Reduced Diversity in the Bacteriome of the Phytophagous Mite *Brevipalpus yothersi* (Acari: Tenuipalpidae)

Oscar E. Ospina 1, Steven E. Massey 2 and Jose Carlos Verle Rodrigues 1,*

1 Center for Excellence in Quarantine and Invasive Species, Agricultural Experimental Station-Río Piedras, Crops and Agro-Environmental Sciences Department, University of Puerto Rico-Mayaguez, 1193 Calle Guayacán, San Juan, PR 00926-1118, USA; ospina.oe@gmail.com
2 Bioinformatics Laboratory, Department of Biology, University of Puerto Rico-Rio Piedras, San Juan, PR 00931-3360, USA; stevenemassey@gmail.com
* Correspondence: jose_carlos@mac.com; Tel.: +1-787-767-9705; Fax: +1-787-756-8329

Academic Editors: Eric W. Riddick and Andrew G. S. Cuthbertson
Received: 27 September 2016; Accepted: 12 December 2016; Published: 20 December 2016

Abstract: Tenuipalpidae comprises mites that transmit viruses to agriculturally important plants. Several tenuipalpid species present parthenogenesis, and in *Brevipalpus yothersi*, the endosymbiont *Cardinium* has been associated with female-only colonies. It is unclear what the bacterial composition of *B. yothersi* is, and how common *Cardinium* is in those microbiomes. We performed a comparative analysis of the bacteriomes in three populations of *B. yothersi* and three additional Tetranychoida species using sequences from V4-fragment of 16S DNA. The bacteriomes were dominated by Bacteroidetes (especially *Cardinium*) and Proteobacteria, showing a remarkably low alpha diversity. *Cardinium* was present in about 22% of all sequences; however, it was not present in *R. indica* and *T. evansi*. In *B. yothersi*, the proportion of *Cardinium* was higher in adults than eggs, suggesting that proliferation of the bacteria could be the result of selective pressures from the host. This hypothesis was further supported because colonies of *B. yothersi* from different populations showed different bacterial assemblages, and bacteriomes from different mite species showed similar abundances of *Cardinium*. A phylogenetic analysis of *Cardinium* revealed that not only specialization but horizontal transmission has been important for this symbiosis. Together, these results represent a glimpse into the evolution of the Tetranychoida and *Cardinium*.

Keywords: bacterial diversity; *Brevipalpus yothersi*; false spider mite; *Cardinium*; habitat filtering; 16S ribosomal RNA; Tetranychoida; *Raoiella indica*; *Tetranychus evansi*; *Oligonychus*

1. Introduction

The mite family Tenuipalpidae has gained prominence in the last two decades as an emerging pest for agricultural and ornamental crops such as citrus, coffee, passion vine, tea, pistachio, and palms [1–3]. Particularly, the genus *Brevipalpus* has emerged as a major pest because of its capacity to transmit virus to crop plants. The most prolific vector species that has been reported is *Brevipalpus phoenicis* Geijskes, for which recent studies indicated that the species has been perpetually misidentified, and that it is actually *B. yothersi* Baker [4,5]. This species was reported to transmit viruses associated with two major cytopathology groups [6] and belonging to at least two virus families [7]. Interestingly, these mites reproduce by asexual thelytoky associated with the “feminizing” endosymbiont *Cardinium* [8,9].

Studies have shown that the presence of endosymbiotic bacteria has the potential to manipulate traits such as nutrition [10,11], immune response [12,13], and reproduction [14,15] in arthropods. In fact, it has been proposed that bacterial symbionts have driven to a large degree the evolution of many...
arthropod taxa by increasing fitness, adaptation, and specialization to different environments [16–18]. Such a close relationship between host and endosymbiont is probably the result of both ecological interactions among symbionts and selective pressures posed by the host [19]. Although literature regarding host-symbiont interactions is extensive, less attention has been directed to the study of these interactions under different conditions imposed by different plant hosts.

The ecological conditions of an arthropod host change along different life stages, which also may alter the conditions in the microhabitat of endosymbionts. It has been reported that bacterial communities in larvae of the mosquito Anopheles gambiae were different from those in adults: Adult A. gambiae showed a decrease in bacterial diversity in comparison to their larvae and there was a drastic shift from a species-rich community to an Enterobacteriaceae-dominated bacteriome [20]. Conversely, the bacterial assemblage of the beetle Agrilus planipennis was significantly more species-rich in the pre-pupae than in both larval and adult stages [21]. In both cases, a specialization in metabolic function of the bacteriome has been suggested as the consequence (or cause) of these changes. Although ontogenic development in insects is different to that in mites, specialization in bacterial diversity is also expected in mites due to equally significant morphological and physiological changes from eggs to adults.

Specialization of the microbiome has also had effects on processes such as reproduction. For example, Wolbachia and Cardinium endosymbionts distort sex ratios and affect fitness and survival in a wide variety of arthropods and other invertebrates [8,9,14,16,22,23]. In fact, it has been shown that elimination of Cardinium results in decreasing survival and reproductive fitness in insects [24]. If the same situation holds true for B. yothersi, it is expected that the vertically-transmitted Cardinium will maintain or increase its abundance at different life stages of the host. To the best of our knowledge, no deep assessment of this kind has been performed for the bacterial communities and abundance of Cardinium in B. yothersi.

We made an assessment of the bacterial communities in populations of B. yothersi obtained from different plant hosts and at different life stages. Understanding the microbiota associated to this species will enhance our knowledge about the evolution of its asexuality, and further develop strategies for its management. Specifically, we defined the composition and abundance of different bacterial groups and tested for changes in abundance of the endosymbiont Cardinium between mite populations and their adult and egg stages.

2. Materials and Methods

2.1. Collection, Identification, and Laboratory Rearing of Mite Species

Specimens of Brevipalpus mites were initially identified as B. phoenicis, but are now recognized as B. yothersi Baker [5]. Those specimens were originally collected from three different hosts in Puerto Rico: Sweet orange (Citrus sinensis, Rutaceae), Tahiti lime (Citrus latifolia, Rutaceae), and glory-bower (Clerodendrum thomsoniae, Lamiaceae). Mite colonies were established by a single egg and maintained in fruit-arenas of “Valencia” sweet orange for 10 generations in an environmental chamber (Precision Model 818; Thermo Scientific, Waltham, MA, USA) at 25 °C and 75% relative humidity [25]. As outgroups, all in the Tetranychinoidea superfamily, we included samples of adult Tetranychus evansi obtained from tomato plants (Solanum lycopersicum, Solanaceae) in the field, as well as colonies of Raoiella indica from coconut (Cocos nucifera, Arecaceae), and an Oligonychus mite (from dry beans, Phaseolus vulgaris, Fabaceae) collected at the Rio Piedras Agricultural Station. For identification, specimens from all species were slide-mounted in Hoyer’s medium and barcoded using previously described primers for the mitochondrial Cytochrome Oxidase Subunit I gene (COI) [25]. Accession numbers for the mites used in this study were deposited at GenBank (Accession numbers: KP180424 to KP180429).
2.2. Genomic DNA Extraction and Pyrosequencing

We extracted total genomic DNA from 20 eggs and 20 mites per colony following the CTAB (Cetyl Trimethyl-Ammonium Bromide) method used in previous studies [25,26]. The DNA extraction manipulations were conducted in a clean laminar flow chamber and we used certified DNA-free tubes and reagents. The mites were washed in 0.05% hypochlorite bleach and rinsed thoroughly with DNA-free water before DNA extraction. We used the primers bac515F (5′-GTGCCAAGCMGCCGCGGTAA-3′) and bac806R (5′-GTGCCAAGCMGCCGCGGTAA-3′) to amplify ≈250 bp of the V4 region coding for part of the 16S Ribosomal Subunit (16S) with the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany). PCR conditions included an initial stage of 3 min at 94 °C, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 5 min. Negative control samples (no mites) were included and processed equally during all steps in order to detect eventual bacterial contamination.

A construct composed by the primers previously mentioned, a linker sequence, and a sample-specific oligonucleotide tag (a.k.a barcode) was incorporated for sequencing on a 454 GS FLX System (Roche, Branford, CT, USA) (Table 1). The inclusion of this linker into the amplicons was made with a second PCR using 1 µL of the previous PCR product under the same conditions. A purification of these PCR products was done with the Agencourt Ampure Beads (Agencourt Bioscience, La Jolla, CA, USA). An estimation of the size and concentration of the amplicons was made by using DNA chips and the Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA). The FLX sequencing solution contained $9.6 \times 10^6$ DNA molecules/µL mixed with 6 million binding beads. This DNA-bead solution was amplified by using emulsion PCR [27] and then, DNA on the binding beads was denatured with NaOH. An FLX sequencing run was performed using a Genome Sequencer FLX System (Roche) on a 70 × 75 GS Picotiter Plate (PTP) by following instructions from the manufacturer.

Table 1. Specific tags used to identify each of the samples after multiplexed FLX sequencing.

| Species                  | Host Plant | Stage | Tag          |
|--------------------------|------------|-------|--------------|
| *Brevipalpus yothersi*   | Glory-bower| eggs  | GAGATCAG     |
|                          |            | adults| GAGTAGAC     |
|                          | Sweet orange| eggs  | GAGATCTC     |
|                          |            | adults| GAGTAGAC     |
|                          | Tahiti lime| eggs  | GAGTACTC     |
|                          |            | adults| GAGTACAG     |
| *Raoiella indica*        | Coconut    | eggs  | GAGTCACT     |
|                          |            | adults| GATGCAG      |
| *Oligonychus sp.*        | Bean       | eggs  | GATGAGCA     |
|                          |            | adults| GATGAGGT     |
| *Tetranychus evansi*     | Tomato     | adults| GAGTAGTG     |

2.3. Data Processing and Quality Control

We performed an initial processing and filtering of the sequence reads by using a pipeline of scripts included in the MacQIIME 1.8.0 distribution [28]. Sequence (.fasta) and quality (.qual) files were obtained from the flowgram file (.sff) generated by the FLX platform using QIIME’s “process_sff.py” script. During the de-multiplexing, we also eliminated the primer, linkers, and barcodes from the reads. Sequences with average Quality Score lower than 25 were discarded from subsequent analysis. This new set of filtered sequences was denoised and aligned in PyNAST v1.2 against the Greengenes reference alignment [29]. Sequences with less than 30% in similarity to the reference alignment were excluded from subsequent analysis. Identification and elimination of chimeric sequences was performed with ChimeraSlayer v4.29.2010 as implemented in MacQIIME and using the template alignment provided by PyNAST.
2.4. Analysis of Bacterial Diversity

We assigned the taxonomy and produced estimations diversity with the pipeline implemented in MacQIIME. Following, we present some of the key points of this pipeline. We made clusters based on a 97% of similarity and identified Operational taxonomic units (OTUs) in the quality-filtered dataset. Each OTU was assigned taxonomy by using the Ribosomal Database Project (RDP) Classifier v2.11 [30] and singletons were discarded from subsequent analyses.

To estimate diversity inside each sample (i.e., alpha diversity) we rarefied the sequences 10 times adding 50 sequences by rarefaction. Averages for the Chao1 and Shannon indexes were calculated based on such rarefactions. Based on the rarefied dataset, we also estimated the unweighted and weighted UniFrac distances, and the Bray-Curtis dissimilarity index. With these estimators, a Principal Coordinate Analysis (PCoA) was performed.

2.5. Phylogenetic Analysis of Cardinium Strains

We extracted from the entire dataset the OTUs assigned as *Cardinium* by the RDP Classifier. Additionally, we obtained representative sequences from all the *Cardinium* available at GenBank regardless of the host where it was isolated. Maximum Parsimony trees were created using the algorithm included in MEGA v6 [31]. The resulting consensus tree was resampled 1000 times by bootstrapping and branches with less than 50% in support were collapsed.

3. Results

3.1. Composition of the Bacteriomes

Sequences were deposited in GenBank Bioproject PRJNA354805. This is the first assessment of the bacterial communities in *Brevipalpus* and other Tetranychidae. Our results show that the bacterial communities of those Tetranychidae were dominated by Bacteroidetes and Proteobacteria. Other groups present in lesser proportions were Actinobacteria, and Firmicutes (Figure 1). Among these, we found OTUs assigned to known bacterial endosymbionts belonging to the genera *Cardinium* (OTUs 33, 190, 233, 314, 335, 445, 457, 460, 547, 625, 805, 843, 888), *Portiera* (OTU 863), *Tremblaya* (OTU 516), and *Wolbachia* (OTU 1043). The most abundant of the endosymbionts was *Cardinium*, being present in about 22% of all sequences; however, it was not present in *R. indica* or *T. evansi* (Table 2). Representative 16S sequences for *Cardinium* and the other endosymbionts are deposited at GenBank (Accession numbers: KX844704 to KX844707). A table with the OTUs for the bacterial taxa found in these mites and their identification numbers is provided as Supplementary Materials (Table S1).

| Mite Species         | Host      | Stage   | Cardinium | Portiera | Tremblaya | Wolbachia |
|----------------------|-----------|---------|-----------|----------|-----------|-----------|
| *Brevipalpus yothersi* | Glory-bower | Eggs 7.9 | -         | -        | -         | -         |
|                      |           | Adults 71.5 | -         | -        | -         | -         |
|                      | Sweet orange | Eggs 11.1 | -         | -        | 0.1       | -         |
|                      |           | Adults 26.5 | -         | -        | -         | -         |
|                      | Tahiti lime | Eggs 6.4 | 0.3       | -        | -         | -         |
|                      |           | Adults 22.2 | -         | -        | -         | -         |
| *Raoiella indica*    | Coconut   | Eggs - | -         | -        | -         | -         |
|                      |           | Adults - | -         | <0.1     | -         | -         |
| *Oligonycus sp.*     | Bean      | Eggs 12.6 | -         | -        | -         | -         |
|                      |           | Adults 90.9 | -         | -        | -         | -         |
| *Tetranychus evansi* | Tomato    | Adults - | -         | -        | -         | -         |
| All the sequences    |          | 22.25   | 0.03      | <0.01    | <0.01     | <0.01     |
were proportionally reduced in adults when compared to eggs. B. yothersi was higher than that of the other mites (Chao1 = 28.1); however, the were the most species-rich sample (Table 3). Nevertheless, the most diverse bacterial community was obtained from R. indica from coconut palms (Shannon = 4.2). Overall, B. yothersi richness (Chao1 = 31.5) was higher than that of the other mites (Chao1 = 28.1); however, the B. yothersi bacteriome was less diverse (Shannon = 2.4) than that of the other species (Shannon = 3.0). When comparing B. yothersi eggs with their adults, we observed that increase of Cardinium was accompanied by a decrease in the proportion of other OTUs. Specifically, OTUs assigned to Pseudomonadaceae and Burkholderiaceae

As suggested by the Chao1 index, endosymbionts in adult Oligonychus sp. from beans were the least species-rich sample (Chao1 = 11.2), and adult B. yothersi from C. aurantifolia (Chao1 = 45.6) were the most species-rich sample (Table 3). Nevertheless, the most diverse bacterial community was obtained from R. indica from coconut palms (Shannon = 4.2). Overall, B. yothersi richness (Chao1 = 31.5) was higher than that of the other mites (Chao1 = 28.1); however, the B. yothersi bacteriome was less diverse (Shannon = 2.4) than that of the other species (Shannon = 3.0). When comparing B. yothersi eggs with their adults, we observed that increase of Cardinium was accompanied by a decrease in the proportion of other OTUs. Specifically, OTUs assigned to Pseudomonadaceae and Burkholderiaceae were proportionally reduced in adults when compared to eggs.

Table 3. Average estimations of bacteria diversity (±standard deviation) based on rarefaction of samples.

| Species          | Host Plant | Stage  | Chao1     | Shannon   |
|------------------|------------|--------|-----------|-----------|
| Brevipalpus yothersi | Glory-bower | eggs   | 100.88 ± 16.95 | 3.32 ± 0.13 |
|                  |            | adults | 94.75 ± 16.26  | 2.79 ± 0.09 |
|                  | Sweet orange | eggs   | 95.20 ± 11.62  | 2.73 ± 0.09 |
|                  |            | adults | 55.93 ± 9.46   | 2.12 ± 0.07 |
|                  | Tahiti lime | eggs   | 97.66 ± 18.88  | 2.36 ± 0.10 |
|                  |            | adults | 146.02 ± 21.25 | 4.24 ± 0.09 |
| Raoiella indica  | Coconut    | eggs   | 82.31 ± 15.13  | 4.41 ± 0.09 |
|                  |            | adults | 115.49 ± 15.59 | 4.74 ± 0.10 |
| Oligonychus sp.  | Bean       | eggs   | 79.46 ± 7.19   | 4.42 ± 0.09 |
|                  |            | adults | 47.17 ± 13.36  | 1.00 ± 0.07 |
| Tetranychus evansi | Tomato     | adults | 54.52 ± 4.45   | 3.66 ± 0.05 |

In general, the bacterial communities containing OTUs assigned to Cardinium were more similar than those lacking the endosymbiont (ANOSIM R_{BrayCurtis/UniFrac} = 0.84/0.59, p < 0.05, Figure 2). Specifically, the bacteriomes of the Oligonychus mite and B. yothersi were more similar among them.
than to those found in the other mite species. Similarity was higher between the less diverse bacterial communities in the eggs of *B. yothersi*, than to the bacteriomes from their respective adults. As expected, mites where *Cardinium* was the predominant taxa (adult *Oligonychus* sp. and adult *B. yothersi* from *C. thomsoniae*) were almost identical. The bacteriome of *T. evansi* adults was more similar to that of *R. indica* than to other bacteriomes in this study.

**Figure 2.** Principal Coordinate Analysis for the dissimilarity among bacterial communities.

### 3.2. Analysis of Cardinium Endosymbionts

The results show that inside each sample of *B. yothersi*, the proportion of OTUs designated as *Cardinium* is higher in adults than in the eggs (Figure 1). In the sample from *C. thomsoniae*, *Cardinium* counts were significantly lower in eggs than in adults (7.9% vs. 71.5%, $\chi^2 = 2600.3$, $p < 0.001$). For the sample obtained from *C. sinensis*, we obtained the same pattern (11.1% vs. 26.5%, $\chi^2 = 322.5$, $p < 0.001$), as well for the mites collected originally on *C. aurantifolia* (6.4% vs. 22.2%, $\chi^2 = 3164.8$, $p < 0.001$). A significant increase in the amount of counts of *Cardinium* sequences were also observed in *Oligonychus* mites obtained from beans. Specifically, *Oligonychus* sp. eggs had 12.6% *Cardinium* sequences whereas the adults had 90.9% ($\chi^2 = 546.4$, $p < 0.001$).

Our phylogenetic analysis of the sequences from *Cardinium* isolated from different hosts showed that bacteria from Hemiptera, Hymenoptera, and mites form a monophyletic clade. Inside this clade, two main groups can be recognized. One of these groups is dominated by *Cardinium* endosymbionts originating from Hemiptera (whiteflies and scale insects) and some Hymenoptera. The second group contains all the *Cardinium* sequences from this study and isolates from other mites and armored scale insects (Diaspididae: Hemiptera). *Cardinium* from copepods and mosquitoes were placed external to the monophyletic clade, suggesting that these arthropods harbor highly differentiated lineages of these bacteria (Figure 3, Supplementary Materials: Figure S1, Table S2).
The low diversity in arthropod microbiomes, if not an artifact of the analyses, could be attributed to the endosymbiont may evoke responses in the host that ultimately favor their proliferation. These advantages conferred by the endosymbiont may result in a decrease of bacteriome diversity and species richness as has been seen in ticks and fleas. In several systems, the dominant endosymbiont confers an advantage in fitness, feeding, or survival to the host [17,24,48]. Even though the immune system may be playing a capital role in shaping the diversity of arthropod microbiomes, there are additional possibilities that we will discuss here.

4. Discussion

4.1. Factors Affecting Diversity in Bacterial Communities

The low number of taxa present in the bacterial communities of the mites in this study is concordant with other studies on arthropod bacteriomes [35,36]. Our results support the idea of a “core bacteriome” on animals [37], represented by Proteobacteria, Bacteroidetes, and Actinobacteria. The fact that bacteriome alpha diversity of these mites is reduced in adults in comparison to eggs reflects the existence of mechanisms that are yet to be described, which are involved in the definition of bacterial assemblages. An increasing number of papers report that competition can be an important driver of reduction of diversity in microbial communities [39–42]. Most of the studies on arthropod gut microbiomes show that competition may even lead to exclusion of taxa by several mechanisms such as the depletion of nutrients [43] and pH changes [44].

In many cases, such as in these mites, the endosymbiont Cardinium increases its relative abundance in the bacterial community possibly by means of competitive exclusion. This would result in a decrease of bacteriome diversity and species richness as has been seen in ticks and fleas [45,46]. However, the host may also shape the bacterial community by generating the conditions for certain bacterial taxa to dominate, a situation known as habitat filtering [47]. In several systems, the dominant endosymbiont confers an advantage in fitness, feeding, or survival to the host [17,24,48]. These advantages conferred by the endosymbiont may evoke responses in the host that ultimately favor their proliferation.
throughout the life of the host. As our results show, the endosymbiont Cardinium was significantly more abundant in adults of B. yothersi and Oligonychus sp. than in their respective eggs. Similarly, Sphingomonas sp. (known endosymbiont in non-arthropod systems [49]) was detected at higher proportions in adults than in eggs of R. indica. We were not able to obtain samples from T. evansi eggs, but we suspect that Erwinia sp. [50] would also be more abundant in the eggs of T. evansi than in the adults.

Environmental factors can also influence the composition of microbial communities. The mites in this study come from different hosts and locations that represent the founding bacterial populations. Although Cardinium was abundantly present in the three B. yothersi colonies, the proportion of Cardinium varied among them. The colony from Glory-bower showed a dominance of Cardinium over other bacterial taxa, which makes this bacterial community more similar to that found in Oligonychus sp. In fleas and ticks that feed on mammals, it has been shown that microbial communities are affected mostly by environmental factors external to the mammal and by mechanisms dictated by the arthropod itself [46,51]. The results in this study are in agreement with the idea that different mite species may converge to have similar bacterial community profiles, and conspecific mites originating from different plant hosts may show different bacterial assemblages.

In this sense, habitat filtering (i.e., original host-plant selection) may be a strong driving force in shaping the bacteriomes of the mites in this study, possibly leading to specialization in the function of these bacterial communities. This filter imposed by the habitat can be seen as the range of conditions and resources that the mite host offers [52]. The host “selects” only a fraction of all the initial diversity, possibly depending upon its physiological requirements. It would also be expected that habitat filtering results in the components of the bacteriome being more phylogenetically related due to host requirements or inability of the symbiont to adapt to different conditions [53–55]. In the mites from this study, most of the bacterial species belong to either Bacteroidetes (such as Cardinium) or Proteobacteria, which indicates a higher degree of phylogenetic relatedness than would be observed in other communities with lower selective pressures. Nevertheless, this work only represents a snapshot of the existing conditions and under natural, non-controlled habitats, different degrees of competition and habitat filtering may occur depending upon the assessed spatial and temporal scale [56].

4.2. Phylogenetic Relationship among Cardinium Strains in Different Systems

The phylogenetic analysis of Cardinium strains failed to show convincing evidence that supports phylogenetic correlation between the evolution of these bacteria and their hosts. Previous works on the distribution of Cardinium indicated that physiological specialization is noticeable due to phylogenetic clustering of Cardinium strains from closely related hosts [57]. Although this idea is not clearly observed in our results, it is possible to infer that Cardinium strains show greater phylogenetic relatedness with the Cardinium strains present in other mites than to hemipterans and hymenopterans. Additionally, Cardinium from copepods and mosquitoes represent highly differentiated lineages from those found in other insects and mites. Although we could not show strong evidence for the idea of the phylogenetic clustering of Cardinium and their hosts, our analysis provides evidence about potential horizontal transmission as an important process in the evolution of Cardinium and their hosts.

5. Conclusions

This study represents the first time that the Next Generation Sequencing approach was used on microbial communities of the agriculturally important Tetranychidae mites [58]. Our results suggest that the bacteriomes of these mites are relatively low in diversity, as in many other arthropods, although diversity was higher in eggs than in adults. The differences between colonies of B. yothersi and similarities among species in regards to the composition of the bacterial assemblages shows that the hosts may be differentially filtering the taxa in their bacteriomes. However, we recognize that competition among bacterial species could also contribute to changes in diversity at different degrees depending upon the assessed temporal and spatial scale. The phylogenetic history of Cardinium
Insects 2016, 7, 80

seems to be driven by physiological specialization of the host and horizontal transmission acting at different degrees. It seems implausible that the asexuality associated with Cardinium in arthropods represents an evolutionary dead end [59,60] on the basis that divergence is still extensive at least for the endosymbiont. Understanding the bacteriome associated with the unique biological system represented by B. yothersi and other Tetranychoidea is certainly an important step towards enhancing knowledge about the evolution of its asexuality, and might further help to develop strategies for its pest management.

Supplementary Materials: The following are available online at www.mdpi.com/2075-4450/7/4/80/s1. Figure S1: Detailed Maximum Parsimony for Cardinium sequences from this study and others, Table S1: OTU table (contains the counts and taxonomic assignation for each OUT in this study), Table S2: Sequences from GenBank published elsewhere that were used in this study for phylogenetic inference in Cardinium.

Acknowledgments: We want to thank the U.S. Department of Agriculture for partial funding of this work (USDA/APHIS 8272-0685 CA). Thanks to Jessica Falero for collecting the initial Brevipalpus populations. Thanks to Jennifer Beard (Queensland Museum) and Ronald Ochoa (USDA-BARC, Beltsville) for sharing the information about the taxonomic status of Brevipalpus mites and comments to improve the manuscript. Thanks to Carl Childers for comments on the original manuscript draft. Thanks to anonymous reviewers for exceptional suggestions to improve the manuscript.

Author Contributions: Jose Rodrigues and Steven Massey conceived and designed the study; Jose Rodrigues prepared the mite colonies and performed data collection; Oscar Ospina and Steven Massey analyzed the data; Oscar Ospina, Jose Rodrigues, and Steven Massey wrote and reviewed the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Kitajima, E.W.; Rezende, J.A.M.; Rodrigues, J.C.V. Passion fruit green spot virus vectored by Brevipalpus phoenicis (Acari: Tenuipalpidae) on passion fruit in Brazil. Exp. Appl. Acarol. 2003, 30, 225–231. [CrossRef] [PubMed]
2. Kitajima, E.W.; Rodrigues, J.C.V.; Freitas-Astua, J. An annotated list of ornamentals naturally found infected by Brevipalpus mite-transmitted viruses. Sci. Agric. 2010, 67, 348–371. [CrossRef]
3. Rodrigues, J.C.V.; Childers, C.C. Brevipalpus mites (Acari: Tenuipalpidae): Vectors of invasive, non-systemic cytoplasmic and nuclear viruses in plants. Exp. Appl. Acarol. 2013, 59, 165–175. [CrossRef] [PubMed]
4. Beard, J.J.; Ochoa, R.; Bauchan, G.R.; Trice, M.D.; Redford, A.J.; Walters, T.W.; Mitter, C. Flat Mites of the World. Available online: http://idtools.org/id/mites/flatmites/ (accessed on 8 September 2016).
5. Beard, J.J.; Ochoa, R.; Braswell, W.E.; Bauchan, G.R. Brevipalpus phoenicis (Geijskes) species complex (Acari: Tenuipalpidae)—A closer look. Zootaxa 2015, 3944, 1–67. [CrossRef] [PubMed]
6. Rodrigues, J.C.V.; Kitajima, E.W.; Childers, C.C.; Chagas, C.M. Citrus leprosis virus vectored by Brevipalpus phoenicis (Acari: Tenuipalpidae) on citrus in Brazil. Exp. Appl. Acarol. 2003, 30, 161–179. [CrossRef] [PubMed]
7. Carstens, E.B. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). Arch. Virol. 2010, 155, 133–146. [CrossRef] [PubMed]
8. Weeks, A.R.; Marec, F.; Breeuwer, J.A.J. A mite species that consists entirely of haploid females. Science 2001, 292, 2479–2482. [CrossRef] [PubMed]
9. Zchori-Fein, E.; Gottlieb, Y.; Kelly, S.E.; Brown, J.K.; Wilson, J.M.; Karr, T.L.; Hunter, M.S. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. Proc. Natl. Acad. Sci. USA 2001, 98, 12555–12560. [CrossRef] [PubMed]
10. Feldhaar, H.; Straka, J.; Krischke, M.; Berthold, K.; Stoll, S.; Mueller, M.J.; Gross, R. Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. BMC Biol. 2007. [CrossRef] [PubMed]
11. Sabree, Z.L.; Kambhampati, S.; Moran, N.A. Nitrogen recycling and nutritional provisioning by Blattabacterium, the cockroach endosymbiont. Proc. Natl. Acad. Sci. USA 2009, 106, 19521–19526. [CrossRef] [PubMed]
12. Oliver, K.M.; Moran, N.A.; Hunter, M.S. Variation in resistance to parasitism in aphids is due to symbiosis not host genotype. Proc. Natl. Acad. Sci. USA 2005, 102, 12795–12800. [CrossRef] [PubMed]
13. Scarborough, C.L.; Ferrari, J.; Godfray, H.C.J. Aphid protected from pathogen by endosymbiont. Science 2005. [CrossRef] [PubMed]
14. Stouthamer, R.; Breeuwer, J.A.J.; Hurst, G.D. Wolbachia pipiens: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* 1999, 53, 71–102. [CrossRef] [PubMed]
15. Pais, R.; Lohs, C.; Wu, Y.; Wang, J.; Aksoy, S. The obligate mutualist Wigglesworthia glossinidia influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Appl. Environ. Microbiol.* 2008, 74, 5965–5974. [CrossRef] [PubMed]
16. Werren, J.H.; Baldi, L.; Clark, M.E. Wolbachia: Master manipulators of invertebrate biology. *Nat. Rev.* 2008, 6, 741–751. [CrossRef] [PubMed]
17. Oliver, K.M.; Degnan, P.H.; Burke, G.R.; Moran, N.A. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol.* 2010, 55, 247–266. [CrossRef] [PubMed]
18. Cordaux, R.; Bouchon, D.; Greve, P. The impact of endosymbionts on the evolution of host sex-determination mechanisms. *Trends Genet.* 2011, 27, 332–341. [CrossRef] [PubMed]
19. Lombardo, M.P. Mutualistic endosymbiotic microbes: An underappreciated benefit of group living. *Behav. Ecol. Sociobiol.* 2008, 62, 479–497. [CrossRef]
20. Wang, Y.; Gilbreath, T.M.; Kukutla, P.; Yan, G.; Xu, J. Dynamic gut microbiome across life history of the stored-product pest Liposcelis bostrychophila (Psocodea: Liposcelididae). *J. Econ. Entomol.* 2008, 101, 1711–1717. [CrossRef]
21. Vasanthakumar, A.; Childers, C.C.; Adams, B.J. Mitochondrial DNA and RAPD polymorphisms in the haploid mite Brevipalpus phoenicis (Acari: Tenuipalpidae). *Exp. Appl. Acarol.* 2004, 34, 275–290. [CrossRef] [PubMed]
22. Hori, M.; Fukano, H.; Suzuki, Y. Uniform amplification of multiple DNAs by emulsion PCR. *Biochem. Biophys. Res. Commun.* 2007, 352, 323–328. [CrossRef] [PubMed]
23. Caporaso, J.G.; Bittinger, K.; Bushman, F.D.; Bushman, J.; Vazquez-Prokopec, G.; Henderson, J.; Goodrich, J.K.; Kuczynski, J.; Stombaugh, J.; Costello, E.K.; Fierer, N.; Knights, D.; Ward, J.; Kreisberg, J.; Harper, K.; Yark, B.; Zhang, N.; Sevinsky, J.R.; Hanke, W.; Judd, A.; Gill, J.; Alpcan, T.; Knights, C.; Kono, A.; Lepp, M.; Liu, T.; Turner, R.; Zaneveld, J.; McDonald, D.; Furr, W.; Gill, S.; Nelson, W.; Gilroy, C.; Osborn, D.; Subramanian, S.; Schweizer, R.; Bell, J.; Vega Thurber, R.; Henrichs, S.; Sogin, M.; Knight, R. Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 2010, 7, 335–336. [CrossRef] [PubMed]
24. Caporaso, J.G.; Bittinger, K.; Bushman, F.D.; Desantis, T.Z.; Andersen, G.L.; Knight, R. PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics* 2010, 26, 266–267. [CrossRef] [PubMed]
25. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 2007, 73, 5261–5267. [CrossRef] [PubMed]
26. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 2013, 30, 2725–2729. [CrossRef] [PubMed]
27. Baumann, P. Biology of bacteriocye-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* 2005, 59, 155–189. [CrossRef] [PubMed]
28. Moran, N.A.; McCutcheon, J.P.; Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* 2008, 42, 165–190. [CrossRef] [PubMed]
29. Mcneill, M.R.; Richards, N.K.; White, J.A.; Laugraud, A. Hidden arsenal: Endosymbionts in arthropods, their role and possible implications for biological control success. *N. Z. Plant Prot.* 2014, 67, 204–212.
30. Moran, N.A.; Hansen, A.K.; Powell, J.E.; Sabree, Z.L. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS ONE* 2012, 7, e36393. [CrossRef] [PubMed]
36. Broderick, N.A.; Raffa, K.F.; Goodman, R.M.; Handelsman, J. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl. Environ. Microbiol.* 2008, 74, 7597–7606. [CrossRef] [PubMed]

37. Jones, R.T.; Sanchez, L.G.; Fierer, N. A cross-taxon analysis of insect-associated bacterial diversity. *PLoS ONE* 2013, 8, e61218. [CrossRef] [PubMed]

38. Juchault, P.; Rigaud, T.; Mocquard, J.-P. Evolution of sex-determining mechanisms in a wild population of *Armadillidium vulgare* Latr. (Crustacea, Isopoda): Competition between two feminizing parasitic sex factors. *Heredity* 1992, 69, 382–390. [CrossRef]

39. Juchault, P.; Rigaud, T.; Mocquard, J.-P. Evolution of sex-determining mechanisms in a wild population of *Armadillidium vulgare* Latr. (Crustacea, Isopoda): Competition between two feminizing parasitic sex factors. *Heredity* 1992, 69, 382–390. [CrossRef]

40. Macaluso, K.R.; Sonenshine, D.E.; Ceraldi, S.M.; Azad, A.F. Ricketsial infection in *Dermacentor variabilis* (Acari: Ixodidae) inhibits transovarial transmission of a second *Rickettsia*. *J. Med. Entomol.* 2002, 39, 809–813. [CrossRef] [PubMed]

41. Kondo, N.; Shimada, M.; Fukatsu, T. Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. *Biol. Lett.* 2005, 1, 488–491. [CrossRef] [PubMed]

42. Hughes, G.L.; Dodson, B.L.; Johnson, R.M.; Murdock, C.C.; Tsujimoto, H.; Suzuki, Y.; Patt, A.A.; Cui, L.; Noss, C.W.; Barry, R.M.; et al. Native microbiome impedes vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. *Proc. Natl. Acad. Sci. USA* 2014, 111, 12498–12503. [CrossRef] [PubMed]

43. Indiragandhi, P.; Anandham, R.; Madhaiyan, M.; Kim, G.H.; Sa, T. Cross-utilization and expression of outer membrane receptor proteins for siderophore uptake by Diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) gut bacteria. *FEMS Microbiol. Lett.* 2011, 320, 239–244. [CrossRef]

44. Pearson, M.; Simpson, S.J.; Ponton, F. Towards an integrated understanding of gut microbiota using insects as model systems. *J. Insect Physiol.* 2014, 69, 12–18. [CrossRef] [PubMed]

45. Levy, R.; Borenstein, E. Metabolic modeling of species interaction in the human microbiome elucidates community-level assembly rules. *Proc. Natl. Acad. Sci. USA* 2013, 110, 12804–12809. [CrossRef] [PubMed]

46. Hawlena, H.; Rynkiewicz, E.; Toh, E.; Alfred, A.; Durden, L.A.; Haslter, M.W.; Nelson, D.E.; Rong, R.; Munro, D.; Dong, Q.; et al. The arthropod, but not the vertebrate host or its environment, dictates bacterial community composition of fleas and ticks. *ISME J.* 2007, 1, 394–402. [CrossRef] [PubMed]

47. Petrov, D.; Bozic, I.; Chiu, W.C.; et al. Positive selection and pleiotropy at the human microbiome. *Science* 2012, 336, 1255–1262. [CrossRef] [PubMed]

48. Levy, R.; Borenstein, E. Metabolic modeling of species interaction in the human microbiome elucidates community-level assembly rules. *Proc. Natl. Acad. Sci. USA* 2013, 110, 12804–12809. [CrossRef] [PubMed]

49. Lee, J.; Shin, S.C.; Kim, S.J.; Kim, B.K.; Hong, S.G.; Kim, E.H.; Park, H.; Lee, H. Draft genome sequence of a *Rickettsia felis*-uninfected and -infected colonized cat fleas, *Ctenocephalides felis*. *ISME J.* 2007, 1, 394–402. [CrossRef] [PubMed]

50. Hawlena, H.; Rynkiewicz, E.; Toh, E.; Alfred, A.; Durden, L.A.; Haslter, M.W.; Nelson, D.E.; Rong, R.; Munro, D.; Dong, Q.; et al. The arthropod, but not the vertebrate host or its environment, dictates bacterial community composition of fleas and ticks. *ISME J.* 2007, 1, 394–402. [CrossRef] [PubMed]

51. Jones, R.T.; Knight, R.; Martin, A.P. Bacterial communities of disease vectors sampled across time, space, and species. *ISME J.* 2010, 4, 223–231. [CrossRef] [PubMed]

52. Costello, E.K.; Stagaman, K.; Dethlefsen, L.; Bohannan, B.J.M.; Relman, D.A. The application of ecological theory toward an understanding of the human microbiome. *Science* 2012, 336, 1255–1262. [CrossRef] [PubMed]

53. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

54. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

55. Costello, E.K.; Stagaman, K.; Dethlefsen, L.; Bohannan, B.J.M.; Relman, D.A. The application of ecological theory toward an understanding of the human microbiome. *Science* 2012, 336, 1255–1262. [CrossRef] [PubMed]

56. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

57. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

58. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

59. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

60. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

61. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

62. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

63. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

64. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

65. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

66. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

67. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

68. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

69. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]

70. Petter-Ridge, J.; Blackwell, M.; Brodie, E.L. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odonotaenius disjunctus*. *ISME J.* 2009, 3, 54–61. [CrossRef] [PubMed]
57. Zchori-Fein, E.; Perlman, S.J. Distribution of the bacterial symbiont Cardinium in arthropods. *Mol. Ecol.* **2004**, 13, 2009–2016. [CrossRef] [PubMed]

58. Chaisiri, K.; McGarry, J.W.; Morand, S.; Makepeace, B.L. Symbiosis in an overlooked microcosm: A systematic review of the bacterial flora of mites. *Parasitology* **2015**, *142*, 1152–1162. [CrossRef] [PubMed]

59. Maynard-Smith, J. *The Evolution of Sex*; Cambridge University Press: Cambridge, UK, 1978.

60. Lynch, M.; Burger, R.; Butcher, D.; Gabriel, W. The mutational meltdown in asexual populations. *J. Hered.* **1993**, *84*, 339–344. [PubMed]