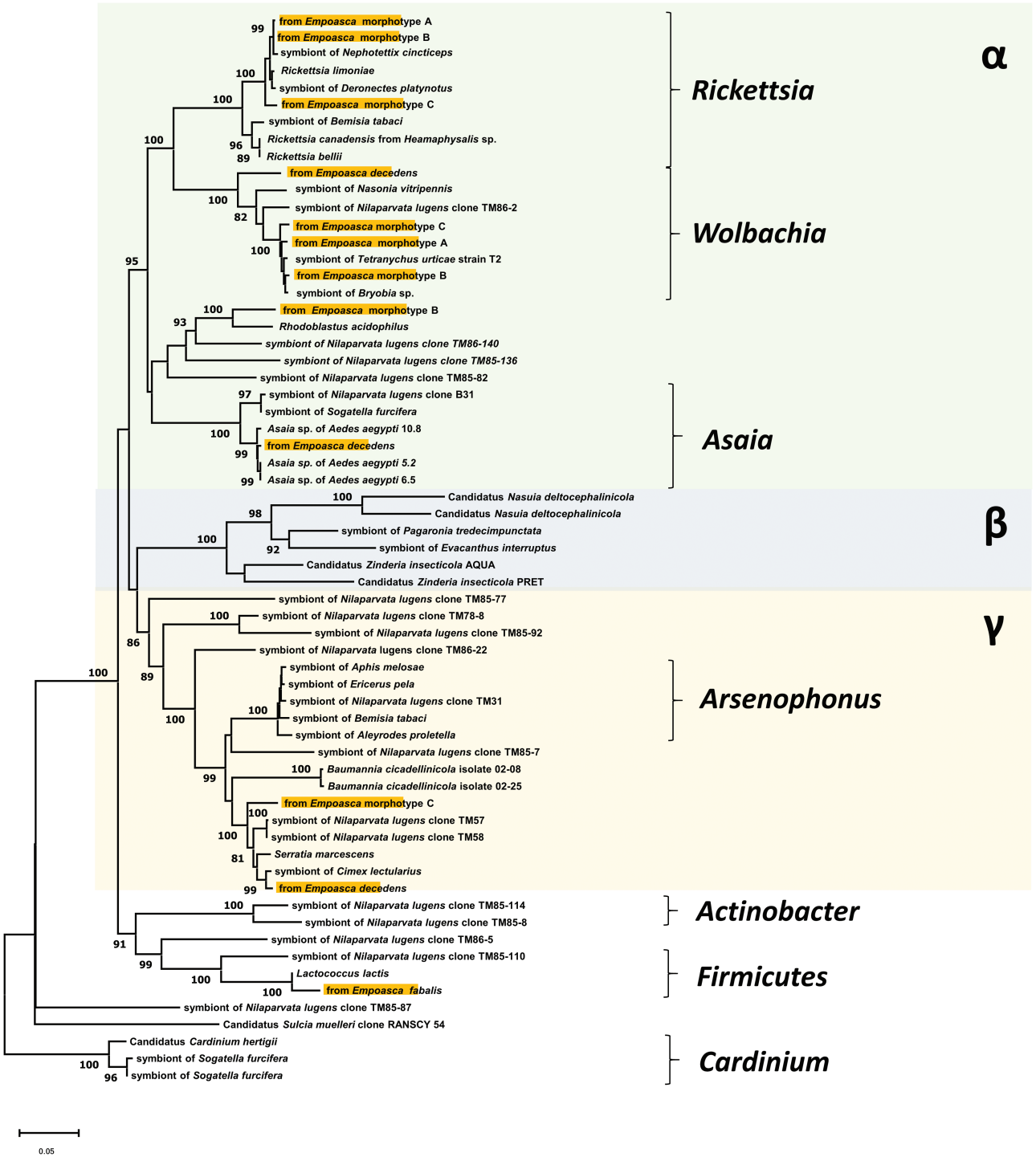

Fig. 2. Bacterial 16s phylogram constructed using the neighbor-joining method as implemented in MEGA X. Colored boxes overlay members of the alpha, beta, and gamma Proteobacteria. The analysis included 63 sequences. There were a total of 1656 positions in the final data set. All positions with gaps and missing data were eliminated. Tamura 3-parameter with a discrete Gamma distribution was used to model differences in evolutionary rate among sites (five categories + G parameter = 0.72). The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogram tree. The numbers on the branches indicate the percentage bootstrap support for the main branches (500 replicates; only bootstrap values of 80% or more are shown). Branch lengths are measured in the number of substitutions per site.

are vital to leafhopper development because they provide for example essential nutrients to their host. Studies on the planthopper *Laodelphax striatellus* showed that treatment with tetracycline affects the structure and composition of microbial communities. In some cases, such as *Wolbachia*, *Bacteroides* Castellani & Chalmers 1919 (Bacteroidales: Bacteroidaceae), and *Abiotrophia* Kawamura et al. 1995 (Lactobacillales: Aerococcaceae), almost 100% of the bacteria can be killed (Zhang et al. 2020), which can lead to a depletion of beneficial bacteria for the host. In the case of the unisexual springtail, *Folsomia candida*, eggs laid by cured

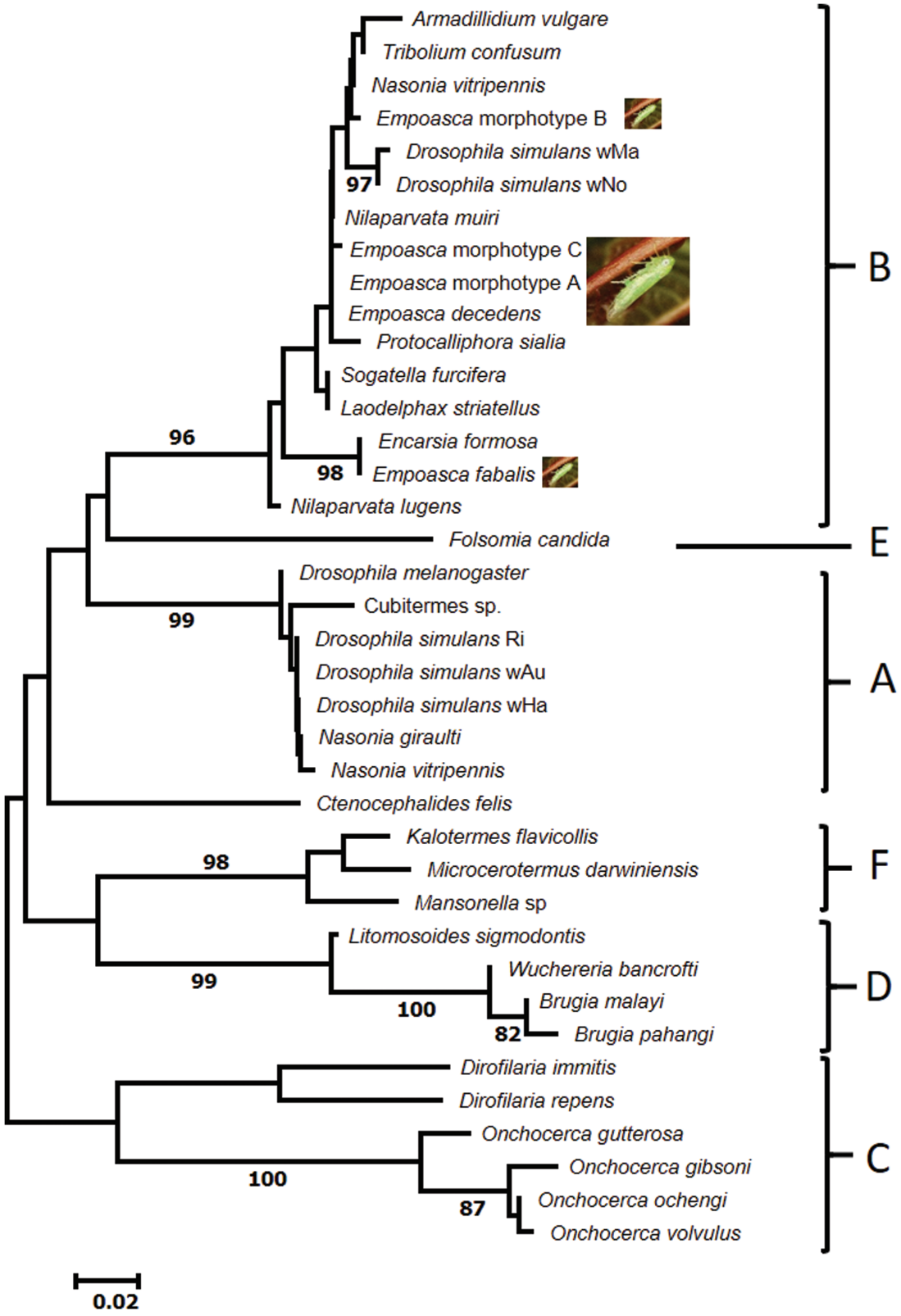

Fig. 3. *Wolbachia* ftsZ phylogram constructed using the neighbor-joining method implemented in MEGA X. The symbionts are indicated by the proper name of their host. Sequences from this study are shown in blue. Major *Wolbachia* supergroup lineages are reported (A–F). Unfortunately, no *Wolbachia* sequence information was obtained of *E. alsiosa*. The analysis included 38 nucleotide sequences. All positions with gaps and missing data were eliminated. A total of 377 positions were in the final dataset. Tamura 3-parameter with a discrete Gamma distribution was used to model differences in evolutionary rate among sites (five categories + G parameter = 0.2082). The numbers on the branches indicate the percentage bootstrap support for the main branches (500 replicates; only bootstrap values of 80% or more are shown). The lengths of the branches are measured in the number of substitutions per site.

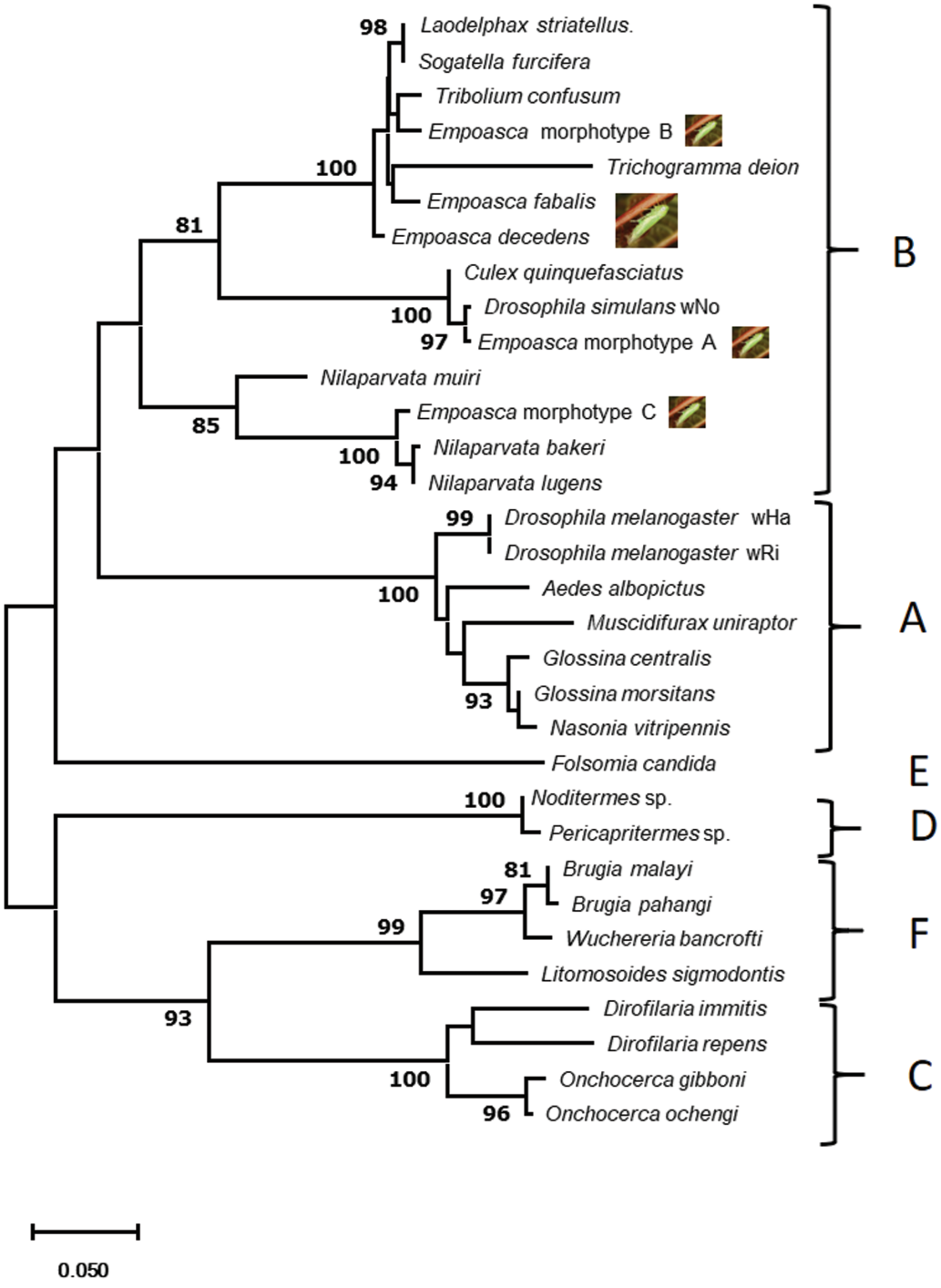

Fig. 4. *Wolbachia* wsp phylogram constructed using the neigbor-joining method implemented in MEGA X. Major *Wolbachia* supergroup lineages are indicated (A–F). Unfortunately, no sequence information of *Wolbachia* was obtained for *E. alsiosa*. Symbionts are indicated by the scientific name of their host. Tamura 3-parameter with a discrete Gamma distribution was used to model differences in evolutionary rate among sites (five categories + G parameter = 0.71). The analysis included 32 sequences. All positions with gaps and missing data were eliminated. There were a total of 482 positions in the final data set. The numbers on the branches indicate the percentage bootstrap support for the main branches (500 replicates; only bootstrap values of 80% or more are shown). The length of the branches is measured in the number of substitutions per site.

females did not hatch or develop without *Wolbachia*, resulting in complete sterility (Frati et al. 2004, Pike and Kingcombe 2009). On the other hand, tetracycline itself inhibits protein synthesis (Dobson and Rattanadechakul, 2001), and thus can have direct lethal or sublethal effects on the host.

The fact that some unisexual *Empoasca* are triploid further complicates studies into the role of bacteria. If bacteria were responsible for the disappearance of males and the cause of parthenogenesis, administration of antibiotics to triploid XXX females would have to result in viable X0 males; however, this is unlikely. The high mortality

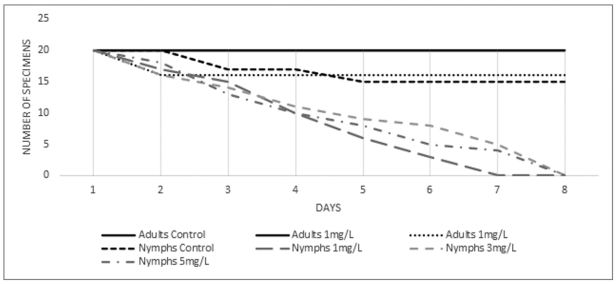

Fig. 5. Treatment with various concentrations of tetracycline. Lines are shown in black for adults and in grey for nymphs. In the adults, the control and the 1 mg/l treatment gave the same results, so the two lines overlap.

of nymphs suggests that production of males after antibiotic treatments is probably not possible. The fact that the unisexual forms are triploid seems to indicate a hybrid origin for morphotype A, but not for morphotypes B and C. One way to test this is to compare the phylogenies of nuclear and mitochondrial genes. Inconsistent phylogenies would indicate a hybrid origin of parthenogenesis. An additional limitation, at least for morphotypes A and B, is that it is not easy to separate the effects of hybridization and polyploidy from those of endosymbionts to explain the origin of clonal variation; however for morphotype C, which is diploid, this may be possible.

Surprisingly, we did not find bacteria in our 16S bacterial screen that are known nutrient endosymbionts of leafhoppers such as *Sulcia* and *Baumannia* (Michalik et al. 2018). It is unlikely that bisexual species and morphotypes of *Empoasca* leafhoppers do not have nutrient symbionts, as their diets and feeding habits are similar to those of other plant and leafhopper species, all of which rely on endosymbionts for essential nutrients not present in the host diet (Moran et al. 2008). Indeed, *Sulcia muelleri* Moran et al 2005 has been found in the potato leafhopper *E. fabae* (Delay, 2013). It is possible that eukaryotic symbionts remain undetected in our PCR, which uses primers designed to anneal to conserved regions of the bacterial 16S rRNA gene. However, our general 16S rDNA primer set should pick *Sulcia* because both primer sequences match exactly with published 16s rDNA *Sulcia* sequences. Alternatively, *Empoasca* could use yeast or other unicellular eukaryotes instead of bacteria (Noda 1977, Sacchi et al. 2008, Michalik et al. 2009).

We postulate that *Wolbachia*, *Rickettsia*, or *Asaia* may have taken on this nutritional role. *Asaia* seems to be the most likely candidate, because of its omnipresence in all *Empoasca* species we screened. It has been reported in bacterial screens of other Hemipteran species such as the small brown planthopper *Nilaparvata lugens* and *Laodelphax striatellus* (Li et al. 2017; Zhang et al. 2019, 2020) and even as intracellular symbiont of the American grapevine leafhopper *Scaphoideus titanus* and the planthopper *Sogatella furcifera* (Gonella et al. 2012; Li et al. 2017, 2020; Zhang et al. 2019). Moreover, in *Sogatella* planthoppers, Li et al (2020) showed with antibiotic treatments that *Asaia* promoted larval development and adult weight, suggesting that it may be a nutritional symbiont. Also, in *Anopheles* Meigen 1818 mosquitoes (Diptera: Culicidae), is essential for larval development (Chouaia et al. 2012, Mitraka et al. 2013).

In leafhoppers and planthoppers, *Wolbachia* can be a facultative (Takiya et al. 2006, Bing et al. 2019) or an obligate symbiont. If we assume that tetracycline successfully removed *Wolbachia* from morphotype C, the higher mortality of nymphs in the antibiotic experiments suggests that the bacterium may be necessary for the development and survival of its host. However, we did not confirm elimination of *Wolbachia* with PCR upon antibiotic treatment, nor did we check whether other bacteria were affected as well. Similar results have been found in the leafhopper species *Empoasca fabae* Harris, 1841, and *Nephotettix cincticeps* (Uhler, 1896) (Hemiptera: Cicadellidae). In these species, nymph mortality was higher after bacteria were removed with antibiotics (DeLay 2013). In *Empoasca fabae*, removal of the symbionts also affected their normal development and reproduction (DeLay 2013). Although *Wolbachia* is unlikely to induce parthenogenesis, *Empoasca* could still benefit from its infection. *Wolbachia* can positively influence host development by increasing their fitness (Weeks et al. 2007, Engelstädter and Hurst 2009, Jaenike 2012), fecundity (Stolk and Stouthamer 1996), or longevity (Dobson et al. 2004). Also, *Rickettsia* may have these effects on the general host fitness (Perlman et al. 2006).

In addition, occasional bacteria were detected in some *Empoasca* species, such as the gamma-proteobacteria of *E. decedens* and morphotype C. They seem closely related to bacteria found in other Hemiptera. Although it is unlikely that they manipulate the mode of reproduction of their host, they may be gut symbionts or secondary endosymbionts that affect host fitness. Also, the bacterium of *E. alsiosa* may not be a contaminant based on its close relationships with *Lactococcus*. It could be a transient bacterium or secondary symbiont as well.

In conclusion, *Rickettsia* may be responsible for the induction of parthenogenesis in *Empoasca* as they are associated with the unisexual morphotypes but are absent in the bisexual species. However, it may be difficult to prove this by treating the host with antibiotic, as *Empoasca* harbor additional bacteria that may be necessary for their survival. They are also polyploids, so it is not possible to know if polyploidy or bacteria are responsible for thelytokous reproduction in these leafhoppers. We found also that *Asaia* bacteria are omnipresent in all *Empoasca* of Madeira and postulate that *Asaia* is a nutritional symbiont of *Empoasca* leafhoppers. The bacterial diversity in *Empoasca*, including intracellular bacteria, omnipresent bacteria, and less frequent bacteria, makes it an interesting system for studying host-bacteria interactions and determining the ecological drivers of the costs and benefits of these associations.

Acknowledgments

We thank Valentina Kuznetsova for critical review of the paper and useful comments on the manuscript. We also thank the reviewers for valuable comments. This study was supported by the Fundação para a Ciência e a Tecnologia (FCT) research project Origin of multiple parthenoforms of *Empoasca* leafhoppers in Madeira Island (PTDC/BIA-BEC/103411/2008). We gratefully acknowledge Nelio Freitas for providing the photos of *Empoasca*.

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