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Worldwide Populations of the Aphid *Aphis craccivora* Are Infected with Diverse Facultative Bacterial Symbionts

Cristina M. Brady · Mark K. Asplen · Nicolas Desneux · George E. Heimpel · Keith R. Hopper · Catherine R. Linnen · Kerry M. Oliver · Jason A. Wulff · Jennifer A. White

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Abstract Facultative bacterial endosymbionts can play an important role in the evolutionary trajectory of their hosts. Aphids (Hemiptera: Aphididae) are infected with a wide variety of facultative endosymbionts that can confer ecologically relevant traits, which in turn may drive microevolutionary processes in a dynamic selective environment. However, relatively little is known about how symbiont diversity is structured in most aphid species. Here, we investigate facultative symbiont species richness and prevalence among worldwide populations of the cowpea aphid, *Aphis craccivora* Koch. We surveyed 44 populations of *A. craccivora*, and detected 11 strains of facultative symbiotic bacteria, representing six genera. There were two significant associations between facultative symbiont and aphid food plant: the symbiont *Arsenophonus* was found at high prevalence in *A. craccivora* populations collected from *Robinia* sp. (locust), whereas the symbiont *Hamiltonella* was almost exclusively found in *A. craccivora* populations from *Medicago sativa* (alfalfa). Aphids collected from these two food plants also had divergent mitochondrial haplotypes, potentially indicating the formation of specialized aphid lineages associated with food plant (host-associated differentiation). The role of facultative symbionts in this process remains to be determined. Overall, observed facultative symbiont prevalence in *A. craccivora* was lower than that of some other well-studied aphids (e.g., *Aphis fabae* and *Acyrthosiphon pisum*), possibly as a consequence of *A. craccivora*'s almost purely parthenogenetic life history. Finally, most (70 %) of the surveyed populations were polymorphic for facultative symbiont infection, indicating that even when symbiont prevalence is relatively low, symbiont-associated phenotypic variation may allow population-level evolutionary responses to local selection.

The nucleotide sequence data reported in this manuscript have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers KF362016–KF362043, with a public release date of September 13, 2013.

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C. M. Brady · J. A. Wulff · J. A. White (✉)
Department of Entomology, University of Kentucky, Lexington, KY 40546, USA
e-mail: jenawhite@uky.edu

M. K. Asplen · G. E. Heimpel
Department of Entomology, University of Minnesota, St. Paul, MN 55108, USA

N. Desneux
French National Institute for Agricultural Research (INRA), 06903 Sophia Antipolis, France

K. R. Hopper
USDA, ARS, Newark, DE 19713, USA

C. R. Linnen
Department of Biology, University of Kentucky, Lexington, KY 40546, USA

K. M. Oliver
Department of Entomology, University of Georgia, Athens, GA 30602, USA

Introduction

Arthropods are frequently infected with maternally transmitted bacteria, which can contribute heritable phenotypic variation important for host ecology. For example, approximately 10 % of insects require infection with bacteria called obligate

symbionts to subsist on diets of plant phloem or xylem [1, 2]. Facultative endosymbionts, in contrast, are not strictly required for host survival and reproduction, but are much more common, infecting most terrestrial arthropods [3–6]. Infection with heritable symbionts can impart dramatic phenotypic effects to their hosts, including influencing host reproduction [7], defense [8], and manipulation of host plant physiology [9]. These symbiont-induced phenotypic effects can, in turn, influence major ecological and evolutionary processes, such as speciation [10, 11], climatic tolerances [12, 13], disease dynamics [14, 15] and host plant associations [16]. Recent examples in *Drosophila neotestacea* [8] and *Bemisia tabaci* [17] have shown that facultative symbionts can drive rapid evolutionary shifts in their hosts.

The frequency of symbiont-driven evolution in arthropods remains unclear [18]. The best-studied facultative symbiont, *Wolbachia*, often manipulates host reproduction to promote its own spread, and can rapidly reach high frequency in host species as a consequence [19, 20]. Bacteria in the genus *Wolbachia* are very common among arthropod species, and have been implicated in causing and/or reinforcing reproductive isolation among sibling species [4, 6, 11, 21]. *Wolbachia* exhibits a bimodal distribution of either high or low infection frequency within host species, rarely showing intermediate levels of infection [4]. This pattern suggests that selective sweeps to high frequency are common but fleeting for *Wolbachia*, and thus difficult to observe.

In contrast, facultative bacterial symbionts in aphids are typically found at intermediate infection frequencies in natural populations (reviewed by Oliver et al. [22]). The extensively studied pea aphid, *Acyrthosiphon pisum*, is host to at least eight different facultative symbionts [23, 24]. While some individual aphids carry only the obligate symbiont *Buchnera aphidicola*, most individual pea aphids are additionally infected with one or more (up to four) facultative symbionts in various combinations [23–25]. These symbionts confer a variety of ecologically relevant phenotypes to *A. pisum*, including protection against parasitism (*Hamiltonella defensa*, *Serratia symbiotica*; [26]), protection against heat shock (*Serratia* [12, 27]), defense against fungal pathogens (*Regiella insecticola*, *Spiroplasma*, *Rickettsia*, and *Rickettsiella* [28–30]), reproductive manipulation (*Spiroplasma* [31]), and modification of body color (*Rickettsiella* [32]). Additionally, the symbiont taxa are differentially distributed in *A. pisum* populations across host plant species [23, 25, 33, 34], indicating that they may act to facilitate or restrict use of some food plant species [16, 35]. Finally, different bacterial strains within these symbiont taxa can differ substantially in the biological properties they impart to their hosts [36]. In summary, vertically transmitted facultative symbionts contribute important phenotypic variation to *A. pisum* that is both heritable and ecologically relevant. *Acyrthosiphon pisum* populations composed of differentially infected individuals are therefore likely to exhibit dynamic, symbiont-driven microevolutionary responses to selection.

Is *A. pisum* extraordinary, or is this level of heritable symbiont diversity common in other groups? Other aphid species can be infected with the same or similar bacterial symbionts as pea aphid, transferred horizontally over evolutionary time [5], presumably via shared natural enemies [37, 38], or shared food plants [39]. While one or a few individuals of many aphid species (~300) have been screened for particular facultative symbionts, only a handful of species have been extensively evaluated with respect to symbiont distribution and diversity (Fig. S1, Table S1). No other aphid species has been shown to be infected with as many symbiont taxa as pea aphid, but then, no other aphid species has been scrutinized as thoroughly. Aphid species with the highest number of specimens examined (e.g., *Sitobion miscanthi*, *Aphis fabae*, and *Aphis gossypii*) also have the highest recorded symbiont richness [40–47], suggesting that estimates of facultative symbiont richness per host species is a function of sampling effort, much as species richness in ecological communities is a function of sampling intensity [48]. In terms of infection frequency, some aphid species are comparable to *A. pisum* (e.g., *Microlophum carnosum*, *Sitobion avenae*; [49, 50]), whereas other species (e.g., *Megoura crassicauda*) have limited symbiont prevalence despite substantial sampling effort [51]. Thus, facultative bacterial symbionts appear to be heterogeneous in prevalence and diversity across aphid species, but we lack a sufficient database to discern whether other factors (e.g., aphid life history traits, phylogenetic relationships) are predictive of facultative symbiont distribution (and potential importance) among aphid taxa.

To start to fill this gap, the goal of the present study was to conduct an in-depth survey of facultative symbiont prevalence and diversity in *Aphis craccivora*, the cowpea aphid. *Aphis craccivora* is cosmopolitan and anholocyclic throughout most of its range, with most populations never going through a sexual reproductive phase [52]. This polyphagous aphid is most common on legumes (Fabaceae), but attacks members of at least 19 other plant families [52]. Previous studies have shown that facultative symbionts from three genera can infect *A. craccivora*: *Arsenophonus*, *Hamiltonella*, and *Serratia* (Table S1; [5, 53–56]). There is evidence, on a limited geographic scale, that facultative symbionts in this aphid are correlated with aphid food plant: Brady and White [53] found that *A. craccivora* populations in Kentucky, USA, on *Medicago sativa* (alfalfa) had a high prevalence of *Hamiltonella*, whereas populations on *Robinia pseudoacacia* (black locust) had a high prevalence of *Arsenophonus*. In the present study, our objectives were to determine (1) whether populations of *A. craccivora* throughout the world exhibited similar associations between host plant usage and symbiont infection, and (2) whether increased sampling effort would increase estimates of symbiont prevalence and diversity within this aphid.

Materials and Methods

Worldwide samples of *A. craccivora* originated from 44 populations from 18 countries and 16 host plants, collected between 2009 and 2012 (Table S2). We defined a population as aphids collected from locations at least 25 km apart, or collected from different host plants at the same location. Field-collected aphids were stored in 95 % ethanol, and given additional ethanol rinses before DNA extraction. DNA was extracted individually from each aphid using DNeasy extraction kits (Qiagen, Valencia, CA, USA) as specified by the manufacturer. When possible, 20 or more aphids were extracted per population, although many populations had fewer specimens available.

We validated aphid identity by randomly selecting at least one aphid per population and amplifying a portion of its mitochondrial COI DNA (Table S3). The amplicons were cleaned using QIAquick PCR Purification Spin Column Kits (Qiagen) and sent to the University of Kentucky AGTC sequencing facility for Sanger sequencing. When aphids exhibiting different symbiont profiles were found within the same population (see below), we obtained COI sequence from aphids with each symbiotype. Resulting sequences were manually inspected in Geneious v 6.0 and compared against the GenBank nucleotide database using Megablast. All populations in the final dataset showed >99 % similarity to *A. craccivora* (accession numbers KF362033–KF362043).

We screened each specimen for facultative symbionts in the genera *Arsenophonus, Hamiltonella*, and *Serratia* (all γ-proteobacteria), which have been detected previously in this species [5, 53], and also for three other facultative symbiont genera: *Regiella* (γ-proteobacteria), *Rickettsia* (α-proteobacteria), and *Spiroplasma* (Mollicutes). These six symbiotic genera are currently considered to be among the most common facultative bacterial associates of aphids [57]. Small scale studies of bacterial diversity in *A. craccivora* using 454-pyrosequencing [53] and denaturing gradient gel electrophoresis (unpublished data) did not detect other potential facultative symbiont taxa in this aphid, but these studies cannot be considered comprehensive. We proceeded with a diagnostic survey of the most common symbionts, but with the explicit understanding that additional symbiont taxa may be uncovered in *A. craccivora* in the future. Diagnostic PCR reactions were conducted using previously published primer sets specific to each symbiont (Table S3) and amplicons were visualized on a 1 % agarose gel stained with Gel-Red (Biotium, Hayward, CA). All assays included positive control reactions with DNA from specimens known to be infected with each symbiont, as well as a DNA-free negative control. All extractions were verified for quality by PCR for the aphid obligate nutritive symbiont *Buchnera aphidicola*. Samples testing negative for *Buchnera* were rescreened, and those that tested negative a second time were discarded from analysis. We additionally screened each extraction for hymenopteran parasitoid 16S ribosomal RNA (Table S3; [58]) and found a significantly lower probability of detecting facultative symbionts in parasitized than unparasitized specimens ($P=0.0001$ using Fisher's exact test). While it is possible that such a pattern reflects differential susceptibility to parasitism between infected and uninfected aphids, it is also possible that developing hymenopteran larvae reduced bacterial titer through consumption, making it more difficult to detect facultative symbionts. Given the higher probability of false negatives within these parasitized aphids, we conservatively opted to exclude parasitized specimens from the primary dataset, but a comparison of symbiont infection frequency between parasitized and unparasitized aphids is available in the supplemental material (Table S4).

To verify symbiont identity, at least one positive individual per population per symbiont was sequenced using the diagnostic primers. Sequences that matched endosymbiotic accessions in GenBank at >95 % identity were considered positive for endosymbiont infection. If the sequences instead matched non-symbiotic clades of environmental bacteria or non-symbiotic members of potentially endosymbiotic clades (e.g., *Serratia marscesens* instead of *Serratia symbiotica*), we attempted to sequence a second putatively positive individual from the same population, and if that specimen also failed to sequence as a symbiotic bacteria, the population was reclassified as negative for the symbiont in question. While it is possible that such sequences corresponded to novel endosymbiotic lineages rather than environmental bacteria, we chose to be conservative and exclude them from our diagnoses. We did not find any evidence for consistent associations that might be indicative of novel endosymbiotic affiliations. *Spiroplasma* diagnostics were particularly prone to yielding false positives that could not be verified by sequencing. To gain additional insight into strain diversity, we also sequenced a longer segment of 16S ribosomal RNA for *Rickettsia*, *Regiella*, and *Serratia* (Table S3), and used multilocus strain type (MLST) primers to amplify three loci (*fbaA*, *ftsK*, *yaeT*) as described by Duron et al. [59] for *Arsenophonus*.

To characterize symbiont diversity within and among aphid populations we calculated several indices of diversity. Each aphid was categorized into one of 13 symbiotypes based on its facultative symbionts. These symbiotypes included categories for single infections of each symbiont type (six types), each observed combination of symbionts in multiply infected individuals (six combinations of two symbionts), and a final category for uninfected individuals. We calculated γ- and α-diversity for the entire dataset as described by Tuomisto [60], considering individual populations as subunits for the α-diversity calculations. We also calculated the Gini–Simpson Index [60] for each population, which indicates the probability that two individuals selected at random would differ in symbiotype. A value of zero indicates a population that is

invariant in symbiotype, whereas non-zero values indicate some variation in symbiotype within a population. For comparison, we also calculated these metrics from recent published datasets for two other aphid species [24, 50]. Finally, we considered the effect of sampling effort on estimated symbiont species richness within *A. craccivora*. Using the same symbiotype categories used for the diversity indices, we calculated a rarefaction curve±95 % CI for the entire dataset using Analytic Rarefaction v. 1.3 (http://strata.uga.edu/software/anRareReadme.html), and compared the observed richness of symbiotypes for each population against the expected richness for that sample size based on the entire dataset.

To compare the prevalence of the symbionts among aphids from different food plants, we used non-parametric Kruskal–Wallis ANOVA (IBM SPSS v.20). This comparison was made among aphids collected from alfalfa (*Medicago sativa*), locust (*Robinia* sp.) and fava (*Vicia fava*) because these plants were represented by multiple populations (>4) and had substantial numbers of aphids (>70). The remaining aphids of known plant origin (223 aphids from 16 populations) were considered collectively as an "Other" category. Aphids with unknown plant associations, such as those collected in suction traps, were excluded from this analysis (73 aphids from six populations).

Because we also obtained mitochondrial COI haplotype data from a subset of aphids per population, we also considered categorical associations between aphid COI haplotype and food plant. To prevent overrepresentation of non-independent clones, this dataset only included multiple aphids from the same population if they were patently not identical; i.e., they were infected with different symbionts or they had different mitochondrial haplotypes. The resulting dataset contained 65 aphids. We then conducted a G-test of independence [61] to evaluate whether the two most prevalent COI haplotypes were significantly associated with aphid food plant. To avoid small expected values among the cells of the contingency table, food plant categories were compressed into just three categories: *M. sativa*, *Robinia* sp., or Other. We further considered associations between symbiont strains and co-inherited aphid COI haplotypes, but low sample sizes per category precluded statistical analysis.

Results

We detected all six facultative symbiont taxa screened for in *A. craccivora* (Table 1). The final dataset included 615 aphids, of which 30 % were infected with *Arsenophonus*, 5 % with *Hamiltonella*, 4 % with *Serratia*, 4 % with *Rickettsia*, 3 % with *Spiroplasma*, and less than 1 % with *Regiella*. Infection of the same individual aphid by multiple facultative symbionts (i.e., superinfection) was rare (1.6 %), and much lower than would be expected by chance based on the infection frequencies of the individual symbionts (expected multiple infection= 6.4 %, $P<0.001$ using Fisher's exact test). Most (54 %) aphids were uninfected by any of the screened endosymbionts.

Despite low infection frequencies overall for most of these symbionts, most populations of *A. craccivora* (80 %) contained at least one individual testing positive for a facultative symbiont (Table 1). Only one quarter of the populations had two symbionts represented, and none contained more than two symbionts. The average Gini–Simpson index per population was 0.24±0.03, indicating that, on average, two aphids drawn from the same population were more likely than not to have the same symbiotype. In contrast, recently screened populations of *A. pisum* and *S. avenae* (Table 2) had Gini–Simpson indices of >0.5, indicating that two aphids drawn at random from the same population were more likely to have different symbiotypes than the same. However, it is noteworthy for *A. craccivora* that only 13/44 (30 %) of populations had Gini–Simpson indices of 0, meaning that the remaining 70 % of populations exhibited at least some within-population variation in symbiotype, generally a mixture of uninfected individuals and individuals infected with a single facultative symbiont. Gamma diversity for the entire dataset was 2.61, whereas within-population alpha diversity was 1.30, showing that a substantial portion of the observed variation in symbiotype was found among, rather than within, populations (2.61–1.30=1.31). Rarefaction analysis confirmed that within-population symbiotype richness was often less than would be expected based on random sampling from the entire dataset: 20/44 (45 %) of the populations exhibited fewer symbiotypes than expected (Fig. 1).

Food Plant Relationships

Some of the variation in symbiont identity and prevalence among *A. craccivora* populations was associated with food plant (Fig. 2). *Arsenophonus* infected aphids from all four food plant categories, but was much more common in aphids collected from *Robinia* sp. than other food plants, and was nearly absent from aphids collected from *M. sativa* (Kruskal–Wallis $\chi^2=11.16$, $df=3$, $P=0.011$). Five of six aphid populations from *Robinia* exhibited high to fixed frequencies of *Arsenophonus*, whereas this symbiont was not detected in the sixth population (Table 1). *Hamiltonella* also showed a significant food plant association, being almost completely absent from aphids collected from any food plants except *M. sativa* ($\chi^2=7.83$, $df=3$, $P=0.049$). The sole exception was a single *Hamiltonella*-infected aphid collected from *Vicia sativa* in Kenchela, Algeria. We detected 11 different aphid COI haplotypes, which were also non-randomly distributed

Table 1 Symbiont distribution among global *Aphis craccivora* populations collected from various host plants

Host plant	Location	*n*	Proportion symbiont infection (and symbiont strain type)							
			A	H	Re	Ri	Se	Sp	Mult	Un
Acacia retinodes	Greece, Poligono	40	0.88 (I)							0.12
Arachis hypogaea	Australia, Indoroopilly	20								1.00
Astragalus huangheensis	China, Langfang	1						1.00		
Chrysanthemum paludosum	China, Langfang	17	0.71 (I)							0.29
Medicago lupulina	Serbia, Mt. Vlasina	5					0.20 (I)			0.80
Medicago polymorpha	Israel, Ramat Yishay	11	0.09 (I)							0.91
Medicago sativa	Algeria, Ghardaia	4								1.00
Medicago sativa	Chile, Santiago	19						0.11		0.89
Medicago sativa	Italy, Perugia	11								1.00
Medicago sativa	Serbia, Baranda	20		0.25 (II)	0.05					0.70
Medicago sativa	Serbia, Jabucki	21			0.14	0.05 (II)				0.81
Medicago sativa	Serbia, Kotraza	16				0.62 (II)				0.38
Medicago sativa	Serbia, Mt. Vlasina	14		0.71 (I)			0.36 (I)		0.29	0.21
Medicago sativa	Spain, Alás	16	0.07 (II)					0.26		0.67
Medicago sativa	USA, Kentucky, Versailles	13		0.85 (I)				0.08	0.08	0.15
Medicago sativa	USA, Nevada, Verdi valley	28	0.14 (I)							0.86
Medicago sativa	USA, Oklahoma, Chickasha	8		0.63 (I)						0.37
Phaseolus radiatus	China, Langfang	12				0.83 (I)				0.17
Phaseolus vulgaris	Taiwan, Tai-chung	20					0.05 (I)			0.95
Robinia pseudoacacia	Algeria, Batna	4								1.00
Robinia sp.	China, N. Beijing	20	1.00 (I)			0.10 (III)			0.10	0.00
Robinia sp.	Iran, Mashhad	6	0.83 (I)							0.17
Robinia pseudoacacia	Serbia, Mt. Dukat	17	0.82 (I)				0.06 (I)		0.06	0.18
Robinia pseudoacacia	Serbia, Mt. Vlasina	17	1.00 (I)							
Robinia pseudoacacia	Spain, Astorga	15	1.00 (I)							
Rosa hybrida	France, Antibes	17	0.94 (I)							0.06
Salvia farinacea	China, Langfang	10	0.90 (I)							0.10
Vicia faba	Algeria, Biskra	14	0.79 (I)					0.07	0.07	0.21
Vicia faba	Algeria, Ghardaia	6	0.83 (I)							0.17
Vicia faba	Chile, Talca	22								1.00
Vicia faba	Egypt, Aswan	20								1.00
Vicia faba	Serbia, Mt. Vlasina	9					0.78 (I)			0.22
Vicia sativa	Algeria, Khenchela	12		0.08 (I)						0.92
Vigna angularis	China, Langfang	17								1.00
Vigna unguiculata	Benin, Calavi	16				0.35 (I)				0.65
Vigna unguiculata	Madagascar, Anja	2					0.50 (I)			0.50
Vigna unguiculata	Nigeria, Jigawa	3								1.00
Vigna unguiculata	Nigeria, Kano	16								1.00
Unknown	Iran, Kermanshah	21	1.00 (I)							
Unknown	Japan, Fukuoka,	17					0.06 (II)			0.94
Unknown	Japan, Nagasaki	3					0.33 (II)			0.67
Unknown, suction trap	USA, Illinois, Urbana	5		0.20			0.60 (II)			0.20
Unknown, suction trap	USA, Iowa, Ames	15					0.13 (II)	0.20	0.07	0.73
Unknown, suction trap	USA, Minnesota, St. Paul	12					0.25 (I)	0.50		0.25

[a] Facultative symbiont abbreviations: *A Arsenophonus*, *H Hamiltonella*, *Re Regiella*, *Ri Rickettsia*, *Se Serratia*, *Sp Spiroplasma*, *Mult* multiple infections in the same specimen, *Un* uninfected

Table 2 Calculated diversity measures for *A. craccivora* and two other aphids from the literature

Species	n[a]	# symbio types[b]	γ Diversity	α Diversity	Gini–Simpson/population	Ref
A. craccivora	615 (44)	13	2.61	1.30	0.24±0.03	–
A. pisum	318 (5)	25	6.57	4.49	0.78±0.04	[24]
S. avenae	50 (4)	7	3.36	2.96	0.68±0.05	[50]

[a] Total aphids (# populations represented). Aphids collected from the same location but from different host plants were considered separate populations

[b] Each observed combination of endosymbionts was included as a symbiotype

across these food plants, with a single predominant haplotype associated with each *M. sativa* and *Robinia* sp. (G=27.6, P <0.001; Table 3, Fig. S2). *Regiella* was exclusively associated with aphids collected from *M. sativa*, but was so rare (only four aphids from two populations in Serbia) that no significant association with food plant could be detected (χ^2=5.04, df= 3, P=0.17). *Rickettsia*, *Serratia*, and *Spiroplasma* were each occasionally found at moderate to high frequency in *A. craccivora* populations (Table 1), but none were significantly associated with food plant category (*Rickettsia* χ^2=0.96, df= 3, P=0.81; *Serratia* χ^2=0.57, df=3, P=0.90; *Spiroplasma* χ^2=3.36, df=3, P=0.34).

We detected multiple strains for four of the six symbiont taxa infecting *A. craccivora*: we found two strains each for *Arsenophonus*, *Hamiltonella*, and *Serratia*, and three strains of *Rickettsia* (Table 4). For the remaining two symbionts, (*Spiroplasma* and *Regiella*) we only had short sequence lengths available, which provided no evidence for multiple strains of these symbionts. For *Arsenophonus*, we sequenced three MLST genes from 37 specimens (representing 12 populations), and found no deviation in sequence at these loci for all but one specimen. The sequence of the dominant strain was nearly identical to that reported from *Arsenophonus* in other *A. craccivora* specimens (3/1,278 bases=0.2 % divergence; [55]). The rare strain diverged from the dominant by 0.7 % (12/1,751 bases), and bore greatest similarity to *Arsenophonus* from *Cactopsylla aleterni* (4/1,322 bases= 0.3 % divergence; [59]). Interestingly, this rare strain came from one of the few *Arsenophonus*-infected aphids collected from *M. sativa*, and was associated with a different aphid COI haplotype than any of the other *Arsenophonus* accessions (Table S5, Fig. S2). This COI haplotype was characteristic of populations of *A. craccivora* in *M. sativa* (Table S5, Fig. S2), whereas the dominant *Arsenophonus* strain was usually associated with a COI haplotype characteristic of aphid populations from *Robinia*, suggesting two independent acquisitions of *Arsenophonus* within *A. craccivora*. Similarly, the three different *Rickettsia* strains were all found in aphids with different COI haplotypes collected from different food plants. In contrast, the two *Hamiltonella* strains both came from aphids with the same COI haplotype that were collected from *M. sativa*.

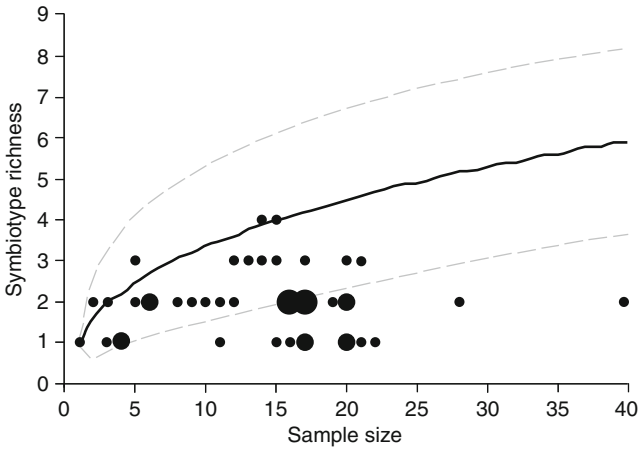

Fig. 1 Rarefaction of complete dataset (n=615 aphids) to determine expected symbiotype richness (*solid dark line*)±95 % CI (*dashed lines*) if only random subsets (n<40) of the full dataset had been examined. *Diamonds* represent observed symbiotype richness for sampled populations; small = 1 population, medium = 2 populations, large = 3 populations with the same value

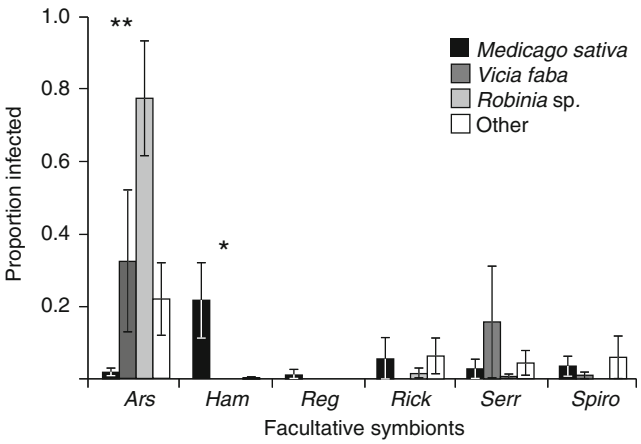

Fig. 2 Mean ± SE proportion symbiont infection of *A. craccivora* collected from various food plants. Ars *Arsenophonus*, Ham *Hamiltonella*, Reg *Regiella*, Rick *Rickettsia*, Serr *Serratia*, Spiro *Spiroplasma*. *Asterisks* indicate significant differences among food plant categories at P<0.05

Table 3 Associations between *A. craccivora* COI haplotype and food plant of origin

	Haplotypes										
	1	2	3	4	5	6	7	8	9	10	11
M. sativa	20	2	1							1	
Robinia sp.	1									9	1
Other	6			4	1	3	3	2	2	9	

Discussion

Worldwide, *A. craccivora* exhibits a high diversity of facultative endosymbionts. We found 11 different strains of facultative symbiotic bacteria, representing six genera. This is probably a conservative estimate, given that our diagnostic approach focused on a subset of common aphid symbionts. It is likely that additional symbionts would be discovered with more intensive scrutiny using techniques that permit the discovery of novel (i.e., unexpected) symbionts, as has happened with the well-studied *A. pisum* (X-type, *Rickettsiella* [32, 62]) and the sweetpotato whitefly, *Bemisia tabaci* (*Hemipteriphilus* [63]). Initial pyrosequencing efforts to characterize bacterial diversity in *A. craccivora* did not detect unexpected or novel facultative symbionts [53], but such efforts were quite limited in scope.

Presuming that additional widespread endosymbionts do not await discovery in *A. craccivora*, facultative symbiont diversity appears to be structured in a substantially different way than described for *A. pisum*. Within populations of *A. craccivora*, we found that variation usually involved the presence/absence of a single symbiont, with most aphids in most populations lacking facultative symbionts. Few populations had more than one facultative symbiont, and no populations had more than two facultative symbionts. Co-infections of two facultative symbionts in individual aphids were rare (<2 %). These patterns contrast sharply with those found in other arthropod species that exhibit high levels of symbiont species richness, such as *A. pisum*, *B. tabaci*, and the chestnut weevil *Curculio sikkimensis*. At least six different facultative symbionts infect each of these insect host species, with the majority of individuals carrying at least one facultative symbiont and many individuals superinfected by multiple symbionts simultaneously [23, 24, 64, 65]. One distinction between *A. craccivora* and these other species is the presence of sexual reproduction, which has been documented as a route for horizontal transmission of endosymbionts in *A. pisum* [66]. Throughout most of its range, reproduction in *A. craccivora* is thought to be exclusively asexual [52], although sexual morphs have been reported and induced in the past [67, 68]. Speculatively, the lack of sexual reproduction in *A. craccivora* may contribute to reduced symbiont prevalence, because one route for lateral symbiont acquisition is unavailable. Additionally, bottlenecks associated with invasive introductions and overwintering source populations in *A. craccivora* likely further limit clonal variation in many populations. Hence, the diverse facultative bacteria associated with *A. craccivora* may be the principle heritable variation occurring among clones in many populations.

Food-plant Relationships

Within a restricted geographic range, Brady and White [53] demonstrated strong and exclusive correlations between two endosymbionts (*Arsenophonus* and *Hamiltonella*) and two food plants (*R. pseudoacacia* and *M. sativa*, respectively). The present, more comprehensive data set showed similar patterns, but illustrated some contrasts between the distribution of the two symbionts. *Arsenophonus* was prevalent in almost all aphid populations collected from *Robinia* sp., but was also present (and sometimes found at high frequency) in aphids collected from other food plants, such as *Chrysanthemum*, *Rosa*, and *Vicia*. In contrast, while *Hamiltonella* was almost exclusively associated with *A. craccivora* collected from *M. sativa*, it was not found at high infection frequencies, and many populations lacked *Hamiltonella* altogether (7/11 populations; Table 1) depicts the relevant data. It is possible that some of the other symbionts in *A. craccivora* may also show association with particular host plants (e.g., *Rickettsia* in aphids from *Phaseolus radiatus*), but the present study did not have sufficient aphid sampling across all food plants to eval-

Table 4 Symbiont strain types found in *A. craccivora*

Symbiont	DNA sequenced	Number of haplotypes	DNA length (bp)	Absolute divergence	% Divergence	Accession numbers
Arsenophonus	23S, *fbaA*, *ftsK*, *yaeT*	2	1,758	12 bp	0.68	KF326018–KF326025
Hamiltonella	16S	2	547	2 bp	0.37	KF326016–KF326017
Rickettsia	16S	3	685	1 vs. 2: 1 bp	0.15	KF326028–KF326030
				1 vs. 3: 4 bp	0.58	
				2 vs. 3: 3 bp	0.43	
Serratia	16S	2	1,138	1 bp	0.09	KF326026–KF326027

uate this possibility. Associations between particular food plants and symbionts have been reported numerous times in *A. pisum*, including associations between *Hamiltonella* and *Medicago* [23, 24, 33, 34, 69–71].

We collected a small amount of aphid genetic data in the course of this study, in the form of mitochondrial COI barcode sequences for verification of aphid identity. While this gene is not a particularly sensitive measure of intraspecific variation within *A. craccivora*, we did gain some additional insights from inspecting the diversity and associations of observed COI haplotypes. First, we saw a significant relationship between aphid COI haplotype and food plant (Table S5). It is not surprising that mitochondria and bacterial symbionts show similar correlations to food plant, given their co-inheritance via maternal cytoplasm. However, it is interesting that within each population, we usually observed only a single COI haplotype, even though aphids with more than one symbiotype were present in the population. This suggests that symbiont gains and losses may occur within food-plant associated aphid lineages. Populations of polyphagous insects feeding upon different food plants have frequently been shown to exhibit genetic differentiation, which has been proposed to be a significant mechanism of evolutionary radiation in insects [72]. *Acyrthosiphon pisum*, for example, has diversified into many genetically distinct host races specialized on herbaceous legumes [71, 73]. It has been suggested that facultative bacterial symbionts may facilitate this specialization by expanding diet breadth [16], although there appear to be complex interactions between aphid genotype, symbiont strain and food plant use [35, 74, 75]. In *A. craccivora*, molecular and morphological evidence also indicate the presence of differentiated host-associated lineages [76–78], and suggest that the taxonomic status of these host races should be considered [76]. As with *A. pisum*, however, it remains to be determined whether differential facultative symbiont infection in *A. craccivora* across host-associated lineages primarily reflects historical associations, or rather is indicative that symbionts have played a causative role in host-associated differentiation [79].

The COI haplotype data in *A. craccivora* also gives a context for interpreting some of the exceptions to our typical associations between facultative symbionts and food plants. *Arsenophonus* was rare in *M. sativa*-infesting populations of *A. craccivora*, but not absent: two *M. sativa* populations of *A. craccivora* had low levels of *Arsenophonus* infection. In Verdi Valley, Nevada, we found the dominant strain of *Arsenophonus* associated with its typical COI haplotype associate, both of which are usually found in *Robinia*-infesting *A. craccivora* populations. Our interpretation is that this was probably a vagrant population established on an atypical host plant, similar to those described by Ferrari et al. [23] for *A. pisum*. In contrast, in Alás, Spain, we found a variant strain type of *Arsenophonus* in association with a COI haplotype that was typical of aphid populations in *M. sativa*, strongly implicating an independent acquisition of *Arsenophonus* by an *M. sativa*-associated lineage of *A. craccivora*. We also suspect that the three *Rickettsia* strains we detected were independently acquired by three different *A. craccivora* lineages, because each was associated with a different COI haplotype.

In conclusion, different lineages of *A. craccivora* are associated with different host plants and different strains of facultative symbionts. The causality of these patterns remains to be determined, but the net result is a high diversity of facultative symbionts for *A. craccivora* in aggregate. Symbiotype diversity within individual populations was low, yet most populations consisted of a mixture of differentially infected individuals, indicating that symbiont-associated variation may have the potential to affect microevolutionary processes on a very local scale, even within this parthenogenetic aphid that apparently exhibits substantial population structuring based on host plant. More generally, we suggest that a strictly parthenogenetic lifestyle may act to limit symbiotype diversity, by restricting the opportunity for sexual transmission of symbionts [66]. In contrast, polyphagy may promote symbiont diversity in such insects by (1) exposing them to a variety of ecological communities that provide different opportunities for horizontal transfer of symbionts from other insects [37, 59] and/or (2) by providing multiple contrasting selective environments in which different symbiotypes may be advantageous [74].

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