FACTORS INFLUENCING THE MAINTENANCE OF A PROTECTIVE SYMBIONT IN THE COW PEA APHID, *APHIS CRACCIVORA*.

by

HANNAH RUBY DYKSTRA

(Under the Direction of Kerry M. Oliver)

ABSTRACT

Many heritable symbionts invade and persist in host populations by conferring benefits, including host protection. In the legume crop pest *Aphis craccivora* (Koch), infection with the heritable symbiont *Hamiltonella defensa* confers protection against parasitoid wasps, an important natural enemy often employed in biological control. Despite this benefit, not all *A. craccivora* carry *H. defensa*. A major aim of this thesis was to estimate the vertical transmission efficiency of *H. defensa* in *A. craccivora* and identify costs that may limit its spread. We found that vertical transmission rates were high but not 100%, and clear costs to infection in population cage studies. We also attempted to better characterize *H. defensa* infection in *A. craccivora*, by examining the genetic diversity of bacterial strains and associated viruses within this system. Overall, we found little symbiont diversity, which is consistent with the aphid being an invasive species with a mostly asexual persistence.

INDEX WORDS: *Hamiltonella defensa*, *Aphis craccivora*, Cowpea aphid, Infection frequency, Defensive mutualism, Endosymbiont, Balancing selection, Tradeoffs, Defensive symbiont

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CHAPTER 1

LITERATURE REVIEW

Introduction

Associations between microorganisms and insects are ubiquitous throughout nature. Many insect species are infected with maternally transmitted bacteria called heritable symbionts (Buchner, 1965; Duron et al., 2008; Moran et al., 2008; Moran et al., 2005c; Russell et al., 2012). It is generally thought that symbionts invade and persist in host populations by manipulating the hosts' reproduction, or providing net fitness benefits (Bull, 1983; Werren, 1997). Many of these associations have persisted over millions of years and led to the specialization and subsequent diversification of many insect groups (Brownlie & Johnson, 2009; Moran et al., 2008; Moran & Telang, 1998). Beneficial heritable symbionts can supplement nutrients (Baumann, 2005; Dixon, 1998; Douglas & Dixon, 1987), widen host diet-breadth (Tsuchida et al., 2004; Tsuchida et al., 2011), provide thermal tolerance (Chen et al., 2000; Russell & Moran, 2006), and confer protection against threats from natural enemies, including pathogens (Ferrari et al., 2004; Hedges et al., 2008; Panteleev et al., 2007; Scarborough et al., 2005; Teixeira et al., 2008), parasites (Jaenike et al., 2010), and parasitoid enemies (Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2009; Xie et al., 2011). These microbes often allow their hosts to persist under otherwise unfavorable conditions.

Heritable Symbionts in Aphids

The heritable symbionts of aphids are classified as either obligate (i.e. required for host survival or reproduction) or facultative (not strictly required by the host) (Oliver et al., 2010). Almost all aphids possess the nutritional symbiont *Buchnera aphidicola (*gamma3-Proteobacteria: Enterobacteriaceae) which supplies the aphid, feeding on a nutrient-poor diet of phloem sap, with essential amino acids (Buchner, 1965; Douglas, 1998). Typical of other obligate symbionts,

Buchnera have reduced genomes (<1Mb) (Moran et al., 2005a), live only within specialized host cells called bacteriocytes, and are strictly maternally transmitted (Douglas, 1998; Hinde, 1971; Munson et al., 1991; Unterman et al., 1989). Like other obligates, the phylogenies of both *Buchnera* and aphids are congruent indicating co-speciation (Wernegreen, 2002).

In addition to their obligate *Buchnera*, many aphids also harbor one or more facultative symbionts (Oliver et al., 2010) and the most common species belong to the Enterobacteriaceae (Chen et al., 1996; Fukatsu et al., 2001; Moran et al., 2005b; Sandstrom et al., 2001; Unterman et al., 1989). Though primarily heritable, facultative symbionts may undergo occasional horizontal transmission within and among species, as seen with phylogenetic analyses and transfection experiments (Ahmed et al., 2013; Buchner, 1965; Moran et al., 2008; Moran et al., 2005b; Moran & Telang, 1998; Oliver et al., 2005; Oliver et al., 2003; Russell et al., 2003; Tsuchida et al., 2011). Facultative symbionts are typically found in multiple host tissues (Darby et al., 2001; Fukatsu et al., 2000; Koga et al., 2003; Moran et al., 2005b; Tsuchida et al., 2005); and molecular diagnostic surveys have shown intermediate infection frequencies in natural populations (e.g. Ferrari et al., 2012; Oliver et al., 2006; Russell et al., 2013; Sandstrom et al., 2001; Simon et al., 2003; Tsuchida et al., 2006; Tsuchida et al., 2002). Some facultative symbionts are associated with mobile genetic elements, which may undergo lateral transfer, potentially moving ecologically important traits within and between symbiont species (Degnan & Moran, 2008b; Moran et al., 2008; Ochman & Moran, 2001; Oliver et al., 2010; Oliver et al., 2005).

Aphids, especially the pea aphid, *Acyrthosiphon pisum*, are important model organisms for studying the effects of heritable bacterial infections. This is the case for several reasons: (1) aphids are infected with a diverse assemblage of heritable symbionts –with the pea aphid commonly associated with eight heritable bacterial species (Russell et al., 2013); (2) most aphids, including the pea aphid, reproduce parthenogenetically during the spring and summer (Dixon, 1998; Harrington, 1994) and clonal lines can be kept indefinitely in the laboratory by mimicking long-day length conditions; and (3) symbiont presence can be manipulated by selectively curing

(i.e. antibiotic diet) particular facultative bacteria (e.g. Chen et al., 2000; Douglas et al., 2006; Koga et al., 2003; Koga et al., 2007), or by infecting aphids with novel symbionts (i.e. transfection) (Oliver et al., 2010). Hence, experimental lines, consisting of aphids sharing the same genetic background but having particular symbiont combinations can be studied such that differences between infected and uninfected aphids in bioassays can be attributed to the symbiont. Such experimental studies in pea aphids have identified diverse benefits to infection with common facultative symbionts. For example, infection with *Regiella insecticola* has been shown to expand aphid diet breadth (Ferrari et al., 2007; Tsuchida et al., 2004) and confer resistance to fungal pathogens (Parker et al., 2013; Scarborough et al., 2005). *Serratia symbiotica* can confer heat tolerance (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006) and possibly compensate for the loss of the nutritional symbiont, *Buchnera* (Koga et al., 2003). Most notable for this study, *Hamiltonella defensa* confers protection against a common natural enemy, the parasitoid *Aphidius ervi* (Hymenoptera: Braconidae), by killing the internally developing wasp larvae (Oliver et al., 2005; Oliver et al., 2003). This defensive phenotype is of great interest since numerous biological programs today employ parasitoids to control herbivore pest populations, and defensive symbionts potentially thwart these efforts (Brewer & Elliott, 2004; Heimpel et al., 2004; Mackauer & Volkl, 1993).

The defensive symbiont H. defensa

Hamiltonella defensa, previously referred to in the literature as "t-type" (e.g. Russell et al., 2003; Sandstrom et al., 2001) or "PABS" (e.g. Darby et al., 2001; Darby et al., 2003; Leonardo & Muiru, 2003; Simon et al., 2003) is named after the evolutionary biologist William D. Hamilton (Moran et al., 2005a). *H. defensa* is a rod-shaped bacterium, <2μm long found extracellularly in the hemocoel, and intracellularly in secondary bacteriocytes or sheath cells associated with the primary bacteriocytes housing *Buchnera* (Darby et al., 2001; Fukatsu et al., 2000; Koga et al., 2003; Moran et al., 2005b; Tsuchida et al., 2005). During a parasitoid attack, a female endoparasitic *Aphidius ervi* wasp injects her egg into an aphid host, where the hatched

larvae develops and feeds for 5 – 8 days, after which the host is killed, and the aphids' cuticle acts as a protectant during the wasp's pupation, where the adult emerges from the aphid 'mummy' (Angalet & Fuester, 1977). During oviposition, the wasp injects venom that causes host castration by degeneration of germarial cells and young embryos (Digilio et al., 2000; Digilio et al., 1998). Wasp cells, known as teratocytes, aid the developing endoparasite by redirecting *Buchnera*-provisioned nutrients to the wasp larvae (Falabella et al., 2005; Falabella et al., 2009; Falabella et al., 2000). *A. pisum* has a weak encapsulation response (Altincicek et al., 2008; Laughton et al., 2011; Oliver et al., 2005), which appears typical of aphids (Carver & Sullivan, 1988). Wasps readily oviposit in *H. defensa*-infected aphids, but fail to complete development in infected, resistant hosts (Oliver et al., 2003).

Although mechanisms underlying symbiont-based protection are unknown, lysogenic lambdoid bacteriophages, called APSEs (*A. pisum secondary endosymbiont*) in the Podoviridae, are required to produce the protective phenotype (Degnan & Moran, 2008a; Moran et al., 2005a; Oliver et al., 2009; van der Wilk et al., 1999). *H. defensa* also possesses a type III secretion system (T3SS) that forms a pilus structure that transports effector proteins for host cell invasion (Degnan et al., 2009; Galan, 1999). Degnan & Moran (2008a) examined APSEs from various species and found genes encoding for cytolethal distending toxin (*cdtB*) homologs (in APSE-2, -6, -7), shiga-toxin (*stx*) homologs (APSE-1, -4, -5), or a YD-repeat (*YDp*) putative toxin (APSE-3) encoded at a variable cassette region—each of these toxins known or suspected to target eukaryotic cells (Degnan & Moran, 2008a). Of the seven variants, three were identified in *A. pisum* (APSE-1, -2, -3), while the other APSE were found in different species, which included a sample of *A. craccivora* (APSE-4) collected from Arizona (Degnan & Moran, 2008a). In *A. pisum*, for example, APSE-2 (*cdtB*) *H. defensa* confers moderate protection, while APSE-3 (*YDp*) *H. defensa* confers high levels or complete protection (Degnan & Moran, 2008b; Oliver et al., 2009; Oliver et al., 2005; Oliver et al., 2003). In work with collaborators, we recently found that

A. craccivora infected with *H. defensa* carrying APSE-4 (*stx*) confers protection against several parasitoid wasps (Asplen et al., unpublished).

Population dynamics of H. defensa

H. defensa is found in about 14% of aphids (Oliver et al., 2010) and is also known to infect the whitefly *Bemisia tabaci* (Clark et al., 1992; Degnan & Moran, 2008b). While facultative in most cases, *H. defensa* may have transitioned to an obligate symbiont in the aphid *Uroleucon ambrosiae*. Although its role is unknown, identical strains of *H. defensa* were found in all *U. ambrosiae* individuals over multiple years and across North America (Degnan & Moran, 2008b; Sandstrom et al., 2001). Despite clear protective benefits identified in lab studies, worldwide surveys find *H. defensa* at intermediate frequencies in natural populations (Brady et al., 2013; Ferrari et al., 2012; Oliver et al., 2006; Russell et al., 2013; Sandstrom et al., 2001; Simon et al., 2003; Tsuchida et al., 2002). In *A. pisum*, population cage studies show that infection frequencies increase rapidly in cages exposed to parasitism by *A. ervi*, but decrease in control cages lacking wasps, suggesting costs to infection when enemies are not present (Oliver et al., 2008). While such tradeoffs are likely important, non-selective factors, including transmission modes and rates, may also influence symbiont dynamics in natural populations (Oliver et al., 2013).

When multiple-infections are present, symbionts can be lost until a single, stable infection is reached (Moran & Dunbar, 2006; Sandstrom et al., 2001). In the laboratory, *H. defensa* occurring as a single infection is maternally transferred at rates approaching 100% in *A. pisum* (Moran & Dunbar, 2006; Weldon et al., 2013). However, transmission rates in the field, and in other aphid species, are mostly unknown. One study in *A. craccivora* reported loss of *H. defensa* (Hopper, 2010). In *A. pisum*, APSE phages can also be inefficiently maternally transmitted, leading to the loss of the protective phenotype and the breakdown of the symbiosis (Oliver et al., 2009; Weldon et al., 2013). In nature, temperature and other abiotic factors likely reduce fidelity of transmission (e.g. Hurst et al., 2001).

While maternal transmission is the primary route for *H. defensa* to reach new hosts, phylogenetic evidence and transfection experiments indicate they undergo occasional horizontal transfer (Degnan & Moran, 2008b; Oliver et al., 2005; Russell et al., 2003; Sandstrom et al., 2001). Little, however, is known about rates and mechanisms of horizontal transmission (Darby & Douglas, 2003). Recent studies show that parasitoids (via contaminated ovipositor), phloem, and sexual transfer are likely routes for horizontal transmission in hemipteran insects (Caspi-Fluger et al., 2012; Gehrer & Vorburger, 2012; Moran & Dunbar, 2006; Oliver et al., 2008). This capacity for lateral transfer of symbionts can lead to the rapid introduction of novel, ecologically important traits, such as resistance to parasitoids in new host species (Oliver et al., 2010). For example, *A. craccivora H. defensa* (APSE-4) was transferred to an *A. pisum* resulting in increased protection against the parasitoid *A. ervi* (Oliver et al., 2005).

PURPOSE OF STUDY

Characterizing H. defensa outside of A. pisum

Much of the work identifying beneficial effects to infection with heritable symbionts has been conducted with *A. pisum* (Oliver et al., 2005; Oliver et al., 2003). Many of the same symbionts infecting *A. pisum*, including *H. defensa*, are found in other aphids (and other insects more generally), yet their roles are typically unknown in these other hosts. *H. defensa* has been documented in at least 17 aphid species (Degnan & Moran, 2008b; Oliver et al., 2010; Russell et al., 2003), including many important pests. *H. defensa* was recently shown to confer protection against parasitoids (*Lysiphlebus fabarum*) in the black bean aphid *Aphis fabae* (Schmid et al., 2012) and was associated with protection against *Binodoxys communis* in the cowpea aphid *A. craccivora* (Desneux et al., 2009). Recent collaborative work using genetically identical aphids with and without *H. defensa* confirmed this symbiont protection in *A. craccivora* against several aphidiiine braconid species (Asplen et al., unpublished). Together, these results indicate that host protection may be a common phenotype associated with *H. defensa*. Although, it does not appear

to protect the grain aphid *Sitobion avenae* (Lukasik et al., 2013), and the Oliver lab recently found strains in *A. pisum* that also conferred no protection (Martinez et al., 2013).

Study Organism

A. craccivora is a highly polyphagous crop pest of legumes (Blackman & Eastop, 2006; Cammell & Way, 1983; Singh & Emden, 1979). Commonly found in both temperate and tropical climates, *A. craccivora* are believed to originate from southern Europe or the Middle East (Blackman & Eastop, 1984; 2006; Singh & Emden, 1979; Van Emden & Harrington, 2007). Many aphid species are sensitive to temperature changes and are only able to reproduce within a limited range, with temperatures above 25°C often reducing aphid fitness (Carroll & Hoyt, 1986; Collins & Leather, 2001; Leather, 1982). However, *A. craccivora* can develop at higher temperatures (up to 29.4°C) with an optimal growing temperature ~25°C (Berberet et al., 2009). Though there have been reports of a few sexual morphs (e.g. Germany, India, and Argentina) (Van Emden & Harrington, 2007), in northern regions, they are thought to be anholocyclic, i.e. only reproducing parthenogenetically, dying off during the winter and recolonizing with migratory aphids from southern locations (or greenhouses) when the milder seasons return (Blackman, 2000; Blackman & Eastop, 2006; Gutierrez et al., 1971). Mothers give birth to live young; and nymphs, noted by their grey appearance (covered in wax), undergo four instar stages until adulthood (Dixon, 1985). Adults are round, shiny black, and ~1.4-2.2mm in length (Blackman & Eastop, 1984; 2006; Stoetzel et al., 1996). It is estimated that *A. craccivora* can travel up to 364 miles in ~11 hours by passive transport (Gutierrez et al., 1974). *A. craccivora* are found early in growing seasons, colonizing young crops within 3 – 5 weeks of their emergence (Farrell, 1976; Obopile, 2006; Ofuya, 1991). They are also known to vector over 30 plant viruses, attacking about 50 crops worldwide (Cammell & Way, 1983; Van Emden & Harrington, 2007). Wilting, stunted growth, and sooty mold fungus are associated with their direct feeding and concentrated amounts of honeydew from heavy aphid densities (Annan et al., 1995; Berberet et al., 2009; Gutierrez et al., 1974; Wu & Thrower, 1981). Field surveys show *A. craccivora* is

associated with a number of facultative symbionts, including *H. defensa*, in natural populations (e.g. Asplen et al., unpublished; Desneux et al., 2009; Russell et al., 2003; Tsuchida et al., 2006; Brady et al., 2013).

Given its worldwide pest status, it is important to understand the role of this protective symbiont in *A. craccivora*, which may influence the efficacy of biological control efforts. In *A. pisum*, no direct fitness costs have been identified with *H. defensa* infections though there appears to be costs to infection in the absence of parasitoid wasps (Oliver et al., 2008). While protective benefits of infection have been characterized in *A. craccivora* (Asplen et al., unpublished), little is known about costs. Similarly, *H. defensa* in *A. pisum* appears to be transmitted with very high fidelity (Moran & Dunbar, 2006; Weldon et al., 2013), yet little is known about transmission rates in *A. craccivora*. Our interests lie in taking previous work in *A. pisum* (tribe Macrosiphini), and applying it to a distantly related aphid, *A. craccivora* (tribe Aphidini) species by beginning to characterize the symbiosis and providing some insight into infection dynamics and life history tradeoffs that shape symbiont-host interaction. We focused on (1) evaluating the factors limiting *H. defensa* spread in *A. craccivora* populations, including vertical transmission efficiency of *H. defensa* and potential costs to infection and, (2) characterizing *H. defensa* infection in this aphid system by estimating symbiont and bacteriophage titers over the aphid lifespan, as well as sequencing relevant bacterial genes to identify haplotypes and their phylogenetic placement among other *H. defensa.*

OBJECTIVES:

CHAPTER 2: Identifying Factors limiting *H. defensa* spread

a. Estimate maternal transmission efficiency

b. Identify costs to infection with *H. defensa*

CHAPTER 3: Characterize *H. defensa* within *A. craccivora*

a. Estimate *H. defensa* and APSE titers

b. Perform genotypic analyses of *H. defensa* & APSE phage

CHAPTER 2

IDENTIFYING FACTORS LIMITING *H. DEFENSA* SPREAD IN *A. CRACCIVORA*

INTRODUCTION

Much of what is known about the protective symbiont *H. defensa* is based on findings from studies, e.g. *in vivo* experiments, with the pea aphid, *A. pisum*. Population cage studies have shown that the protective benefits conferred by *H. defensa* to *A. pisum* under the threat of parasitoid attack is a major force driving the spread of this symbiont, while costs in the absence of wasps potentially limit its spread (Oliver et al., 2008). The intermediate infection frequency *H. defensa* is found at in natural populations (e.g. Russell et al., 2013) is thought to reflect this tradeoff (e.g. Oliver et al., 2008). Specific costs have not been identified, but infection conceivably results in constitutive costs, as *H. defensa* likely relies on the aphid and the aphid's obligate symbiont *Buchnera* for nutrition (Degnan et al., 2009). Alternatively, costs could be induced when the symbiont is mobilized to destroy the wasp if, for example, the symbiont produced toxins harmful to aphid tissue (Oliver et al., 2013). Also, symbiont maintenance can be influenced by non-selective factors, including transmission modes and rates, as well as drift and migration (Oliver et al., 2013). Though no vertical transmission losses of single infections of *H. defensa* in lab-reared *A. pisum* have been reported (Moran & Dunbar, 2006; Weldon et al., 2013), transmission failures in nature may be higher due to abiotic factors like temperature (e.g. Hurst et al., 2001; Oliver et al., 2013).

Even though *H. defensa* has been detected in ~14% of aphid species, little is known about the symbiosis outside of *A. pisum* (Oliver et al., 2010). Recent collaborative work found that *H. defensa* with bacteriophage APSE also confers protection against parasitoids in the cowpea aphid *Aphis craccivora* (Asplen et al., unpublished; Desneux et al., 2009), and is found at intermediate frequencies in natural populations (Brady et al., 2013). To further characterize the role of *H.*

defensa, we evaluated factors limiting the symbiont spread in *A. craccivora*. We estimated transmission rates for *H. defensa* and its bacteriophage APSE. We assessed for fitness costs associated with infection by measuring four fitness parameters and performing population cage experiments in controlled genetic backgrounds (Oliver et al., 2008; Russell & Moran, 2006). Considering the potential influence of temperature on transmission fidelity and costs, experiments were conducted at both 20° and 25°C.

METHODS AND MATERIALS

a. Experimental Aphids

We reared field-collected *A. craccivora* on *Vicia faba* (fava bean) as this is a suitable food source regardless of the host plant this aphid is collected from (Berg, 1984). Fava seedlings were grown in a temperature controlled greenhouse (University of GA) at roughly 22°C and seeds were planted in 3.5" plastic pots containing Pro-Mix BX professional growing mix and watered as needed. Clonal lines were initiated from single parthenogenetic *A. craccivora* females (see **Appendix A** for list of clones) and each line then held in replicate cup cages (inverted Solo clear plastic cups vented with a mesh top over a 3.5" plant pot) in Model I41LLVL Percival biological incubators (Percival Scientific, Inc.) at both 20° and 25°C with a 16h:8h Light-Dark (L:D) cycle. The long-day conditions imitate summer photoperiod to ensure maintenance of parthenogenetic reproduction.

DNA Preparation

Two methods were used to extract DNA for molecular work from *A. craccivora* samples. In cases requiring stable DNA (e.g. sample storage or sequencing) we used the proprietary E.Z.N.A.™ Insect DNA isolation kit (Omega Bio-Tek) following the manufacturer's instructions. For one extraction, 10-15 aphids were collected and for the initial cell lysis stage, samples were incubated at 60°C for 30min. The concentration of eluted DNA was measured with a NanoDrop® Spectrophotometer (Thermo Scientific) and extractions with > 15ng/µL were stored at 4°C (if

used for testing within 3 months) or -20°C. Kit extractions are expensive and time consuming when performed on a large number of samples, so for a more rapid approach we prepared DNA using a 'squish' extraction method from Gloor et al. (1993) for immediate use (e.g. in diagnostic PCR for transmission assays and population cages). Individual whole aphids were ground, with a pipette tip, in a lysis buffer (10mM Tris-Cl pH 8.2, 1mM EDTA, 25mM NaCl) and proteinase K (20mg/mL) (1µL per 100µL buffer). Samples were then incubated at 40°C for 35min, then 95°C for 2min30s, and held at 4°C until use.

Screening Aphid Populations for Symbiont Presence

Diverse and, sometimes, unexpected symbiont lineages, have been discovered within field-collected aphids (e.g. Guay et al., 2009; Haynes et al., 2003; Russell et al., 2013; Tsuchida et al., 2010). To ensure the presence of only *H. defensa*, we used a 'universal' 16S rRNA screening technique known as Denaturing Gradient Gel Electrophoresis (DGGE) to detect all bacteria present in each of our aphid lines. With DGGE, PCR amplified fragments from different bacteria (or even those containing a single base pair difference) migrate at different rates when exposed to a linear gradient of chemical denaturants (Sheffield et al., 1989). Bands can then be extracted from the gel, Sanger sequenced, and identified using the BLASTn algorithm against the "non-redundant" GenBank (NCBI) nucleotide database.

For DGGE, our *A. craccivora* lab line samples were screened with primers 356F (5'-GC CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3') and 517R (5'-ATT ACC GCG GCT GCT GG-3') to amplify the variable V3 region of 16rDNA (Muyzer et al., 1993). Primer 356F contains a 40 nucleotide 'GC-clamp' at its 5' end, to help stabilize the resulting DNA fragments to differentiate single base changes (Sheffield et al., 1989). 20µL PCR reactions were made with PCR-grade water, 10X PCR Buffer (200mM Tris-HCl pH 8.4, 500mM KCl), $MgCl_2$ (1.5 mM), dNTPs (200µM), APEX HotStart *Taq* DNA Polymerase (5U/µL) (Genesee Scientific, San Diego CA), both primers (5µM each), and whole DNA template (~300ng). The following PCR conditions were used: 95°C for 15min to

activate the enzyme; then 20 cycles of 94°C for 1min, a 65° – 55°C touchdown for 1min (-0.5°C/cycle) and 72°C for 1min; followed by 25 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1min; and a final 72°C for 8min followed by a 4°C hold (Muyzer et al., 1993).

We casted 6.5% acrylamide gels with a gradient of 40 to 65% denaturant of 7M urea and 40% (v/v) formamide, and PCR products were run at 70V for 16.5h in 1X TAE buffer (60°C) using a CBS Scientific DGGE system (Del Mar, CA). A subset of bands were excised, eluted in 50µL of PCR-grade water, and then incubated at 37°C for 30min prior to repeating the same DGGE PCR procedure with the same primers as outlined above. PCR products were then cleaned with an Omega Bio-Tek (Norcross, GA) E.Z.N.A. Cycle Pure Kit (per manufacturer's instructions) and single band products were confirmed with gel electrophoresis. These products were then sequenced in the forward direction by Eurofins MWG Operon (Huntsville, AL).

Creating Experimental Lines

DGGE confirmed the presence of *H. defensa* in six out of ten *A. craccivora* lines screened, with no other co-infections of facultative symbionts found. Diagnostic PCR also confirmed infection with *H. defensa* and bacteriophage APSE (details below). Using a technique from Douglas et al. (2006), cohorts in three *A. craccivora* lines (Ac1, SV1, & LL1) were selectively cured of *H. defensa*. This controls for potential fitness variation due to genetic background, and any differences found in subsequent bioassays can be attributed to the symbiont presence (Oliver et al., 2010). Briefly, ten 4th instar aphids from each clonal line fed on an artificial diet (Koga et al., 2007) mixed with an antibiotic cocktail of 50µg/mL each of gentamycin, cefotaxime, and ampicillin for three days. We pipetted the antibiotic-treated diet into a small Petri dish (35mm diameter) covered with Parafilm M stretched thoroughly across the dish's opening. This dish was inverted and placed in a larger Petri dish so that aphids could feed through the membrane via their stylet. Clonal lines were maintained on separate Petri dishes and after three days of feeding, aphids were individually placed on *V. faba* plants and any offspring observed the following three days were discarded. Later offspring were collected and infection

absence confirmed using diagnostic PCR. All experimental lines were started from single parthenogenetic females, and those cured were designated with an "–ab" following their clone name (e.g. Ac1ab). Cured lines were not used in any experiment until at least ten generations passed to eliminate any residual effects from antibiotic treatments (Douglas et al., 2006; Koga et al., 2003).

b. Estimating Maternal Transmission Efficiency

To estimate vertical transmission rates of *H. defensa* in *A. craccivora*, a total of 601 3rd instar offspring from 57 known infected mothers were screened with diagnostic PCR for the presence of *H. defensa*. Additionally, a subset of offspring were then screened for APSE presence. We estimated transmission rates using a total of four clonal lines. In June 2011, clonal lines Ac1, Ac21, and Ac17 were sampled at 25°C, and additional lines were sampled between July and August 2012 (Ac1 at 25°C, SV1 at both 20° and 25°C). All cohorts were started with 4th instar aphids individually placed in polystyrene petri dishes with freshly clipped *V. faba* leaves and left to reproduce for several days. Offspring were sampled every 2 – 3 days until the mother died. Leaves were added every 1 – 2 days, and petri dishes were changed as needed if any visible dew present on the bottom of the dish. 3rd instar offspring were collected in 0.2µL test tubes and DNA prepped with the squish extraction method.

To assess *H. defensa* presence, we amplified *H. defensa dnaK* gene fragments using primers T70F2 (5'-GGT TCA GAA AAA AGT GGC AG-3') and T70R2 (5'- CGA GCG AAA GAG GAG TGA-3') (from Moran et al., 2005a). A subset of the offspring sampled were then evaluated for APSE presence with the P28 gene using primers APSEP28F (5'-TGA TAA AAG CGG ATA ATG CC-3') and APSEP28R (5'-GCG TTT GTC ATA CTG AAA AGG-3') (Moran et al., 2005a). We confirmed DNA amplification and extraction viability by amplifying fragments of the aphid gene ef1α (primers 213F 5'-CCG TGG AGA TGC ACC ACG AAG C-3', 214R 5'-AGC AGC TCC CTT GGG TGG GT-3') and *Buchnera* (*dnaK*, primers 215F 5'-CCA ACA GCT GCG GCA CTT GC-3', 216R 5'- TCA CCT CCA AGA TGG GTG TCT CCA-3').

Primers were designed using Geneious Pro 5.4.6 (Biomatters Ltd). All diagnostic PCR were performed on a Roche LightCycler® 480 II, and procedures adapted from established protocols (Moran et al., 2005a; Oliver et al., 2009). Each 10µL reaction contained 5µL SYBR Green I Master (Molecular Probes, Inc., Eugene, OR, USA), 1µL (5µM) each of forward and reverse primer, 2µL of PCR-grade water, and 1µL DNA template. PCR included a pre-incubation of 95°C for 5min; 45 cycles of amplification at 95°C for 10s, annealing temperature decreasing from 68°C to 55°C at 18°C increments, 72°C for 10s; a melting curve at 95°C for 5s, 65°C for 1min, then to 97°C; and a final hold at 40°C. Each PCR run also included positive and negative controls.

c. Identifying potential fitness costs in H .defensa-infected A. craccivora

Using the antibiotic-cured experimental lines, we conducted fitness assays to evaluate if *H. defensa* infection influenced development time, fecundity, size, and survival (e.g. Oliver et al., 2006; Russell & Moran, 2006). All fitness assays were performed at both 20° and 25°C treatments and within each incubator, cohort cup cages were kept on the same tray and routinely rotated to reduce potential positional effects. To further control for variation, all experiments were started with same-aged aphids on ~2 week old *V. faba* plants. Due to the small size of *A. craccivora* offspring, aphids were observed under a magnifying lamp.

Using newborns in three clonal backgrounds, we first measured *A. craccivora* development time from birth to their first reproduction (TFR) and adult fresh weight (FW) for those with (Ac1, SV1, LL1) and without (Ac1ab, SV1ab, LL1ab) *H. defensa.* For logistic reasons, some replicates were started at 6am (±1.5h) and others at 8pm (±1.5h) for each clonal line/treatment. Starting on day 4, cup cages were monitored at 2h intervals for reproduction, at which point adults were immediately weighed on a Micro Scale, and then discarded. To reduce effects of crowding, we limited the number of newborns per cup cage to no more than twelve, and all offspring born during the experiment were removed immediately.

We then assessed cumulative fecundity and survivorship for two infected (SV1, Ac1) and uninfected (SV1ab, Ac1ab) lines. Five nymphs (48±3h) were placed in each *V. faba* cup cage and held in replicate (n=8) at both temperature treatments. Once aphids reached adulthood, the number of offspring and adults (alive & dead) were monitored at 2 – 3 day intervals. All offspring were removed with tweezers, and best efforts were taken to avoid disturbing the adult aphids. All cages were lined with Fisherbrand® weighing paper (4" by 4") to catch any aphids dropping from the plant and falling into the soil—reducing mortality for those fallen aphids and allowing for easier offspring monitoring. At ~12 – 15 days, due to plant senescence, adult aphids were carefully transferred to a fresh plant. The cumulative fecundity analysis excluded cup cages where all five adult aphids were dead on or prior to day 8. These cages, however, were not excluded from the survivorship analysis.

d. Population Cages

To determine if costs to infection were more apparent in the presence of competition, we conducted population cage experiments. Three replicate cages (BugDorm® 25cm x 25cm x 25cm) were maintained at both treatments (n= 6 total) each containing four *V. faba* plants of similar age and size. All cages were initiated with an equal number (80) of same-aged 3rd instar aphid nymphs in one clonal background (Ac1), but 50% were infected with *H. defensa*, and 50% were uninfected. Sixty ~3rd instar nymphs were randomly collected from variable plant parts in each cage at the start of the experiment and subsequently screened every 2 – 3 weeks. Due to the amount of time required to prepare DNA and perform PCR reactions, testing occurred over two day intervals where samples from 25°C cages were collected, squish extractions (180 samples total) and two Roche LightCycler® runs (dxPCR) were performed on the first day. 20°C cages were then sampled and tested the following day. We determined sample infection status with the same *H. defensa dnaK* primers, preparation, and procedures used to evaluate maternal transmission (T70F2 & T70R2 from Moran et al., 2005a).

There was a 24h difference between treatments, however, we staggered planting and aphid rearing so that plants and aphids used at both temperatures were grown under the same conditions and were of the same age at Time 0. At 25°C, cages were tested at eight time points; cages at 20°C were only tested at six because samples could not be taken on day 40 without decreasing the cage populations drastically. We were able to screen aphids on day 58 when cage populations had increased and sampling would not negatively affect the next testing day. Cages were checked every 2 – 3 days for plant quality and rotated throughout the incubator. Dead plants were removed and changed as needed. If some *A. craccivora* were still attached to wilting plants, the base of the stem was clipped and left in the cage to reduce the number of aphids removed during plant changes. The cut end of the plant would be secured into the dirt, close to the newly added plant, and those dead leaves were then removed (~2 days later) once *A. craccivora* had moved onto the young *V. faba*. Cages and plants were watered and changed as needed.

e. Data Analysis

All statistical analyses were performed using the JMP v. 9.0.3 64-bit platform (SAS Institute Inc., Cary, NC, 1989–2010). At each temperature, uninfected and infected lines with the same clonal background were analyzed for significant differences. We performed a Chi-square test on maternal transmission data, and logistic regression to analyze survivorship up to day 13. The development time, estimated by TFR, were non-normally distributed, so a non-parametric Wilcoxon rank sum analysis was performed. Both fresh weight (FW) and cumulative fecundity (per cup cage) data had a log-normal distribution so all were log-transformed prior to ANOVA tests. Our population cage experiment resulted in data non-normally distributed so all ratios were logit transformed, and a linear regression performed for each treatment with a t-test to evaluate whether the regression slopes significantly differed from zero. A likelihood ratios Chi square test was then used to evaluate differences between the start and the final time points in the population cages.

RESULTS

a. Maternal transmission loss in A. craccivora

Overall, the maternal transmission rate of *H. defensa* in *A. craccivora* (**Table 2.1**) were high at 99.33% (n=601). All *H. defensa*-infected aphids screened for APSE tested positive for the bacteriophage (n=341, 100%). Of the 601 offspring evaluated, only four aphids, from the same line (SV1), tested negative for *H. defensa*. These negatives were confirmed with additional diagnostic PCR screens and tests verifying that DNA extractions were viable. All four transmission failures occurred at 25°C, but given the small number of negatives it is not surprising that a Pearson Chi-square test found no relationship between frequency of transmission and clonal lines (at 25°C only, $\chi^2_{3,434}$= 5.751, p= 0.1244), nor between temperature (SV1 at 20° & 25°C, $\chi^2_{1,256}$= 1.748, p= 0.1861).

Table 2.1. Vertical Transmission in *A. craccivora*. The number of aphids tested positive out of the total tested per line for the gene *dnaK* for *H. defensa* and P28 for phage at 20° and 25°C (T).

	T	Ac21	Ac1	Ac17	SV1	Total
H. defensa	25°C	84/84	119/119	52/52	175/179	**430/434**
	20°C	---	90/90	---	77/77	**167/167**
APSE	25°C	---	---	---	175/175	**175/175**
	20°C	---	89/89	---	77/77	**166/166**

b. Identifying Potential Fitness Costs Associated with H. defensa

If *H. defensa* imposes infection costs, then we would expect to see longer development times, smaller adult weights, greater mortality, or fewer offspring in infected aphids. With respect to fresh weight, we found no significant differences between infected and uninfected *A. craccivora* at 25°C, and only a single clone (LL1) exhibited significant differences (t_{76}= 5.104,

p<0.0001) at 20°C (see **Table 2.2**). In this case, aphids from the infected line were smaller, suggesting a cost to infection with *H. defensa*.

Table 2.2. Back-transformed Fresh Weight (mg) at 20° and 25°C. Log-transformed weights of *H. defensa* infected and uninfected lines were compared with an ANOVA t-test (p-value*** represents significance); the number of aphids (n), range, mean, and standard error (s.e.) values are presented.

T	Line	n	Range	Mean ± s.e.	p-value
20°C	AC1	18	0.109 – 0.297	0.169925 ± 0.07542	0.9723
	AC1ab	27	0.092 – 0.385	0.16778 ± 0.06158	
	LL1	34	0.095 – 0.254	0.161347 ± 0.04395	<0.0001***
	LL1ab	44	0.123 – 0.408	0.217512 ± 0.03863	
	SV1	16	0.093 – 0.369	0.171307 ± 0.07914	0.4396
	SV1ab	16	0.09 – 0.2817	0.159311 ± 0.07914	
25°C	AC1	39	0.07 – 0.269	0.136068 ± 0.04836	0.1472
	AC1ab	51	0.08 – 0.285	0.149688 ± 0.04229	
	LL1	40	0.079 – 0.322	0.159502 ± 0.04646	0.6414
	LL1ab	44	0.078 – 0.311	0.163523 ± 0.04430	
	SV1	36	0.102 – 0.262	0.17201 ± 0.04357	0.8553
	SV1ab	31	0.106 – 0.34	0.176842 ± 0.04695	

In our TFR assays (**Table 2.3**), the LL1 clone showed a marginally significant (p=0.0405) increase in development time relative to the uninfected control at 20°C. There was a stronger response observed between infected and uninfected Ac1 aphids at 25°C (p = 0.0001). In both cases, development times were longer in *H. defensa*-infected aphids, consistent with infection costs.

Table 2.3. Time to first reproduction (hours) at 20° and 25°C. *H. defensa* infected and uninfected clonal lines were compared using Wilcoxon rank sum (p<0.05*; p<0.001**). The table includes Quartile1 (Q1) and 3 (Q3), and the number of aphids (n).

T	Line	n	Range	Q1	Median	Q3	p-value
20°C	**AC1**	18	153.5 – 252	163.375	207	224	0.7716
	AC1ab	27	120.5 – 269	149.5	212	232.5	
	LL1	34	121.5 – 252	202	213	230	0.0405*
	LL1ab	44	121.5 – 232.5	188.5	206	213	
	SV1	16	120.5 – 249	131.25	218.75	235.625	0.1995
	SV1ab	16	191.5 – 269	205.5	220.75	235.125	
25°C	**AC1**	39	119.5 – 205	130.5	161	175	0.0001**
	AC1ab	51	119.5 – 169	119.5	130.5	147	
	LL1	40	122.5 – 179.67	125.5	130.75	154	0.5802
	LL1ab	44	122.5 – 203	125	130.75	143.5	
	SV1	36	119.5 – 180.5	126.5	138.5	157.125	0.8599
	SV1ab	31	122.5 – 180	122.5	135	147	

While a comparison of the infected and uninfected cohorts revealed no significant differences in the cumulative fecundity for either line at 20°C, the *H. defensa*-carrying Ac1 line produced significantly more offspring ($\bar{x}$= 131.21) than their uninfected Ac1ab counterpart ($\bar{x}$= 86.66) at 25°C (P = 0.0300) (see **Table 2.4**). The logistic regression analysis of aphid survivorship revealed that all clonal lines at 25°C, and most at 20°C, were more likely to survive up to day 13 when they were infected with *H. defensa* (**Table 2.5**). The only exception was SV1 at 20°C, which showed no significant differences in lifespan associated with infection (X^2=0.15, p-value= 0.7023).

Table 2.4. Back-transformed cumulative fecundity per aphid cage (N) in two clonal backgrounds at 20° and 25°C; comparisons were made using an ANOVA t-test ($p<0.05$*).

T	Line	N	Range	Mean ± s.e.	p-value
20°C	Ac1	15	21 – 148	60.51 ± 0.11424	0.5983
	Ac1ab	16	13 – 261	69.09 ± 0.10686	
	Sv1	15	18 – 171	48.36 ± 0.16392	0.3147
	Sv1ab	15	12 – 229	64.48 ± 0.15836	
25°C	Ac1	13	47 – 228	131.21 ± 0.14017	0.0300*
	Ac1ab	14	43 – 258	86.66 ± 0.13507	
	Sv1	12	29 – 229	90.32 ± 0.15866	0.3081
	Sv1ab	10	56 – 231	118.26 ± 0.17380	

Table 2.5. Logistic regression (X^2 $p<0.05$*) of *H. defensa* (HAM) and its effect on aphid survivorship (up to day 13) comparing uninfected and infected lines at each temperature; N represents the number of cup cages per line.

T	Line	N	Regression Equation	X^2	p-value
20°C	Ac1	15	Y = 1.018 - 0.454 HAM	5.8	0.016*
	Ac1ab	16			
	Sv1	15	Y = 1.154 + 0.073 HAM	0.15	0.7023
	Sv1ab	15			
25°C	Ac1	13	Y = 0.375 - 0.509 HAM	8.1	0.0044*
	Ac1ab	15			
	Sv1	12	Y = 0.293 - 0.476 HAM	6.02	0.0141*
	Sv1ab	11			

d. Population Cages Reveal clear Cost

Figure 2.1 shows the mean frequency of *H. defensa* in our *A. craccivora* population cages for each time point. There was a significant decline in infection frequencies over time at both 20° ($p<0.0001$) and 25°C ($p<0.0001$) for line Ac1 (**Table 2.6**). However, symbiont loss occurred earlier and was completely lost from the population at 20°C (see **Figure 2.1**).

This decrease in *H. defensa* over time indicates costs to infection, which may be more harmful at lower temperatures. There was a significant difference of final infection frequencies from the start of the experiment (Time 0) to day 93 at 20°C (likelihood ratio X^2= 155.3483, p-value <.0001) and to day 114 at 25°C (likelihood ratio X^2= 76.6038501, p-value <0.0001).

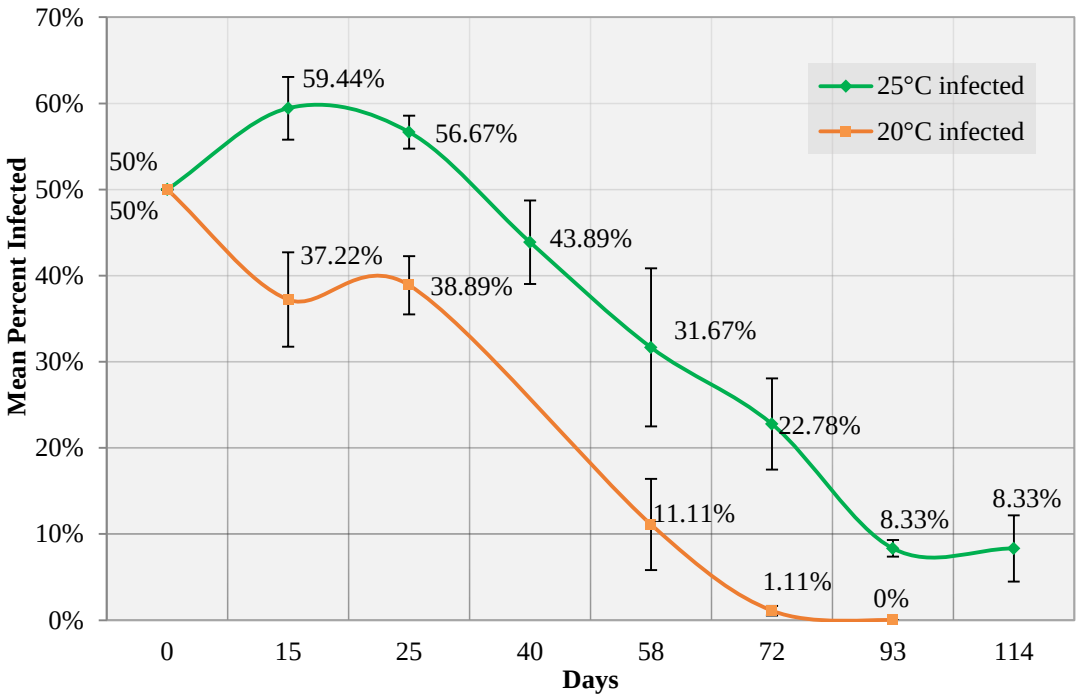

Figure 2.1. The frequency of *H. defensa* infection over time (Days) at 20° (orange) and 25°C (green). For Time 0, all cages started with 50% infected aphids (for both temperatures). For each time point, error bars are presented. Cages at 20°C were screened up to day 93, where no aphids were positive for symbiont presence.

Table 2.6. Linear regression of logit (infection frequency) over time. Slopes for each cage (6 total) and mean slopes for replicate cages at each temperature are included in the table; t-test, p-values*** represent significance; n= data points. ‡ Slope is through day 58; † Slope is through day 72; Slopes at 25°C, through day 114.

T	Cage	Slope	R^2	n	Mean Slope	p-values
20°C	4‡	-0.05349	0.916519	14	-0.0516359	p<0.0001***
	5†	-0.0533	0.941883			
	6†	-0.04872	0.756261			
25°C	1	-0.02604	0.812523	24	-0.0290507	p<0.0001***
	2	-0.03796	0.93586			
	3	-0.02315	0.821796			

DISCUSSION

In the cowpea aphid *A. craccivora*, we identified clear costs to infection associated with the bacterial symbiont *H. defensa*, which may explain why this protective symbiont is not found at higher frequencies in natural populations. Costs were most evident in population cages, where uninfected aphids out-competed those with *H. defensa* resulting in the decline in infection frequency over time to very low levels in cages held at both 20° and 25°C (**Figure 2.1**). However, when we investigated specific fitness parameters, using cup cage assays where aphids were not reared in direct competition, we failed to identify consistent costs among clones and treatments. In the aphid line SV1 there were no significant differences between infected and uninfected aphids among most parameters other than an *increase* in survivorship at 25°C (**Table 2.5**). Other clones showed some costs to infection; *H. defensa*-infected LL1 had delayed development time and reduced mass, but only at one temperature (20°C). A few showed some benefits and costs at the same time like at 25°C where Ac1-infected aphids exhibited delayed development time, yet greater cumulative fecundity. Our results are similar to published work in *A. pisum*, which also failed to identify specific costs to *H. defensa* infection, but found clear costs in population cages (e.g. Oliver et al., 2008; Russell & Moran, 2006). The discrepancy between cup and population cage assays may be due to rearing lines separately, which can create statistical noise not present when different aphid lines are subjected to identical conditions and allowing small fitness differences to be detected (Oliver et al., 2008).

We also documented maternal transmission failures in some of our *H. defensa*-infected aphids. This could also influence symbiont loss within natural populations. It is unlikely that transmission failure contributed to the decline in *H. defensa* frequencies in our population cage studies given the rate of transmission loss observed was 0.67%. Oliver et al. (2008) also considered inefficient transmission an unlikely factor influencing symbiont loss within their population cages given the fidelity of transmission seen in lab-reared *A. pisum* approaches 100%. With *A. craccivora*, however, we observed multiple, anecdotal symbiont loss in the lab due to transmission failures (also reported in Hopper, 2010). Over the course of two years, *H. defensa* was completely lost in four of the six of our lab-reared colonies. This contrasts with rates in *A. pisum*, where no instances of *H. defensa* loss have been recorded across numerous lab-held lines with many maintained for 10+ years (Moran & Dunbar, 2006; Weldon et al., 2013).

Within *A. craccivora*, *H. defensa* confers protection against parasitoid wasps (Asplen et al., unpublished), but there is a cost to infection which leads to reduced symbiont frequencies within a population. Like in *A. pisum*, there appears to be a tradeoff to infection (Oliver et al., 2008). Transmission rates may be substantially lower in the field, due to high or low temperatures (reviewed in Russell et al., 2013) and may be an important factor limiting symbiont spread in natural *A. craccivora* populations, more so than in *A. pisum*.

CHAPTER 3

CHARACTERIZING *H. DEFENSA* WITHIN *A. CRACCIVORA*

INTRODUCTION

Many heritable symbionts, including *H. defensa*, are uncultivable, and hence not amenable to standard microbiological diagnostic tools. However, genomic studies, combined with in vivo experiments, have led to important insights into symbiont function and lifestyle (e.g. Moran et al., 2008; Oliver et al., 2010). Such studies on facultative symbionts, for example, have identified pathways likely involved in protection, and the presence of mobile genetic elements (e.g. bacteriophages) important in the regulation and maintenance of the symbiosis (Degnan & Moran, 2008a; Moran et al., 2005a; Oliver et al., 2009; Oliver et al., 2005; Russell et al., 2003; Weldon et al., 2013).

Recent parasitism assays found that the cowpea aphid *A. craccivora* infected with *H. defensa* carrying the APSE-4 bacteriophage conferred high levels of protection against two *Binodoxys* parasitoid species (Asplen et al., unpublished). A previous study showed that a strain of *H. defensa* transfected from *A. craccivora* into the pea aphid *A. pisum* conferred its new host protection against the parasitoid wasp *A. ervi* (Oliver et al., 2005). Currently only one *H. defensa* strain and APSE haplotype from *A. craccivora* has been characterized (Degnan & Moran, 2008a; b; Moran et al., 2005a). While sequencing additional *H. defensa* or APSE genomes was beyond the scope of this thesis, we hoped to gain insight into the biology of *H. defensa* found in *A. craccivora* by examining strain variation with a Multilocus Sequence Typing method for bacteria using sequences of internal fragments from conserved genes (Maiden et al., 1998). This loci data can also be used in phylogenetic analyses to infer horizontal transfer of symbiont movement among hosts. In collaboration with S.R. Weldon, we sequenced a portion of the variable region of APSE, which encodes for eukaryotic toxins and correlates with levels of symbiont-based

protection provided in *A. pisum* (Degnan & Moran, 2008a; b; Oliver et al., 2009; Oliver et al., 2005; Oliver et al., 2003).

H. defensa's extracellular persistence in the hemolymph may also be important for the protective phenotype, given endoparasitic wasps typically develop in the aphid hemocoel, and are bathed by hemolymph. However, published microscopy studies reveal that the location of *H. defensa* may vary extracellularly or intracellularly (in bacteriocytes and sheath cells) depending on the host (Darby et al., 2001; Moran et al., 2005b; Sandstrom et al., 2001). In addition to tropism, the protective phenotype may also be influenced by symbiont densities within the aphid host, with levels high enough to confer protective benefits, yet low enough to limit fitness costs (Jaenike, 2012; Weldon et al., 2013). Not only is the bacteriophage APSE presence required to confer the protective phenotype against parasitoid wasps (Degnan & Moran, 2008a; Moran et al., 2005a; Oliver et al., 2009; van der Wilk et al., 1999), but it may also regulate symbiont densities within the host as one study found when the phage was lost, *H. defensa* densities increased and reduced *A. pisum* fitness (Weldon et al., 2013). To further characterize the *H. defensa* associated with *A. craccivora*, we performed some basic microscopy and quantitative PCR (qPCR) estimates of *H. defensa* and APSE over time.

METHODS AND MATERIALS

a. Examining A. craccivora hemolymph

H. defensa taken from *A. craccivora* has been successfully transferred and established in a novel host (Russell et al., 2003), suggesting symbiont presence within the hemolymph. To confirm this, we adapted techniques for collecting *A. craccivora* hemolymph and slide preparation from Fukatsu et al. (2000) and Tsuchida et al. (2005). We first confirmed that *H. defensa* was the only symbiont infecting clone Ac1 (see Chapter 2 methods). We then surface-sterilized aphids from line Ac1 with 70% ETOH and their legs were clipped with sterilized tweezer scissors to extract hemolymph. From the wound site we collected the clear exudate into ~75µL of 1X PBS buffer (2g Sodium Chloride, 0.05g Potassium Chloride, 0.36g Sodium

Phosphate Dibasic Ahydrous, 0.06g Potassium Phosphate Monobasic, 200mL of distilled water, 7.4) on a mounting slide. Gut associated bacteria or environmental contaminants are unlikely to be present at high titers in the hemolymph. Samples were then fixed with 4% paraformaldehyde and incubated for ~30min at 4°C. Slides were then stained with ~25µL of VECTASHIELD® Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA), containing 4'-6-diamidino-2-phenylindole (DAPI, 1.5µg/mL), and hemolymph observed with an ultraviolet filter on an EOS fluorimetry (Olympus) microscope.

b. Estimating APSE phage and H. defensa abundance

We estimated symbiont abundances in *A. craccivora* using real-time quantitative PCR. All of the *H. defensa* in our experimental lines carried APSE-4 so phage titers were also estimated. Two clonal lines (Ac1 & Sv1) were evaluated at 20° and 25°C as temperature can influence within-host densities in other insect systems (e.g. Hurst et al., 2000). Newborns (± 2h) from apterous adults were reared in cup cages, consisting of an inverted clear Solo cup (mesh-top vent) over a 3.5" potted ~2 week old *V. faba* plants. Cup cages were held, in replicate, at both treatments in Model I41LLVL Percival biological incubators (Percival Scientific, Inc.) with a 16h:8h Light-Dark (L:D) cycle.

At different stages of nymphal and adult development (48, 96, 144 and 216h), samples for each time point were homogenized in a 20µL reaction of squish buffer (10mM Tris-Cl pH 8.2, 1mM EDTA, 25mM NaCl) and proteinase K (20mg/mL; 1µL per 100µL buffer) (Gloor et al., 1993); then incubated in the Eppendorf® Thermal MasterCycler at 40°C for 35min, 95°C for 2min30s, and with a final hold at 4°C. Due to the small size of 48h old *A. craccivora*, two nymphs were included per 20µL squish extraction, but final values presented represent per aphid titers.

Single copy bacterial (*dnak*) and phage (P28) genes were amplified using a Roche LightCycler® 480 II to estimate the number of bacterial cells or phage genomic copies present in each aphid (Moran et al., 2005a). All 10µL reactions contained 5µL SYBR Green I Master

(Molecular Probes, Inc., Eugene, OR, USA), 1µL (5µM) each of forward and reverse primer (see **Table 3.1**), 2µL of PCR-grade water, and 1µL DNA template (Moran et al., 2005a; Oliver et al., 2009). Master mixes were prepared in a laminar flow cabinet with indirect lighting to reduce SYBR Green I degradation caused by light exposure (Zipper et al., 2004). PCR included: 95°C for 5min; 45 cycles at 95°C for 10s, annealing temperature decreasing from 68°C to 55°C at 18°C increments, 72°C for 10s; 95°C for 5s, 65°C for 1min, then to 97°C; and a final hold at 40°C (Moran et al., 2005a). Amplifications were analyzed with an external 'absolute' standard curve produced from serial dilutions of 1E02 to 1E09 (Oliver et al., 2006). To calibrate the standard curve, each plate contained three standards (1E07 concentration) and two negative controls (with a template of water) for each targeted gene.

Table 3.1. Primer sequences for Titers. Primer sequences and their references: (Moran et al., 2005a) ⫫; Chapter 2$_{\Delta}$ of this thesis.

Primer Name	Sequence (5' to 3')	Target
T70F2 T70R2	F: GGTTCAGAAAAAAGTGGCAG R: CGAGCGAAAGAGGAGTGA	*dnak* (*H. defensa*) ⫫
APSEP28F APSEP28R	F: TGATAAAAGCGGATAATGCC R: GCGTTTGTCATACTGAAAAGG	P28 gene (APSE) ⫫
213F 214R	F: CCGTGGAGATGCACCACGAAGC R: AGCAGCTCCCTTGGGTGGGT	aphid gene ef1α $_{\Delta}$

All *H. defensa* and phage values were corrected to account for differences in extraction efficiencies and aphid size using the ef1α gene. All samples were screened using ef1α primers (213F, 214R) and the Roche LightCycler® 480 generated crossing point (Cp) data. A 'relative' standard curve was created for the aphid ef1α gene with the experimental sample (SV1, *#D9-12*) with the highest Cp value, which was serially diluted (5-fold) and run in triplicate along with two negative controls to create a standard curve. Rather than calculate relative quantifications for

ef1α, we employed the Pfaffl Method (Pfaffl, 2001) to correct for variation when standard curve efficiency values differ (*H. defensa dnak* 2.018, APSE P28 2.013, ef1α 1.908). All normalized concentrations for *dnak* and P28 for each time point were calibrated by the sample with the lowest ef1α Cp value.

c. Examining strain variation in H. defensa and APSE from A. craccivora

Lines investigated and DNA preparation

We sampled *H. defensa* strain diversity from our six lab-held infected colonies (designated as *KO* samples, all from the U.S.) and 16 additional lines, collected from the U.S. and Serbia (provided by Jennifer White, Univ. of KY, designated *JW* samples), see **Table 3.2**. DNA extractions were prepared with either the E.Z.N.A. Insect DNA Isolation Kit (Omega Bio-Tek) or DNeasy blood and tissue kit (Qiagen) following the manufacturer's protocols.

Table 3.2. *KO* and *JW* samples.

	N	Location
KO	4	Tucson, AZ
	1	Landlord, KY
	1	Shaker Village, KY
JW	1	Yuma, AZ
	6	Chickasha, OK
	2	Serbia, Baranda
	1	Urbana, IL
	1	Ankeny, IA
	1	Serbia, Baranda
	4	Serbia, Mt. Vlasina

H. defensa and phage Diversity in A. craccivora

To identify symbiont diversity, we used primers and PCR protocols previously established by Degnan and Moran (2008b). We Sanger sequenced, in both directions, six *H. defensa* housekeeping genes (accD, dnaA, hrpA, recJ, ptsI, and rpoS) and two genes involved with the type three secretion system (invC, spaP). We sequenced all eight genes for our *KO* lab-

lines and five genes for the *JW* samples (spaP, ptsI, invC, rpoS, and hrpA). Primers were designed with Geneious Pro V.5.4.6 (Biomatters, Ltd.) to amplify the phage-encoded toxin region, thought to contribute to wasp mortality, flanked by the highly conserved core genes (e.g. P3) of the APSE genome (Degnan & Moran, 2008a). A portion of this variable cassette region (VCR) of APSE was sequenced (modified from Degnan & Moran, 2008a; b) from region P5 to P10. Primers for *H. defensa* are listed in **Table 3.3**, and those for phage are in **Table 3.4**.

We prepared 30µL PCR reactions containing 15µL of 2X GoTaq® Hot Start Colorless Master Mix (Promega, Madison, WI, USA) (2X Colorless GoTaq® Reaction Buffer pH 8.5, 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, and 4mM $MgCl_2$); 3µL (5µM) each of the forward and reverse primers; 6-7.5µL of PCR-grade water; and 1.5-3µL DNA template (~9-15ng/µL). For sequencing *H. defensa* genes, PCR conditions included 1 cycle at 94°C for 2min; 11 cycles at 94°C for 30s; a touchdown from 58°C to 46°C for 50s, 72°C for 1.5min; then 25 cycles at 94°C for 30s, 46°C for 50s, 72°C for 1.5min; 72°C for 5min; and samples were stored at 4°C until gel electrophoresis. PCR for the APSE-4 variable cassette region included 1 cycle at 94°C for 4min; 25 cycles of 94°C for 1min, 50°C for 1min, 72°C for 2min; 72°C for 10min, and a hold 14°C. For PCR with APSE-2 there was 1 cycle at 94°C for 2min; 35 cycles of 94°C for 30s, 60°C for 45s, 72°C for 30s; 5min at 72°C; and a final hold at 4°C.

PCR products were run on 1% agarose 1XTBE gels at 90V for ~45min. Each gel included a GeneRuler™ 100bp DNA ladder (3µL) and a negative PCR control. Gels were prepared in one of two ways: pre-stained with the addition of Biotum Gel Red nucleic acid dye (Biotum, Hayward, CA) prior to gel casting (0.5µL/100mL 1XTBE); or stained post-electrophoresis for 15-20min in a sealed container of Biotum Gel Red (1:100 µL in 1X TBE). Stained gels were viewed under UV light with a gel imaging system (BioRad), and band lengths were scored according to their alignment relative to the ladder. PCR products were cleaned with Fermentas (Glen Burnie, MD) FastAP ™ (0.5µL) and Exonuclease I (0.3µL), incubated for 10min at 37°C and 5min at 75°C, and then sent for automated Sanger sequencing (Eurofins

MWG Operon, Huntsville, Al, USA). The forward and reverse sequences were manually inspected for mismatched base pair assignments, ambiguities, and poor quality ends trimmed using Geneious Pro V.5.4.6 software (Biomatters Ltd.).

Table 3.3. Primer sequences and their target bacterial genes (Degnan & Moran, 2008b).

Loci	Sequence (5' to 3')	Target
accD 291/832	F: TTCTGGAGCACAAAAAGACAC R: AAGGTTCAGGTTGATGAGTCAG	carboxyl transferase subunit ß
dnaA 146/1209	F: GTTTTGTTTTGGATTGGGTTC R: TCAGGAAGACTATGATTGGTGAG	transcriptional dual regulator
hrpA 106/984	F: AAACCCAATCTGACAAAAATAGG R: TAACTCTTCGGCTTCTGACAAC	ATP-dependent helicase
invC 65/834	F: CACCCCCTTCTTATTTACATCG R: GGTATTCACCCAGTTCAACAAAC	T3SS ATP Synthase
ptsI 181/709	F: ATTTTACGGGCTTCTGCTTTTG R: CTTCGGTGGTTGATTGACTCAG	phosphotransferase enzyme I
recJ 44/1012	F: ATCCGCTCTCAGAAACATACC R: GATGACATAAATCCAATGCCTC	5'→3' exonuclease
rpoS 165/862	F: AGTAGAAAATGTCACACAGCG R: GGGCTCGTAACCTAATAATCC	RNA polymerase sigma-38
spaP 37/528	F: TTTTCAACATTATTACCCTTCGTC R: CTCATCATCATCATCCCCAAC	T3SS inner membrane component

Table 2.4. Primers for the APSE variable cassette region (VCR) (modified from Degnan & Moran, 2008a; b).

Loci	Sequence (5’ to 3’)	VCR
APSE4 P5/P8	F: AGACATGGACCCCGAGGTGA R: TCGCCTACTACAATACCTACCTGGC	P5 to P8
APSE4 P9/P10	F: ACCTGGACCCATCAAAGAGAGTTCA R: AACGTAGCAAGGCCAGGCGG	P9 to P10
APSE2 VR5	F:GCACTAAGCGATGTTATCGGCATAGAA R: TGCCAACTATACTCTCTCACTGTTCCA	P5

We took representative sequences for each bacterial strain (no variation within strains was detected) found and estimated pairwise divergences in relation to the published sequences of

5ATac, the strain of *H. defensa* taken from *A. pisum*, but originating from *A. craccivora* (Degnan & Moran, 2008a). Complementary nucleotide sequences for individual genes were aligned, gaps removed, and uploaded in PHYLIP format and analyzed with codeml in PAML v4.7 (Yang, 2007) (seqtype=1, runmode=-2).

H. defensa Phylogenetic Trees

We constructed phylogenetic trees using the three distinct strains identified in *A. craccivora* combined with homologous sequences found in Genbank (NCBI) for 24 *H. defensa* and three related species from the Enterobacteriaceae—*Escherichia coli*, *Yersenia enterocolitica*, and *Salmonella enterica* subsp. *enterica serovar typhimurium* (for list of Accession numbers to those collected from Genbank, see **Appendix B**). Sequence alignments were made for each gene (invC, ptsI, spaP, and rpoS) in Geneious Pro v6.1.2 (Biomatters, Ltd.) using the global alignment algorithm. Uneven ends were trimmed flush and the four loci were then concatenated and realigned. A prior study found no evidence of recombination for these loci (Degnan & Moran, 2008b). The best-fit model of nucleotide evolution was chosen with the Akaike information criterion (AIC) using jModelTest 2.1.3 (Darriba et al., 2012; Guindon & Gascuel, 2003). We generated maximum likelihood trees with 100 bootstrap replicates using PhyML 3.0 (Guindon et al., 2010) and Bayesian trees using MrBayes v3.2.1 (Huelsenbeck & Ronquist 2001) with *E. coli* as the root and the generalized time-reversible model (with gamma rate variation, subsampling frequency 200, and burn-in length 110,000 generations). We also built a second intraspecies-only Bayesian tree from a multilocus alignment of the same *H. defensa* sequences, but excluding the enteric outgroups, and rooted to a strain from the whitefly, *Bemisia tabaci*, using the Hasegawa, Kishino, and Yano 1985 model (+ invariable sites).

Evaluating Aphid Genetic Background

Given the anholocylcic (only reproducing asexually) life cycle of this aphid, we wanted to determine if there were any easily detected variations among our *A. craccivora* clones, which may influence symbiont and phage diversity. We used four existing microsatellite (MSAT)

markers known to amplify polymorphic non-coding DNA regions (short tandem repeats) in the related aphid species *Rhopalosiphum padi* (Simon et al., 2001) and *Aphis gossypii* (Vanlerberghe-Masutti et al., 1999) to increase the likelihood of amplification in *A. craccivora* (Squirrell et al., 2003; Wilson et al., 2004). In addition to the *22* samples infected with *H. defensa*, DNA extractions of four naturally uninfected aphid lines (designated $KO^{\emptyset}$) were also prepared as mentioned above. PCR products (see **Table 3.5** for primers and reaction conditions) were run on 1.5% agarose gels in 1X TBE buffer at 100V for ~50min. PCR reactions producing products of the expected length were cleaned and sequenced. We used the Blastn function on the NCBI database to verify all amplified products matched reference sequences from the original papers (see **Appendix C** for list).

Table 3.5. MSAT primers & PCR specifications. PCR conditions taken from † Harrison and Mondor (2011) and also ‡Wilson et al. (2004); Δ *Rhopalosiphum padi* (Simon et al., 2001), and ╬ *Aphis gossypii* (Vanlerberghe-Masutti et al., 1999) are the reference organisms.

Loci	Primer Sequence (5'-3')	PCR
R5.10 Δ	F: CCGACTAAGCTTAATATTGTTTG R: CGGTTCGGAGAACATAAGAG	Denaturation 94°C for 2m; one cycle of 62°C 30s, 72°C 45s, 94°C 15s; one cycle of 61 30s, 72°C 45s, 94°C 15s; one cycle of 59°C 30s, 72°C 45s, 94°C 15s; one cycle of 57°C 30s, 72°C 45s, 94°C 15s; 30 cycles of 55°C 30s, 72°C 45s, 94°C 15s; final extension 72C for 2min ‡
Ago53 ╬	F: TGACGAACGTGGTTAGTCGT R: GGCATAACGTCCTAGTCACA	Denaturation at 94°C for 4min; 35 cycles of 30s at 94°C, 35s at 58°C, and 45s at 72°C; and a final 10min extension step at 72°C †
Ago59 ╬	F: GCGAGTGGTATTCGCTTAGT R: GTTACCCTCGACGATTGCGT	
Ago126 ╬	F: GGTACATTCGTGTCGATTT R: TAAACGAAAAAACCACGTAC	

RESULTS

a. H. defensa present within the hemolymph

With fluorescent stereomicroscopy, we were able to confirm the extracellular presence of *H. defensa* in *A. craccivora* hemolymph, see **Figure 3.1**. Within the aphid line Ac1, a clone known to only harbor *H. defensa* and no other facultative symbiont (see Chapter 2), rod-shaped bacteria (~2μm long) were seen typically as individuals or in pairs throughout the hemolymph, similar to how *H. defensa* is described in *A. pisum* (Darby et al., 2001; Moran et al., 2005b; Sandstrom et al., 2001). Within *A. craccivora*, *H. defensa* is found freely throughout the hemolymph in the same location where wasp larvae would also reside within the aphid host.

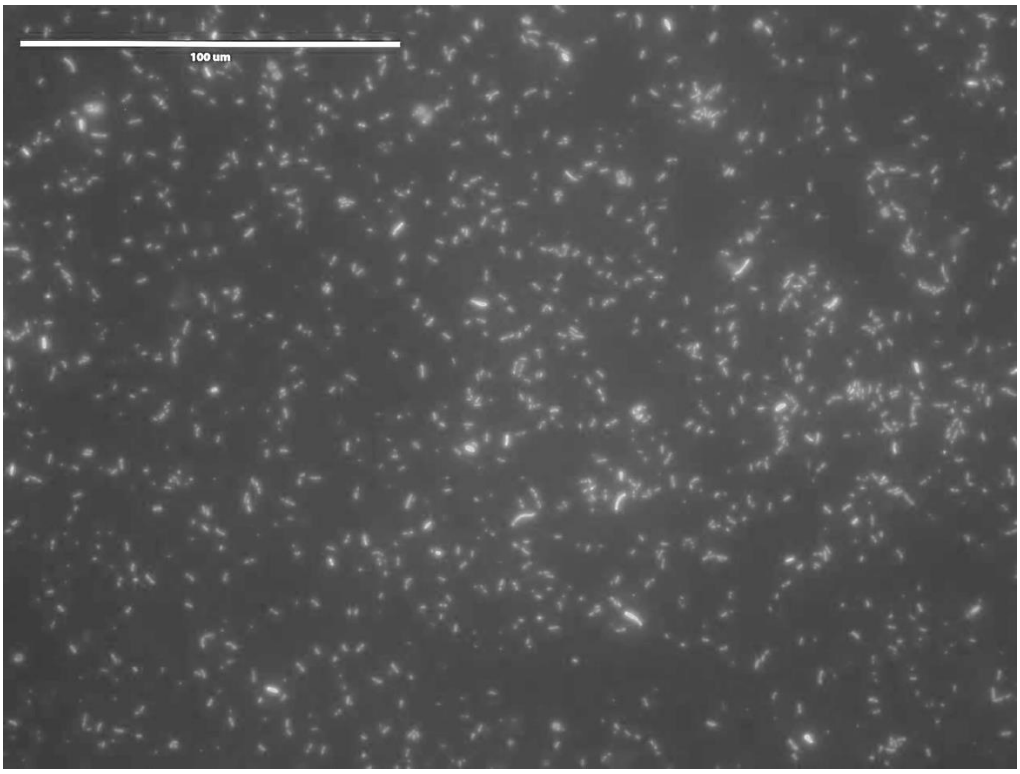

Figure 3.1. *H. defensa* DAPI stain. Wet mount of hemolymph extracted from three *A. craccivora*. The bacteria can be seen as individual rods or in chained pairs.

b. H. defensa and Phage Abundance in A. craccivora

We estimated the abundances of APSE and *H. defensa* in aphids at several developmental stages using qPCR. Overall, we saw increases in both *H. defensa* and APSE titers from 48h old nymphs (~2nd instar) to 9d old adults (**Figure 3.2 A-D**). Looking at the overall mean trend and at each age in line Ac1, there was always more phage genomic copy (ratio range: 4.8 to 36.9 APSE per *H. defensa*) with slightly higher ratios found at 25°C compared to 20°C (**Figure 3.3**). In line SV1 the ratios of phage to *H. defensa* were lower than the Ac1 line, but mean ratios at 25°C were greater than 20°C in 2d, 4d, and 9d old aphids (ratio range: 10 to 43.9). In 6d old SV1 aphids, 20°C had more phage present (16.3 ratio difference).

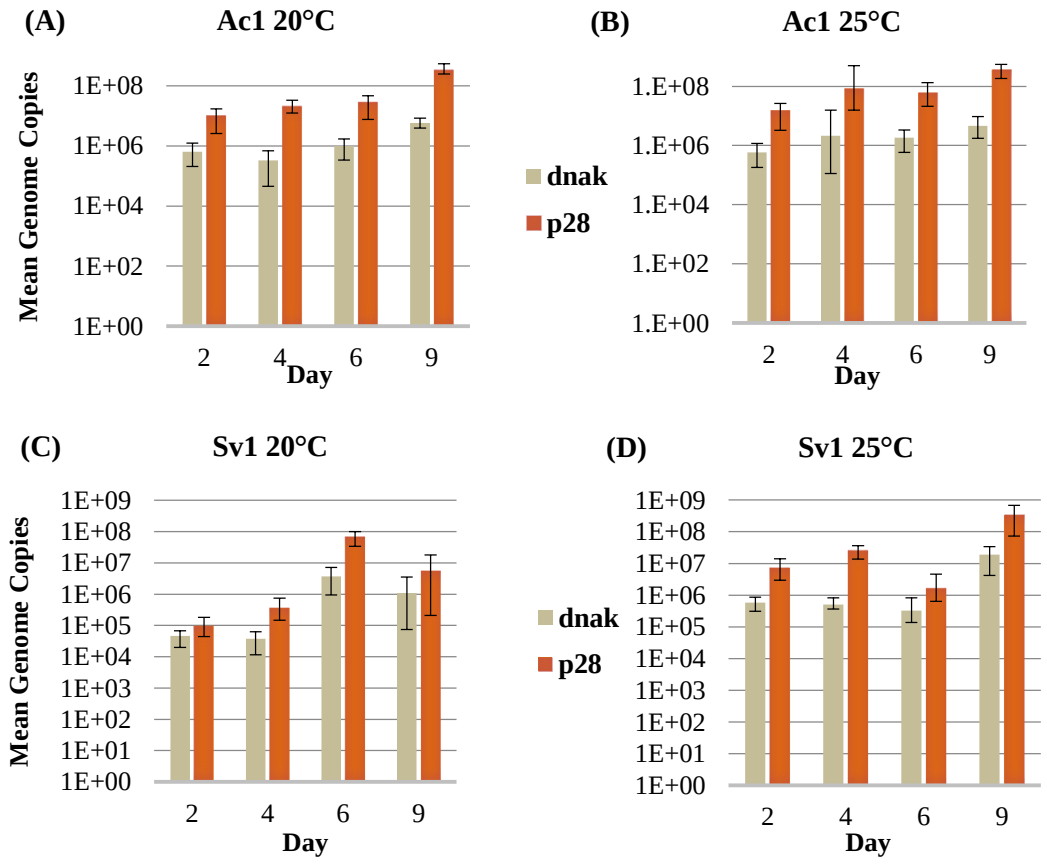

Figure 3.2 (A-D). Bacterial & Phage Titers. Mean genomic copies (y-axis) of *H. defensa* (*dnak*) and APSE (P28) for Ac1 (A-B) and Sv1 (C-D) at 20° and 25°C. Values represent whole aphid (n=8). Bars represent range.

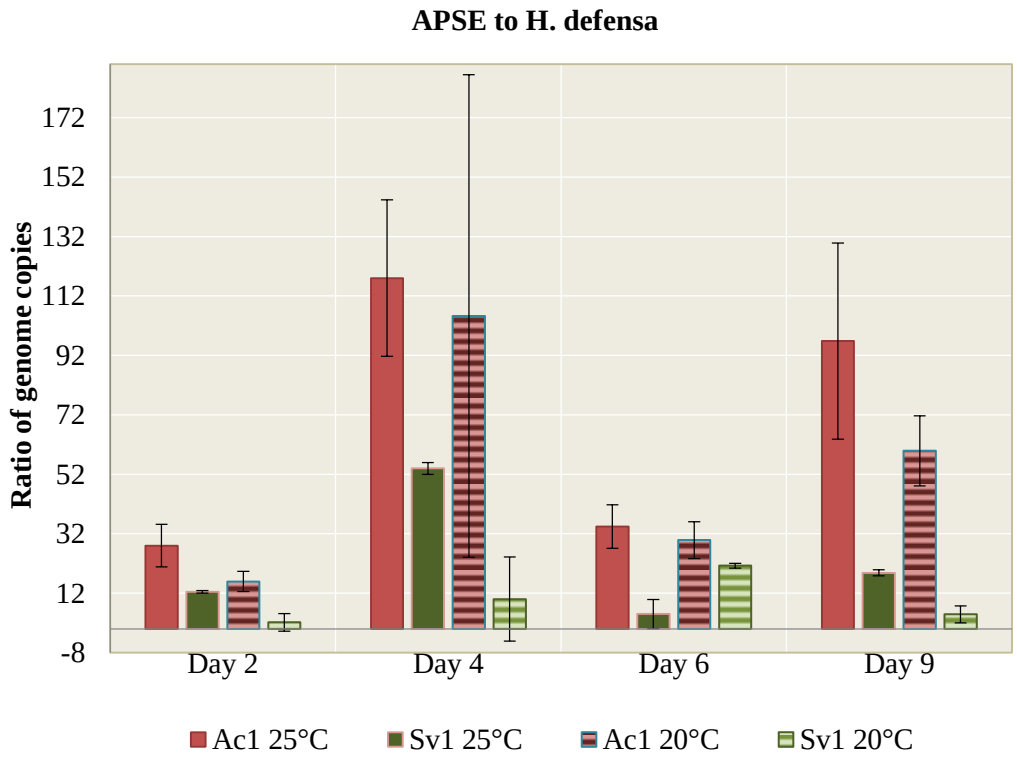

Figure 3.3. Mean Ratio of APSE copies to *H. defensa* copies. For each column, the mean ratio (n=8); bars represent 95% confidence intervals.

c. Assessing Symbiont Diversity in A. craccivora

H. defensa Diversity

We detected three distinct *H. defensa* strains (*str.*) among the 22 *H. defensa* infected *A. craccivora* clones evaluated. Out of the 22 samples screened, 19 comprised *str. AcHd1* 100% identical to each other and the previously published 5ATac sequence. Two of the Serbian clones were infected with a 2nd variant (*str. AcHd2*), and the 3rd (*AcHd*3) came from a single clone collected in Arizona. Gene sequences, overall, were highly similar (≥98.6%) and dN/dS values were low (**Table 3.6**) across all five loci.

Table 3.6. *H. defensa* pairwise sequence comparisons relative to the reference gene sequences of 5ATac. % Identity is the percentage of identical bases per residue. dN/dS values were calculated in CODEml.

Gene	strain	% Identity	dN	dS	dN/dS
rpoS	*str. 1*	100	0	0	0.2586
	str. 2	98.6	0.0097	0.0322	0.3029
	str. 3	98.8	0.0077	0.0333	0.2314
ptsI	*str. 1*	100	0	0	0.0425
	str. 2	99.2	0.0025	0.0323	0.0773
	str. 3	99.6	0	0.0217	0.001
invC	*str. 1*	100	0	0	0.0686
	str. 2	99.6	0	0.022	0.001
	str. 3	99.7	0	0.0146	0.001
hrpA	*str. 1*	100	0	0	0.4769
	str. 2	99.5	0.004	0.0084	0.473
	str. 3	99.5	0.002	0.0127	0.1571
spaP	*str. 1*	100	0	0	0.001
	str. 2	99.4	0.003	0.0164	0.1842
	str. 3	99.6	0	0.0207	0.001

Phage Diversity

We performed diagnostic PCR with primers specific for the shiga-like toxin (*stx*) associated with APSE-4 (N=21), and sequenced the variable cassette region for six samples. We confirmed *stx* encoding APSE in all *str. AcHd1* but not for *AcHd2* or *AcHd3*. This suggests that *AcHd1* all likely carry APSE-4, but ongoing work to identify phage-types using a multilocus sequence typing approach will confirm this. The variable cassette region for both *AcHd2* and *AcHd3*, however, amplified with primers diagnostic for cytolethal distending toxin subunit B (*cdtB*), which is associated with other APSEs, including APSE-2 found in *A. pisum*. Currently, the APSEs in *A. craccivora* are being further characterized with additional sequencing within the variable cassette region.

A. craccivora Clonal Structure

With MSATs, we distinguished two *A. craccivora* clonal variants among infected and uninfected lines at each loci: a majority type found in both the U.S. and Serbia (variant *a*, n=22),

and a second only identified in a few aphids from Serbia (*b*, n=4). Variant *b* aphids were collected from Mt. Vlasina, and they harbored the *H. defensa str. AcHd1*. In limited sampling, we found no correlation between aphid clonal genotype and *H. defensa* strain (**Table 3.7**).

Table 3.7. Clone DNA sequence results for aphid (MSAT), *H. defensa* (*AcHd*), and the variable cassette region for APSE (VCR). Those (*) VCR regions were sequenced. *N* is the number of clones. No tests were performed where gray boxes present.

	N	Location	MSAT	*AcHd*	VCR
KO	4	Tucson, AZ	*a*	*str. 1*	APSE-4*
	1	Landlord, KY	*a*	*str. 1*	APSE-4*
	1	Shaker Village, KY	*a*	*str. 1*	APSE-4*
JW	1	Yuma, AZ	*a*	*str. 3*	APSE-2 like
	6	Chickasha, OK	*a*	*str. 1*	APSE-4
	2	Serbia, Baranda	*a*	*str. 2*	APSE-2 like
	1	Urbana, IL	*a*	*str. 1*	APSE-4
	1	Ankeny, IA	*a*	*str. 1*	APSE-4
	1	Serbia, Baranda	*a*	*str. 1*	APSE-4
	4	Serbia, Mt. Vlasina	*b*	*str. 1*	APSE-4
$KO^{Ø}$	4	Tucson, AZ	*a*		

Phylogenies of H. defensa strains

Our phylogenies were built using concatenated sequences (at four loci) of 24 *H. defensa* strains and three related enteric bacterial species. The Maximum likelihood (**Appendix D**) and Bayesian phylogenies recovered similar topologies to each other and an earlier phylogeny of this symbiont (Degnan & Moran, 2008b). Only the Bayesian tree is presented (**Figure 3.4**), which shows that the three *A. craccivora* strains (in orange, **Figure 3.4**) did not cluster together. That these strains each cluster in distinct clades highly suggest independent acquisitions of *H. defensa* via lateral transfer from other host species. The *A. craccivora* strains also did not cluster together on the intraspecies tree (**Appendix E**).

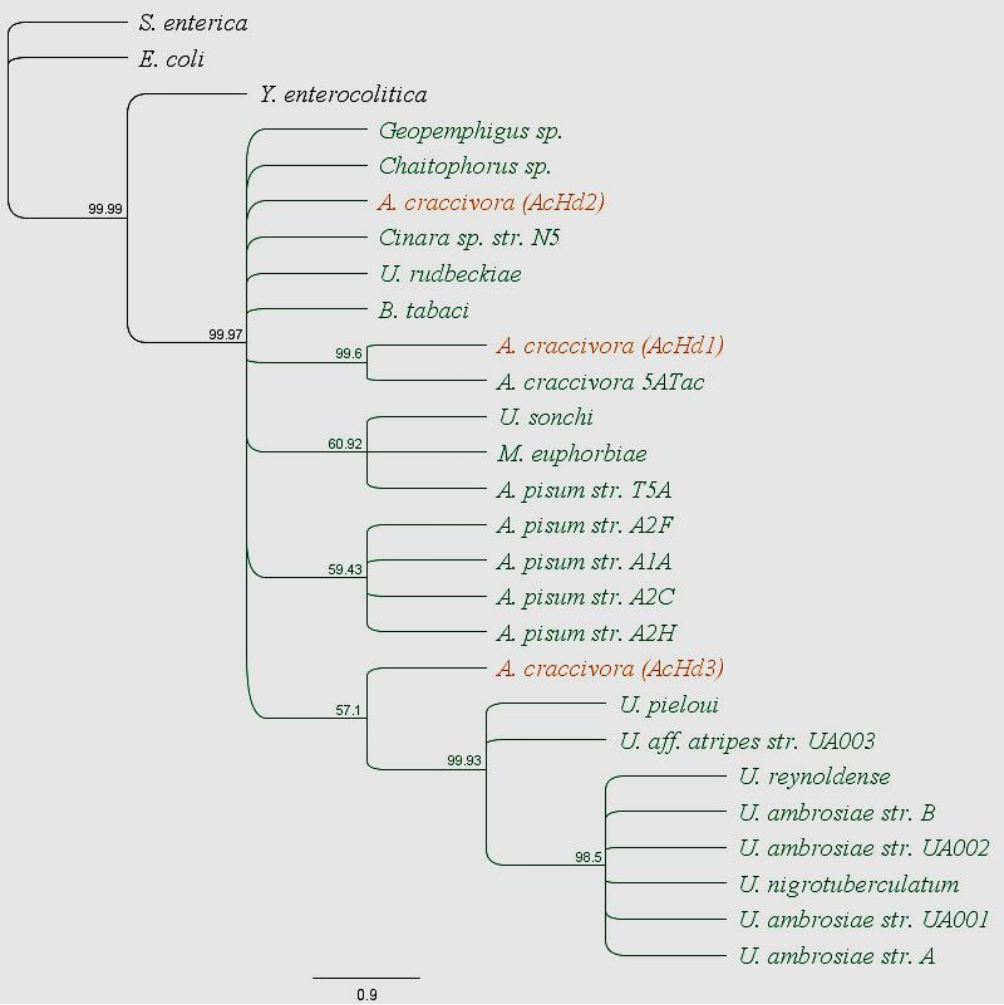

Figure 3.4. Multilocus Bayesian phylogeny of *H. defensa* and related enteric bacteria (in black) with posterior probabilities of four loci (ptsI, invC, spaP, rpoS). *H. defensa* names (green & orange) correspond to their host.

DISCUSSION

Limited Clonal and H. defensa Diversity in A. craccivora

We performed a range of diagnostics to further characterize the symbiont *H. defensa* found in the cowpea aphid *A. craccivora*. Using a multilocus sequencing approach we examined strain diversity among 22 *H. defensa*-infected clones, but found only three distinct variants. The majority of clones examined 19/22 harbored a single strain type. Phylogenetic analyses, using both Bayesian and maximum likelihood algorithms, recovered similar tree topologies and placed

each of these three strains within distinct clades indicating independent infections of *H. defensa* into *A. craccivora* likely arising from lateral transfer from other host species. This is typical of facultative heritable symbionts and in contrast to the pattern of strict co-speciation and co-cladogenesis seen in obligate symbionts like *Buchnera* (Moran et al., 2008). The vast majority (14/15) of N. American strains were all one type (*AcHd1*), but though our sampling was more limited, *H. defensa* diversity appears to be higher in *A. pisum* where Russell et al. (2013) found multiple *H. defensa* strains within and among five aphid populations in three geographic locales (PA, UT, WI). In pea aphids, only three APSE variants have been recorded in limited sampling (Degnan & Moran, 2008a). We found at least two APSE variants in *A. craccivora*, one being the previously identified APSE-4 characterized by a shiga-like primary toxin (*stx*) and a second encoding a different toxin (*cdtB*) similar to APSE-2 found in *A. pisum* (Degnan & Moran, 2008a). The *H. defensa* (from 5ATac) with APSE-4 is known to improve *A. pisum* survivability at elevated temperatures (Russell & Moran, 2006) and confer resistance to parasitoids (Asplen et al., unpublished; Oliver et al., 2005). APSE-2s have been shown to confer moderate levels of protection against parasitism in *A. pisum* (Oliver et al., 2003) so this *H. defensa* strain may also confer protection in *A. craccivora*; something worthy of testing in future trials.

Further work is ongoing to characterize the APSEs associated with *H. defensa* strains infecting *A. craccivora*. The preliminary evidence that some *A. craccivora* harbor APSE-2-like phages suggests that the virus may have moved laterally between these two aphids that share host plants. Further work is needed to confirm this, but phylogenetic studies indicate that APSEs move laterally within *H. defensa* in *A. pisum* (Degnan & Moran, 2008b) and it is plausible they occasionally move among host species as well. By sequencing microsatellite markers we also found little evidence of clonal diversity in *A. craccivora*. The latter may not be surprising given the anholocyclic lifecycle across most of its range, as well as genetic bottlenecks associated with introductions to novel regions (Gutierrez et al., 1974; Lockwood et al., 2007). These effects likely contribute to the limited diversity of *H. defensa* within this species. However, given that heritable

symbionts can jump into novel hosts, they have the potential to comprise much of the heritable genetic variation associated with asexual invasive species.

Tissue Tropism and Symbiont Titers

Through stereomicroscopy using DAPI staining, we confirmed *H. defensa* persists in the hemolymph of *A. craccivora*. Tissue tropism is likely important given this symbiont's role in preventing successful parasitoid attacks. The occurrence of free-living *H. defensa* throughout the hemolymph allows for direct contact with wasp tissue.

We estimated phage and *H. defensa* abundances in two *A. craccivora* lines throughout aphid development at both 20° and 25°C. In all lines and treatments, we observed *H. defensa* and APSE titers increase with aphid age (**Fig. 3.2 A-D**) as seen in *A. pisum* (Martinez et al., submitted; Weldon et al., 2013). We also found that APSE titers exceeded those of *H. defensa* (as seen in *A. pisum*), and the APSE-*H. defensa* ratio varied with aphid genotype, temperature, and over time. In *A. pisum*, APSEs play a role in regulating the protective symbionts probably through lytic activity (Weldon et al., 2013). More study is needed in *A. craccivora* to examine the role of APSE-4 in influencing symbiont abundance. Compared to *A. pisum, H. defensa* densities are generally lower at equivalent time points. For example, 2d old *A. pisum* were found to have ~1E07 *H. defensa* per aphid (Weldon et al., 2013) while *A. craccivora* values were all two orders of magnitude lower (max. value= 6.41E05 in Ac1 at 20°C). The cowpea aphid is much smaller than *A. pisum* so lower titers are to be expected as these symbionts occur extracellularly within the hemocoel in both species.

Overall, the role of *H. defensa* in both *A. pisum* and *A. craccivora* appears similar. *H. defensa* protects both aphid species from attack by braconid parasitoids (e.g. Asplen et al., unpublished; Oliver et al., 2003; Oliver et al., 2005) and costs to infection in the absence of parasitoid pressure have been identified in both systems (see Chapter 2; Oliver et al., 2008). It appears host protection is a phenotype often associated with *H. defensa* infection, though not all

strains confer protection like those unprotective strains identified in both the grain aphid *Sitobion avenae* (Lukasik et al., 2013) and in *A. pisum* (Martinez et al., 2013). There is also evidence of horizontal transmission of *H. defensa* in both aphid systems, with ramifications for biological control efforts for invasive pests like *A. craccivora.* Further study is needed to understand *H. defensa* symbioses in other invasive species.

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APPENDICES

A. Collection data for *A. craccivora* clones. N= number of clonal lines; *KO* samples were lab-reared and *JW* DNA samples were provided by Jennifer White (University of Kentucky). ¥DGGE confirmed *H. defensa* presence for *KO* samples, and we confirmed *JW* samples were infected using qPCR.

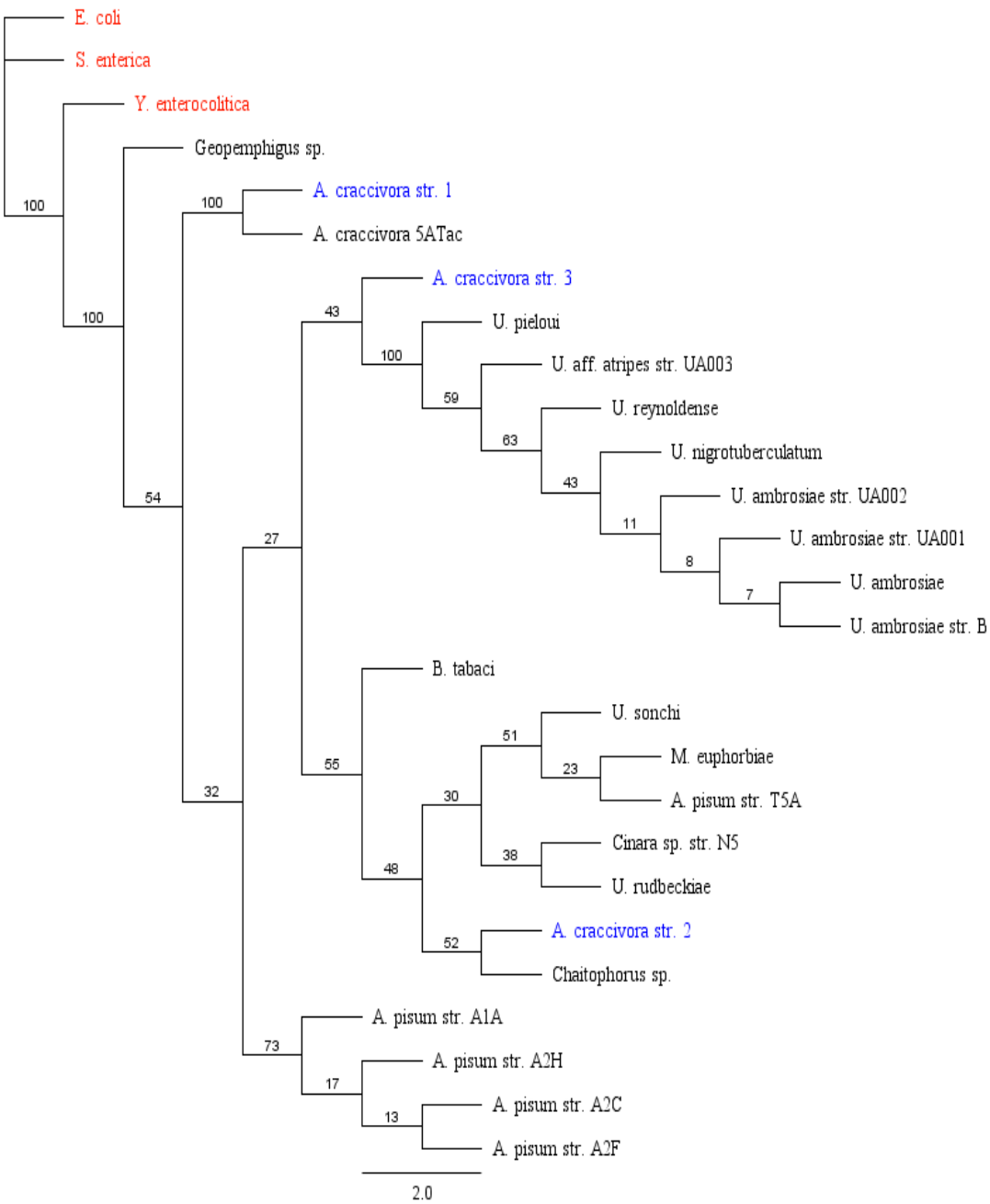
Samples	**N**	**Location**	**Year**	**Host Plant**	***H. defensa*¥**
KO	1	Tucson, AZ	1999	*Medicago sativa*	+
	3	Tucson, AZ	2009-2010	*Medicago sativa*	+
	1	Landlord, KY	2011	*Medicago sativa*	+
	1	Shaker Village, KY	2011	*Medicago sativa*	+
JW	4	Tucson, AZ	2009-2010	*Medicago sativa*	(-)
	1	Yuma, AZ	2010	*Medicago sativa*	+
	6	Chickasha, OK	2011	*Medicago sativa*	+
	3	Serbia, Baranda	2011	*Medicago sativa*	+
	4	Serbia, Mt. Vlasina	2011	*Medicago sativa*	+
	1	Urbana, IL	2010	*SUCTION TRAP*	+
	1	Ankeny, IA	2009	Medicago sativa	+

B. Multilocus Accession Numbers. Sequences were used to generate *H. defensa* phylogenies.

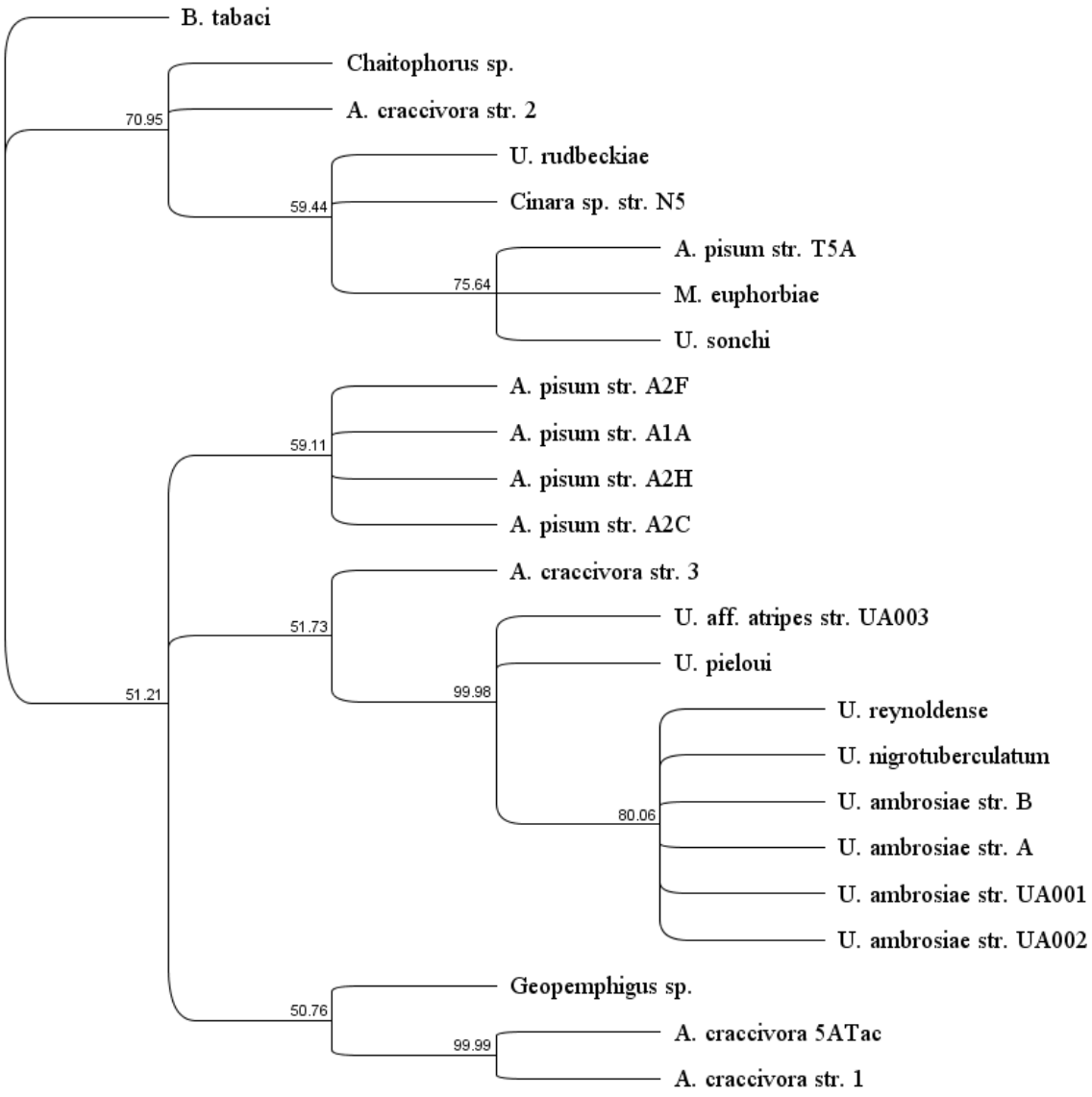
Bacterial loci	***ptsI***	***rpoS***	***spaP***	***invC***
Alignment length	504 bp	675bp	465 bp	684 bp
H. defensa				
Ac. pisum str. 5AT	EU021860	EU021727	EU021954	EU021932
Ac. pisum str. A1A	EU021861	EU021728	EU021955	EU021933
Ac. pisum str. A2C	EU021862	EU021729	EU021956	EU021934
Ac. pisum str. A2F	EU021863	EU021730	EU021957	EU021935
Ac. pisum str. A2H	EU021864	EU021731	EU021958	EU021936
Ap. craccivora str. 5ATac	EU021865	EU021732	EU021959	EU021937
B. tabaci bv. B	EU021866	EU021733	EU021960	EU021938
Geopemphigus sp.	EU021867	EU021734	EU021961	EU021939
M. euphorbiae	EU021868	EU021735	EU021962	EU021940
U. ambrosiae str. A	EU021869	EU021736	EU021963	EU021941
U. ambrosiae str. B	EU021870	EU021737	EU021964	EU021942
U. ambrosiae str. UA001	EU021871	EU021738	EU021965	EU021943
U. ambrosiae str. UA002	EU021872	EU021739	EU021966	EU021944
U. sp. near *atripes*	EU021873	EU021740	EU021967	EU021945
U. nigrotuberculatum	EU021874	EU021741	EU021968	EU021946
U. pieloui	EU021875	EU021742	EU021969	EU021947
U. rudbeckiae	EU021876	EU021743	EU021970	EU021948
U. sonchi	EU021877	EU021744	EU021971	EU021949
U. reynoldense	EU021879	EU021746	EU021972	EU021951
Chaitophorus sp.	EU021881	EU021748	EU021974	EU021952
Cinara sp.	EU021882	EU021749	EU021975	EU021953
E. coli O157:H7 str. EDL933	NC_002655			
S. enterica subsp. enterica serovar Typhimurium str. LT2	NC_003197			
Y. enterocolitica str. 8081	NC_008800			

C. MSATs. We sequenced 26 aphid DNA samples at four microsatellite (MSAT) loci (base pair, bp, sequence length range) and only found two variant clones: Var. *a* (22/26) & Var. *b* (4/26). To verify sequence products, representative clones for each variant were run through the nBLAST (NCBI) search. The matching Accession #'s, the expected values, and % nucleotide similarity are included in the table below.

MSAT	Length	BLAST MatchMatch	Var.	n	Expect	Similarity
R5.10	209-250 bp	AF277462	*a*	1	3.00E-39	83%
			b	1	3.00E-49	83%
Ago53	75-118 bp	AF092526	*a*	2	1.00E-34 to 2.00E-26	91-97%
			b	1	1.00E-34	91%
Ago59	119-149 bp	AF092527	*a*	1	1.00E-45	98%
			b	1	3.00E-42	99%
Ago126	108-160 bp	AF092532	*b*	1	7.00E-44	88%
			a	3	3.00E-26 to 5.00E-14	86-87%



D. Multilocus Maximum-Likelihood. ML Phylogeny for *H. defensa* with bootstrap support. All *H. defensa* names correspond to their host (blue and black); outgroups are in red.



E. Bayesian phylogeny of *H. defensa*-only. Variant strains are named according their host and are rooted to *B. tabaci*. Posterior probabilities are also included.