

Heritable Endosymbionts of *Drosophila*

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ABSTRACT

Although heritable microorganisms are increasingly recognized as widespread in insects, no systematic screens for such symbionts have been conducted in *Drosophila* species (the primary insect genetic models for studies of evolution, development, and innate immunity). Previous efforts screened relatively few *Drosophila* lineages, mainly for *Wolbachia*. We conducted an extensive survey of potentially heritable endosymbionts from any bacterial lineage via PCR screens of mature ovaries in 181 recently collected fly strains representing 35 species from 11 species groups. Due to our fly sampling methods however, we are likely to have missed fly strains infected with sex ratio distorting endosymbionts. Only *Wolbachia* and *Spiroplasma*, both widespread in insects, were confirmed as symbionts. These findings indicate that in contrast to some other insect groups, other heritable symbionts are uncommon in *Drosophila* species, possibly reflecting a robust innate immune response that eliminates many bacteria. A more extensive survey targeted these two symbiont types through diagnostic PCR in 1225 strains representing 225 species from 32 species groups. Of these, 19 species were infected by *Wolbachia* while only three species had *Spiroplasma*. Several new strains of *Wolbachia* and *Spiroplasma* were discovered, including ones divergent from any reported to date. The phylogenetic distribution of *Wolbachia* and *Spiroplasma* in *Drosophila* is discussed.

INTRODUCTION

The extent of symbiotic associations in animals is prompting a new evaluation of the role of microorganisms, particularly Bacteria, in animal development, ecology and

evolution (BACKHED *et al.* 2005; MCFALL-NGAI 2002). Although pathogenic infections are more intensively studied, recent studies of divergent groups including mollusks, nematodes, annelids, insects, and mammals reveal that chronic, non-invasive associations with particular bacterial lineages are common and are often beneficial or even required for the development and reproduction of hosts (BACKHED *et al.* 2005; BAUMANN 2005; BRUMMEL *et al.* 2004; MCFALL-NGAI 2002; NELSON and FISHER 1995; TAYLOR *et al.* 2005). Beneficial effects include dietary supplementation through biosynthesis of needed nutrients, developmental interactions that prime the immune system, improved tolerance to thermal stress, and defenses against natural enemies. At the same time, many chronic infectious agents have subtle deleterious effects on hosts, blurring the distinction between pathogenic and mutualistic associations.

Of particular interest are heritable microorganisms, which are especially widespread in insects (see BUCHNER 1965; JEYAPRAKASH and HOY 2000; MORAN *et al.* 2005b; WERREN *et al.* 1995a). Many of these are mutualistic, but some exert distinctive effects on host reproduction, such as biasing sex ratio, effecting parthenogenesis, or causing incompatibility in crosses with uninfected strains of the same host species (WERREN *et al.* 1995b).

The genus *Drosophila* provides the primary insect genetic model system for studies of evolution and diversification (POWELL 1997) and for studies of infectious processes and immunity (MYLONAKIS and ABALLAY 2005). *Drosophila* species lack so-called “primary symbionts” (ancient obligate associations in which symbionts occupy specialized host organs, BUCHNER 1965), but they do form facultative associations with maternally transmitted symbionts that undergo occasional horizontal transfer into naïve hosts.

Despite the broad interest in *Drosophila* for ecological, evolutionary and genetic studies, and the recent investigations of heritable symbionts in insects generally, few *Drosophila* species have been screened for the presence of heritable endosymbionts. Indeed some associations have been discovered in the course of recent genomic sequencing projects, in which symbiont DNA has been intermixed with that of the hosts (SALZBERG *et al.* 2005a; SALZBERG *et al.* 2005b). The little deliberate screening that has been performed has been restricted mostly to *Wolbachia pipientis*, estimated to infect up to 70% of insect species, from many insect orders (JEYAPRAKASH and HOY 2000). In *Drosophila*, most screening for *Wolbachia* has concentrated on long-term laboratory cultures (BOURTZIS *et al.* 1996; CLARK *et al.* 2005; GIORDANO *et al.* 1995; MILLER and RIEGLER 2006; SALZBERG *et al.* 2005a; SALZBERG *et al.* 2005b; WERREN and JAENIKE 1995; WERREN *et al.* 1995a; WERREN *et al.* 1995b; ZHOU *et al.* 1998) as opposed to natural populations (CHARLAT *et al.* 2004; DYER and JAENIKE 2005; HAINE *et al.* 2005; JAENIKE *et al.* 2003; MONTENEGRO *et al.* 2006; VAVRE *et al.* 1999; VENETI *et al.* 2004). Furthermore, there has been considerable phylogenetic bias in the species screened. Of the 69 species of the family Drosophilidae for which *Wolbachia* screening results have been published, 68 belong to the genus *Drosophila* (Figure 1). Of these, 41 belong to the subgenus *Sophophora*, which has approximately 500 species, including *D. melanogaster*, while only 26 belong to the larger subgenus *Drosophila*, which has approximately 1500 species, excluding the Hawaiian *Drosophila* and the *Scaptomyza* (MARKOW and O'GRADY 2006). Of the 20 species infected with *Wolbachia*, 17 belong to the subgenus *Sophophora* while only three belong to the subgenus *Drosophila*. Thus, uneven

taxonomic sampling could underlie the observation that 17 of the 20 genus *Drosophila* species reported to harbor *Wolbachia* are from the subgenus *Sophophora*.

The only other heritable symbiont group reported for *Drosophila* species is *Spiroplasma* (MONTENEGRO *et al.* 2006; MONTENEGRO *et al.* 2005; WILLIAMSON and POULSON 1979; WILLIAMSON *et al.* 1999), which, along with related bacteria in the phylum Mollicutes, is widespread in insect hosts (GASPARICH 2002) and which sometimes causes son-killing in infected females (ANBUTSU and FUKATSU 2003; MONTENEGRO *et al.* 2005; VENETI *et al.* 2005). In *Drosophila*, *Spiroplasma* infections are currently documented in five species of the subgenus *Sophophora*—*D. melanogaster* (MONTENEGRO *et al.* 2005); and *D. willistoni*, *D. nebulosa*, *D. paulistorum*, and *D. equinoxialis* (WILLIAMSON and POULSON 1979; WILLIAMSON *et al.* 1999); and four species of the subgenus *Drosophila*—*D. hydei* (OTA *et al.* 1979); and *D. neocardini*, *D. paraguayensis*, and *D. ornatifrons* (MONTENEGRO *et al.* 2006).

Examples of sex ratio bias or male-killing have been reported for a few other *Drosophila* species (reviewed in ANBUTSU and FUKATSU 2003), but the causative agents have not been identified. Other bacterial groups, including the Gammaproteobacteria (e.g., MORAN *et al.* 2005a) and the phylum Bacteroidetes (ZCHORI-FEIN and PERLMAN 2004), are also common as opportunistic heritable symbionts of insects, having major effects on reproduction, but their extent in *Drosophila* is not known. However, these bacterial groups also include transient colonizers of external surfaces and/or guts of insects; as a result, PCR amplification of whole insect DNA extracts with primers diagnostic to these groups would give little information regarding heritable symbiotic associations. One way to circumvent this problem is to screen extracts from dissected

ovaries, the tissue most likely to harbor any heritable endosymbionts, but this procedure is time-consuming. *Wolbachia* lacks closely related free-living or facultative counterparts, making its detection feasible by screening whole insect extracts with *Wolbachia*-specific PCR primers.

Given the array of heritable endosymbionts reported in other insects and the limited information about *Drosophila*, we have addressed the following questions: (1) What are the frequency and diversity of heritable bacterial endosymbionts in natural populations of *Drosophila* species? and (2) Are there any detectable phylogenetic patterns affecting endosymbiont infection within the genus *Drosophila*? Due to our fly sampling procedure, we likely would have missed sex-ratio distorting bacteria but should have detected bacteria that cause other effects on their hosts. Our initial PCR screen, using several sets of both “universal” and taxonomically restricted bacterial primers with DNA from dissected ovarioles, focused upon newly established isofemale strains from natural populations of 35 species (from 11 species groups) representing both major *Drosophila* subgenera (Figure 1). Because this initial screening revealed only the presence of *Wolbachia* and *Spiroplasma*, symbionts that can be definitively diagnosed using DNA extracted from whole flies, we then examined the distribution of these two symbionts across a total of 223 species from the Tucson *Drosophila* Species Stock Center.

MATERIALS AND METHODS

***Drosophila* species and strains screened**

Over 4700 individual flies from over 1500 strains were screened, with usable results obtained for 1401 strains. The complete list of strains, their origin and screening procedures employed are provided as supplemental material (Appendix 1;

<http://www.genetics.org/supplemental>) and in a online database

(<http://amadeus.biosci.arizona.edu/~bjn/flyendo/index.php>).

Fly strains for exhaustive screening of ovarian tissues: For the screening of heritable endosymbionts from any lineage of Bacteria, we used primarily recently collected (< 2 yr) isofemale lines. Our rationale was based on our aim of detecting heritable symbionts present in wild fly populations (but possibly lost or acquired in long-term lab stocks). At the same time, isofemale lines were retained with the aim of having their progeny for further studies if endosymbionts were encountered and to validate fly species identification by a combination of examination of male genitalia and molecular approaches. For most species, identification could be confirmed by sequencing of fragments of the mitochondrial genes *cytochrome oxidase I* (FOLMER *et al.* 1994) and *II* (LIU and BECKENBACH 1992), while 1Kb of sequence flanking the X-linked microsatellite locus *X008* was required to discriminate *D. pseudoobscura* from *D. persimilis* (MACHADO *et al.* 2002), and a fragment of *xanthine dehydrogenase (xdh)* (MJ-Xdh-798 5'-GAGCCAGACATTGGTGGAG-3' and MJ-Xdh-1496 5'-AAGTAGGACTTGTGCTCGATGG-3'; L. Matzkin, unpublished) was sequenced to distinguish among certain members of the repleta species group.

Fly strains for targeted screens: For the screening of Wolbachia and Spiroplasma only, we used both recent and older collections, mostly derived from the Tucson Stock Center. We screened 223 species from the Tucson Stock Center (including most of the species used in the exhaustive screens). At the time of screening, this represented effectively all species in the collection that were neither in quarantine nor in the critical care unit. For the majority of the species, more than one strain was available and screened.

DNA Extraction

Ovarian dissection and DNA extraction: We examined 3–4 females per fly strain. In order to ensure that ovaries contained large numbers of mature oocytes, mature females were placed in freshly yeasted vials for 2–3 days prior to dissection. Each female was anesthetized with CO₂, surface-sterilized in 95% ethanol, and dissected under sterile Phosphate buffer. Ovaries were extracted carefully with sterile forceps (making sure the gut was not broken during the dissection), rinsed briefly in 0.5% bleach (0.03% sodium hypochlorite final concentration) and in sterile water, placed in a sterile microtube, frozen immediately in liquid nitrogen, and kept at -80° until DNA extraction. DNA was extracted with the DNEasy Kit (Qiagen, Valencia, CA) following the protocol for Gram-positive bacteria (i.e., lysozyme extraction). Two separate elutions in AE Buffer of 30ul were performed for each sample.

Whole-fly DNA extraction for targeted screens: We examined three females per fly strain. Females were anesthetized with CO₂; each female was placed individually into the well of a 96-well PCR plate on dry ice and kept at -80° until extraction. Each fly was crushed on ice with a sterile pestle in 48ul of Squish Buffer (10mM Tris-HCl; 1mM EDTA; 25mM NaCl) and 2ul Proteinase K (20mg/ml) (GLOOR *et al.* 1993). This was then incubated 30 min at 37° and 5 min at 95°.

PCR screening/Sequencing

For ovary extracts, we conducted PCR screens with each of the seven primer pairs listed in Table 1. The first two should amplify ~1400 bp of the 16S ribosomal RNA gene of most Eubacteria. The 559F–35R pair should amplify ~1000 bp of the 3' end on the 16S rRNA gene, the intergenic spacer (ITS or IGS) which varies in size, and the first ~35 bp

of the 23S gene. However, it should not amplify the relatively few bacteria for which the 16S and 23S rRNA genes are not in the same operon, such as *Buchnera* (TAMAS *et al.* 2002) and *Wolbachia pipientis* (FOSTER *et al.* 2005; WU *et al.* 2004). To increase our chances of detecting endosymbionts, we also used four additional primer pairs, each of which amplifies a specific group of bacteria known to include heritable endosymbionts of insects (i.e., Bacteroidetes, Spiroplasma and several Gram positive, Wolbachia, Cytophaga-like organisms; see Table 1).

We included positive and negative controls for every PCR run. PCR runs with failed positive controls or with positive negative controls were excluded from the results. The quality of each ovary DNA extract was assessed by amplification of the fly's mitochondrial *Cytochrome Oxidase I* (mtCOI) gene. Templates that were negative for this PCR were excluded from the results.

To assess presence of endosymbionts, we conducted an initial PCR screening (12.5µl PCR reaction) for all samples. Samples that were scored as positive in the first PCR were then subjected to a second PCR reaction (50µl total volume) for confirmation and sequencing. Extremely weak amplifications that did not yield enough template for sequencing were regarded as negative.

Both strands of each PCR product were directly sequenced with an ABI 3700 at the University of Arizona's Genomics Analysis & Technology Core Facility. If sequence results were unclear, suggesting that more than one sequence type or multiple PCR fragments were present, then PCR products were cloned and then sequenced (~3 clones/PCR fragment/individual). Sequences were assembled and edited with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI).

Identification of Bacteria

We used Blastn (ALTSCHUL *et al.* 1997) and/or Classifier (COLE *et al.* 2005) to determine the identity of bacterial sequences. If a sequence was $\geq 98\%$ identical to a sequence found in GenBank, it was assigned to that bacterial species or group.

Interpretation of Screening Results

Flies were scored as infected by an endosymbiont if at least one individual of that line yielded a positive PCR result that was confirmed with a second PCR and, in most cases, a sequence. However, due to the possibility of contamination by free-living or facultative bacteria not known to be heritable symbionts of arthropods (e.g., *Escherichia coli*, *Pseudomonas*), flies that yielded positive PCR and sequences for these bacteria were not scored as infected. In the majority of cases, three individual flies from an infected line gave PCR results for a given primer pair, and a sequence was obtained from at least one individual.

Alignment and phylogenetic analyses

To investigate the phylogenetic affinities of endosymbionts found in this study, we conducted phylogenetic analyses of the 16S rRNA gene. For Wolbachia analyses, we included published sequences (at least 1340 bp-long) representing the highest Blastn hits to new haplotypes and representatives of most Wolbachia supergroups. For Spiroplasma analyses, we included the highest Blastn hits, and published sequences of related lineages and outgroups based on GASPARICH *et al.* (2004). Sequences were aligned by eye in MacClade 4.06 (MADDISON and MADDISON 2003). Unalignable characters were excluded from phylogenetic analyses. Our alignments have been deposited in TreeBase (<http://www.treebase.org/treebase>) under accession numbers SN2737-10782 and

SN2737-10783. We used PAUP*4.0b10 (SWOFFORD 1998) to construct a Neighbor-joining (NJ) tree under the Kimura-2-parameter model (KIMURA 1980) of *Drosophila mtCOI* sequences for verification of species identity.

Given the evidence for widespread recombination in *Wolbachia* (BALDO *et al.* 2006), particularly within the *wsp* gene (BALDO *et al.* 2005; REUTER and KELLER 2003; WERREN and BARTOS 2001), we did not attempt to construct phylogenetic relationships using this gene, as they would probably not reflect the true phylogenetic history of the strains. We used PAUP* and Modeltest 3.7 (POSADA and CRANDALL 1998) to infer the most appropriate model of sequence evolution for 16S rRNA gene of *Wolbachia* and *Spiroplasma* haplotypes. We conducted Maximum Likelihood heuristic searches assuming the models selected above. As a measure of support for our phylogenetic inferences, we used MrBayes 3.1.2 (HUELSENBECK and RONQUIST 2001) to obtain Bayesian posterior probabilities. Four simultaneous Monte Carlo Markov Chains were run for 10,000,000 cycles, and sampled every 100 cycles, under a model that included a substitution rate for each type of transition and transversion (General Time Reversible), a proportion of invariable sites, and a Gamma distribution of rates across sites. Posterior probabilities for each node were obtained from a consensus of trees excluding the initial set of cycles preceding convergence on stable likelihood values (i.e., the ‘burn in’).

Nomenclature

All the sequences obtained were compared to GenBank sequences by Blastn. If the most similar sequence in the database was not identical to our sequence, then our sequence was regarded as a new haplotype. For *Wolbachia* sequences, we named new

haplotypes according to the host species. If a haplotype was not new (i.e., 100% identical to a sequence in GenBank), it was labeled with a previously assigned name.

RESULTS

Exhaustive screening of flies from natural populations for heritable symbionts: We examined ovaries of 181 fly strains from 35 species in 11 species groups (Appendix 2), aiming to discover all possible maternally transmitted bacterial symbionts. According to our scoring criteria, three of the seven primer pairs used for screening produced no positive results (Table 2). Because all samples included in the results gave positive reactions for the DNA isolations (based on the PCR of *mtCOI*), and positive template controls were run for every primer pair, these negative results show that these bacteria, including *Cardinium* (Cytophaga-like) and other Bacteroidetes, were absent from all samples. Furthermore, in cases in which the more “universal” primers produced products, indicating presence of some bacterial type in the sample, the sequenced products almost always corresponded to either *Wolbachia* or *Spiroplasma*, which were also revealed by the corresponding diagnostic primer screens. Thus, screening with the 10F-1507R and/or 27F-1492R universal primer pairs demonstrated the presence of *Wolbachia* in eight species (Table 3) from the *melanogaster* and *willistoni* species groups (subgenus *Sophophora*). We also detected the presence of *Spiroplasma* in one species (*D. hydei*) with the 27F-1492R primer pair (Table 4).

Several known heritable endosymbionts of insects are within the Enterobacteriaceae (Gammaproteobacteria), and some screens were designed to detect members of this group. Screening with the 559F-35R universal primer set, which spans the intergenic

spacer of the rRNA operon and thus excludes Wolbachia which lacks an intact operon (WU *et al.* 2004), revealed the presence of proteobacterial sequences in several ovary extracts. Some of these were identified with Blastn and Classifier as particular species or genera (e.g., *E. coli*, *Pseudomonas*, *Sphingomonas*). These sequences probably reflect contamination or opportunistic pathogenic infection of the tissue or extract. A few others were identified as Enterobacteriaceae, but the genus could not be identified based on the DNA sequence; these sequences could represent heritable symbionts. However, in most cases we found these sequences in only one individual per line, and these bacteria were not detected with either of the two other universal primer pairs for the 16S rRNA gene. We adopted a conservative criterion and disregarded them as heritable endosymbionts. Screening with group-specific primers revealed the presence of Wolbachia in the same eight species as with the universal primers as well as in one additional species (*D. tropicalis*; Table 3), and the presence of Spiroplasma in *D. hydei* and *D. mojavensis* (Table 4); the Spiroplasma strains from *D. mojavensis* were not detected with any of the “universal” primer pairs. We found no evidence of other bacterial groups with the other group-specific primers (Table 2).

Targeted screens for Wolbachia and Spiroplasma: We screened for the presence of Wolbachia and Spiroplasma with group-specific primers in whole-fly DNA extracts from 1255 strains from approximately 223 species representing 32 species groups of the family Drosophilidae, most within the genus *Drosophila* (Appendix 2; Figure 1A). We found evidence of Wolbachia in 16 species from the genus *Drosophila* (representing four species groups) and in one species from each, the genus *Scaptomyza* and the genus *Scaptodrosophila* (Table 3; Figure 1B). We found evidence for Spiroplasma in *D. hydei*,

D. mojavenensis, and *D. aldrichi*, all within the repleta species group (Table 4; Figure 1B).

All positive findings were confirmed with sequencing, and all positives were found in more than one independently extracted fly from the line.

Frequency of endosymbionts: Wolbachia was much more common than Spiroplasma.

Wolbachia was detected in 8.0% of all species examined, whereas Spiroplasma occurred in only 1.3% of species. Within Wolbachia-infected species, 62% of strains examined were infected (Table 3). In contrast, within Spiroplasma-infected species, only 11% of strains were infected (Table 4). Spiroplasma was most frequent in *D. hydei* where it occurred in 27% of the strains examined.

Diversity of Wolbachia: Based on a dataset of 1374 bp of the 16S rRNA sequences, we found six haplotypes of Wolbachia, three of which had not been reported before in any organism. Most of our phylogenetic analyses of the Wolbachia 16S rRNA gene included only taxa for which at least 1340 bp were available, but a subset of the analyses were conducted on a shorter dataset (820 bp) to allow for inclusion of other lineages. Similarly, in a subset of the analyses, we removed the Wolbachia lineages that appeared most divergent to the *Drosophila*-associated Wolbachia strains, in order to reduce the effects of mutational saturation on our phylogenetic inferences. Most of the substitution models employed in the Maximum Likelihood and Bayesian analyses included a different rate for almost every type of substitution, unequal base frequencies, as well as a specific proportion of invariable sites and a specific gamma shape parameter (discrete approximation; four categories) for rate differences among sites.

A consensus of our phylogenetic analyses (Figure 2) shows *wPana* (from *D. pseudoananassae*) within what is typically regarded as the B supergroup of Wolbachia,

along with *wNo* (based on a shorter sequence; not shown) and *wMau* strains of *D. simulans*. *wNik* (from *D. nikananu*) appeared to be most closely related to the *Wolbachia* strains from the sandfly *Phlebotomus* and the spider *Diaea*. However, in the absence of the *Diaea* strain (a shorter ~820 bp sequence that was only included in a subset of the analyses) there was little or no support for the relationship between the *wNik* and the haplotype from *Phlebotomus*. Nevertheless, inclusion of the *Diaea* sequence resulted in very high support for this relationship (96% Bayesian posterior probability). *wTak*, the haplotype from an unidentified *Drosophila* species of *takahashii* subgroup, appeared to be related to the E supergroup found in springtails, but support for this relationship was low (55–79%). In our analyses, the E supergroup fell within what is traditionally recognized as the A supergroup with 85–96% support, and thus, the A supergroup was not monophyletic. The strain from *D. willistoni* (*wWil*) was identical to the consensus of the trace archives of the *D. willistoni* whole genome shotgun sequencing project that contain fragments of the 16S rRNA gene, but was otherwise unknown and unnamed. *wWil* was very similar to other *Drosophila*-associated *Wolbachia*; *wRi* and *wMel* (also found in our screenings), as well as to *wHa*, and *wAu*, all of which were previously assigned to the A supergroup (BALDO *et al.* 2005; MERCOT and CHARLAT 2004).

Based on the highly variable *wsp* gene, we found 14 haplotypes (GenBank Accession Nos. DQ412091–DQ412111; Table 3). Of these, seven had been reported in *Drosophila* before, and seven were new haplotypes for *Drosophila* (i.e., *wBai*, *wBic*, *wNik*, *wPana*, *wPse*, *wTak*, and *wSto*). Although *wsp* is known to undergo widespread recombination in *Wolbachia* (BALDO *et al.* 2005), near-identical sequences likely reflect close relationship. Of these new haplotypes, three were very similar to other *Drosophila*-associated

Wolbachia: the haplotype of *Scaptodrosophila stonei* (wSto) was 99% identical to that from *D. septentrionalis* (wSpt; AY620209); the haplotype from *D. bicornuta* (wBic) was 99% identical to that from *D. bifasciata* (AJ271112; a male-killer) as well as to other non-Drosophila insects; and in agreement with the phylogenetic analyses of the 16S rRNA gene. The haplotype from *D. pseudonananassae* (wPana) was 99% identical to B-clade sequences from *D. simulans* (wMa-AF020069 and wNo-AF020074). Four strains were quite different from anything reported from Drosophila before: that from *D. baimaii* (wBai) was 99% similar to Wolbachia from another dipteran (*Pseudacteon curvatus*; family Phoridae; AY878108); that from *D. pseudotakahashii* (wPse) was 99% similar to Wolbachia from fig and gall wasps and a heteropteran (AY567677, AY095154, AB109568); that from a species in the takahashii subgroup (wTak) was 99% similar to haplotypes from lice (AY331130); and that from *D. nikananu* (wNik) was very distinct from any Wolbachia reported to date, showing only 83% similarity to the closest sequence in GenBank, from a scarabid beetle host (*Onthophagus vaulogerii*; AY157683).

Diversity of Spiroplasma: Based on a 439 bp-long fragment of the 16S rRNA gene of Spiroplasma, we found four Spiroplasma haplotypes in the lines examined (Table 4; Figure 3). Our phylogenetic analyses were restricted to this portion of the 16S rRNA gene because we could not obtain PCR products of the *D. mojavensis* strains with the “universal” primer pairs, which targeted a longer fragment (~1500 bp), and because *D. aldrichi* was only included in the targeted surveys for Wolbachia and Spiroplasma, which examined a shorter fragment of the 16S rRNA gene of Spiroplasma. Each of the three species of Drosophila had a different haplotype, and two different haplotypes were observed in *D. hydei*: haplotype 1 was the most common in *D. hydei* while haplotype 2

only occurred in one line. Haplotype 1 was sister to *S. poulsonii*, from *D. willistoni* (subgenus *Sophophora*). These in turn were closely related to *Spiroplasma* from a tick and to *S. insolitum*, which infects flowers and insects. Haplotype 2 of *D. hydei* was closely related to the *Spiroplasma* found in *D. mojavensis* and *D. aldrichi* (also members of the *repleta* species group; subgenus *Drosophila*). These in turn were most closely related (83% Bayesian posterior probability) to *S. citri*, *S. phoeniceum*, *S. melliferum*, which occur in plants and insects.

DISCUSSION

Prior to this work, there had been no systematic survey of heritable endosymbionts, other than *Wolbachia*, in *Drosophila* species. By examining 181 fly strains (from 35 species; 11 species groups) in the genus *Drosophila*, for presence in ovarioles of endosymbionts from any lineage of Bacteria (Figure 1), we have gained a more complete picture of the nature and scope of heritable endosymbiotic infections in this group of organisms. The most striking and unexpected result is that only two kinds of heritable endosymbionts were detected in these samples: *Wolbachia* and *Spiroplasma*. We contrast this to some other insects, which possess a variety of bacterial symbionts, with high representation of Gammaproteobacteria (e.g., BAUMANN 2005). While our results imply a relatively low incidence of other heritable symbionts that do not cause sex-ratio distortion in *Drosophila*, such infections may occur in some populations or species. A previous study, based on PCR screenings of different tissues of *D. paulistorum* including ovaries, reports presence of a *Proteus*-like bacterium (Enterobacteriaceae; Gammaproteobacteria) (MILLER *et al.* 1995). As mentioned above, we did detect presence of Enterobacteriaceae

in some of our ovary extracts, but disregarded them as heritable endosymbionts because these occurrences were very sporadic and their DNA sequences did not allow a more specific identification. Although it is possible that some of these are truly heritable endosymbionts, our results suggest that if bacterial groups other than *Wolbachia* and *Spiroplasma* indeed associate with *Drosophila*, they do not appear to be widespread.

Absence of other heritable endosymbionts may reflect a robust innate immune response that eliminates infections by most bacterial groups. *Drosophila* species are saprophytic, utilizing necrotic plant material as feeding and breeding sites. Their niche is filled with a high diversity of microorganisms, many of which they consume along with the necrotic plant tissue. Exposure to microorganisms at all stages of their life cycle could have shaped the *Drosophila* immune system to resist infection by most bacteria. Indeed, *Drosophila* uses efficient mechanisms to prevent microbial infection (HOFFMANN 2003; TZOU *et al.* 2002). Insect groups that are more prone to associations with heritable bacteria, such as sap-feeding insect families (BAUMANN 2005), may have less robust immune mechanisms, particularly against Gram-negative bacteria that replicate in the hemocoel. Currently, little is known about the immune system of sap-feeding insects such as aphids and relatives, although aphids are reported to have an attenuated encapsulation response (MACKAUER 1986).

Despite the apparently robust immune system of *Drosophila*, *Wolbachia* and *Spiroplasma* have managed to invade a few species. One possible reason is that both *Spiroplasma* and *Wolbachia* appear to avoid recognition by innate immune systems (BOURTZIS *et al.* 2000; HURST *et al.* 2003). Previous work reported 20 out of 69 *Drosophilid* species examined to be infected with *Wolbachia*. Our study more than triples

the number of Drosophilid species screened for Wolbachia and Spiroplasma. Our results revealed infections in nine additional species of the family Drosophilidae, including members of three groups previously unknown to have Wolbachia: cardini group (*D. arawakana*) in the genus *Drosophila*; and members of two other genera, *Scaptomyza* (*S. pallida*), and *Scaptodrosophila* (*S. stonei*). In some cases, infection by Wolbachia has persisted through decades (up to ~60 years) of laboratory culture in the stock center, as reported by previous studies (CLARK *et al.* 2005; RIEGLER *et al.* 2005).

While previous studies had examined several Drosophilid species for Wolbachia, very few species had been surveyed for Spiroplasma, revealing nine *Drosophila* species infected by this bacterium: *D. willistoni*, *D. paulistorum*, *D. nebulosa*, and *D. equinoxialis* (willistoni species group; subgenus Sophophora); *D. melanogaster* (melanogaster species group; subgenus Sophophora); *D. paraguayensis*, *D. ornatifrons*, *D. neocardini* (MONTENEGRO *et al.* 2006), and *D. hydei* (OTA *et al.* 1979; tripunctata, guarani, cardini, and repleta groups, respectively; subgenus *Drosophila*), but this last one had not been confirmed by DNA sequencing. Our results revealed infections in *D. hydei*, and in two additional members of the repleta group in which Spiroplasma had not been reported previously (*D. aldrichi* and *D. mojavenis*), but not in any of the other species groups reported before. This is probably due to the fact that most of the *Drosophila*-associated Spiroplasma strains reported to date are male killers, which were not likely to be found in our fly samples for reasons discussed above.

Frequency of Wolbachia and Spiroplasma: Wolbachia was more common than Spiroplasma. Overall infection rates with Wolbachia were found to be low (8% and 12% of examined species; present study and all studies, respectively), compared to Wolbachia

infection rates of insect species in general: 16.9–22% (using standard PCR, WERREN *et al.* 1995a; WERREN and WINDSOR 2000) and 70% (using Long-PCR, JEYAPRAKASH and HOY 2000). Observation of low infection rates in our study could reflect a sampling bias. Many of our samples were derived from isofemale lines or old lab strains, which were unlikely to include male killers (including some Wolbachia, Spiroplasma, and other heritable bacteria). Indeed, we did not detect endosymbionts in several species in which male-killers had been reported before (i.e., *D. bifasciata*, *D. prosaltans*, *D. paulistorum*, *D. equinoxialis*, *D. nebulosa*, and *D. robusta*; CAVALCANTI *et al.* 1957; IKEDA 1970; MAGNI 1953; POULSON 1966; WILLIAMSON and POULSON 1979). However, our procedures would have enabled detection of symbionts causing cytoplasmic incompatibility (the most widely documented Wolbachia phenotype) or mutualistic phenotypes.

Phylogenetic distribution of Wolbachia and Spiroplasma: Wolbachia and Spiroplasma appear to be concentrated in certain Drosophilid groups. For example, based on previous work and the present study, 242 species have been screened for Wolbachia (Figure 1), most of which (229) belong to the genus *Drosophila*. Of these, 86 species belong to the subgenus *Sophophora* and 143 to the larger subgenus *Drosophila*. However, Wolbachia infections have been detected in 23 species of the subgenus *Sophophora* (out of 86; 27%), compared to only four species of the subgenus *Drosophila* (out of 143; 3%). Thus, Wolbachia is much more common in the subgenus *Sophophora* than in the subgenus *Drosophila* (G-test = 29.7; $p = 4.9 \times 10^{-8}$; d.f. = 1). This difference remains highly significant if only species with at least three tested strains are included. The proportion of Wolbachia-infected species in the subgenus *Sophophora* more closely

reflects the overall proportion of Wolbachia-infected insect species (16.9%–70; depending on the screening method), while the proportion of infected species in the subgenus *Drosophila* is much lower. Whether or not this reflects resistance to Wolbachia in some *Drosophila* groups remains to be determined.

Far fewer species (11 out of 228; based on the present and past studies) are infected with *Spiroplasma*, and these fall into six species groups within the genus *Drosophila*. In contrast to Wolbachia, no significant difference was observed between the subgenus *Sophophora* and subgenus *Drosophila* in the distribution of *Spiroplasma* (G-test = 0.17; p = 0.7; d.f. = 1). Interestingly, one of the groups that harbored *Spiroplasma* (the repleta group; subgenus *Drosophila*), which is well represented in our study (51 species), did not harbor any Wolbachia, but three of its species harbored *Spiroplasma*.

Diversity of Wolbachia: Our screening revealed new haplotypes of Wolbachia and *Spiroplasma*, including some similar to previously reported haplotypes from *Drosophila*. Other Wolbachia strains were not similar to any previously reported in *Drosophila*, but similar to ones reported from divergent taxa such as other dipterans, hymenopterans, heteropterans, and lice, providing further evidence that Wolbachia has been horizontally transmitted among very divergent taxa (HEATH *et al.* 1999; STEVENS *et al.* 2001). One of the haplotypes (*wNik*) was distinct from any reported to date, both in 16S rRNA and in *wsp* sequence. Another (*wTak*) appeared closely related to the E clade (from springtails) based on the 16S rRNA gene, but its *wsp* sequence was 99% identical to a haplotype from lice regarded as a member of the B clade (KYEI-POKU *et al.* 2005). This disagreement between genes is expected due to the widespread recombination reported among and within several Wolbachia genes (BALDO *et al.* 2006). *wNik* and *wTak* were

divergent from any haplotypes reported from *Drosophila*, but examination of multiple loci (e.g., BORDENSTEIN and ROSENGAUS 2005; CASIRAGHI *et al.* 2005) may be necessary to accurately infer their phylogenetic affinities. In this regard, not recovering a monophyletic A supergroup may be the result of lack of phylogenetic signal in the 16S rRNA gene.

Occurrence of wWil (A supergroup) in both *D. tropicalis* and *D. willistoni* may reflect a recent horizontal transfer between these closely related species. Because, the two infected *D. tropicalis* strains were collected at the same locality as one of the infected *D. willistoni* strains, suggesting horizontal transmission due to habitat sharing, as reported for closely related species of the obscura species group (HAINE *et al.* 2005). A previous study that used diagnostic PCR primers to distinguish the A and B supergroups of *Wolbachia* reported infection by B supergroup *Wolbachia* in *D. tropicalis*, although this was not corroborated by DNA sequencing. This species may associate with *Wolbachia* from both supergroups, as observed in *D. simulans* (reviewed by MERCOT and CHARLAT 2004).

Diversity of Spiroplasma: Our study also revealed new strains of *Spiroplasma*.

Haplotype 1 (from *D. hydei*) was closely related to the type strain of *S. poulsonii* from *D. willistoni* (Figure 3) as well as to the strains from *D. nebulosa* and *D. melanogaster* (results not shown; based on a different portion of the 16S rRNA sequence). *D. hydei* (subgenus *Drosophila*) is very distantly related to *D. willistoni*, *D. nebulosa*, and *D. melanogaster* (subgenus *Sophophora*) suggesting that horizontal transfer may have occurred in the recent past between these divergent groups. Indeed, the high similarity between the sequence from *D. nebulosa* (*willistoni* group) and a Brazilian strain from *D.*

melanogaster (melanogaster group) has been attributed to a recent horizontal transfer from the New World native *D. nebulosa* to the Old World native *D. melanogaster* (MONTENEGRO *et al.* 2005). The clade formed by *S. poulsonii* and haplotype 1 is most closely related to spiroplasmas found in ticks (unknown transmission mode), and in flowers and insects (horizontally transmitted). The other three haplotypes of *Spiroplasma*, all found in members of the repleta group (subgenus *Drosophila*) fall into a separate monophyletic group, whose closest relatives are *S. citri*, *S. phoeniceum*, and *S. melliferum*, horizontally transmitted pathogens of plants (the first two) and honeybees (the latter). The lack of monophyly of our haplotypes indicates that *Spiroplasma* invaded *Drosophila* at least twice. Despite belonging to two separate clades, all the *Drosophila*-derived haplotypes fell into the Citri–Melliferum–Insolitum–277F clade defined by GASPARICH ET AL. (2004).

With few exceptions (EBBERT 1991; YAMADA *et al.* 1982), strains of *S. poulsonii* cause son-killing in *D. willistoni*, *D. nebulosa*, *D. melanogaster*, *D. neocardini*, *D. paraguayensis*, and *D. ornatifrons*, and in species to which they have been artificially transferred (EBBERT 1991; EBBERT 1995; MONTENEGRO *et al.* 2006; MONTENEGRO *et al.* 2005; WILLIAMSON and POULSON 1979; WILLIAMSON *et al.* 1999). Our preliminary results suggest that its close relative, haplotype 1, does not cause son-killing in *D. hydei*; *D. hydei* was previously reported (OTA ET AL. 1979) to harbor a non-male-killing strain, of unknown relationship to the *Spiroplasma* of our study. The other three haplotypes (2–4) also show no evidence of son-killing, suggesting that none of the *Spiroplasma* strains associated with repleta group flies cause son-killing. Whether these *Spiroplasma* strains

are incapable of killing males or the repleta group flies are resistant to male-killing is unknown.

We observed no cases of co-infection of one individual fly or strain by more than one Wolbachia or Spiroplasma strain and no cases of co-infection by Wolbachia and Spiroplasma. Co-infection by more than one Wolbachia strain has been reported in several organisms (for example MILLER and RIEGLER 2006; PERROT-MINNOT *et al.* 1996; VAVRE *et al.* 1999; WERREN *et al.* 1995b), and co-infection by Wolbachia and Spiroplasma has been reported only in *D. melanogaster* (MONTENEGRO *et al.* 2005).

Conclusion: Our study triples the number of *Drosophila* species screened for Wolbachia, vastly increases the screening for Spiroplasma, and is the first broad screening aimed at discovery of heritable symbionts from any bacterial phylum. Our finding of low symbiont diversity in the sampled *Drosophila* species suggests significant differences among insect groups in their basic proclivities for symbioses, with *Drosophila* possibly presenting more obstacles to the establishment of intimate associations. Some insight into reasons for this difference may be found in comparisons of gene inventories of *Drosophila* species with those of other arthropods, made possible by ongoing genome sequencing efforts. Our findings also raise the question of the nature of the phenotypic effects of Wolbachia and Spiroplasma in the newly discovered host species. These symbionts could play a major evolutionary role, as certain kinds of phenotypes can result in infections sweeping through populations with major consequences for levels of polymorphism and fixation of alleles (e.g., DEAN *et al.* 2003; RIEGLER *et al.* 2005).

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LITERATURE CITED

- ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG *et al.*, 1997
Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs. *Nucleic Acids Res.* **25**: 3389–3402.
- ANBUTSU, H., and T. FUKATSU, 2003 Population dynamics of male-killing and non-male-
killing *Spiroplasmas* in *Drosophila melanogaster*. *Appl. Environ. Microbiol.* **69**:
1428–1434.
- BACKHED, F., R. LEY, J. SONNENBURG, D. PETERSON and J. GORDON, 2005 Host-
bacterial mutualism in the human intestine. *Science* **307**: 1915-1920.
- BALDO, L., S. BORDENSTEIN, J. J. WERNEGREN and J. H. WERREN, 2006 Widespread
recombination throughout *Wolbachia* genomes. *Mol. Biol. Evol.* **23**: 437-449.
- BALDO, L., N. LO and J. H. WERREN, 2005 Mosaic nature of the *Wolbachia* surface
protein. *J. Bacteriol.* **187**: 5406-5418.
- BAUMANN, P., 2005 Biology of bacteriocyte-associated endosymbionts of plant sap-
sucking insects. *Annu. Rev. Microbiol.* **59**: 155-189.
- BORDENSTEIN, S., and R. ROSENGAUS, 2005 Discovery of a novel *Wolbachia* supergroup
in isoptera. *Curr. Microbiol.* **51**: 393-398.
- BOURTZIS, K., A. NIRGIANAKI, G. MARKAKIS and C. SAVAKIS, 1996 *Wolbachia* infection
and cytoplasmic incompatibility in *Drosophila* species. *Genetics* **144**: 1063-1073.
- BOURTZIS, K., M. M. PETTIGREW and S. L. O'NEILL, 2000 *Wolbachia* neither induces nor
suppresses transcripts encoding antimicrobial peptides. *Insect Mol. Biol.* **9**: 635-
639.

- BRUMMEL, T., A. CHING, L. SEROUDE, A. F. SIMON and S. BENZER, 2004 *Drosophila* lifespan enhancement by exogenous bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 12974-12979.
- BUCHNER, P., 1965 *Endosymbiosis of Animals with Plant Microorganisms*. Interscience, New York.
- CASIRAGHI, M., S. R. BORDENSTEIN, L. BALDO, N. LO, T. BENINATI *et al.*, 2005 Phylogeny of *Wolbachia pipientis* based on *gltA*, *groEL* and *ftsZ* gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. *Microbiology* **151**: 4015–4022.
- CAVALCANTI, A. G. L., D. N. FALCÃO and L. E. CASTRO, 1957 “Sex-ratio” in *Drosophila prosaltans*—a character due to interaction between nuclear genes and cytoplasmic factors. *American Naturalist* **91**: 321–325.
- CHARLAT, S., J. W. BALLARD and H. MERCOT, 2004 What maintains noncytoplasmic incompatibility inducing *Wolbachia* in their hosts: a case study from a natural *Drosophila yakuba* population. *Journal of Evolutionary Biology* **17**: 322--330.
- CLARK, M. E., C. L. ANDERSON, J. CANDE and T. L. KARR, 2005 Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. *Genetics* **170**: 1667-1675.
- COLE, J. R., B. CHAI, R. FARRIS, Q. WANG, S. KULAM *et al.*, 2005 The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* **33**: D294-D296.

- DEAN, M. D., K. J. BALLARD, A. GLASS and J. W. O. BALLARD, 2003 Influence of two *Wolbachia* strains on population structure of east African *Drosophila simulans*. *Genetics* **165**: 1959–1969.
- DYER, K., and J. JAENIKE, 2005 Evolutionary dynamics of a spatially structured host-parasite association: *Drosophila innubila* and male-killing *Wolbachia*. *Evolution* **59**: 1518–1528.
- EBBERT, M. A., 1991 The interaction phenotype in the *Drosophila willistoni* spiroplasma symbiosis. *Evolution* **45**: 971–988.
- EBBERT, M. A., 1995 Variable effects of crowding on *Drosophila* hosts of male-lethal and non-male-lethal spiroplasmas in laboratory populations. *Heredity* **74**: 227–240.
- FOLMER, O., M. BLACK, W. HOEH, R. LUTZ AND R. VRIJENHOEK, 1994 DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**: 294-299.
- FOSTER, J., M. GANATRA, I. KAMAL, J. WARE, K. MAKAROVA *et al.*, 2005 The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biology* **3**: e121.
- FUKATSU, T., and N. NIKOH, 2000 Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **66**: 643–650.
- GASPARICH, G. E., 2002 Spiroplasmas: Evolution, adaptation and diversity. *Front. Biosci.* **7**: D619-D640.

- GASPARICH, G. E., R. F. WHITCOMB, D. DODGE, F. E. FRENCH, J. GLASS *et al.*, 2004 The genus *Spiroplasma* and its non-helical descendants: phylogenetic classification, correlation with phenotype and roots of the *Mycoplasma mycoides* clade. *Int. J. Syst. Evol. Microbiol.* **54**: 893–918.
- GIORDANO, R., S. L. O'NEILL and H. M. ROBERTSON, 1995 Wolbachia infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. *Genetics* **140**: 1307-1317.
- GLOOR, G., C. R. PRESTON, D. M. JOHNSON-SCHLITZ, N. A. NASSIF, R. W. PHILLIS *et al.*, 1993 Type I repressors of P element mobility. *Genetics* **135**: 81-95.
- HAINE, E. R., N. J. PICKUP and J. M. COOK, 2005 Horizontal transmission of *Wolbachia* in a *Drosophila* community. *Ecol. Entom.* **30**: 464-472.
- HEATH, B. D., R. D. J. BUTCHER, W. G. F. WHITFIELD and S. F. HUBBARD, 1999 Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. *Curr. Biol.* **9**: 313-316.
- HOFFMANN, J. A., 2003 The immune response of *Drosophila*. *Nature* **426**: 33-38.
- HUELSENBECK, J. P., and R. RONQUIST, 2001 MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.
- HURST, G. D. D., H. ANBUTSU, M. KITSUKAKE and T. FUKATSU, 2003 Hidden from the host: *Spiroplasma* bacteria infecting *Drosophila* do not cause an immune response, but are suppressed by ectopic immune activation. *Insect Mol. Biol.* **12**: 93-97.
- IKEDA, H., 1970 The cytoplasmically-inherited 'sex-ratio' condition in natural and experimental populations of *Drosophila bifasciata*. *Genetics* **65**: 311-333.

- JAENIKE, J., K. A. DYER and L. K. REED, 2003 Within-population structure of competition and the dynamics of male-killing *Wolbachia*. *Evol. Ecol. Res.* **5**: 1023–1036.
- JEYAPRAKASH, A., and M. A. HOY, 2000 Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* **9**: 393–405.
- KIMURA, M., 1980 A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120.
- KYEI-POKU, G. K., D. D. COLWELL, P. COGHLIN, B. BENKEL and K. D. FLOATE, 2005 On the ubiquity and phylogeny of *Wolbachia* in lice. *Mol. Ecol.* **14**: 285–294.
- LANE, D. J., 1991 16S/23S rRNA sequencing, pp. 115–175 in *Nucleic acid techniques in bacterial systematics*, edited by E. STACKEBRANDT and M. GOODFELLOW. John Wiley and Sons, New York, NY.
- LIU, H., and A. T. BECKENBACH, 1992 Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* **1**: 41–52.
- MACHADO, C. A., R. M. KLIMAN, J. MARKERT and J. HEY, 2002 Inferring the history of speciation from multilocus DNA sequence data: the case of *Drosophila pseudoobscura* and close relatives. *Mol. Biol. Evol.* **19**: 472–488.
- MACKAUER, M., 1986 Growth and developmental interactions in some aphids and their hymenopterous parasites. *J. Insect Physiol.* **32**: 275–280.
- MADDISON, D. R., and W. MADDISON, 2003 MacClade 4: Analysis of phylogeny and character evolution, pp. Sinauer Associates, Sunderland, Massachusetts.

- MAGNI, G. E., 1953 Sex-ratio; a non-Mendelian character in *Drosophila bifasciata*.
Nature **172**: 81.
- MARKOW, T. A., and P. M. O'GRADY, 2006 *Drosophila: A guide to species identification and use*. Academic Press Elsevier, London.
- McFALL-NGAI, M., 2002 Unseen forces: the influence of bacteria on animal development. Dev. Biol. **242**: 1-14.
- MERCOT, H., and S. CHARLAT, 2004 *Wolbachia* infections in *Drosophila melanogaster* and *D. simulans*: polymorphism and levels of cytoplasmic incompatibility. Genetica **120**: 51–59.
- MILLER, S. G., B. C. CAMPBELL, J. BECNEL and L. EHRMAN, 1995 Bacterial entomopathogens from the *Drosophila paulistorum* semispecies complex. J. Invertebr. Pathol. **65**: 125-131.
- MILLER, W., and M. RIEGLER, 2006 Evolutionary dynamics of wAu-like *Wolbachia* variants in Neotropical *Drosophila* spp. Appl. Environ. Microbiol. **72**: 826–835.
- MONTENEGRO, H., L. HATADANI, H. MEDEIROS and L. KLACZKO, 2006 Male killing in three species of the tripunctata radiation of *Drosophila* (Diptera: Drosophilidae). J. Zool. Syst. Evol. Res. **44**: 130-135.
- MONTENEGRO, H., V. N. SOLFERINI, L. B. KLACZKO and G. D. D. HURST, 2005 Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*. Insect Mol. Biol. **14**: 281-288.
- MORAN, N. A., C. DALE, H. DUNBAR, W. A. SMITH and H. OCHMAN, 2003 Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. Environ. Microbiol. **5**: 116–112.

- MORAN, N. A., J. RUSSELL, T. FUKATSU and R. KOGA, 2005a Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.* **71**: 3302-3310.
- MORAN, N. A., P. TRAN and N. M. GERARDO, 2005b Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum *Bacteroidetes*. *Appl. Environ. Microbiol.* **71**: 8802-8810.
- MUNSON, M. A., P. BAUMANN, M. A. CLARK, N. A. MORAN, D. J. VOEGTLIN *et al.*, 1991 Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *J. Bacteriol.* **173**: 6321-6324.
- MYLONAKIS, E., and A. ABALLAY, 2005 Worms and flies as genetically tractable animal models to study host-pathogen interactions. *Infect. Immun.* **73**: 3833–3841.
- NELSON, D. C., and C. R. FISHER, 1995 Chemoautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps, pp. 125-167 in *Microbiology of Deep-Sea Hydrothermal Vent Habitats*, edited by D. M. KARL. CRC Press, Boca Raton.
- OTA, T., M. KAWABE, K. OISHI and D. F. POULSON, 1979 Non-male-killing spiroplasmas in *Drosophila hydei*. *J. Hered.* **70**: 211–213.
- PERROT-MINNOT, M. J., L. R. GUO and J. H. WERREN, 1996 Single and double Infections with *Wolbachia* in the parasitic wasp *Nasonia vitripennis*: Effects on Compatibility. *Genetics* **143**: 961-972.
- POSADA, D., and K. A. CRANDALL, 1998 Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

- POULSON, D., 1966 Further cases of maternal SR in *Drosophila* species. *Drosoph. Inf. Serv.* **41**: 77.
- POWELL, J., 1997 *Progress and prospects in evolutionary biology: the Drosophila model*. Oxford University Press, NY.
- REUTER, M., and L. KELLER, 2003 High levels of multiple *Wolbachia* infection and recombination in the ant *Formica exsecta*. *Mol. Biol. Evol.* **20**: 748-753.
- RIEGLER, M., M. SIDHU, W. MILLER and S. O'NEILL, 2005 Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Curr. Biol.* **15**: 1428-1433.
- RUSSELL, J. A., A. LATORRE, B. SABATER-MUNOZ, A. MOYA and N. A. MORAN, 2003 Side-stepping symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol.* **12**: 1061-1075.
- SALZBERG, S., J. DUNNING HOTOPP, A. DELCHER, M. POP, D. SMITH *et al.*, 2005a Correction: Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. *Genome Biol.* **6**: 402.
- SALZBERG, S., J. DUNNING HOTOPP, A. DELCHER, M. POP, D. SMITH *et al.*, 2005b Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. *Genome Biol.* **6**: R23.
- STEVENS, L., R. GIORDANO and R. F. FIALHO, 2001 Male-killing, nematode infections, bacteriophage infection, and virulence of cytoplasmic bacteria in the genus *Wolbachia*. *Annu. Rev. Ecol. Syst.* **32**: 519-545.
- SWOFFORD, D. L., 1998 PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), pp. Sinauer Associates, Sunderland, MA.

- TAMAS, I., L. KLASSON, B. CANBACK, A. K. NASLUND, A. S. ERIKSSON *et al.*, 2002 50 million years of genomic stasis in endosymbiotic bacteria. *Science* **296**: 2376-2379.
- TAYLOR, M. J., C. BANDI and A. HOERAUF, 2005 *Wolbachia* bacterial endosymbionts of filarial nematodes, pp. 245-284 in *Advances in Parasitology*, Vol 60.
- TZOU, P., E. DE GREGORIO and B. LEMAITRE, 2002 How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. *Curr. Opin. Microbiol.* **5**: 102-110.
- VAVRE, F., F. FLEURY, D. LEPETIT, P. FOUILLET and M. BOULETREAU, 1999 Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Mol. Biol. Evol.* **16**: 1711-1723.
- VENETI, Z., J. K. BENTLEY, T. KOANA, H. R. BRAIG and G. D. HURST, 2005 A functional dosage compensation complex required for male killing in *Drosophila*. *Science* **307**: 1461-1463.
- VENETI, Z., M. TODA and G. HURST, 2004 Host resistance does not explain variation in incidence of male-killing bacteria in *Drosophila bifasciata*. *BMC Evol. Biol.* **4**: 52.
- WEEKS, A. R., R. VELTEN and R. STOUTHAMER, 2003 Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc. R. Soc. Lond. B. Biol. Sci.* **270**: 1857–1865.
- WERREN, J., and J. JAENIKE, 1995 *Wolbachia* and cytoplasmic incompatibility in mycophagous *Drosophila* and their relatives. *Heredity* **75**: 320-326.

- WERREN, J. H., and J. D. BARTOS, 2001 Recombination in *Wolbachia*. *Curr. Biol.* **11**: 431-435.
- WERREN, J. H., D. WINDSOR and L. GAO, 1995a Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B. Biol. Sci.* **262**: 197-204.
- WERREN, J. H., and D. M. WINDSOR, 2000 *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. Lond. B. Biol. Sci.* **267**: 1277–1285.
- WERREN, J. H., W. ZHANG and L. R. GUO, 1995b Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc. R. Soc. Lond. B. Biol. Sci.* **261**: 55–63.
- WILLIAMSON, D. L., and D. F. POULSON, 1979 Sex ratio organisms (Spiroplasmas) of *Drosophila*, pp. 175–208 in *The Mycoplasmas*, edited by R. F. WHITCOMB and J. G. TULLY. Academic Press, New York.
- WILLIAMSON, D. L., B. SAKAGUCHI, K. J. HACKETT, R. F. WHITCOMB, J. G. TULLY *et al.*, 1999 *Spiroplasma poulsonii* sp. nov., a new species associated with male-lethality in *Drosophila willistoni*, a neotropical species of fruit fly. *Int. J. Syst. Bacteriol.* **49**: 611–618.
- WU, M., L. V. SUN, J. VAMATHEVAN, M. RIEGLER, R. DEBOY *et al.*, 2004 Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. *PLoS Biology* **2**: e69.
- YAMADA, M., S. NAWA and T. K. WATANABE, 1982 A mutant of SR organism (SRO) in *Drosophila* that does not kill the host males. *Jpn. J. Genet.* **57**: 301–305.

ZCHORI-FEIN, E., and S. J. PERLMAN, 2004 Distribution of the bacterial symbiont

Cardinium in arthropods. Mol. Ecol. **13**: 2009-2016.

ZHOU, W. G., F. ROUSSET and S. O'NEILL, 1998 Phylogeny and PCR-based classification

of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. B. Biol. Sci.

265: 509–515.

Table 1. Primer pairs used for PCR screening

Primer pair (5' to 3')	Target Gene (fragment size)	Target Group	Annealing Temp [Mg+]
10F AGTTTGATCATGGCTCAGATTG ^a	16S rRNA	Most Bacteria	60°C
1507R TACCTTGTTACGACTTCACCCCAG ^a	(~1500bp)		[1.5mM]
27F GAGAGTTTGATCCTGGCTCAG ^b	16S rRNA	Most Bacteria	55°C
1492R GGTTACCTTGTTACGACTT ^b	(~1470 bp)		[1.5mM]
559F CGTGCCAGCAGCCGCGGTAATAC ^d	16S-ITS-35R	Most Bacteria	58°C
35R CCTTCATCGCCTCTGACTGC ^c	(> 1000 bp)	(not Wolbachia)	[1.5mM]
10FF AGAGTTTGATCATGGCTCAGGATG ^d	16S rRNA	Cytophaga-	58°C
1370R CGTATTCACCGGATCATGGC ^e	(~ 1300 bp)	Flavobacterium- Bacteroidetes	[4.5mM]
63F GCCTAATACATGCAAGTCGAAC ^c	16S rRNA	Spiroplasma and	55°C
TKSSp TAGCCGTGGCTTTCTGGTAA ^f	(~450 bp)	several Gram+	[1.5mM]
WspF TGGTCCAATAAGTGATGAAGAACTAGCTA ^g	<i>wsp</i>	Wolbachia	touchdown
wspR AAAAATTAAACGCTACTCCAGCTTCTGCAC ^g	(~600 bp)		65–55°C [1.5mM]
CLOf GCGGTGTAAAATGAGCGTG ^h	16S rRNA	Cytophaga-	57°C
CLOr1 ACCTMTTCTTAAGCTCAAGCCT ^h	(~450 bp)	like organism	[1.5mM]
LCO-1490 GGTCAACAAATCATAAAGATATTGG ⁱ	mitochondrial	Most	45°C
HCO-2198 TAAACTTCAGGGTGACCAAAAAATCA ⁱ	COI	invertebrates	[5mM]

^a (MUNSON *et al.* 1991); ^b (LANE 1991); ^c unpublished; ^d (RUSSELL *et al.* 2003); ^e (MORAN *et al.* 2003); ^f (FUKATSU and NIKOH 2000); ^g (JEYAPRAKASH and HOY 2000); ^h (WEEKS *et al.* 2003); ⁱ (FOLMER *et al.* 1994).

Table 2. Number of species and strains scored as positive for each of the primer sets
(see Materials and Methods).

Primer pair	Tissue examined	Number of species that were positive ^a	Total number of species examined	Number of strains that were positive	Total number of strains examined
Universal 16S (10F–1507R)	ovaries	4W	35	24W	181
Universal 16S (27F–1492R)	ovaries	8W 1S	35	36W 5S	181
Universal 16S–23S (559F–35R)	ovaries	0	35	0	181
<i>Bacteroidetes</i> 16S (10FF–1370R)	ovaries	0	35	0	181
<i>Cardinium</i> and near relatives (CLOf1–CLOr1)	ovaries	0	35	0	181
<i>Spiroplasma</i> 16S (63F–TKSSsp)	ovaries & whole flies	3	225	18	1401
<i>Wolbachia</i> wsp	ovaries & whole flies	19	225	271	1401

^a W: sequence corresponded to *Wolbachia* 16S gene; S: sequence corresponded to *Spiroplasma* 16S gene.

Table 3. Species found positive for infection, with Wolbachia and Wolbachia haplotypes found

Species group or Genus	Species	Strains examined			Wolbachia infected			16S		
		Whole			Whole			% strains	<i>wsp</i> haplotypes	haplotypes
		Ovaries	flies	Total	Ovaries	flies	Total infected			
cardini	<i>D. arawakana</i>		71	71		63	63	89	2 <i>w</i> Wil; 56 <i>w</i> Spt	
melanogaster	<i>D. ananassae</i>	3	17	20	3	7	10	50	7 <i>w</i> Ri; 3 <i>w</i> Spt	3 <i>w</i> Ri ^{b,c}
melanogaster	<i>D. baimaii</i>		2	2		1	1	50	1 <i>w</i> Bai (new)	
melanogaster	<i>D. bicornuta</i>		2	2		2	2	100	2 <i>w</i> Bic (new)	
melanogaster	<i>D. mauritiana</i>		3	3		2	2	67	2 <i>w</i> No	
melanogaster	<i>D. melanogaster</i>	11	51	60	10	37	45	75	37 <i>w</i> Mel	8 <i>w</i> Mel ^a
melanogaster	<i>D. nikananu</i>	1	2	2	1	1	1	50	1 <i>w</i> Nik (new)	1 <i>w</i> Nik ^b (new)
melanogaster	<i>D. pseudoananassae</i>	8		8	3		3	38	3 <i>w</i> Pana	3 <i>w</i> Pana ^b
melanogaster	<i>D. pseudotakahashii</i>		2	2		1	1	50	1 <i>w</i> Pse (new)	
melanogaster	<i>D. quadraria</i>		1	1		1	1	100	1 <i>w</i> Ri	
melanogaster	<i>D. sechellia</i>		3	3		1	1	33	1 <i>w</i> Ha	

melanogaster	<i>D. simulans</i>	18	30	48	18	13	31	65	2 wMel; 27 wRi	16 wRi ^{b,c}
melanogaster	takahashii subgroup	1		1	1		1	100	1 wTak	1 wTak ^a
melanogaster	<i>D. teissieri</i>	1	2	2	1	2	2	100	2 wSpt	1 wMel ^a
saltans	<i>D. sturtevantii</i>		7	7		2	2	29	2 wStv MI	
willistoni	<i>D. tropicalis</i>	1	3	3	1	2	2	67	2 wWil	
willistoni	<i>D. willistoni</i>	1	195	196	1	100	101	52	78 wWil	1 wWil ^a (new)
Scaptodrosophila	<i>Scaptodrosophila stonei</i>		2	2		1	1	50	1 wSto (new)	
Scaptomyza	<i>Scaptomyza pallida</i>		1	1		1	1	100	1 wSpt	
Total				434			271	62		

^a PCR product obtained with 27F-1492R primer pair only.

^b PCR product obtained with both 27F-1492R and 10F-1507R primer pairs.

^c Sequence is one bp different from another wRi strain (AY833061).

Table 4. Species found positive for infection with *Spiroplasma* and *Spiroplasma* haplotypes found

Species group	Species	Strains examined			<i>Spiroplasma</i> infected			%	Spiraplasma 16S haplotype
		Ovaries	Whole flies	Total	Ovaries	Whole flies	Total		
repleta	<i>D. aldrichi</i>		12	12		1	1	8	2 haplotype_3 ^a
repleta	<i>D. hydei</i>	9	32	33	7	9	9	27	8 haplotype_1 ^b ; 1 haplotype_2 ^b
repleta	<i>D. mojavensis</i>	7	114	121	3	5	8	7	8 haplotype_4 ^a
	Total			166			18	11	

^a PCR product obtained with 63F-TksspR primer pair only.

^b PCR product obtained with both 63F-TksspR and 27F-1492R primer pairs.

FIGURE LEGENDS

Figure 1 (A) Phylogenetic distribution of Drosophilid species screened for endosymbiotic bacteria. Phylogenetic relationships among most of the species groups and genera in the subfamily Drosophilinae are based on MARKOW AND O'GRADY (2006). Capitalized taxon names represent genera other than the genus *Drosophila*. Non-capitalized names are species groups within the genus *Drosophila*; the subgenus to which they belong is also indicated. For each taxon, numbers represent (from left to right, respectively): number of species screened for mainly for *Wolbachia* in previous studies, number of species screened for all groups of bacteria (i.e., ovary extracts) in the present study, number of species screened specifically for *Wolbachia* and *Spiroplasma* (i.e., whole flies) in the present study, the combined total number of species screened in the present study, the total species screened for *Wolbachia* based on previous and the present study.

(B) Species found to be positive for *Wolbachia* or *Spiroplasma* from (left to right) previous studies, the present study, the combined total, first for *Wolbachia* and then for *Spiroplasma*. Previous studies: ^aBOURTZIS *et al.* (1996); ^bCHARLAT *et al.* (2004); ^cCLARK *et al.* (2005); ^dDYER and JAENIKE (2005); ^eGIORDANO *et al.* (1995); ^fHAINE *et al.* (2005); ^gJAENIKE *et al.* (2003); ^hMILLER and RIEGLER (2006); ⁱMONTENEGRO *et al.* (2005); ^jMONTENEGRO *et al.* (2006); ^kSALZBERG *et al.* (2005a; 2005b); ^lVAVRE *et al.* (1999); ^mVENETI *et al.* (2004); ⁿWERREN and JAENIKE (1995); ^oWERREN *et al.* (1995a; 1995b); ^pWILLIAMSON and POULSON (1979); ^qWILLIAMSON *et al.* (1999); ^rZHOU *et al.* (1998).

Figure 2. Consensus of trees based on 16S rRNA gene of *Wolbachia* lineages inferred by Maximum likelihood and Bayesian analyses. Numbers indicate the range of Bayesian

posterior probabilities obtained under different sets of taxa and characters. Recognized Wolbachia supergroups are indicated by capital letters (A–F). Taxon names indicate the host species and are followed by the GenBank Accession number. Orange labels are haplotypes observed in the present study but reported before; red labels had never been reported before; and blue labels are other Wolbachia strains associated with *Drosophila* reported in previous studies. Most analyses were based on a 1374 bp-long dataset that included only taxa for which at least 1340 bp were available, but a subset of analyses included a taxon with a shorter sequence. Lineages from the D and C supergroups were removed sequentially in a subset of the analyses.

Figure 3. Maximum likelihood phylogeny of *Spiroplasma* 16S rRNA gene based on 439 characters. Haplotypes associated with *Drosophila* found in the present study are indicated in red (hap 1–4), while the one found in a previous study is indicated in blue. Tree was rooted with *Spiroplasma ixodetis*. Numbers next to nodes indicate Bayesian posterior probabilities (> 50%). Numbers next to taxon labels correspond to GenBank accessions, and host organism is indicated in parentheses.

A. SAMPLING				B. RESULTS						
Previous Studies Wolbachia only	All Bacteria Ovar Screens	Wolbachia Whole Fly Screens	TOTAL SPECIES Current Study Spiroplasma	TOTAL SPECIES All Studies	WOLBACHIA		SPIROPLASMA			TOTAL SPECIES
					Previous Studies	Current Study	TOTAL SPECIES	Previous Studies	Current Study	
CHYMOMYZA			1	1	1			—		—
SCAPTODROSOPHILA ^a	1		5	5	5		1	1		—
obscura ^{a,f,h,m}	8	6	9	10	15	3		3		—
melanogaster ^{a,b,c,e,f,h,k,l,o,r}	19	12	50	51	52	8	13	14	1	1
williston ^{a,h,k,o,p,q}	9	3	8	8	9	3	2	3	4	4
saltans ^{a,h,o}	5		9	9	10	3	1	3		—
ZAPRIONUS			4	4	4			—		—
Drosilopha ^f	1	1	1	1	1			—		—
SCAPTOMYZA			2	2	2		1	1		—
modified mouthparts			4	4	4			—		—
picture wing			2	2	2			—		—
virilis ^a	2	1	12	12	12			—		—
repleta ^a	6	6	51	51	51			—	1	3
dreyfusi			1	1	1			—		—
canalinae			1	1	1			—		—
bromeliae			1	1	1			—		—
mesophragmatica			2	2	2			—		—
robusta		1	3	3	3			—		—
melanica			4	4	4			—		—
nannoptera		1	3	3	3			—		—
tumiditarsus			1	1	1			—		—
immigrans ^{a,f}	3	2	12	12	12			—		—
pallidipennis			1	1	1			—		—
funebris ^f	1		2	2	2			—		—
guarani ⁱ	1		3	3	4			—	1	1
calloptera			1	1	1			—		—
cardini ⁱ	1	1	13	13	14		1	1	1	1
quinaria ^{a,d,n}	8		6	6	11	2		2		—
tripunctata ^{i,n}	2		6	6	7			—	1	1
testacea ^{f,n}	5	1	1	1	6	1		1		—
polychaeta			3	3	3			—		—
HIRTODROSOPHILA			1	1	1			—		—
TOTAL SPECIES	72	35	223	225	245	20	19	29	9	3
TOTAL SPECIES GPS.	15	11	32	32	34	6	7	9	6	1

Fig. 2



