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Multiple introductions of the *Spiroplasma* bacterial endosymbiont into *Drosophila*

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Abstract

Bacterial endosymbionts are common in insects and can have dramatic effects on their host's evolution. So far, the only heritable symbionts found in *Drosophila* have been *Wolbachia* and *Spiroplasma*. While the incidence and effects of *Wolbachia* have been studied extensively, the prevalence and significance of *Spiroplasma* infections in *Drosophila* are less clear. These small, gram-positive, helical bacteria infect a diverse array of plant and arthropod hosts, conferring a variety of fitness effects. Male-killing *Spiroplasma* are known from certain *Drosophila* species; however, in others, *Spiroplasma* appear not to affect sex ratio. Previous studies have identified different *Spiroplasma* haplotypes in *Drosophila* populations, although no extensive surveys have yet been reported. We used a multilocus sequence analysis to reconstruct a robust *Spiroplasma* endosymbiont phylogeny, assess genetic diversity, and look for evidence of recombination. Six loci were sequenced from over 65 *Spiroplasma*-infected individuals from nine different *Drosophila* species. Analysis of these sequences reveals at least five separate introductions of four phylogenetically distinct *Spiroplasma* haplotypes, indicating that more extensive sampling will likely reveal an even greater *Spiroplasma* endosymbiont diversity. Patterns of variation in *Drosophila* mitochondrial haplotypes in *Spiroplasma*-infected and uninfected flies imply imperfect vertical transmission in host populations and possible horizontal transmission.

Keywords: bacteria, host–parasite interaction, microbial biology, species interactions

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Introduction

Microorganisms that live in close association with animals, plants and other taxa have a diverse array of effects on their partners, ranging from mutualistic to parasitic. Insects, in particular, form relationships with a variety of bacterial endosymbionts (Buchner 1965). Species of the genus *Drosophila*, despite serving as important model organisms in evolutionary biology, only recently have been screened for heritable bacterial endosymbionts. A large-scale survey across the genus revealed that *Drosophila*, unlike many other insects, harbour only *Wolbachia* and *Spiroplasma* as heritable endosymbionts (Mateos *et al*. 2006). While the incidence and effects of *Wolbachia* in *Drosophila* have been studied extensively (Werren 1997; McGraw & O'Neill 2004), the prevalence and significance of *Spiroplasma* infections in *Drosophila* are far less clear.

Spiroplasma are small, gram-positive, wall-less, helical bacteria (Whitcomb & Tully 1982; Williamson 1998). A few *Spiroplasma* are agronomically important plant pathogens causing corn stunt (*Spiroplasma kunkelii*) and citrus stubborn disease (*Spiroplasma citri*) (Bove 1997). However, *Spiroplasma* also infect a wide array of arthropod hosts (Gasparich *et al*. 2004) in which they have diverse effects: they can be mutualistic (Ebbert & Nault 2001), pathogenic (Bove 1997), or sex-ratio distorters (Williamson & Poulson 1979; Goodacre *et al*. 2006; Tinsley & Majerus 2006). Initial reports of *Spiroplasma* in *Drosophila* species involved male killing, in which male offspring die during embryogenesis (Williamson

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Table 1 *Spiroplasma*-infected *Drosophila* used in this study

Subgenera	Species group	Species	No. of individuals	Localities sampled
Drosophila	*repleta*	*D. hydei*	19	San Carlos, Mexico
			9	Magdalena, Mexico
			5	Tucson, Arizona
			2	San Pablo Etla, Mexico
			2	Organ Pipe National Monument, Arizona
Drosophila	*repleta*	*D. mojavensis*	5	Organ Pipe National Monument, Arizona
			2	Santa Catalina Island, California
			2	San Carlos, Mexico
Drosophila	*repleta*	*D. aldrichi*	7	Tucson, Arizona
Sophophora	*melanogaster*	*D. ananassae*	1	Africa
			1	Hawaii
Sophophora	*melanogaster*	*D. atripex*	1	Africa
Drosophila	*repleta*	*D. wheeleri*	6	Tucson, Arizona
Sophophora	*melanogaster*	*D. melanogaster*	1	Uganda (Pool *et al*. 2006)
Sophophora	*melanogaster*	*D. simulans*	1	San Carlos, Mexico
Drosophila	*quinaria*	*D. tenebrosa*	5	Santa Catalina Mountains, Arizona

& Poulson 1979). Numerous other *Drosophila* species, however, are infected with spiroplasmas that do not cause male killing, and their fitness effects are unknown (Kageyama *et al*. 2006; Mateos *et al*. 2006; T. Watts, N.A. Moran, T.A. Markow, unpublished data).

Knowledge of the diversity of *Spiroplasma* infecting *Drosophila* is key to fully understanding the consequences of harbouring this endosymbiont. Fitness effects, positive or negative, can vary depending on the particular bacterial strain (e.g. Werren 1997; Pfarr & Hoerauf 2005; Degnan & Moran 2008). Elucidation of evolutionary relationships also will provide insight into whether *Spiroplasma* is an ancient infection followed by co-divergence between host and bacteria, as is common for beneficial endosymbionts (Shigenobu *et al*. 2000; Akman *et al*. 2002; Tamas *et al*. 2002; van Ham *et al*. 2003), or whether multiple introductions have occurred via horizontal transmission as seen for reproductive parasites such as *Wolbachia* (Werren & Bartos 2001; Baldo *et al*. 2006). Thus far, *Spiroplasma* infections have been observed in 16 *Drosophila* species. Male-killing spiroplasmas are known to infect *Drosophila willistoni*, *D. nebulosa*, *D. paulistorum*, and *D. equinoxialis* of the willistoni species group (Williamson & Poulson 1979), likely *D. ornatifrons*, *D. neocardini*, and *D. paraguayensis* of the tripunctata group (Montenegro *et al*. 2006a), as well as *D. melanogaster* (Montenegro *et al*. 2005; Pool *et al*. 2006). Non-male-killing spiroplasmas infect *Drosophila hydei* (Ota *et al*. 1979; Mateos *et al*. 2006) *D. aldrichi*, *D. mojavensis* (Mateos *et al*. 2006), *D. wheeleri*, *D. tenebrosa* (T. Watts, N.A. Moran, T.A. Markow, unpublished data), *D. simulans*, *D. atripex*, and *D. ananassae* (T.A. Markow, unpublished). Previous phylogenetic analyses have revealed close relationships among several male-killing spiroplasmas and the non-male-killing spiroplasmas infecting some *D. hydei* (Montenegro *et al*. 2005; Kageyama *et al*. 2006) while Mateos *et al*. (2006) explored the relationships of the non-male-killing spiroplasmas infecting other *D. hydei*, *D. aldrichi* and *D. mojavensis*. The evolutionary relationships, however, of other newly discovered spiroplasmas remain poorly understood, as do the relationships of the male-killing to other non-male-killing *Drosophila* spiroplasmas.

Population processes, such as horizontal transmission and recombination, also are little known for *Spiroplasma* in *Drosophila* and other arthropod species where it is a vertically transmitted endosymbiont (Majerus *et al*. 1999). Recombination could obscure true infection histories for phylogenetic relationships determined by a single locus (Holmes *et al*. 1999; Feil & Spratt 2001), could affect the adaptive potential of the spiroplasma genome, and lend insight into the dynamics of the *Drosophila/Spiroplasma* symbiosis. We used a multilocus sequencing approach to address the following questions: (i) what are the evolutionary relationships of the *Spiroplasma* infecting *Drosophila*? (ii) how many introductions of *Spiroplasma* have occurred in *Drosophila*? (iii) is there any recombination? and (iv) what is the association between host mitochondrial haplotype and spiroplasma infection, and what are implications for the relative roles of vertical and horizontal transmission within *Drosophila* populations?

Materials and methods

Samples of Drosophila

Sixty-nine infected individuals from nine *Drosophila* species were examined (Table 1). Most individuals were sampled in 2005–2007 from natural populations in western North America. Others were obtained from the Tucson *Drosophila*

Table 2 Primers and annealing conditions for each locus

Locus	Product	Primer	Sequence	Annealing temperature
16S	Ribosomal	23F	CTCAGGATGAACGCTGGCGGCAT	
	RNA partial	TKSS	Fukatsu *et al*. 2001;	
		16STF1	GGTCTTCGGATTGTAAAGGTCTG	65–48 C touchdown
		16STR1	GGTGTGTACAAGACCCGAGAA	
ITS	Internal	ITS-N2	Majerus *et al*. 1999;	
	Transcribed	ITS-N55		65–48 C touchdown
	Spacer			
RpoB	RNA	RpoBF3	GGNTTTATTGAAACACCATAYCGTC	
	Polymerase B	RpoBR2	GCATGTAATTTATCATCAACCATGTGTG	63–53 C touchdown
		RpoBF1	ATGGATCAAACAAATCCATTAGCAGA	
		RpoBR4	CTTTGTTTCCATGGCGTCCAGCC	
ParE	DNA	ParEF2	GGAAAATTTGGTGGTGATGG	63–53 C touchdown
	Topoisomerase	ParER2	TGGCATTAATCATTACATTAATTTCT	
FtsZ	Cell division	FtsZF2	TGAACAAGTCGCGTCAATAAA	63–53 C touchdown
	Protein	FtsZR3	CCACCAGTAACATTAATAATAGCATCA	
FruR	Partial fructose	FruF	Montenegro *et al*. 2000	58–48 C touchdown
	Operon	FruR		

Stock Center or recently collected in other parts of the world. For some individuals, isofemale lines were established to assay for the male-killing phenotype.

DNA extractions were performed as in Mateos *et al*. (2006) or Gloor & Engels (1992), and 2 L was used as template in a 25- L polymerase chain reaction (PCR), using PCR methods as in Mateos *et al*. (2006). PCR cycling conditions were an initial denature of 3 min 94 C, followed by 30 s 94 C, 45 s 68 C, 45 s 72 C; annealing temperature was lowered 1.0 C per cycle for 15 cycles, then kept for 20 cycles at 48 C. Variations in cycling conditions as well as primer sequences for the various loci are listed in Table 2. PCR products were directly sequenced in both directions using amplification primers and an ABI 3730 sequencer at the Genomics and Analysis Technology Core Facility at the University of Arizona.

Sequencing

Spiroplasma *multilocus sequencing.* Six loci were chosen to compare *Drosophila* spiroplasmas to other sequenced spiroplasmas, to detect phylogenetic incongruence among loci, and to increase phylogenetic resolution. The 16S ribosomal RNA (rRNA) and internal transcribed spacer (ITS) loci were selected because these conserved loci have been sequenced for numerous other spiroplasmas. The remaining genes, *rpoB* (RNA polymerase B), *ftsZ (*cell-division protein), *parE* (DNA topisomerase), and *fruR (*partial fructose operon) are more rapidly evolving bacterial housekeeping genes that are good phylogenetic markers because they are unlikely to be under positive selection and are likely to be orthologous among all spiroplasmas (Welch *et al*. 2002; Dunning-Hotopp *et al*. 2006).

The partially assembled *Spiroplasma citri* genome was used to locate several of the genes and confirm that they are in different chromosomal regions. Additionally, *rpoB* and *parE* have been sequenced for other *Spiroplasma* species, allowing for elucidation at multiple loci of the relationships of *Drosophila* spiroplasmas to those infecting other organisms. Finally, we sequenced a small portion (~400 bp) of the fructose operon (*fru*), previously found to be a variable locus in other *Drosophila* spiroplasma studies (Montenegro *et al*. 2005).

Amplification of each locus was attempted for all infected *Drosophila,* followed by sequencing. For those not amplifying after two attempts, primers were redesigned for re-amplification. A complete listing of *Drosophila* samples used and their amplification success is provided in Table S1, Supporting information. GenBank Accession numbers are FJ656998–FJ657372.

Drosophila *mitochondrial DNA sequencing.* To detect variable mitochondrial sequences within populations of *Drosophila hydei*, the partial cytochrome oxidase II (COII) locus was sequenced (600 bp) (PCR conditions were as in Folmer *et al*. 1994) as well as a 600 bp of the AT-rich region (primers and PCR conditions as in Brehm *et al*. 2004). Twenty infected and 30 uninfected flies roughly reflecting the proportion of infected individuals in this species (T. Watts, N.A. Moran, T.A. Markow, unpublished data) were sequenced for these regions. These flies were from five localities throughout the Sonoran Desert and southern Arizona (Table 1). For *Drosophila mojavensis*, the cytochrome oxidase I (COI) locus was sufficiently variable and was sequenced for 30 infected and 40 uninfected individuals from three localities (Table 1). GenBank Accession numbers are FJ656811–FJ656997.

Table 3 Features of the six loci used in this study

Locus	Number of alleles	Number of sites	Number of polymorphic sites	Nucleotide diversity per site	GC content	Ka/Ks	Recombination
16S rRNA	7	1252	205	0.034	49%	N/A	None
ITS	7	202	51	0.034	30%	N/A	None
RpoB	8	1292	182	0.094	34%	0.038	Outgroup
ParE	5	933	155	0.082	32%	0.096	None
FtsZ	5	886	140	0.077	38%	0.085	None
FruR	5	327	72	0.108	32%	0.253	None

Sequence analysis and phylogenetics

The sequences were cleaned in Sequencher 4.5 (Gene Codes), aligned using Muscle (Edgar 2004), and adjusted in the SeAl manual alignment program (Rambaut 1996). Additional *Spiroplasma* sequences were downloaded from National Center for Biotechnology Information (NCBI). These sequences included the highest blast hits for the different haplotypes at each locus and other related *Spiroplasma* species based on published *Spiroplasma* phylogenies (Gasparich *et al*. 2004; Regassa & Gasparich 2006). The outgroup species for the more conserved 16S rRNA, ITS, and *rpoB* was the most closely related species with a full genome sequenced, *Mycoplasma mycoides*. For the more rapidly evolving *ftsZ*, *parE*, and *fruR*, the *M. mycoides* sequences were too divergent to reasonably align, and the most closely related *Spiroplasma* species outside the groups of interest were used. Where none was available, the tree was midpoint rooted.

Phylogenetic analyses were performed individually on each locus as well as on combinations of loci. Distance-based (neighbour-joining) phylogenetic reconstructions with 1000 bootstrap replicates were performed using PAUP* 4.0b10 (Swofford 1998). Shimodaira–Hasegawa (SH) tests were run to compare the likelihood score of the best tree for the data set of each locus against the likelihood of the topology of every other locus. The SH tests were run using full optimization and 1000 bootstrap replicates in PAUP. Bayesian phylogenetic analyses were performed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001). Bayesian analyses were run for 10 000 000 generations on four simultaneous Monte Carlo Markov chains using the general time reversible model, collecting trees every 100 generations. The first 5000 trees were discarded as 'burn-in'.

DnaSP (Rozas *et al*. 2003) was used to calculate population genetic parameters such as nucleotide diversity, GC content, average Ka/Ks, and recombination. Additionally, recombination within the alignments of each individual locus was detected with Genconv (Sawyer 1989). Haplotype networks were constructed using the TCS program (Clement *et al*. 2000) while Arlequin (Schneider *et al*. 2000) was used to build minimal spanning trees.

Results

Genetic diversity of Spiroplasma *infecting* Drosophila

Spiroplasma from all nine *Drosophila* species amplified for the 16S rRNA, ITS, and *rpoB* loci. For *parE*, *ftsZ*, and *fruR*, the spiroplasmas infecting *Drosophila atripex*, *D. ananassae*, and *D. tenebrosa* did not amplify after multiple attempts. The inability to amplify these loci after several attempts with multiple primer sets likely reflects the large sequence divergence at these more rapidly evolving loci. *Spiroplasma* infecting *Drosophila simulans* amplified only for 16S rRNA, *rpoB*, and *ftsZ*.

A basic description of the genetic diversity indices is given in Table 3. Amplified loci ranged from 327–1252 bp in length with an average of 35% G + C content. Levels of nucleotide diversity and sequence divergence were different at each of the six loci, with the 16S rRNA locus being the most conserved and the *fruR* locus having the highest nucleotide diversity. The average pairwise Ka/Ks for protein-coding loci ranged from 0.038 to 0.253, reflecting purifying selection. Only one *Spiroplasma* haplotype was found to infect each *Drosophila* species except for the case of *Drosophila hydei*, which contained two. The same *Spiroplasma* haplotype infects both *Drosophila aldrichi* and *D. wheeleri*.

Same phylogenetic pattern seen across loci indicates a lack of recombination

Similar evolutionary relationships are seen among the *Drosophila* spiroplasmas at each locus (Figs 1, 2, and 3) indicating an absence of intergenic recombination. No statistically significant phylogenetic incongruence was found at any pairwise comparison between loci (Table S2, Supporting information). Furthermore, no intragenic recombination was detected within any locus, with the exception of a possible recombinant in *Spiroplasma chrysicola*. Given that recombination was not detected, the loci were concatenated,

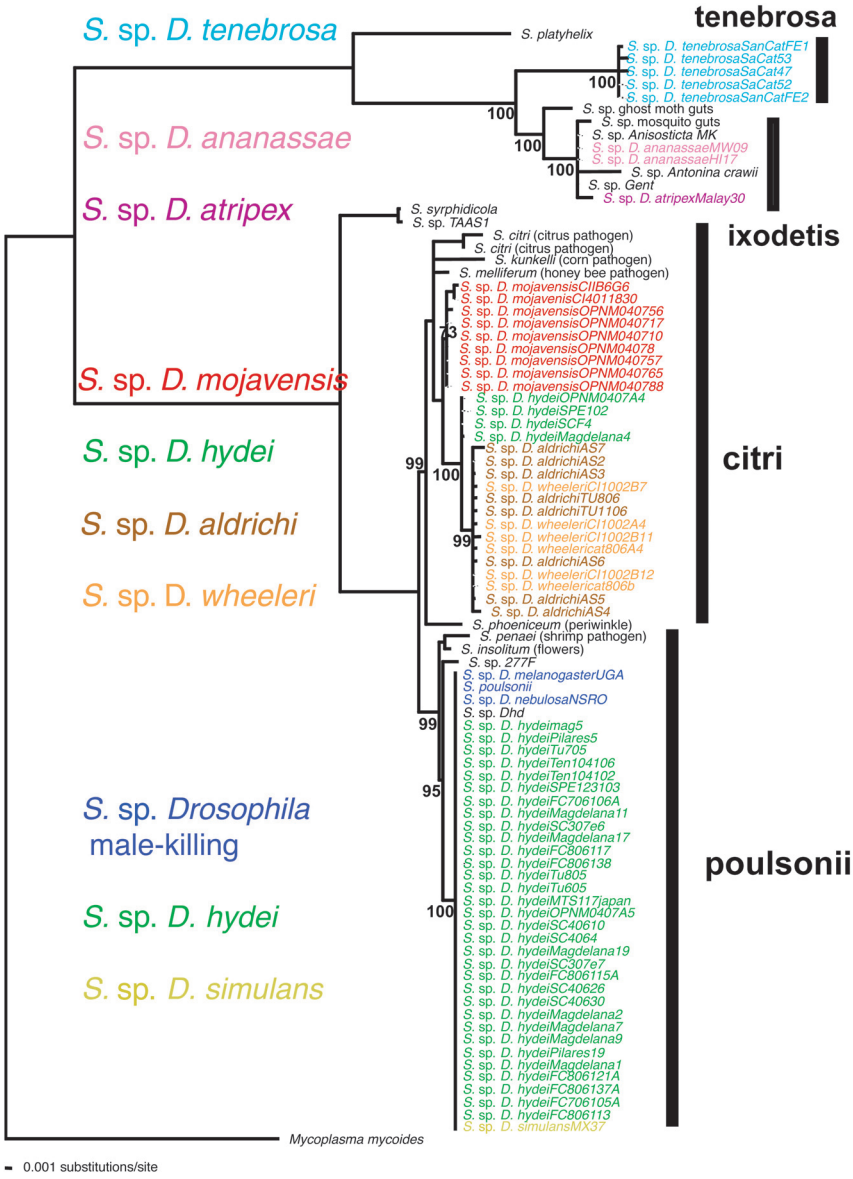

Fig. 1 Bayesian phylogeny based on *Spiroplasma* 16S rRNA gene. *Spiroplasma* infecting different *Drosophila* species in different colours. Support for clades given as Bayesian posterior probabilities. The spiroplasmas infecting *Drosophila* fall into four distinct clades, which are labelled in bold type with black bars.

and the resultant tree with only unique spiroplasma haplotypes is shown in Fig. 4.

Drosophila *spiroplasmas fall into four distinct phylogenetic clades*

A Bayesian phylogenetic tree based on 1252 bp of 16S rRNA from all 69 individuals (Fig. 1) is representative of the evolutionary relationships at each locus. The *Spiroplasma* infecting *Drosophila* (denoted *S.* sp. *Drosophila*) fall into four distinct clades with high bootstrap support. The clade containing the *Spiroplasma poulsonii* of *Drosophila willistoni* also contains the spiroplasmas infecting 32 *D. hydei* individuals from various locales from North America, as well as one from Japan. Additionally, the same *Spiroplasma* haplotype infects *D. simulans*. Within the citri clade, about 2% sequence divergent from those of the poulsonii clade, is another group of spiroplasmas infecting four *D. hydei* individuals as well as *D. aldrichi*, *D. wheeleri*, and *D. mojavensis*. Contained within this citri clade are three well-supported spiroplasma groups: *Spiroplasma* sp. *Drosophila mojavensis, S.* sp. *D.hydei, and S.* sp. *D. wheeleri/D. aldrichi*. The remaining two clades in which *Drosophila* spiroplasmas are found, the ixodetis and tenebrosa clades, show about 12% sequence divergence from the poulsonii and citri clades. Falling into the ixodetis clade are the spiroplasmas infecting *D. atripex* and *D. ananassae* from Africa as well as a *D. ananassae* from Hawaii. Finally, the spiroplasmas infecting *D. tenebrosa* fall

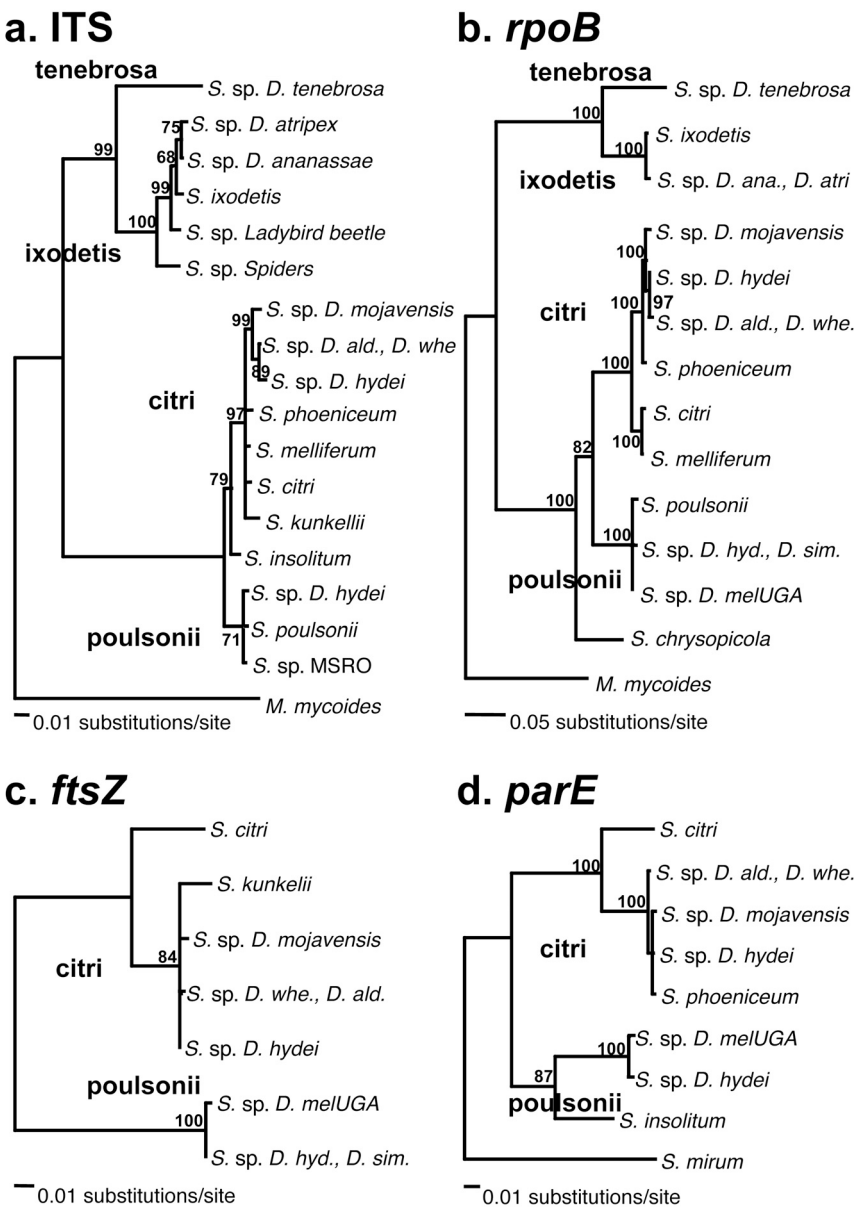

Fig. 2 Bayesian phylogenies based on *Spiroplasma* loci ITS, *rpoB*, *ftsZ*, and *parE*. Identical *Spiroplasma* haplotypes condensed at each locus. ITS and *rpoB* are rooted with *Mycoplasma mycoides*, while *ftsZ* and *parE* are midpoint rooted. Support for clades given as Bayesian posterior probabilities. The major clades into which the *Drosophila* spiroplasmas fall are labelled in bold type. Abbreviations: *D. whe.* (*D. wheeleri*), *D. ald.* (*D. aldrichi*), *D. moj.* (*D. mojavensis*), *D. sim.* (*D. simulans*), *D. melUGA* (male-killing *Spiroplasma* infecting *Drosophila melanogaster* from Uganda). The same phylogenetic pattern is seen across all loci.

into a distinct clade, most closely related to the ixodetis clade, but nonetheless separated by an average 3% sequence divergence.

At least five separate introductions of Spiroplasma *into* Drosophila

Spiroplasmas found to infect *Drosophila* are not monophyletic. The *Drosophila* spiroplasmas in each clade are more closely related to those infecting other organisms than they are to those infecting other *Drosophila*. For example, the *Drosophila* spiroplasmas in the poulsonii clade are most closely related to *Spiroplasma phoencium*, prevalent on flower and plant surfaces (Bove 1997), as well as *Spiroplasma penaei*, a pathogen of shrimp (Fig. 1). Another major group of *Drosophila Spiroplasma* haplotypes is more closely related to *S. citri* and *S. kunkelii*, plant pathogens, than to the *Drosophila* spiroplasmas in the poulsonii clade. A third group is most closely related to the *Spiroplasma* of the ixodetis tick, several species of spider and ladybird beetles. Finally, the spiroplasmas infecting *D. tenebrosa* are different from any *Spiroplasma* species represented thus far in GenBank. At the ITS locus, where additional sequences are available, the *D. tenebrosa* spiroplasma appears to be most closely related to that infecting spiders, although it is still > 4% sequence different from any previously sequenced *Spiroplasma*. Thus, each clade represents a separate introduction into *Drosophila* hosts. Furthermore, two very different spiroplasmas infect

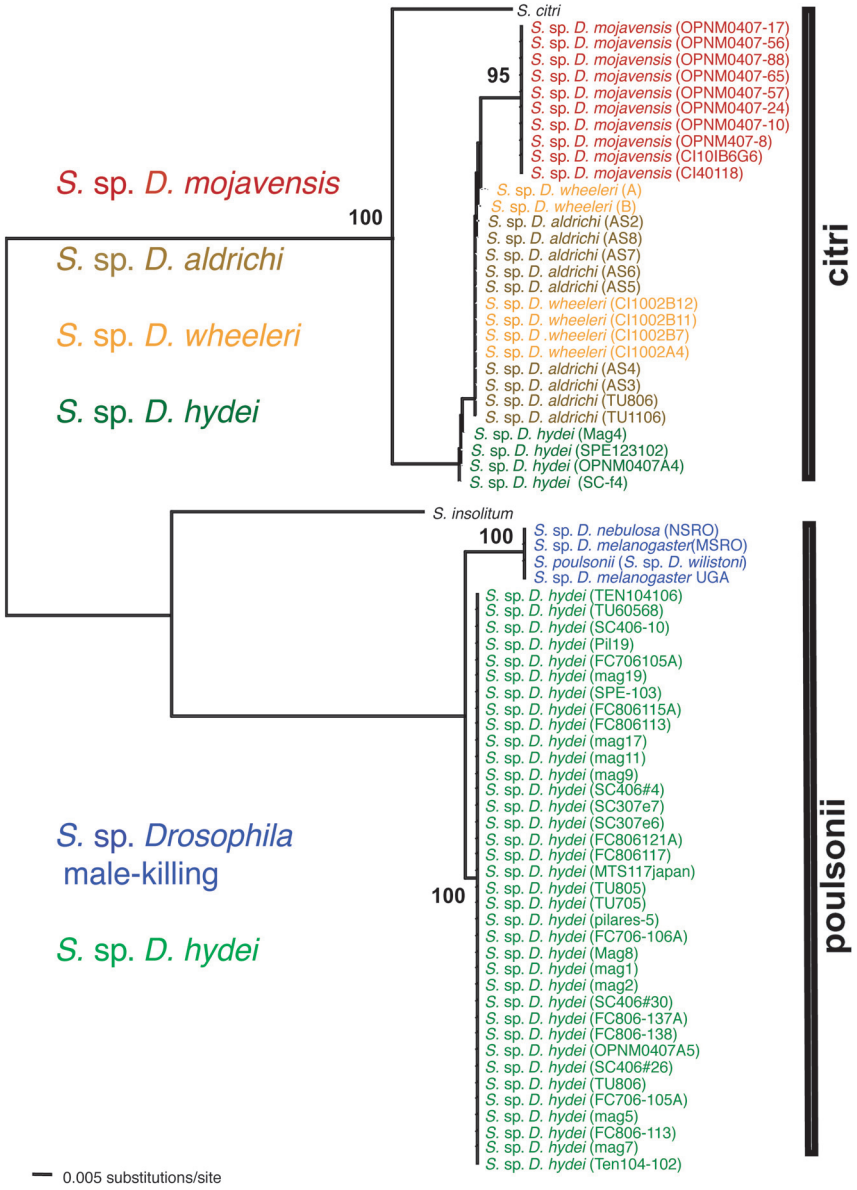

Fig. 3 Bayesian phylogeny based on the fruR locus. *Spiroplasma* infecting different *Drosophila* species in different colours. Support for clades given as Bayesian posterior probabilities. The male-killing spiroplasmas fall into a single well-supported clade and are a small proportion of the spiroplasma diversity sampled thus far.

D. hydei; those in the poulsonii clade that infect the majority of *D. hydei* individuals, and those in the citri clade, found in only four *D. hydei* individuals. The phylogenetic relationships of the *Spiroplasma*-infected *Drosophila* species used in this study are denoted in Fig. 5.

Relationships between the male-killing and non-male-killing spiroplasmas

Only one male-killing spiroplasma, that infecting *Drosophila melanogaster*, was available for sequencing at all loci, although others were represented by 16S rRNA and *fruR* sequences in GenBank. Male-killing spiroplasmas infecting *D. melanogaster* and *D. nebulosa* have 16S rRNA sequences identical to that of the non-male killers infecting *D. hydei*. At the five other loci, however, the male killer from *D. melanogaster* has a haplotype different from the *D. hydei* spiroplasmas. At the *fruR* locus (Fig. 3.), the male-killing *Spiroplasma* all group together with strong bootstrap support and are clearly separate from the non-male killers in *D. hydei*, with a 2% sequence divergence between the haplotypes.

Spiroplasma *infections within populations*

We looked for associations between *Drosophila* mitochondrial haplotype and spiroplasma infection. If an infection has occurred recently and is maintained in the population due to a high fidelity of vertical transmission within descendant matrilines, we expect a particular spiroplasma infection to be associated with one or only a few mitochondrial

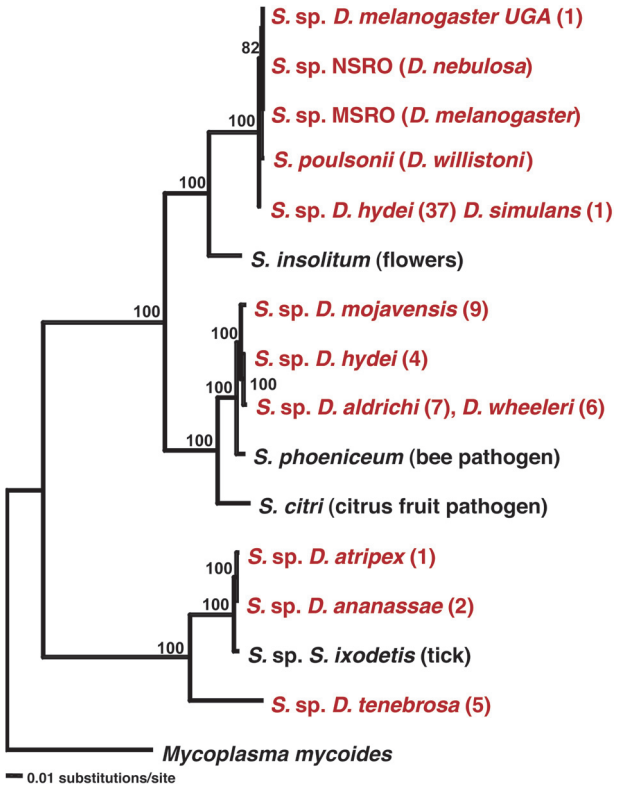

Fig. 4 Bayesian phylogeny based on concatenated sequences of multiple spiroplasma loci. Identical *Spiroplasma* haplotypes are condensed, and the number of individuals with each haplotype is given in parenthesis following the haplotype name. *Drosophila* spiroplasmas coloured in red. Support for clades given as Bayesian posterior probabilities.

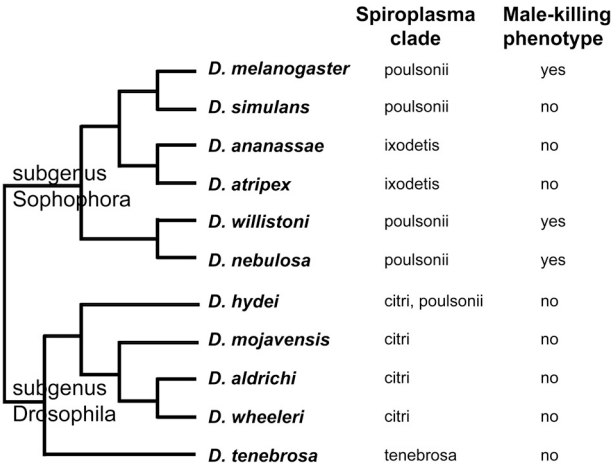

Fig. 5 Cladogram of *Spiroplasma*-infected *Drosophila* species used in this study. *Drosophila* species relationships based on Markow & O'Grady (2005). The clade of *Spiroplasma* infecting each *Drosophila* species, as well as its male-killing phenotype, is denoted.

haplotypes within a population. Alternatively, spiroplasma infection affecting all or most mitochondrial haplotypes would suggest an older infection followed by loss in some lineages and/or frequent horizontal transmission of spiroplasmas among individuals in the populations. We were able to test these predictions in two *Drosophila* species.

For *D. mojavensis*, 81 individuals from three populations [Organ Pipe National Monument (OPNM), San Carlos (SC) and Catalina Island (CI)] belong to 14 total haplotypes forming three distinct clusters (Fig. 6a). The CI flies form a separate cluster with only two mitochondrial haplotypes. Flies with both haplotypes were both *Spiroplasma* infected and *Spiroplasma* uninfected. The *D. mojavensis* mitochondrial haplotypes from SC and OPNM of mainland Sonora are intermixed in the two remaining groups. One cluster contains a prevalent mitochondrial haplotype (containing more than 20 individuals) that belongs to both infected and uninfected flies. Other mitochondrial haplotypes in this clade also contain both infected and uninfected flies. The other cluster, however, contains mitochondrial haplotypes consisting of mostly uninfected flies. Only one individual in this group of haplotypes is infected. In total, spiroplasma is associated with seven of the 14 mitochondrial haplotypes in the population. A majority of the sampled individuals, both infected and uninfected, fall into two haplotypes. This lack of a strong association of infection status with mitochondrial haplotype is consistent with either an older infection followed by loss or horizontal transmission.

The 53 *D. hydei* sampled contain two types of *Spiroplasma*, the poulsonii clade and the citri clade. For this species, both the COII and AT-rich region of the mitochondrial genome had limited sequence variation, despite the wide geographical sampling. Only 12 closely related haplotypes are shown in the haplotype network (Fig. 6b), and both infected and uninfected *D. hydei* have these haplotypes. The citri clade *Spiroplasma* is associated with only two connected *Drosophila* haplotypes. The poulsonii clade *Spiroplasma* infects most of the other haplotypes. Similar to the pattern seen in *D. mojavensis*, this distribution of infection is consistent with horizontal transmission, or an older infection with subsequent loss.

Discussion

Phylogenetic analyses show at least five separate introductions of four distinct clades of *Spiroplasma* into *Drosophila*. This surprising amount of spiroplasma diversity was discovered despite limited sampling. The majority of samples in this study were collected from only the western part of North America, yet, in addition to finding citri and poulsonii spiroplasmas, we identified a very divergent *Spiroplasma* infecting *Drosophila tenebrosa*. Its most closely related *Spiroplasma* species is *S. ixodetis*, although it is still 3–15% divergent from *S. ixodetis* at various loci. Our limited sampling of *Drosophila* outside of western North America

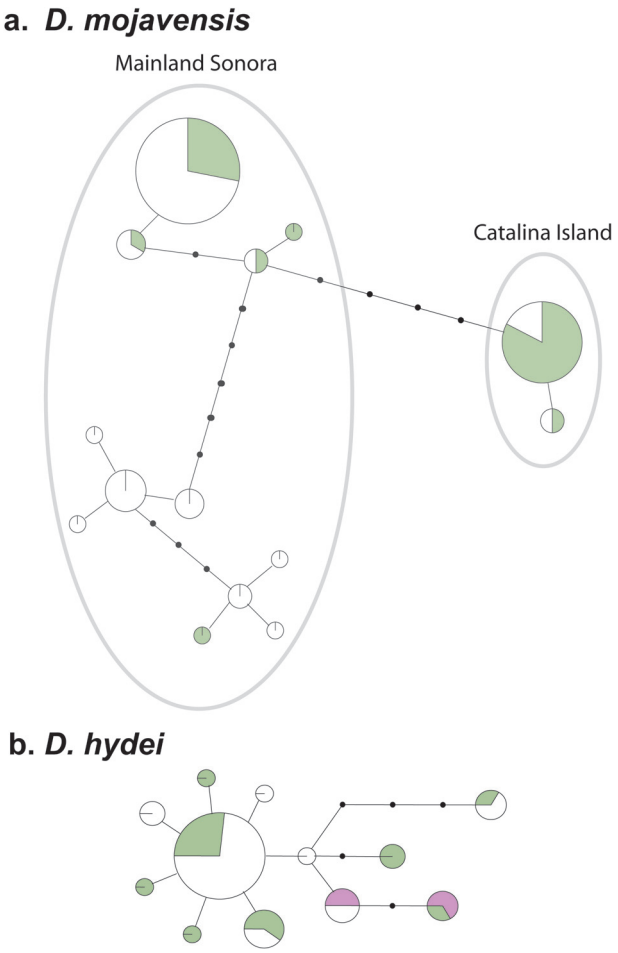

Fig. 6 Minimal spanning haplotype network of *Drosophila* mitochondrial loci. The size of the open circles reflects the number of individuals with each haplotype. Each dot connecting haplotypes represent a single mutational step. The proportion of infected *Drosophila* for each haplotype is shaded. (a) *Cytochrome oxidase I* network for *Drosophila mojavensis*. (b) Combined COII and AT-rich region network for *Drosophila hydei*. Individuals infected with the poulsonii-type *Spiroplasma* are shaded in green, while individuals infected with the citri-type *Spiroplasma* are shaded in purple.

identified *Drosophila* infected with ixodetis-type spiroplasmas from Africa. Recent screening of arthropods for a few *Spiroplasma* strains uncovered additional spiroplasma hosts (Duron *et al*. 2008), and it is likely that a wider geographical and taxonomic sampling of *Drosophila* will reveal an even greater diversity of this prevalent bacterium.

The four divergent clades of *Drosophila* spiroplasmas represent four separate introductions, as the closest relatives for each clade are spiroplasmas infecting other organisms. Furthermore, *Drosophila hydei* appears to have been infected at least twice, by spiroplasmas from two different clades. Five separate introductions is a minimum estimate, and more horizontal transmission events have likely occurred. For example, *D. hydei* and *D. simulans* spiroplasmas display identical haplotypes at every locus. This low divergence is inconsistent with a single ancient infection pre-dating the split of these two species, estimated at over 50 million years (Tamura *et al*. 2004). For some of the other more closely related *Drosophila* species, it is unclear whether shared infections are ancient or recent introductions. For example, *Drosophila mojavensis*, *D. aldrichi*, and *D. wheeleri* all are in the repleta species group, and *D. aldrichi* and *D. wheeleri* are closely related sister species, so an older infection of the three is possible. More extensive sampling of related species would resolve the pattern, although other evidence, such as the lack of genetic variation in the *Spiroplasma* infecting each *Drosophila* species, suggests that horizontal transmission is more likely. This lack of variation, sometimes extending over a large geographical region, suggests that each infection is recent and has rapidly spread.

A potential mechanism for horizontal transmission, mites, has been demonstrated in a laboratory setting (Jaenike *et al*. 2007). In addition, *Spiroplasma* are common gut bacteria in many insects, and plant surfaces, with deposited faecal matter, have been found to act as a reservoir for spiroplasmas (Bove 1997). Both *Spiroplasma citri* and *S. kunkelli* are vectored by leafhoppers, and thus these spiroplasmas have the ability to be picked up by insects and horizontally transmitted. Furthermore, the *D. mojavensis*, *D. aldrichi*, *D. wheeleri*, and *D. hydei* sampled have sympatric ranges at many of the collection sites and breed in similar cactus rots (Ruiz & Heed 1988). Many arthropods use cactus rots as breeding sites, and consequently these rots may also serve as reservoirs for spiroplasma.

To investigate *Spiroplasma* transmission within *Drosophila* populations, we assessed patterns of variation in *Drosophila* mitochondrial haplotypes in *Spiroplasma*-infected and uninfected flies. We expected to find strong associations between spiroplasma infection and a particular *Drosophila* mitochondrial haplotype, suggestive of a recent infection maintained in the population by strict vertical transmission. We did not find this pattern for either *D. hydei* or *D. mojavensis* populations. In exploring the association of *Drosophila* haplotype and infection, however, we were only able to look at populations infected with the non-male-killing spiroplasmas. We would expect an even stronger association with a male-killing spiroplasma infection and mitochondrial haplotype, as this mechanism increases the chance of vertical transmission.

For *D. mojavensis*, spiroplasma is associated with 7 of 14 total haplotypes in three sampled populations. A majority of the sampled individuals, both infected and uninfected, had two of these haplotypes. For the CI population, the prevalence of infection is 60% (T. Watts, N.A. Moran, T.A. Markow, unpublished data), and the diversity of mitochondrial haplotypes is low, with only two sampled. Given that this small, isolated population likely underwent a

bottleneck (Reed *et al*. 2007), the prevalence may reflect infection status of the few flies colonizing the island. Machado *et al*. (2007), however, found higher levels of genetic diversity in the CI *Drosophila* at nuclear loci, and postulated that the lack of mitochondrial diversity may be due to a mitochondrial sweep. Reproductive parasites such as *Wolbachia* often cause mitochondrial sweeps (Jiggins 2003; Engelstadter & Hurst 2007) and such sweeps suggest the presence of some kind of reproductive manipulation or strong fitness advantages for infected females. For the Sonoran *D. mojavensis*, the diversity of mitochondrial haplotypes was higher. Given that infection is associated with only a subset of Sonoran haplotypes, but that those haplotypes are two of the three total haplotype groups, *Spiroplasma* may be an older infection in *D. mojavensis* that was subsequently lost in the third group before its diversification. In this case, *Spiroplasma* may be maintained solely by a high fidelity of vertical transmission, with some loss. Alternatively, horizontal transmission may be spreading the infection among susceptible *Drosophila*, with those individuals in the uninfected group *Spiroplasma* resistant.

In the *D. hydei* populations, the citri-type *Spiroplasma* appears to be a relatively recent infection maintained by vertical transmission, as the four individuals that have this *Spiroplasma* type have two very similar mitochondrial haplotypes. The uninfected individuals with this haplotype may have lost the spiroplasma infection, or the mitochondrial loci may lack sufficient resolution to fully distinguish matrilines. The four individuals infected with the citri-type *Spiroplasma* each were collected from different geographical regions. As *D. hydei* is a cosmopolitan species (Markow & O'Grady 2005), the spread of this infection throughout the range of collection is not unexpected. For the *D. hydei* infected with the poulsonii-type spiroplasmas, there may have been an ancient infection before the diversification of haplotypes followed by loss of the infection from many individuals of each haplotype. If spiroplasma only were vertically maintained, all the while undergoing loss from all haplotypes, the infection is likely to have been lost completely in some cases in the absence of some fitness benefit. Populations of *D. hydei* in Japan, however, have been documented to maintain high population prevalence levels (25–46%) over the course of 30 years (Kageyama *et al*. 2006), even though the fidelity of vertical transmission of this spiroplasma is low at the colder temperatures these populations experience (Osaka *et al*. 2008). Thus, it is also possible that some horizontal transmission is maintaining *Spiroplasma* in *D. hydei* populations.

We found no evidence for recombination among *Drosophila* spiroplasmas from different clades. Any recombination among spiroplasma strains infecting a single species may have been undetected because of the lack of intraspecific genetic diversity. Alternatively, recombination may not be possible in these bacteria. Several *S. citri* strains contain a truncated, nonfunctional, *recA* gene. In *Escherichia coli* and other bacteria, *recA* is responsible for promoting homologous recombination and recombinatorial DNA repair (Kowalczykowski 2000). In fact, *S. citri* has been shown to be more sensitive to ultraviolet damage than other closely related bacterial taxa with a functional *recA* gene (Marais *et al*. 1996b). Other pathways exist, however, such as recombination involving extrachromosomal DNA such as plasmids and bacteriophage known to occur in various *S. citri* strains (Barroso & Labarere 1988; Marais *et al*. 1996a). A lack of recombination may suggest that horizontal transmission rarely causes co-infection or that co-infections are not stable. Both the citri and poulsonii haplotypes are circulating in the *D. hydei* populations of San Carlos, Magdalena, and OPNM, so if *Spiroplasma* is horizontally transmitted, co-infection is possible.

The strains of *Spiroplasma* that cause male-killing group together separated from the non-male-killing *Spiroplasma* infecting *D. hydei* and *D. simulans* with high bootstrap support. This is consistent with suggestions made for a single origin for male-killing spiroplasmas in *Drosophila* (Montenegro *et al*. 2005; Pool *et al*. 2006). This phylogenetic pattern is seen at 16S rRNA, *fruR* and *spoT (*data not shown), the loci for which sequences from the male-killing *Spiroplasma* infecting willistoni group *Drosophila* were available for comparison. Interestingly, a different species of *Spiroplasma, S. ixodetis,* is known to cause male killing in the ladybird beetle (Tinsley & Majerus 2006) and the butterfly (Jiggins *et al*. 2000). This strain of *Spiroplasma* is most closely related to the spiroplasmas infecting *Drosophila ananassae* and *D. atripex*, which have been stably maintained in the laboratory with no evidence of male killing.

Conclusions

Drosophila are infected with four very different types of spiroplasmas, the majority of which do not cause male killing. Given that our sampling was limited to western North America, a wider geographical and taxonomic sampling of *Drosophila* will undoubtedly reveal still other types of *Spiroplasma*, each of which could potentially have different fitness consequences for their *Drosophila* hosts. The existence of multiple introductions implies that horizontal transmission has played an important role in the distribution of *Spiroplasma* in *Drosophila.* Additionally, patterns of variation in *Drosophila* mitochondrial haplotypes in *Spiroplasma*-infected and uninfected flies imply imperfect vertical transmission in host populations and possible horizontal transmission. Further exploration of the roles and mechanisms of vertical and horizontal transmission of the different spiroplasma strains can also help determine conditions under which this endosymbiont persists in *Drosophila* populations. Finally, our multilocus analysis supports clonality in *Spiroplasma* infecting *Drosophila*, despite evidence

for horizontal transmission. Thus *Spiroplasma* may be more similar to beneficial bacteria trapped in their hosts with no opportunity for recombination. Although previous studies have not found strong fitness consequences of spiroplasma infection in the laboratory (Ebbert 1991; Kageyama *et al*. 2006; Montenegro *et al*. 2006b), conditionally beneficial fitness effects may help to explain its distribution in host populations.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 *Spiroplasma*-infected *Drosophila* individuals used in this study. The male-killing phenotype for each individual is listed, as well as the spiroplasma haplotype amplifed at each locus. N/S, not available (did not amplify)

Table S2 Shimodaira–Hasegawa test results able of the difference in –Ln values for the best tree of each data set vs. the topology from every other loci, followed by the statistical significance of the difference (*P* value)

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