Characterizing Variation in the Abundance of the Milkweed Aphid Symbiont *Arsenophonus*

Kylie Schofield, Laramy Enders

Abstract

Microorganisms can influence plant-insect interactions, as well as insect physiology. The bacterial genus *Arsenophonus* is a widespread insect symbiont and is associated with numerous ecological roles. It is known that *Arsenophonus* occurs in aphids and acts as a facultative symbiont. However, it is unknown how *Arsenophonus* varies across aphid species or the roles it plays. Previous research investigating three common milkweeds aphids suggested that *Arsenophonus* occurred in higher abundance in *Aphis asclepiadis* compared to *Aphis nerii* or *Myzocallis asclepiadis*. The goal of this study was to investigate if there were differences in *Arsenophonus* relative abundance across the three common milkweed aphids by (1) developing a methodology (i.e., qPCR assay) to estimate relative abundance of the symbiont in the milkweed aphids and (2) run the assay on field caught populations to quantify relative abundance differences of *Arsenophonus.* Here I show that *Arsenophonus* occurred in higher titer levels in *A. asclepiadis* than *A. nerii* or *M. asclepiadis.* Variation in *Arsenophonus* abundance across aphid species is likely a result of host plant specificity and has possibly lead to variations between the aphid species ecology. Overall, this study was an important initial step towards developing qPCR-based methods for quantifying differences in *Arsenophonus* titer levels across three common milkweed aphid species. There are numerous follow up studies that could further investigate strain, location, and roles of *Arsenophonus* in aphids.

Keywords: *Arsenophonus, Aphis asclepiadis, Aphis nerii, Myzocallis asclepiadis*, endosymbiont, common milkweed

Introduction

Microbes are ubiquitous in the environment and can shape plant-insect interactions in numerous ways (Mayoral-Peña et. al. 2020). Plants may utilize microbes to acquire and translocate (i.e., roots to shoots) nutrients, which can lead to increased growth and improved recovery from damage (e.g., herbivory) (Hansen & Enders, 2022). Plants can also use microbes to prime themselves against herbivores by increasing resistance and producing defensive chemicals (Hansen & Enders, 2022). From an insect's perspective, microbes can supplement essential nutrients not acquired through feeding (i.e., obligate symbionts) (Hansen & Enders, 2022). They can also facilitate an insect's use of a host plant by aiding in plant material digestion and detoxification of a plants defense chemicals (Enders et al., 2022; Hammer & Bowers, 2015; Hansen & Enders, 2022). Microorganisms can also shape insect physiology in many ways. Insect facultative symbionts are not necessary for their hosts survival, but are able to alter the host's performance, behavior, and fitness. They can impact host plant specificity, as

well as resistance to thermal fluctuations, parasitoids, and pathogens (Karimi et al., 2019). For example, high bacterial symbiont abundance in cereal aphids provides thermal tolerance (Majeed et al., 2022). However, it remains largely unknown how microbes vary across insects and what roles they play. Even for well-studied insect symbiotic microbes (e.g., *Buchnera, Wolbachia, Rickettsia* and *Arsenophonus*) there are still knowledge gaps and unknowns that need to be addressed.

Arsenophonus are a genus of bacteria with a diverse host range and ecological versatility. It has been found in a few plant species, but is mostly considered an insect associated intracellular symbiont, occurring in a wide variety of insect hosts (e.g., triatomine bugs, ant lions, parasitic wasps, psyllids, ticks, bees, lice, louse fly, date palm hopper, whitefly, aphids), as well as a few plant species (Chang et al., 2022; Nováková et al., 2009). *Arsenophonus* can spread via vertical (i.e., parent to offspring) and horizontal (i.e., between individuals) transmission and is primarily considered to be a facultative symbiont, but few strains may act as obligate symbionts (Karimi et al., 2019). This genus of bacteria has several roles that may impact a host's performance, resistance, and fitness; all are highly dependent on host species and microbe strain. In parasitoid wasps, *Arsenophonus* will alter the hosts reproductive behavior via male-killing, which can lead to distorted sex-ratios (Jousselin et al., 2013; Karimi et al., 2019; Nováková et al., 2009). When associated with a particular strain of *Arsenophonus*, brown plant hoppers experienced decreased insecticide resistance. *Arsenophonus* can also provide resistance against entomopathogenic fungi (Chang et al., 2022). When dosed with antibiotics to kill off *Arsenophonus*, date palm hoppers experienced slower developmental rates in various life stages, decreased nymph survival, and lower adult longevity (Karimi et al., 2019). Soybean aphids also have increased fitness when infected with *Arsenophonus* (Chang et al., 2022) and is thought to be a facultative symbiont in aphids in general. However, it is still largely unclear what role *Arsenophonus* plays in the ecology or physiology of many aphid species, or to what extent symbiont abundance may vary across aphid species.

Previous research investigating the microbial symbiont communities of three aphid species (*Aphis nerii, Aphis asclepiadis,* and *Myzocallis asclepiadis*), that can coexist on common milkweed, suggests *A. asclepiadis* have higher titer levels of *Arsenophonus* than either *A. nerii* or *M. asclepiadis* (Enders et al., 2022). It is currently unknown if *Arsenophonus* is a facultative symbiont of milkweed aphids or whether it's impact on aphid host varies across species. The aim of this study is to address whether there are differences in relative abundance of *Arsenophonus* occurring in the 3 common milkweed aphid species. The objectives for this study were (1) to develop a methodology (i.e., qPCR assay) to estimate the relative abundance of *Arsenophonus* endosymbiont in milkweed aphids and (2) quantify the relative abundance of *Arsenophonus* from field caught milkweed aphids using the qPCR assay. I hypothesized that *A. asclepiadis* would have higher titer levels of the endosymbiont *Arsenophonus* than both *A. nerii* and *M. asclepiadis*.

Methods

Sample collection and processing

The focal species of this study were 3 common milkweed aphids: *A. asclepiadis, A. nerii,* and *M. asclepiadis*. *A. nerii* are broad specialist (> 50 host plants), *A. asclepiadis* are narrow specialist (< 10 host plants), and *M. asclepiadis* is monophagous, only feeding on common milkweed (Enders et al., 2022). These three species of aphids coexist on common milkweed plants (*Asclepias syriaca*). (Smith et al., 2008). Adult *A. nerii* typically have bright lemon-yellow bodies and black appendages. Adult *A. asclepiadis* can be yellow- to pale- to olive-green with mottling. *M. asclepiadis* can be yellow- to brown-green with yellow/orange/red patches and black markings (Smith et al., 2008). Both *Aphis* species feed on apical leaves in groups, while *M. asclepiadis* usually feed dispersed, underneath lower leaves (Smith et al., 2008). Both *Aphis* species can have 2 adult morphs (unwinged and winged), while *M. asclepiadis* only have winged adults (Smith et al., 2008).

Aphids were collected on 08/16/2022 from a subset of sites used by Enders et. Al. 2022 in West Lafayette, IN. Locations were chosen by the presence of at least one common milkweed plant, with at least 5 aphids present. One to three leaf samples were collected from each plant with aphids present and were stored in freezer bags. If more than one plant was collected from at an individual site, then samples were pooled together. Species were stored separately, unless multiple species were found on the same plant/leaf sample. In these cases, mixed samples were placed in a sperate bag. Each bag was labeled with location/GPS, aphid species (single species or mixed), and date. Aphid samples were identified, via http://www.aphidsonworldsplants.info/, and then sorted by location and species into 1.5 ml tubes containing 100% molecular ethanol and then stored in a -20 C freezer until ready for analysis.

DNA extraction and gel electrophoresis

For objective 1, 12 DNA samples were prepared (5 *A. asclepiadis* per 1.5 ml tube). The aphids were collected previously by Dr. Laramy Enders, in 2017, and stored in a -20 C freezer until their use. For objective 2, 24 DNA samples were prepared (8 samples per aphid species [*A. nerii, A. asclepiadis,* and *M. asclepiadis*], 1 aphid per each 1.5 ml tube). Enough samples for each objective were made to have a few extra of each species. A Dneasy Blood and Tissue Kit was used and quick-start protocol was followed, with a few exceptions. For step 1, the crushed aphids were incubated at 56 C for 30 minutes. For step 8, the DNA was eluted with 50 ul Buffer AE (objective 1) and 20 ul (objective 2. To confirm enough DNA was extracted for objective 1, the samples (6 ul) were run through a 50 ml, 1% agarose gel at 85 Volts for 45 minutes. The gels were visualized using the BioRad Gel Doc EZ Imager machine and Image Lab software. Adequate DNA extraction material was confirmed by the presence of solid bands and minimal to no streaking. All DNA extraction samples were kept in a -20 C freezer until they were used for PCR and or qPCR.

Primers

The first objective was to develop a qPCR assay to estimate the relative abundance of the *Arsenophonus* endosymbiont in milkweed aphids. In order to account for individual aphid extraction efficiencies (i.e., differences in total DNA concentration extracted per individual aphid) and to later calculate relative abundance of *Arsenophonus*, it was necessary to test both the *Arsenophonus* gene and an aphid housekeeping gene (Ef1a). Both genes are single copy

genes and can be used to estimate the number of *Arsenophonus* and aphid genomes present in a DNA extraction. *Arsenophonus* titer levels for each individual aphid can be calculated as a relative measure of *Arsenophonus* gene copy numbers compared to the copy number of aphid genes (Ef1a).

In total, three *Arsenophonus* primers were tested (Ars_1, Ars_2, and Ars_3) and three Ef1a primers were tested (Ef1a_1, Ef1a_2, and Ef1a_3). The *Arsenophonus* primer template was a 150 bp segment of the 16s rRNA gene that was previously identified in Enders et.al. 2022 as a dominant strain found in *A. asclepiadis*. The template used to design the Ef1a primers was a sequence from *Aphis craccivora* (GenBank: MG252361.1). The program Primer BLAST on the National Center for Biotechnology Information GenBank database was used for primer design. The parameters used for design of primers for both *Arsenophonus* and Ef1a included PCR product size (min = 70, max = 150) and primer melting temperatures I (min = 57 C, opt = 60 C, max = 62 C). Each primer being tested came as a set of a forward and reverse primer. Additional information on each primer can be found in Supplemental document (Table 1).

PCR for Primer Optimization

Arsenophonus primers (Ars_1, Ars_2, and Ars_3) and Ef1a primers (ef1a_1, ef1a_2, and ef1a_3) were tested on 10/29/2022 and 12/15/2022, respectively. A Promega GOTaq PCR kit was used and the standard protocol was followed. The MasterMix recipe was created for each primer using the Promega GOTaq MasterMix Calculator. The same recipe was calculated and used for each primer, which can be found in the supplemental document (Table 2). Reagent amounts were calculated based on the number of total reactions; in this case, the reaction amount was calculated for 5 reactions per primer, with 3 DNA extraction samples, 1 positive control and 1 extra to account for any pipetting errors. Working solutions/aliquots of each primer set and dNTPs were made from stock solutions. All PCR reagents were then thawed (on ice) and the MasterMix was made. PCR reactions were set up for a total of 20 ul per PCR tube, with 4 ul of DNA extraction and 16 ul of appropriate PCR MasterMix (specific to the primer being tested). Negative controls contained 4 ul of nuclease free water instead of DNA extractions. PCR reactions were run in a Bio-Rad T100 Thermal Cycler with the following protocol: (1) Taq activation/denaturation @ 95 C for 2 min, (2) PCR cycling, repeat for 30 cycles (denaturation @ 95 C for 30 sec, annealing @ 60 C for 30 sec, and elongation @ 72 C for 1 min), (3) final extension @ 72 C for 5 min, and (4) Hold @ 4 C for infinite time (until removed from machine). The PCR samples were run through the gel electrophoresis protocol stated previously. Primers were further tested, via qPCR, if there were shown to amplify properly (i.e., singular bands, no smearing).

qPCR for Primer Optimization

Based on the PCR gel electrophoresis results, all *Arsenophonus* primers and two Ef1a primers (ef1a_1 and ef1a_3) were further tested using qPCR on 11/5/2022 and 12/16/2022, respectively. The iTaq Universal SYBR Green Supermix protocol was followed. The reaction mix recipe (supplemental document, Table 3) was calculated based on the number of reactions being tested. To test primer efficiency and whether the primers would amplify across a range of starting DNA concentrations, serial dilutions (1:10, 1:100, 1:1000) were made for each DNA sample (two *A. asclepiadis* DNA samples per primer). Each DNA sample and dilution had three

technical replicates. Each primer set had two negative control technical replicates. Into the wells of a clear qPCR plate, 18 ul reaction mix and 2 ul of DNA samples and dilutions were pipetted into the appropriate wells and mixed via pipetting up and down 2-3 times. After sealing and removing any air bubbles, plates were loaded into the Bio-Rad C1000 Touch Thermal Cycler CFX96 Real-Time System. Plates were ran using the following qPCR thermal cycling protocol: (1) polymerase activation and DNA denaturation @ 95 C for 2 min, (2) amplification (denaturation @ 95 C for 5 sec, annealing and extension @ 60 C for 30 sec), (3) repeat amplification steps 39x, and (4) melt curve analysis (65-95 C, increase by 0.5 C increments every 2-5 sec).

Primers were selected for further use based on PCR gel electrophoresis and qPCR for primer optimization results. Stated previously, PCR results for ef1a_2 showed little to no amplification (i.e., no clear bands) and was not further tested. After analyzing the qPCR for primer optimization results (i.e., amplification curve, standard curve, and melt peak), ef1a_3 and Ars_3 were eliminated from selection. Ars_1 and Ars_2 had similar results and thus I decided to move forward with Ars_2.

qPCR of Field Caught Aphids using Selected Primers

Objective 2 involved running a qPCR assay on field caught milkweed aphids (*A. asclepiadis, A. nerii,* and *M. asclepiadis*) using the optimized primers that were selected as a result of objective one (i.e., Ars_2 & ef1a_1). All qPCR protocols stated previously (i.e., iTaq Universal SYBR Green Supermix protocol and qPCR thermal cycling protocol) were followed, with the exception of using field caught samples instead of serial dilutions. Fifteen DNA extraction samples were selected; five samples per aphid species. Both primers (Ars_2 and ef1a_1) were used on each sample and each primer and sample grouping had two technical replicates. Each primer and aphid species grouping had two negative control technical replicates.

Data analysis and visualization

For objective 1, data visualization of qPCR reactions (i.e., amplification curve, standard curve, and melt peaks) were done via the Bio-Rad CFX Maestro program. The amplification curve depicts if a primer would amplify across a range of starting DNA concentrations. An ideal amplification curve would show evenly spaced and tight technical replicates groupings of each dilution (Taylor et al., 2015). The standard curve depicts how efficient a primer is amplifying DNA every cycle (i.e., efficiency value) and the coefficient of determination (i.e., R^2). An ideal efficiency value is between 80 and 110%. The R^2 value indicates how linear the data is and can give an idea if the efficiency is consistent across different DNA starting concentrations. A low R^2 value indicates wide variation in efficiency, whereas a high R^2 value indicates little variation; ideal R^2 values are ≥ 0.980 (Taylor et al., 2015). Melt peaks will give an indication if a single PCR product is being amplified (i.e., a single peak), which is desirable (Taylor et al., 2015).

For objective 2, qPCR data was extracted from the Bio-Rad CFX Maestro program and was further analyzed and visualized through Rstudio (version 2022.12.0+353). Code for analysis and visualization can be found in the Supplemental document. Averages of each sample's technical replicate Cq values, for both primers, were taken, with one exception. The second technical replicate for sample 15 did not amplify Ars_2; thus, the first technical replicate value

was used for sample 15 instead of an average of Ars_2 Cq values. Relative abundance of *Arsenophonus* was estimated using the equation $RA = 2^{-\Delta Cq}$, wherein Cq = (*Arsenophonus* Cq – Ef1a Cq). *Arsenophonus* relative abundance was calibrated to Ef1a to account for individual extraction efficiencies (i.e., differences in total DNA concentration extracted per individual aphid) by multiplying each samples RA by a correction factor. The correction factor for each sample was found by dividing the maximum Ef1a Cq by each sample Ef1a Cq value. Using the resulting corrected RA values, averages and standard errors for each species were calculated and used to create a bar plot examining differences in *Arsenophonus* relative abundance.

A non-parametric Kruskall-Wallis test was performed to indicate if there was at least one statistically significant different median of the corrected RA values of *Arsenophonus* between the aphid species. To examine pairwise comparisons of corrected RA values between each aphid species, a pairwise Wilcoxon rank sum test was performed. Lastly, percent difference of mean corrected RA values between aphid species were calculated to get an idea of the effect size of how different the means are between aphids.

Results

PCR gel electrophoresis

All three *Arsenophonus* primers (Ars_1, Ars_2, and Ars_3) (Figure 1) and ef1a_1(Figure 2) had clear bands with little to no smearing, meaning the primers were amplifying the DNA. Ef1a_3 had visible bands, but were not very clear, meaning there was some amplification, but not a lot. Ef1a_2 did not amplify any DNA (i.e., no clear bands visible) and was not further tested using qPCR.

qPCR for Primer optimization

Ars_1, Ars_2, Ars_3 and ef1a_1 all successfully amplified across a range of starting DNA concentrations indicated by the evenly spaced and tight groupings of the serial dilutions shown in figure 3 and 4. Although ef1a_3 was tested using qPCR, it did not amply and was eliminated from primer selection. Ars_1, Ars_2, and ef1a_1 primers were within the desirable efficiency value and R^2 value ranges (i.e., efficiency value between 80 and 110% and R^2 value ≥ 0.980) (Figure 5 and 6). Ars_3 primer had a too high efficiency value and too low R^2 value and was eliminated from further primer selection. Ars_1, Ars_2, and ef1a_1 each had a single melt peak with little to no variation, indicating a single PCR product being amplified (Figure 7 and 8). Ars_3 had a lot of variation in the melt peak and was eliminated from primer selection. Although Ars_1 and Ars_2 had similar results, due to time constraints of the study, only one *Arsenophonus* primer was chosen between the two (i.e., Ars_2).

qPCR of Field Caught Aphids using Selected Primers

Using the selected primers for the qPCR assay from objective one on field caught aphids indicated *Arsenophonus* titer levels were highest in *A. asclepiadis* (Figure 9). Between the aphid species corrected RA values, at least one median was statistically significantly different (Kruskall-Wallis test; p-value = 0.003735). Pairwise comparisons between the milkweed aphid species indicated each species *Arsenophonus* titer levels were significantly different from one another (Pairwise Wilcoxon rank sum test). Comparisons between *A. asclepiadis* and *A. nerri*

were approaching significance with a p-value of 0.056, whereas comparisons between *A. asclepiadis : M. asclepiadis* and *A. nerii : M. asclepiadis* were well below 0.05 (i.e., both p-values = 0.024). Percent difference of means showed that *A. asclepiadis* had 78.22% higher RA of *Arsenophonus* than *A. nerii* and 154.14% higher RA than *M. asclepiadis*. *A. nerii* had 108.68% higher RA of *Arsenophonus* than *M. asclepiadis*.

Discussion

Overall, I successfully developed a qPCR assay to measure relative abundance of the *Arsenophonus* endosymbiont in the three common milkweed aphids: *A. asclepiadis, A. nerii,* and *M. asclepiadis*. Initial assessment of field populations supports both the results reported by Enders et. Al. (2022) and my hypothesis that *A. asclepiadis* have higher titer levels of *Arsenophonus* endosymbiont than *A. nerii* and *M. asclepiadis*.

The variation in *Arsenophonus* titer levels could be a result of each aphid species host plant specificity, which can shape microbial communities in herbivores (Enders et al., 2022). Different host plant ranges could lead to variation in microbial communities just by the host interacting and acquiring microbes from different plants and different environments. *Arsenophonus* functionality for aphids is still largely unknown. However, the different titer levels of *Arsenophonus* could possibly contribute to variations between the aphid species ecology (e.g., host plant breadth, mutualisms with ants, predation, and parasitism rates). Out of the three milkweed aphids, *A. asclepiadis* is the most often tended to by ants (Enders et al., 2022). *Arsenophonus* could play a role in ant-aphid mutualistic interactions. However, further research needs to be conducted to investigate that line of thought. *Arsenophonus* could also possibly impart some sort of additional fitness benefit; for example, it could possibly help aid in resistance to secondary plant defense compounds present in the milkweed plant or other host plants it may feed on.

One major limitation of this study is that when optimizing primers in objective one, only *A. asclepiadis* DNA extraction samples were used. While this would lead to an accurate representation of *Arsenophonus* in *A. asclepiadis*, it could have led to an under-estimation of *Arsenophonus* in both *A. nerii* and *M. asclepiadis.* *Arsenophonus* are also very diverse in terms of strains, which can impart specific responses for hosts (Nováková et al., 2009). The *Arsenophonus* template used for the primers was a dominant strain specific to *A. asclepiadis*, thus the primers used may not have accurately estimated Arsenophonus relative abundance in the other species if the they contained different strains. Future studies should optimize primers for each species being tested to find accurate representations of endosymbiont relative abundances, as well as examine dominant *Arsenophonus* strains in different species. Another limitation of this study is the small sample size used when testing the qPCR assay on field caught aphids. Additional testing should be conducted using a larger sample size of field caught individuals across multiple locations and collected from different host plants where possible.

In conclusion, this study takes important first steps towards developing qPCR-based methods for quantifying differences in titer levels of bacterial symbiont *Arsenophonus* across milkweed aphid species. This work confirms there are higher relative abundances of *Arsenophonus* in *A. asclepiadis* compared to the other two species. *Arsenophonus* infection rates and prevalence are dependent on environmental temperature (Chang et al., 2022). For

example, honeybees infected with *Arsenophonus*, in the UK, had increased prevalence from spring until fall and then would lose the endosymbiont during the winter (Chang et al., 2022). It therefore might be interesting to see if geographic location (e.g., latitude and longitude over large scales) and or temperature changes over the course of a season alter Arsenophonus abundance in the milkweed aphids and to what effect. Additionally, to follow up on these results, utilizing some well-established tests, such as green fluorescent protein (GFP) fluorescence microscopy, aphid genome sequencing, and antibiotic curing, would help scientists to further understand the many unknowns surrounding *Arsenophonus*.

Figures

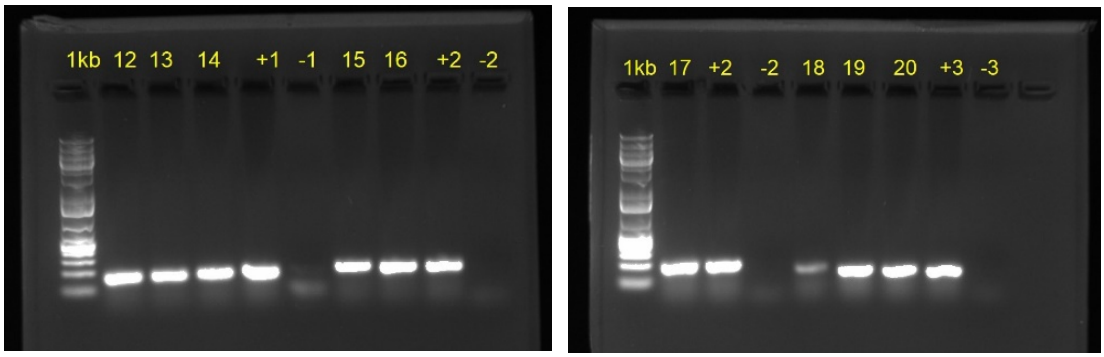

Figure 1. PCR gel electrophoresis of *Arsenophonus* primers. Ars_1 and controls, wells: 12-14, +1, and -1. Ars_2 and controls, wells: 15-17, +2, and -2. Ars_3 and controls, wells: 18-20, +3, and -3.

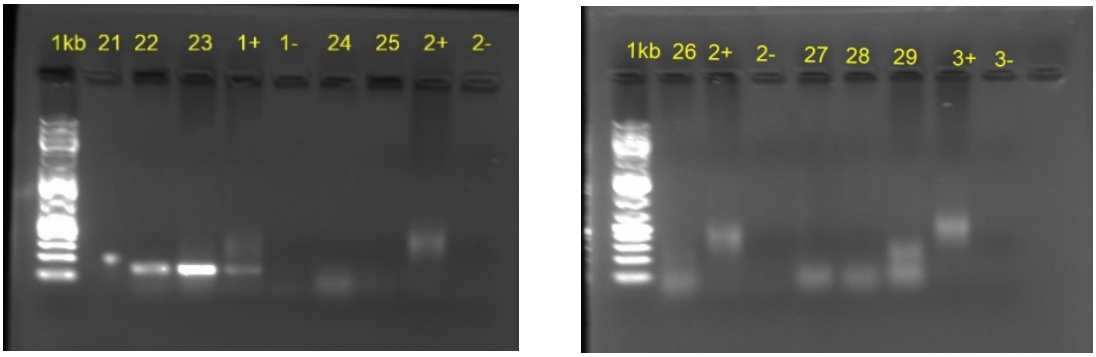

Figure 2. PCR gel electrophoresis of Ef1a primers. Ef1a_1 and controls, wells: 21-23, 1+, and 1-. Ef1a_2 and controls, wells: 24-26, 2+, and 2-. Ef1a_3 and controls, wells: 27-29, 3+, and 3-.

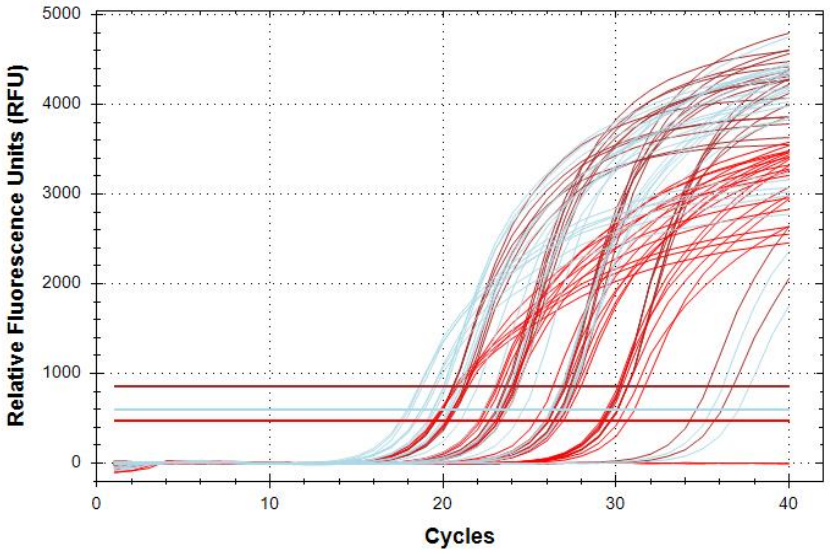

Figure 3. Amplification curve of *Arsenophonus* primers (Ars_1 = dark red, Ars_2 = light red, and Ars_3 = light blue) across series of dilutions (1x, 1:10x, 1:100x, and 1:1000x).

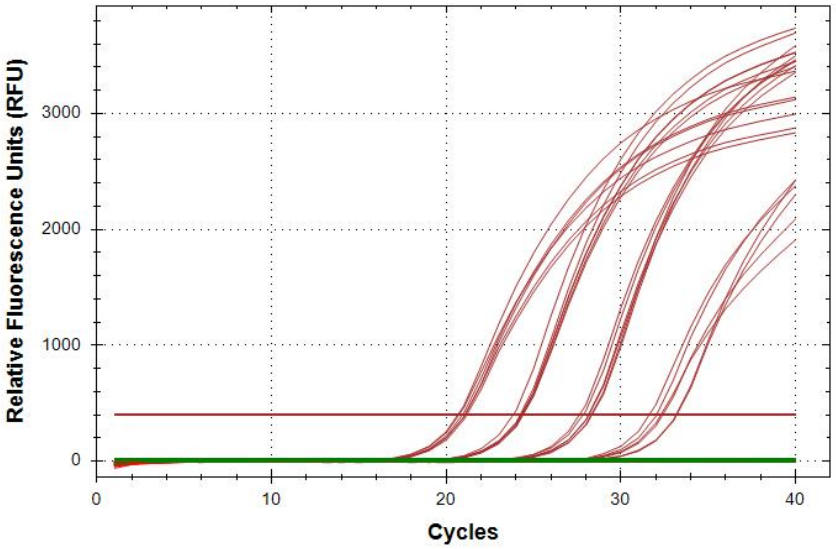

Figure 4. Amplification curve of Ef1a primers (ef1a_1 = dark red, ef1a_3* = light red) across series of dilutions (1x, 1:10x, 1:100x, and 1:1000x). Ef1a_3 did not amplify.

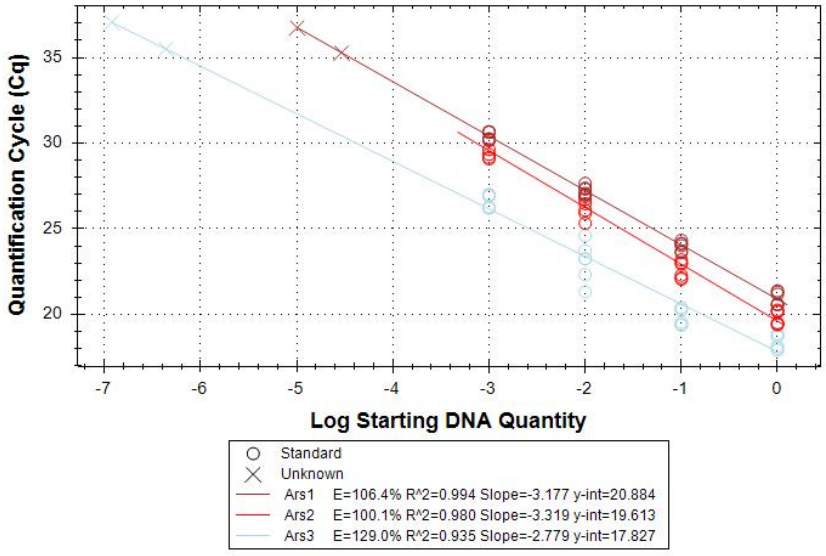

Figure 5. Standard curve of *Arsenophonus* primers (Ars_1 = dark red, Ars_2 = light red, and Ars_3 = light blue).

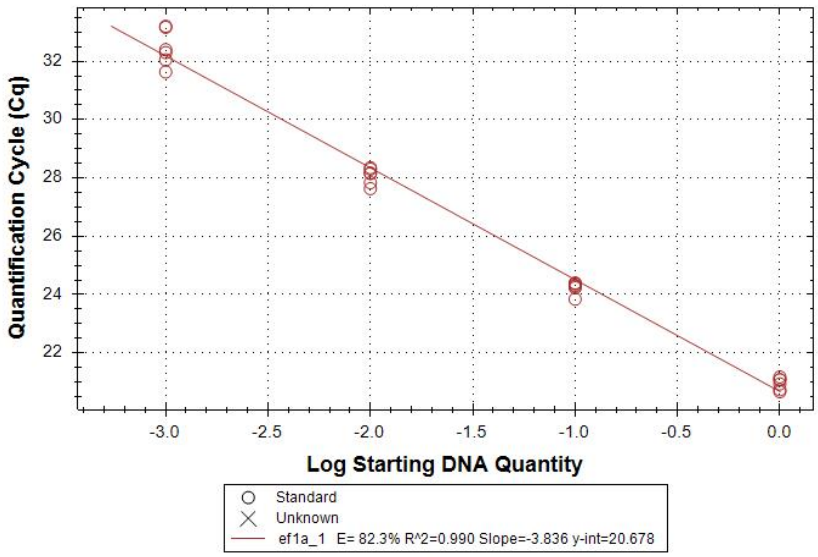

Figure 6. Standard curve of Ef1a primer (ef1a_1 = dark red).

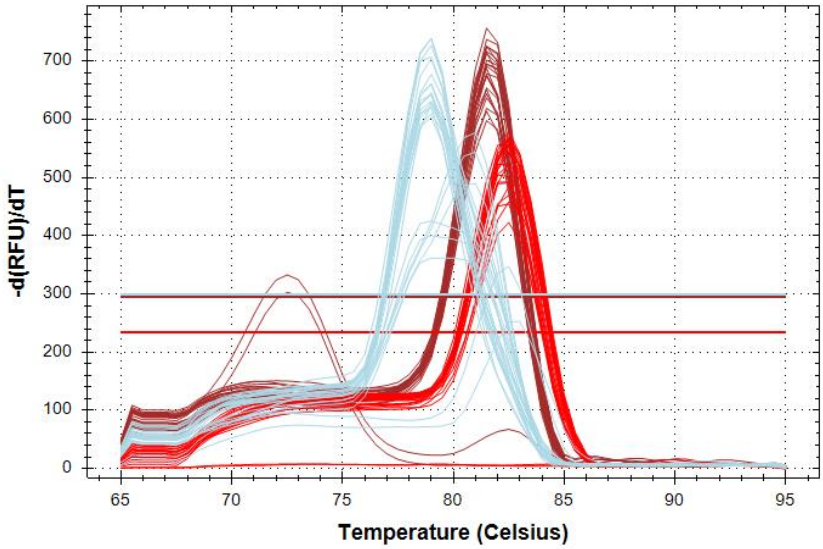

Figure 7. Melt peak curves of *Arsenophonus* primers (Ars_1 = dark red, Ars_2 = light red, and Ars_3 = light blue).

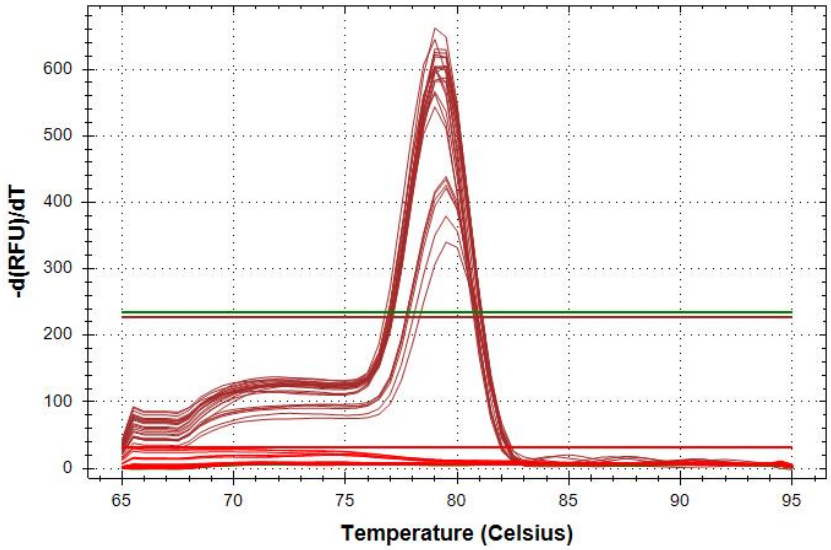

Figure 8. Melt peak curve of Ef1a primers (ef1a_1 = dark red, ef1a_3* = light red). Ef1a_3 did not amplify.

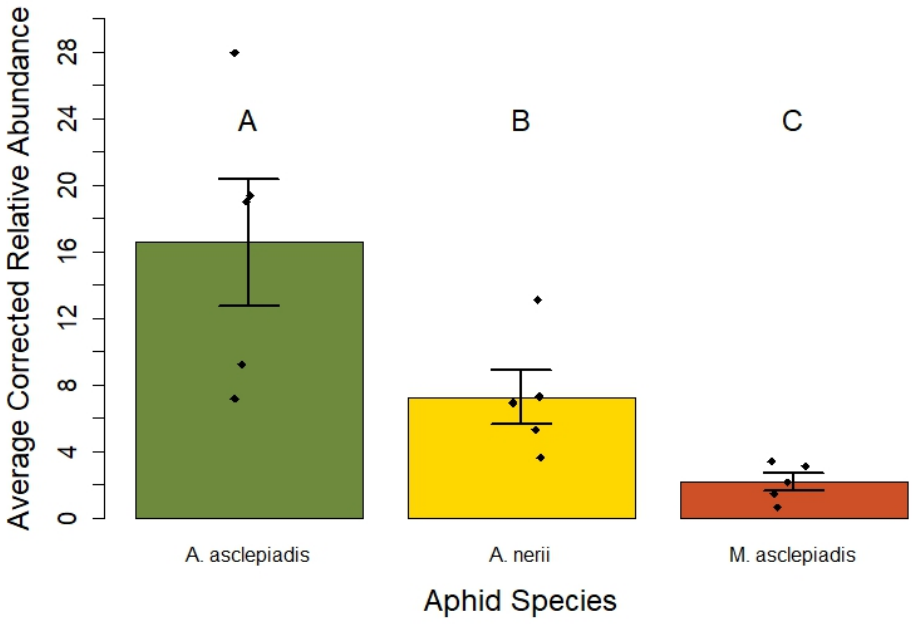

Figure 9. *Arsenophonus* relative abundance titer levels highest in *A. asclepiadis*. Results of qPCR assay run on field caught milkweed aphids. Letters (A, B, and C) indicate p-values below 0.1 (Pairwise Wilcoxon rank sum test).

References

Mayoral-Peña, Z., Álvarez-Martínez, R., Fornoni, J., & Garrido, E. (2020). The Extended Microbiota: How Microbes Shape Plant-Insect Interactions. In Evolutionary Ecology of Plant-Herbivore Interaction (pp. 135–146). Springer International Publishing. https://doi.org/10.1007/978-3-030-46012-9_7

Chang, C. Y., Sun, X. W., Tian, P. P., Miao, N. H., Zhang, Y. L., & Liu, X. D. (2022). Plant secondary metabolite and temperature determine the prevalence of Arsenophonus endosymbionts in aphid populations. *Environmental Microbiology*, *24*(8), 3764–3776. https://doi.org/10.1111/1462-2920.15929

Enders, L., Hansen, T., Brichler, K., Couture, J., & French, E. (2022). Aphid species specializing on milkweed harbor taxonomically similar bacterial communities that differ in richness and relative abundance of core symbionts. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-25554-y

Hammer, T. J., & Bowers, M. D. (2015). Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*, *179*(1), 1–14. https://doi.org/10.1007/s00442-015-3327-1

Hansen, T. E., & Enders, L. S. (2022). Host Plant Species Influences the Composition of Milkweed and Monarch Microbiomes. Frontiers in Microbiology, 13, 840078–840078. https://doi.org/10.3389/fmicb.2022.840078

Jousselin, E., Cœur d'Acier, A., Vanlerberghe-Masutti, F., & Duron, O. (2013). Evolution and diversity of Arsenophonus endosymbionts in aphids. Molecular Ecology, 22(1), 260–270. https://doi.org/10.1111/mec.12092

Karimi, S., Seyahooei, M. A., Izadi, H., Bagheri, A., & Khodaygan, P. (2019). Effect of Arsenophonus Endosymbiont Elimination on Fitness of the Date Palm Hopper, Ommatissus lybicus (Hemiptera: Tropiduchidae). Environmental Entomology, 48(3), 614–622. https://doi.org/10.1093/ee/nvz047

Majeed, M. Z., Sayed, S., Bo, Z., Raza, A., & Ma, C.-S. (2022). Bacterial Symbionts Confer Thermal Tolerance to Cereal Aphids Rhopalosiphum padi and Sitobion avenae. Insects (Basel, Switzerland), 13(3), 231–. https://doi.org/10.3390/insects13030231

Nováková, E., Hypša, V., & Moran, N. A. (2009). Arsenophonus, an emerging clade of intracellular symbionts with a broad host distribution. BMC Microbiology, 9(1), 143–143. https://doi.org/10.1186/1471-2180-9-143

Smith, R. A., Mooney, K. A., & Agrawal, A. A. (2008). Coexistence of three specialist aphids on common milkweed, Asclepias syriaca. *Ecology*, *89*(8), 2187–2196. https://doi.org/10.1890/07-1441.1

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2015). A Practical Approach to RT-pPCR - Publishing Data That Conform to the MIQE Guidelines. *Bio-Rad Laboratories, Ince.* , *Bulletin 5859*.