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EXPRESSION OF CYTOPLASMIC INCOMPATIBILITY IN *DROSOPHILA SIMULANS* AND ITS IMPACT ON INFECTION FREQUENCIES AND DISTRIBUTION OF *WOLBACHIA PIPIENTIS*

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Abstract.—The aim of this study is to examine the expression of cytoplasmic incompatibility and investigate the distribution and population frequencies of *Wolbachia pipientis* strains in *Drosophila simulans*. Nucleotide sequence data from 16S rDNA and a *Wolbachia* surface protein coding sequence and cytoplasmic incompatibility assays identify four distinct *Wolbachia* strains: *w*Ha, *w*Ri, *w*Ma, and *w*Au. The levels of cytoplasmic incompatibility between six lines carrying these strains of bacteria and three control lines without bacteria are characterized. Flies infected with *w*Ha and *w*Ri are bidirectionally incompatible, and males that carry either strain can only successfully produce normal numbers of offspring with females carrying the same bacterial strain. Males infected with *w*Au do not express incompatibility. Males infected with the *w*Ma strain express intermediate incompatibility when mated to females with no bacteria and no incompatibility with females with any other *Wolbachia* strain. We conduct polymerase chain reaction/restriction fragment length polymorphism assays to distinguish the strain of *Wolbachia* and the mitochondrial haplotype to survey populations for each type and associations between them. *Drosophila simulans* is known to have three major mitochondrial haplotypes (*si*I, *si*II, and *si*III) and two subtypes (*si*IIA and *si*IIB). All infected lines of the *si*I haplotype carry *w*Ha, *w*No, or both; *w*Ma and *w*No are closely related and it is not clear whether they are distinct strains or variants of the same strain. Infected lines with the *si*IIA haplotype harbor *w*Ri and the *si*IIB haplotype carries *w*Au. The *w*Ma infection is found in *si*III haplotype lines. The phenotypic expression of cytoplasmic incompatibility and its relation to between-population differences in frequencies of *Wolbachia* infection are discussed.

Key words.—Cytoplasmic incompatibility, *Drosophila simulans*, geographic variation, symbiont, *Wolbachia pipientis*.

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The α-proteobacteria *Wolbachia pipientis* can affect the inheritance patterns of its host and has the potential to be used as a biocontrol agent. This manuscript aims to examine the expression of cytoplasmic incompatibility (CI) and the distribution and population frequencies of *Wolbachia* strains that infect *Drosophila simulans*. We compare these results with previously collected data and discuss how *Wolbachia* infections may influence mitochondrial diversity, which serves as a companion field of study (Ballard 2000a,b). We intend this to serve as a stepping stone to investigating *Wolbachia* strains that theoretically should have different transmission fidelity, potential to be modified by the environment, or effects on host fitness.

Wolbachia are a group of maternally inherited intracellular bacteria that have been found in a number of invertebrate species and can have dramatic affects on reproduction in their hosts (Werren 1997; Bourtzis and O'Neill 1998). *Drosophila simulans* is a cosmopolitan species that is known to have three major mitochondrial haplotypes (*si*I, *si*II, and *si*III; Solignac and Monnerot 1986; Baba-Aïssa et al. 1988) and two subtypes (*si*IIA and *si*IIB; Ballard 2000b) that appear to be nonrandomly associated with each bacterial strain (Montchamp-Moreau et al. 1991). In the present study, we identify four strains of *Wolbachia* carried by *D. simulans* and assay the phenotypic effect that different strains have on CI. We connect CI variation of different strains with the population level frequencies and geographic distribution of *Wolbachia* in *D. simulans*. Investigating the effect of *Wolbachia* on gene flow in the host is an important step to understanding the distribution of the parasite and potentially each mitochondrial type.

In *D. simulans*, CI may be expressed when a male harboring a strain of *Wolbachia* mates with a female that does not carry that same strain or is uninfected. Sperm enters the egg normally, but defects in fertilization begin as early as pronuclear interactions (Lassy and Karr 1996), causing a reduction in egg hatchability (Hoffmann et al. 1986). Compelling evidence that *Wolbachia* causes CI came from treating infected lines with antibiotics to cure the fly line of the bacteria. Crosses between treated females and infected, untreated males expressed CI (Hoffmann and Turelli 1988). Boyle et al. (1993) further supported the view that *Wolbachia* causes CI by microinjecting *Wolbachia* from *D. simulans* into an uninfected *D. melanogaster* line. Bacterial densities in cells and expression of CI were lower in the injected *D. melanogaster* line. Boyle et al. (1993) subsequently took the *Wolbachia*-infected cytoplasm out of the *D. melanogaster* line and placed it into an uninfected *D. simulans* line, which caused bacterial densities and expression of CI to be similar to the levels of the original line.

Environmental and physiological factors can affect the expression of CI. Infected males exposed to stress as larvae (e.g., low nutrition level, heat) have lowered expression (Hoffmann et al. 1986; Clancy and Hoffmann 1998; Feder et al. 1999). It is not known if these effects are due to reduced bacterial load in the host or to physiological influences of stress on the bacteria or host, although this is a subject of ongoing scrutiny. Older males, or males that have multiply mated, also have reduced expression of CI (Turelli and Hoffmann 1995; Karr et al. 1998). This may result from a decline in bacterial densities with age and multiple matings. Binnington and Hoffmann (1989) found that young males from a *Wolbachia*-infected line harbor the bacteria in the testes, whereas older males of the same line do not.

Wolbachia strain differences also affect the expression of CI in *D. simulans,* and this is reflected in the geographic distribution of the bacteria. O'Neill and Karr (1990) found reciprocal incompatibility in crosses between *D. simulans* lines collected in California and Hawaii (USA). Lines that carry *Wolbachia* but do not express incompatibility or express low levels of incompatibility have been found in Australia (Hoffmann et al. 1994), Ecuador (Turelli and Hoffmann 1995), Florida (USA; Ballard et al. 1996), and Tanzania (Merçot and Poinsot 1998a). Multiple infections within a single fly also arise in nature. Merçot et al. (1995) found lines of *D. simulans* in the Seychelles and New Caledonia that are infected with two strains of *Wolbachia* and are bidirectionally incompatible with flies collected from California. We do not include multiply infected lines in the CI assays described here.

In this study, we characterize *Wolbachia* genotypes using DNA sequences (16S rDNA and a *Wolbachia* surface protein coding gene, *wsp*) in 12 infected lines. These loci were chosen to permit comparison with previous studies, to maximize sequence variation, and to facilitate phylogenetic investigations. The *Wolbachia* strains are then mapped onto the mitochondrial genealogy from these isofemale lines. We test incompatibility levels between six infected lines and three uninfected controls. Additional *wsp* sequences were obtained from lines from Florida, and the Seychelles to identify the *Wolbachia* infecting these populations. Finally, population-level frequencies of *Wolbachia* infection are linked with the mitochondrial haplotype of the host and the strength of incompatibility.

Materials and Methods

The Fly Lines

Isofemale lines were established from eight females from Reunion and 181 females from Madagascar caught in March 1998. Of the Madagascar females, 31 lines were collected at Joffreville, 42 at Antaninarivo, 81 at Antsirabe, 17 at Ambositra, and 10 at Ranomafana. Most of the Hawaiian lines were collected in December 1998, 18 lines from Oahu and 62 from Kauai. Collections were also sent to us by Ken Kaneshiro (n = 14) and David Baer (n = 4) from Oahu in July 1998. Four lines were collected in Tahiti during July 1998. Lines were sorted in the field and as many isofemale lines as possible were established there. The genital arch of the male offspring was checked to confirm each line was *D. simulans.*

Three previously collected lines were used in this study. These lines were selected to link this study with previous work on well-studied lines and to increase geographic diversity. Two lines, DSR and DSW, were collected by A. A. Hoffmann in 1984. DSR was collected near Riverside, California, and DSW was collected near Watsonville, California (Hoffmann et al. 1986; O'Neill and Karr 1990; Turelli and Hoffmann 1991; Lassy and Karr 1996). Line NC48 was collected in Noumea, New Caledonia (Baba-Aïssa et al. 1988; Merçot and Poinsot 1998b).

In *D. simulans,* there are three mitochondrial haplotypes, *si*I, *si*II, and *si*III (Solignac and Monnerot 1986). For experimental analysis, three lines from each haplotype were picked to represent the diversity of *D. simulans.* Two *Wolbachia*-infected lines and one uninfected line from each haplotype were chosen for the phenotypic expression of incompatibility study (Table 1). This design is limited because the two bacteria strains found in the *si*II haplotype are not replicated. The CI expression of these strains are well documented and our data corroborate published results (Hoffmann et al. 1986, 1994; O'Neill and Karr 1990). However, to minimize this limitation, we replicated any engaging results with fly lines hosting complementary bacterial strains. An alternative approach would have been to include multiple representatives of each *Wolbachia* strain. This alternate design, however, would cause significant logistical problems that would restrict sample sizes of replicates obtainable.

Table 1. Nomenclature of fly lines and bacteria strains used in molecular and phenotypic analysis.

Fly line	Fly origin[1]	Bacteria strain	Mitochondrial haplotype
HW09	1	*w*Ha	*si*I
TT01	1	*w*Ha	*si*I
DSR	3	*w*Ri	*si*II
MD225	1	*w*Au	*si*II
MD199	1	*w*Ma	*si*III
RU07	1	*w*Ma	*si*III
NC48T	2	*w*−	*si*I
DSW	3	*w*−	*si*II
MD111	1	*w*−	*si*III

[1] Fly origins: 1, this study; 2, collected by F. Baba-Aïssa and tetracycline treated for this study; 3, Hoffman and Turelli (1988).

We used naturally uninfected controls wherever possible (Hoffman et al. 1986; Merçot and Poinsot 1998b). Tetracycline treatment may not cure all individuals and is likely to kill symbiotic bacteria in the gut. Removal of symbiotic bacteria may directly influence the fitness of the flies and thus, observed CI expression. NC48, originally infected with two lines of *Wolbachia* (see below), was tetracycline treated to rid the line of the bacterial infection more than five generations before the experiment began (O'Neill and Karr 1990). This was necessary, because we had no wild-caught uninfected lines from this haplotype (*si*I). The tetracycline-treated NC48 line is referred to as NC48T.

DNA Extraction, Polymerase Chain Reaction, and DNA Sequencing

DNA was isolated using the fixed tissue protocol from Gentra's (Minneapolis, MN) PureGene® Kit by extracting three whole, adult *D. simulans* at a time. The 16S rDNA was amplified following O'Neill et al. (1992) and *wsp* following Zhou et al. (1998). Both strands were sequenced using Taq-Big Dye Deoxy Terminator Cycle sequencing (Applied Biosystems, Foster City, CA), using 55 ng of template DNA and 20 ng of primer. In each case, two internal primers and the amplification primers were employed. The internal primers for 16S rDNA were COV1 (5′ACGGGATTTCACTTTTAACTTA3′) and COV6 (5′TAATAAGTTAAAAGTGAAATCC3′). For the *wsp* locus, the internal primers were *wsp*719R (5′GAGTGATAGGCATATCTTCAAT3′), and *wsp*697F (5′AATTGAAGATATGCCTATCACT3′). The latter positions were numbered with respect to GenBank acquisition number AF020070.

NC48 is doubly infected with *Wolbachia* and the polymerase chain reaction (PCR) amplicons were cloned prior to sequencing. Here we simply designate the alternate sequences as NC48A and NC48B. We also obtained *Wolbachia* sequences from a doubly infected line from the Seychelles that is employed in Carracedo et al. (1998). PCR amplicons were cut with restriction enzymes and electrophoresed. Larger fragments were cut from the gel and sequenced.

Sequences were imported into Sequencher® (Gene Codes, Ann Arbor, MI), the chromatograms verified, and consensus sequences for each line and gene were determined. The 16S rDNA sequences were aligned to each other without ambiguity. To facilitate alignment, the *wsp* sequences were aligned against a large European Molecular Biology Laboratory (EMBL) dataset that consists of 28*wsp* DNA sequences (Zhou et al. 1998, ftp://ftp.ebi.ac.uk/pub/databases/embl/align; EMBL accession number DS32273).

Sequences were exported into PAUP* (Swofford 1998) and a single data matrix was constructed. The 16S rDNA sequence of *Anaplasma marginale* (a closely related bacteria, GenBank accession number M60313) was employed as the outgroup (O'Neill et al. 1992). There is currently no suitable outgroup sequence for the *wsp* locus (Zhou et al. 1998) and it was defined as being unknown. In an attempt to represent the number of insertion and deletion (indel) events accurately, each presumed event was parsimoniously scored by inserting a ''1'' into the matrix at appropriate sites. Gaps were then treated as missing data.

Following the procedure of conditional data combination (Bull et al. 1993), the data were divided into two process partitions: the 16S rDNA and the *wsp* locus. The incongruence-length-difference (ILD) test (Farris et al. 1995) was employed to test the null hypothesis that the two partitions are evolving under homogeneous processes. Bootstrapping (Efron 1982; Felsenstein 1985) was used to test monophyly. When the outgroup was included, 1000 pseudosamples were generated to estimate the bootstrap proportions.

Ballard (2000b) sequenced intron 1 of the duplicated locus of *alcohol dehydrogenase* and the complete mitochondrial genomes of two lines of *D. melanogaster* and 22 lines of *D. simulans.* We constructed a mitochondrial genealogy from the two lines of *D. melanogaster* and 13 of *D. simulans,* as described above. (GenBank accession numbers: AF200828, AF200829, AF200834, AF200836, AF200838–AF200841, AF200843–AF200845, AF200848, AF200850–AF200852). The 13 lines of *D. simulans* included the lines employed for the CI assays and represented the diversity within each of the three major mitochondrial haplotypes. We then mapped the strains of *Wolbachia* onto the well-supported genealogy of fruit fly mtDNA.

Cytoplasmic Incompatibility Assay

Drosophila simulans lines were raised at constant temperature (25°C) and density (30 larvae per vial). They were collected as virgins (4-h intervals between collections) and aged for 2 days. Males and females from specific lines were then introduced to a vial of molasses food (10% molasses and 2% agar in water, seeded with a streak of 5 μl of 5 g of yeast diluted in 20 ml of water). They were given 24 h to mate, at which time the male was discarded and the female transferred to a new vial. Females were given fresh vials for four consecutive 24-h periods, and the number of eggs laid were counted within 8 h of the transfer. Between 26 h and 36 h after the transfer, the number of eggs left unhatched was counted. The expression of cytoplasmic incompatibility was quantified as the number of eggs left unhatched in the second counting period divided by the total number of eggs laid.

A preliminary analysis was conducted to determine the appropriate number of days to count eggs. This analysis considered the CI of compatible crosses (males and females from the same line) over four consecutive days. On successive days, the means and variances of the percent eggs unhatched in these compatible crosses increased. To test significance of differences in variances the Brown-Forsythe test was used. Variances differed significantly between days if four days of data were used ($F = 11.07$, $P < 0.0001$), if three days were used ($F = 6.37$, $P < 0.002$), but not if two days were used ($F = 2.76$, $P = 0.099$). Thus, all CI data presented here are the estimated hatchability of eggs laid the first two days after the male was discarded. All possible reciprocal crosses of the *D. simulans* lines were made, with 17 ± 5 replicate pairs per cross. Because the distribution of data depart from normality, we report the results as median and 10th/90th quantiles. These statistical measures assume no underlying distribution.

To test differences in expression of CI between lines, the nonparametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test was used (Sokal and Rohlf 1995, pp. 446–447). We discuss the trends between fly lines and bacterial strains in CI. Wilcoxon rank sum tests, with Bonferroni correction, were then used to investigate specific questions. An alternative is to conduct parametric analyses of variance (ANOVAs) and then subject the data to multiple post hoc *t*-tests to see which pairs of lines are different. However, CI data are not normally distributed and the distribution is of biological importance. Moreover, we had 81 crosses to compare with each other ($n = 6561$), and correcting for the large number of multiple comparisons has the potential to mask true biological signals.

We set an arbitrary threshold of egg unhatchability, over 70%, to define a cross as incompatible. The lowest value observed in this category was 83% eggs unhatched. Values below 30% unhatchability were considered compatible (30% being the highest realized value), and between 30% and 70% as equivocal. The results from the *w*Ma strain were equivocal and two additional lines were selected to replicate these assays (see Results).

At the end of the cytoplasmic incompatibility study, all fly lines were checked with the polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) protocol to verify that they still carried their original strain of bacteria. All infected lines maintained their bacterial strain throughout the experiment.

CI data were analyzed using the JMP software package (vers. 3.2.2 for the MacIntosh; SAS Institute 1995).

Population Survey

DNA was extracted from 295 isofemale lines and *wsp* primers employed to test for the presence/absence of *Wolbachia*

infection. A PCR/RFLP strategy was employed to distinguish four *Wolbachia* cytotypes and three major mitochondrial haplotypes of *D. simulans*.

Wolbachia

In the population surveys, we included samples of greater than 20 isofemale lines per locality (see Table 5). Forty-three isofemale lines included in this study are not in the population survey. If an extract from an isofemale line did not amplify, DNA was re-extracted from three individuals from that same line and the assay repeated. In one case, an infected line was not detected by the original PCR. It is possible that lines infected with low densities of bacteria would not be identified as infected. In this study, we do not quantify bacterial densities. The *wsp* locus is highly variable and it is possible that these primers do not bind to some divergent strains of *Wolbachia*. To examine this further, we used the conserved 16S rDNA primers on a subset of lines. No lines shown to be uninfected with *wsp* primers were observed to be infected when tested with the 16S rDNA primers.

The *wsp* amplicons were cut with restriction enzymes to distinguish each strain. *Dpn*II cuts *w*Ma into four fragments (56-, 118-, 146-, and 282-bp fragments), *w*Au into three fragments (77-, 135-, and 420-bp), *w*Ri into two fragments (194- and 396-bp), but does not cut *w*Ha. If there was any ambiguity in distinguishing lines infected with *w*Ma and *w*Au strains of *Wolbachia*, an uncut amplicon was digested with *Hin*dIII. *Hin*dIII cuts *w*Ma (139- and 467-bp fragments), but not *w*Au. Suspected *w*Ha lines were digested with *Dde*I, which cuts *w*Ha into two distinct fragments (254- and 375-bp fragments). All lines generated *wsp* restriction profiles that were consistent with the expected profile.

Mitochondrial haplotypes

The primers 5′CATACACAACATATATTTGCTCA3′ (4455+) and 5′GGCTTCAATTAAAGAATAAGGG3′ (6201−) were used to amplify a mitochondrial fragment between 1737 bp and 1766 bp. The PCR reaction was divided into two aliquots. One aliquot was cut with *Dde*I and the other with *Hin*fI. *Dde*I cuts *si*I into four fragments (24-, 38-, 841-, and 863-bp), *si*II into four fragments (24-, 38-, 728-, and 947-bp), and *si*III into three fragments (24-, 38-, and 1675-bp). *Hin*fI cuts *si*I into four fragments (146-, 241-, 498-, and 881-bp), *si*II into three fragments (241-, 644-, and 882-bp), and *si*III into four fragments (146-, 241-, 498-, and 852-bp). Classification of the *si*IIA and *si*IIB haplotypes followed Ballard (2000b).

Nomenclature

Our nomenclature is provided in Table 1. Names proceeded by MD were collected in Madagascar and the RU lines on Reunion. The fly lines HW09 and TT01 are from our Hawaiian and Tahitian collections, respectively. The fly lines DSR and DSW were collected in California (Hoffmann and Turelli 1988). NC48T is the tetracycline-treated line from New Caledonia (Baba-Aïssa et al. 1988; Merçot and Poinsot 1998b).

It is the convention to name the *Wolbachia* strain with a *w* (for *Wolbachia*), followed by capital letters that represent the geographical location where the strain was first described (Rousset and de Stordeur 1994). Thus, the *Wolbachia* strain in DSR is called *w*Ri, after Riverside, California (Hoffmann et al. 1986). The *w*Ha strain was first identified from lines collected in Hawaii (O'Neill and Karr 1990). The *Wolbachia* strain *w*Au was found in *D. simulans* in Australia (Hoffmann et al. 1994), whereas *w*Ma was first described in lines collected from Mont d'Ambre in Madagascar (Rousset and Solignac 1995; F. Rousset, pers. comm.). The *w*No and *w*Ha strain doubly infect most lines in Noumea, New Caledonia, and in the Seychelles Islands (Merçot et al 1995; Rousset and Solignac 1995). The *w*Ki strain infects lines from Mount Kiliminjaro, Tanzania (Merçot and Poinsot 1998a). We employ *w*- for an uninfected line of flies.

Results

Sequence Analysis

The 16S rDNA *Wolbachia* sequence data from HW09, TT01, NC48A, and one of the Seychelles sequences (*w*Ha) are identical to each other and to the sequence from the Hawaiian *D. simulans* of Braig et al. (1998; GenBank accession number X61769). There is one nucleotide difference between these sequences and that of Rousset et al. (1992; GenBank accession number X64265). The *wsp* sequence from HW09, TT01, and NC48A is 576 bp long and identical to that described by Braig et al. (1998; GenBank accession number AF020068). The *Wolbachia* sequences obtained from DSR (*w*Ri) are identical to the published sequences (16S rDNA: GenBank accession numbers X64265 and X61770; *wsp*: GenBank accession number AF020070). The 16S rDNA sequences from MD225 and MD106 (*w*Au) are identical to *w*Ri and the 588-bp *wsp* sequences are identical to the *wsp* *w*Au sequence in GenBank accession number AF020067. The *w*Ma and *w*No strains are very similar and differ by a single nucleotide. The 16S rDNA sequences from RU07, RU13, MD112, and MD199 have two substitutions relative to that identified in GenBank as *w*Ma (X64266; position 161 A→G, position 418 G→A). The NC48B 16S rDNA sequence also has these two differences, plus a T→C substitution at position 440. This substitution is shared by a 16S rDNA sequence from the Seychelles line and the *w*No 16S rDNA sequence in GenBank (X64267). The *wsp* sequences from the lines carrying the *w*Ma strain of bacteria are 558 bp long and are homosequential with the *w*No sequences in NC48B and the Seychelles line. They have a 2-bp insertion (AA) at positions 498 and 499 relative to GenBank accession number AF020074.

Phylogenetic Analysis

Wolbachia

The 16S rDNA data was alignable without ambiguity. Four ambiguous regions at the *wsp* locus (positions 61–119, 211–251, 375–404, and 517–571) were deleted before the phylogenetic analysis. A total of 848 bp of 16S r DNA (14 parsimony-informative characters) and 418 bp of *wsp* (70 parsimony-informative characters) sequence data were included.

The ILD test (excluding the outgroup) showed that the two

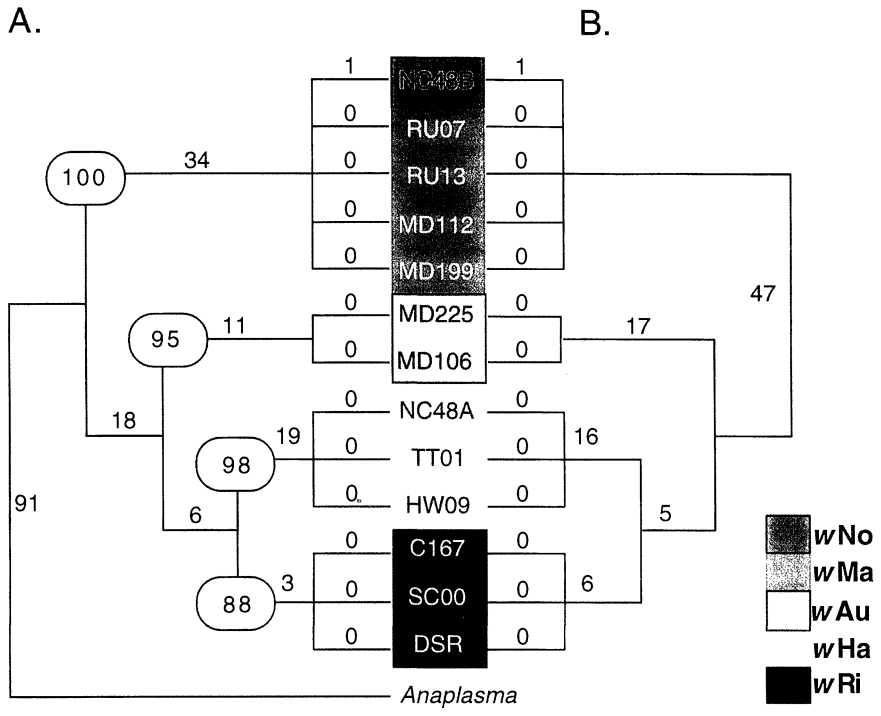

FIG. 1. Phylogenetic analysis of 848 bp of 16S rDNA and 418 bp of the *wsp* locus of *Wolbachia pipientis* found in *Drosophila simulans* (center). The incongruence-length-difference test (excluding the outgroup) showed that the two partitions are not evolving under significantly different processes (92 steps, $P = 1.0$). As a consequence, the data remain combined for phylogenetic analysis. Phylogenetic analysis using parsimony found a single most parsimonious tree of 183 steps (consistency index = 0.97) when the outgroup (*Wolbachia* from *Anaplasma*) is included (A) and 92 steps (consistency index = 0.95) when it was excluded (B). Bootstrap proportions from 1000 replicates and the resulting proportions (> 70%) are placed in the circles in the nodes. Numbers above each line denote the branch length.

partitions are not evolving under significantly different processes (183 steps, $P = 1.0$). Following the procedure of conditional data combination (Bull et al. 1993), the data remain combined for phylogenetic analysis.

Phylogenetic analysis using parsimony found a single most parsimonious tree of 183 steps (consistency index = 0.97) when the outgroup was included (Fig. 1A) and 92 steps (consistency index = 0.95) when it was excluded (Fig. 1B). When the outgroup was included LogDet/paralinear minimum evolution analysis (ME-tree = 0.17288) generates the same topology. However, these data do not robustly determine the phylogenetic affinities of the *Wolbachia* strains. The most parsimonious reconstruction suggests that *w*Ri and *w*Ha are sister strains and topology dependent-permutation test probability (T-PTP) testing (Faith and Cranston 1991) supports monophyly of this clade (T-TPT = 0.01, one step). However, only one step is required to decay the *w*Ri and *w*Ha clade, and this result is only supported by 52% of bootstrap pseudoreplicates.

Mapping Wolbachia onto the mitochondrial lineage

The alignment of the 15 mitochondrial genomes was straightforward for the majority of the sequence. However, it was not possible to unequivocally determine the indels between positions 5535 and 5584 and 6022 and 6047. As a consequence, 74 bp of 15,034 bp were deleted from all analyses. Five equally parsimonious trees were found of length 1042 steps (Fig. 2). The strict consensus tree has the same topology as that presented by Ballard (2000b). We then employed the criterion of parsimony to map *Wolbachia* strain onto the phylogeny. These data suggest that there is a high degree of congruence between the strain of *Wolbachia* and the mitochondrial haplotype, with the caveat that *Wolbachia* may be lost from some lines (Merçot et al. 1995; Van Meer and Stouthamer 1999). The three *si*I lines are infected with the *w*Ha strain, however, NC48 was doubly infected and also carried *w*No. Two strains of *Wolbachia* infect the *si*II haplotype. The *w*Ri strain infects the *si*IIA haplotype, whereas the *w*Au strain infects the *si*IIB haplotype. Three of the four *si*III lines are infected with the *w*Ma strain.

Cytoplasmic Incompatibility Assay

The expression of CI between each of the fly lines is listed in Table 2. To statistically investigate CI, we performed a series of Kruskal-Wallis tests serially removing the lines that express highest levels of incompatibility (Table 3). We followed the two-way tests with one-way Wilcoxon tests as appropriate. This facilitates critical examination of the data and minimizes the number of tests. A schematic representation of incompatibility interactions between fly lines that have different infection status is illustrated in Figure 3. Briefly, all lines are self-compatible. The lines infected with the strains *w*Ha and *w*Ri are bidirectionally incompatible with each other and unidirectionally incompatible with all other lines. Males infected with *w*Ma are heterogeneous in their

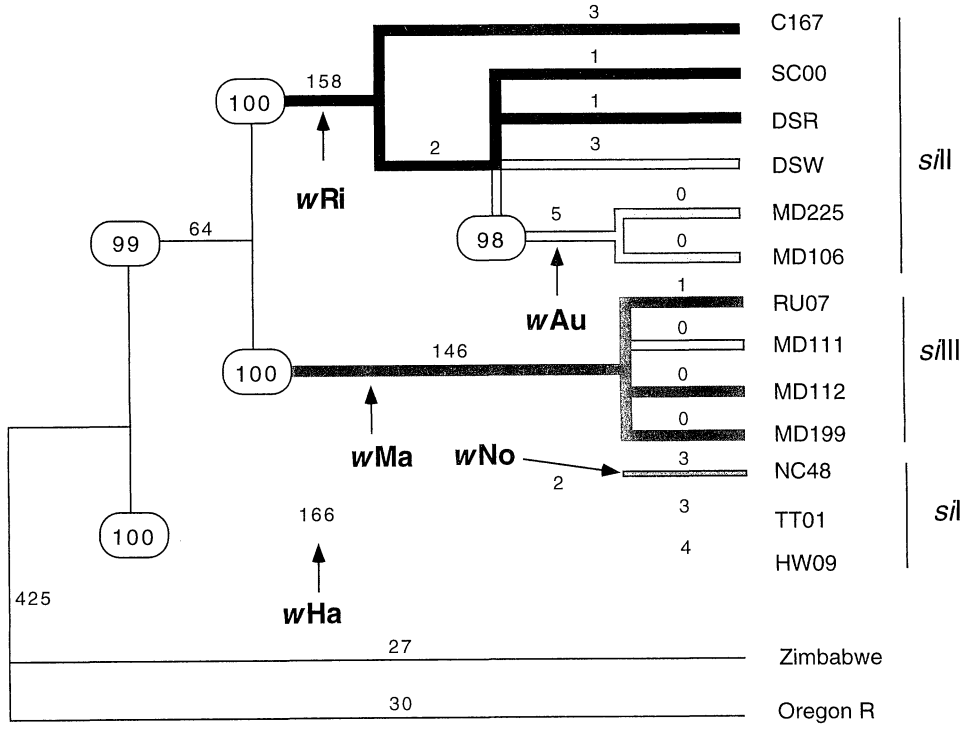

FIG. 2. The four strains of *Wolbachia* are mapped onto the mitochondrial genealogy. The open bars designate uninfected lines. Note that NC48 is doubly infected with *w*Ha and *w*Ma. *Drosophila melanogaster* is the designated outgroup. A total of 14,958 bp were included: 14,029 characters are constant, 46 variable characters are parsimony uninformative, and 883 variable characters are parsimony informative. Five equally parsimonious trees of length 1042 steps were found. These trees have a consistency index of 0.92. The number of substitutions is shown above each branch. The datasets were bootstrapped 1000 times and the resulting proportions (> 70%) are shown in circles.

expression of CI, whereas *w*Au-infected males do not express CI.

Overall, CI differs significantly among males and females from different lines, and there is a significant interaction between the male and the female of the cross (Table 3A). First, we removed the lines infected with the *w*Ha (HW09 and TT01) and *w*Ri (DSR) strains of *Wolbachia.* As previously reported, the bacterial strains *w*Ha and *w*Ri are bidirectionally incompatible with each other and unidirectionally incompatible with all other lines (O'Neill and Karr 1990). The two lines infected with *w*Ha exhibit similar patterns of cytoplasmic incompatibility ($\chi^2 = 8.83$, df = 1, $P = 0.69$ for males following Bonferoni correction; ($\chi^2 = 10.90$, df = 1, $P = 0.61$ for females).

The Kruskal-Wallis comparison of the six remaining lines (infected MD199, RU07, MD225 and uninfected NC48T, MD111, and DSW) shows significant male and female effects (Table 3B). Males infected with *w*Ma (MD199 and RU07) exhibit heterogeneous patterns of CI and there is no significant male effect following removal of them (Table 3C). Males infected with *w*Ma expressed CI differently when mated to uninfected or to infected females ($\chi^2 = 67.61$, df = 1, $P < 0.001$). The *w*Ma-harboring males are compatible with females that carry any other strain of bacteria and with one of the uninfected controls (MD111). These males are at least partially incompatible with two uninfected females (NC48T and DSW). Nigro (1991) reported that *w*Ma-infected males express CI when crossed with the uninfected females, whereas Rousset and Solignac (1995) reported that the cross is compatible. To further investigate this pattern, we repeated the CI assay with two additional lines infected with *w*Ma (MD99 and RU13 males by NC48T, DSW, and MD111 females; Table 4). The results did not resolve the discrepancy. Males carrying *w*Ma are either compatible, incompatible, or equivocal when mated to uninfected females (Tables 2, 4). One potential explanation for these results is that there is heterogeneity in the density of *Wolbachia* in *w*Ma males. An alternative is that *w*Ma is, in fact, two or more distinct strains.

There is a significant female effect when *w*Au-bearing and uninfected lines are compared (Table 3C). When NC48T is removed, there is no significant effect (Table 3D). When uninfected MD111 males were mated with NC48T females, 60% of the eggs did not hatch. These females may have been suffering reduced fitness as a result of tetracycline treatment more than five generations before the assay.

The *w*Au strain from Madagascar (MD225) does not express CI. A Wilcoxon sign rank test showed that MD225 males and females do not exhibit significantly different levels of incompatibility than naturally uninfected males or females, respectively ($\chi^2 = 28.66$, df = 3, $P = 0.81$ for males; $\chi^2 = 44.46$, df = 3, $P = 0.06$ for females). This result has previously been shown with lines of *D. simulans* collected from Australia (Hoffmann et al. 1994).

A single conundrum remains from the results of the CI assays that is not easily explained. In three of four CI assays, *w*Ma-bearing males were compatible with what we assume were uninfected females (MD199, RU07, and MD99 males by MD111 females). However, RU13 males mated to MD111

TABLE 2. Expression of cytoplasmic incompatibility (i.e., median percent eggs unhatched) and 10th and 90th quartiles between fly lines and bacteria strains (in parentheses). Each value represents the female line at the top crossed with the male line at the left.

Male fly line			Female fly line									
Mitochondrial haplotype	Bacteria strain		HW09 *siI* *w*Ha	TT01 *siI* *w*Ha	DSR *siII* *w*Ri	MD225 *siII* *w*Au	MD199 *siIII* *w*Ma	RU07 *siIII* *w*Ma	NC48T *siI* *w*−	DSW *siII* *w*−	MD111 *siIII* *w*−
HW09	*siI*	*w*Ha	0.8 (0.0–72.0)	2.3 (0.0–37.4)	100.0 (96.6–100.0)	100.0 (92.3–100.0)	100.0 (100.0–100.0)	100.0 (100.0–100.0)	100.0 (99.9–100.0)	100.0 (59.4–100.0)	98.4 (97.1–100.0)
TT01	*siI*	*w*Ha	0.0 (0.0–1.4)	2.3 (0.0–13.0)	100.0 (94.8–100.0)	97.5 (80.5–100.0)	98.2 (88.3–100.0)	99.1 (77.2–100.0)	100.0 (100.0–100.0)	100.0 (48.9–100.0)	98.8 (19.5–100.0)
DSR	*siII*	*w*Ri	97.1 (90.7–100.0)	96.8 (84.5–100.0)	4.4 (0.0–12.3)	100.0 (98.4–100.0)	100.0 (100.0–100.0)	99.3 (96.9–100.0)	100.0 (84.6–100.0)	99.5 (96.6–100.0)	99.2 (54.3–100.0)
MD225	*siII*	*w*Au	2.5 (0.0–75.0)	11.7 (0.0–69.6)	2.6 (0.0–20.9)	4.2 (0.0–79.2)	4.2 (0.0–52.7)	9.4 (0.9–78.1)	23.8 (8.1–46.5)	6.6 (0.2–12.5)	3.2 (0.9–100.0)
MD199	*siIII*	*w*Ma	0.0 (0.0–100.0)	0.3 (0.0–16.0)	0.8 (0.0–2.8)	7.9 (1.9–29.3)	1.1 (0.0–87.7)	1.1 (0.0–57.1)	30.9 (10.3–100.0)	50.9 (5.8–80.4)	2.1 (0.0–42.7)
RU07	*siIII*	*w*Ma	0.0 (0.0–0.8)	10.8 (0.4–100.0)	0.7 (0.0–13.9)	11.4 (0.7–49.9)	2.2 (0.0–58.5)	1.9 (0.0–31.6)	100.0 (100.0–100.0)	90.8 (41.8–100.0)	3.5 (0.9–95.0)
NC48T	*siI*	*w*−	0.7 (0.0–46.1)	2.3 (0.4–41.9)	3.5 (0.0–9.7)	2.3 (0.0–81.7)	0.0 (0.0–1.5)	0.8 (0.0–23.4)	4.8 (0.0–10.2)	2.4 (0.4–57.4)	0.5 (0.0–32.1)
DSW	*siII*	*w*−	0.0 (0.0–22.1)	1.4 (0.0–12.3)	1.2 (0.0–5.8)	1.6 (0.0–50.5)	0.9 (0.0–19.8)	3.0 (0.0–31.4)	16.3 (7.0–32.0)	2.1 (0.0–7.3)	2.6 (1.1–18.3)
MD111	*siIII*	*w*−	0.0 (0.0–35.9)	0.0 (0.0–3.3)	0.8 (0.0–8.5)	5.2 (0.9–22.1)	5.9 (0.0–50.5)	0.4 (0.0–36.7)	74.4 (23.2–92.9)	6.8 (0.0–7.4)	2.8 (0.0–11.2)

TABLE 3. Kruskal-Wallis tests of cytoplasmic incompatibility (percent eggs unhatched) in males and females from different lines. (A) All lines; (B) comparison of six lines (MD199, RU07, NC48T, MD225, MD111, and DSW); (C) comparison of four lines (NC48T, MD225, MD111, and DSW); and (D) comparison of three lines (MD225, MD111, and DSW).

Source	df	SS	*H*	*P*
A.				
Male	8	57778639	41.29	<0.001
Female	8	21420431	153.07	<0.001
Male × female	64	30958260	221.23	<0.001
Error	1233	73575800		
B.				
Male	5	2071403	16.31	0.006
Female	5	2931001	23.08	<0.001
Male × female	25	2201852	3.47	1.0
Error	650	22991150		
C.				
Male	3	90162	4.83	0.18
Female	3	290597	15.58	0.001
Male × female	9	117457	2.10	0.98
Error	298	2227550		
D.				
Male	2	15376	2.50	0.29
Female	2	21533	3.50	0.17
Male × female	4	19087	1.55	0.19
Error	196	637098		

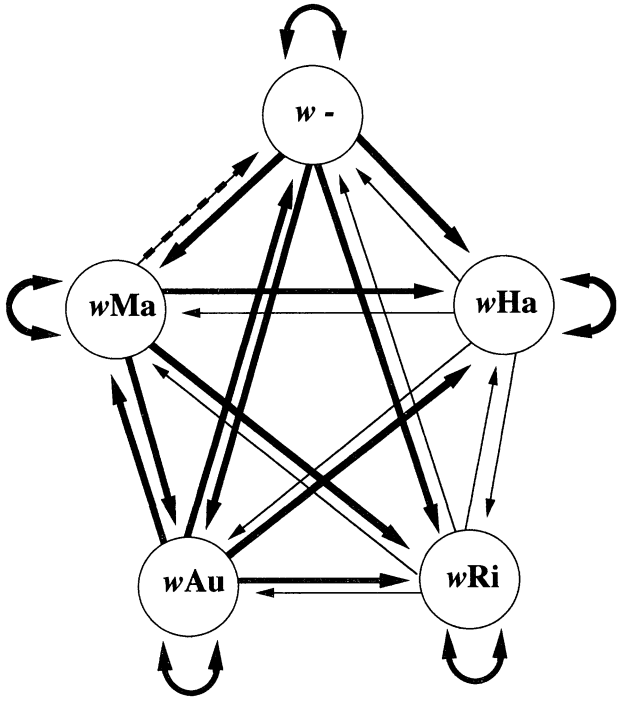

FIG. 3. Schematic of cytoplasmic incompatibility between *Drosophila simulans* lines that either carries one of four strains of *Wolbachia* or is uninfected. Arrows go from males to females. The thickness of the line represents the level of incompatibility. Thin lines represent incompatibility, thick lines represent compatibility, and dashed lines represent variable expression of incompatibility.

TABLE 4. Expression of cytoplasmic incompatibility (median percent eggs unhatched) and 10th and 90th quantiles (in parentheses) between fly lines and bacteria strains of crosses made to check the *w*Ma pattern. Each value represents the female line at the top crossed with the male line to the left.

Male fly line			Female fly line		
	Mitochondrial haplotype	Bacteria strain	NC48T *si*I *w*−	DSW *si*II *w*−	MD111 *si*III *w*−
MD99	*si*III	*w*Ma	100.0 (10.2–100.0)	6.2 (3.4–96.7)	4.3 (23.1–100.0)
RU13	*si*III	*w*Ma	42.4 (22.1–100.0)	100.0 (68.9–100.0)	100.0 (75.4–100.0)

females are 100% incompatible. To verify the infection status of MD111, PCR amplification of DNA from nine flies tested at three primer:template ratios with both genes did not suggest the line was infected. It remains possible that MD111 is heterogeneous for infection, it carries a strain of *Wolbachia* that is not detected by the 16S rDNA and *wsp* primers, or that it is infected with an additional microorganism.

Frequency of Wolbachia in D. simulans

The frequency of *Wolbachia* differs dramatically between populations, and the patterns of these differences are consistent within *Wolbachia* strain and their expression of CI. Population-level estimates of the frequency of infection are summarized in Figure 4.

In this study, all 102 isofemale lines collected from islands in the Pacific Ocean (Hawaii and Tahiti) carry the *si*I mitochondrial haplotype and the *w*Ha strain of *Wolbachia* (Table 5). This reinforces the result that the Pacific island populations are infected at high frequency with *w*Ha (Turelli and Hoffmann 1995; Fig. 4). Doubly infected lines of the *si*I mitochondrial type have been reported from New Caledonia and the Seychelles (Merçot et al. 1995; Merçot and Poinsot 1998b).

We did not conduct a population survey of lines infected with the *w*Ri strain. Turelli et al. (1992) found Californian lines with *w*Ri are of the *si*II haplotype. This infection/haplotype variant spread up the western coast of North America and is typically stable at high (~95%) frequencies.

In Madagascar, the *w*Au strain is found in the *si*IIB haplotype at frequencies of about 56% (Table 5). In no case was a *si*III fly line infected with the *w*Au strain of *Wolbachia*. Hoffmann et al. (1994) described populations of *D. simulans* infected with *w*Au along the eastern coast of Australia that are infected with *Wolbachia* at levels below 20% (a subset of their population level survey is illustrated in Fig. 4). Here we report that the Florida population studied by Ballard et al. (1996), in which 40% of the lines were infected, carry *w*Au.

In Madagascar *w*Ma is found at a frequency of about 31% in flies that carry the *si*III haplotype, and never in lines with the *si*II haplotype (Fig. 4, Table 5). This is the first report of the population-level frequency of *w*Ma. If bacteria densities are highly variable in *w*Ma-carrying individuals, this estimate may be low. Merçot and Poinsot (1998a) found that 18% of *D. simulans* from Mount Kilimanjaro, Tanzania, are infected with *Wolbachia* (*w*Ki). H. Merçot (pers. comm.) reports that those lines carry the *si*III mitochondrial haplotype.

DISCUSSION

The difficulty of defining *Wolbachia* strains has both taxonomic and evolutionary implications. Our recent collections of *D. simulans* are infected with at least four strains of *Wolbachia*. Merçot and Poinsot (1998b) suggested that *D. simulans* is also infected with the *w*No strain. The *w*Ma and *w*No strains differ by a single substitution in the 16S rDNA and are homosequential at the *wsp* locus. Thus, it is not clear whether they are distinct strains or variants of the same strain. To our knowledge, CI assays have not been conducted between *w*Ma- and *w*No-infected fly lines. This assay, in addition to sequencing additional *Wolbachia* loci, may help to

TABLE 5. *Wolbachia* infections in mitochondrial haplotypes of *Drosophila simulans*.

				*Si*I		*si*IIA	*si*IIB		*si*III	
Ocean	Island	Site	*N*	*w*Ha	*w*−	*w*−	*w*Au	*w*−	*w*Ma	*w*−
Pacific	Kauai	Kuku Park	62	62	—	—	—	—	—	—
	Oahu	Honolulu	36	36	—	—	—	—	—	—
	Tahiti	Papeete[1]	4	4	—	—	—	—	—	—
Indian	Madagascar	Joffreville	31	—	—	—	11	8	5	7
		Antannarivo	42	—	—	2	18	10	—	12
		Antsirabe	81	—	—	3	31	25	5	17
		Ambositra[1]	17	—	—	—	9	6	1	1
		Ranomafana[1]	10	—	—	—	4	3	2	1
	Reunion	St. Pierre/St. Dennis	8	—	—	—	—	1	5	2
Total[2]			291	102	0	5	73	53	18	40

[1] These sites were not used in the population-level survey because less than 20 isofemales lines were established.

[2] The lines DSW (*si*II, *w*−), DSR (*si*II, *w*Ri), NC48 (*si*I, Ha and *w*Ma double infection), and Seychelles (*si*I, *w*Ha and *w*Ma double infection) included in this study are not presented in this table.

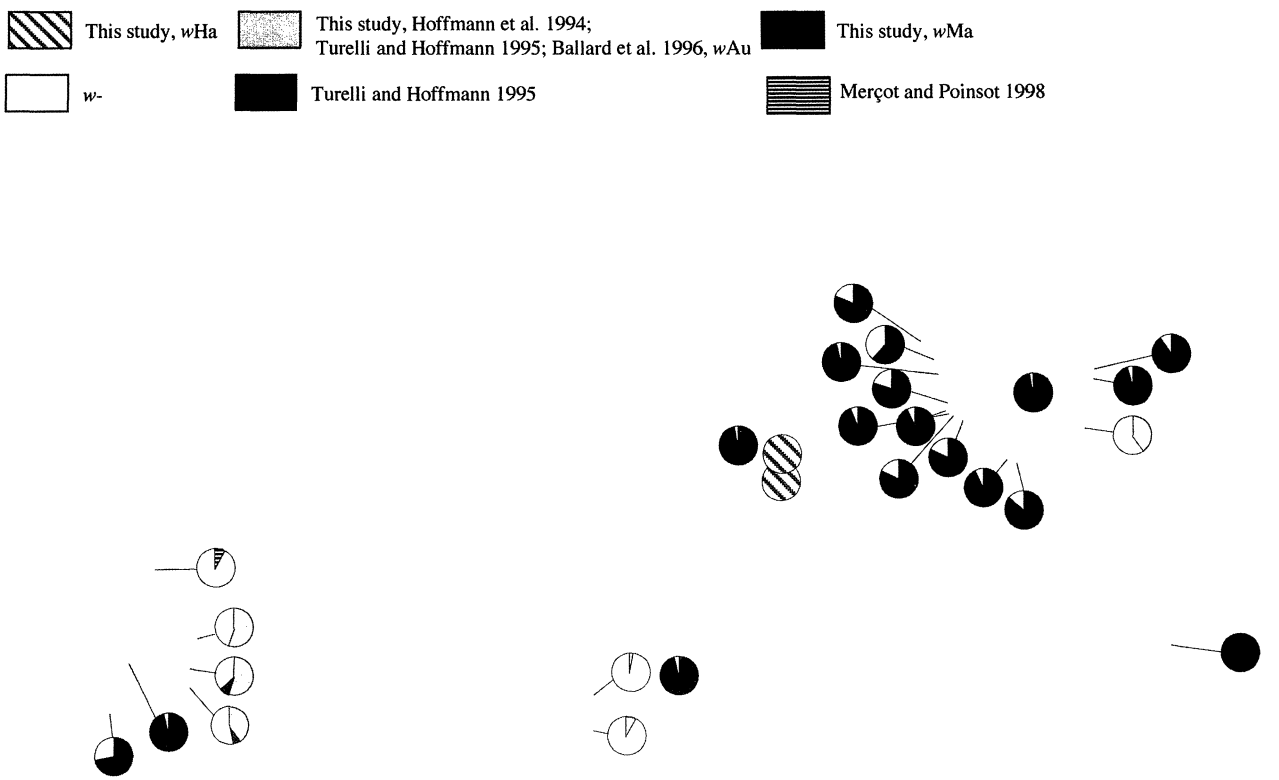

FIG. 4. Frequencies of *Wolbachia* in populations of *Drosophila simulans* reported in the literature and here. Only populations in which greater than 20 individuals were tested are reported.

resolve whether *w*Ma and *w*No are distinct. Merçot and Poinsot (1998a) suggest that a sixth strain of *Wolbachia* (*w*Ki) may also infect *D. simulans*. Lines carrying this strain were not available for inclusion in this study.

It is also not clear whether *w*Au and *w*Me (the latter from *D. melanogaster*) are the same strain or if they are distinct strains and *w*Au has the rescuing phenotype. Bourtzis et al. (1998) suggest that naturally infected females from Australia (carrying *w*Au) are immune to the incompatibility when bred to male *D. simulans* that have been microinjected with a *Wolbachia* strain carried in *D. melanogaster*—implying that the *w*Au infection rescues the incompatible cross. The *w*Au and *w*Me strains of *Wolbachia* are homosequential at the 16S rDNA locus (A. C. James and J. W. O. Ballard, unpub. data) and differ by 5 bp at the *wsp* locus (Zhou et al. 1998). Thus, the phylogenetic affinity of the strains may influence the potential to rescue. In this study, *w*Au-carrying females were unable to rescue the cross with males carrying either *w*Ri or *w*Ha. However, the crosses of *w*Au females with males carrying *w*Ma are compatible.

All *D. simulans* that have been collected in Hawaii and Tahiti carry the *si*I mitochondrial type. Of these, 154 of 155 are infected with the *w*Ha strain of bacteria (Turelli and Hoffmann 1995; this study). This infection causes high incompatibility between males that carry *w*Ha and females that are uninfected or carry a different strain of *Wolbachia*. Thus, any immigrant or environmentally cured female in these populations would not be able to find a compatible male. An uninfected male would be able to produce offspring with females from these populations, but all of the offspring would carry both the maternal mitochondrial type and bacteria and, thus, populations polytypic for bacteria infection would not be created.

It is curious that all lines collected from New Caledonia and some from the Seychelles have the *si*I haplotype and a majority of these are doubly infected with *w*Ha and *w*No (Merçot et al. 1995). Turelli and Hoffman (1995) did not distinguish *Wolbachia* strains in their survey, but note that 96% of individuals collected from Noumea, New Caledonia, are infected. Merçot and Poinsot (1998b) reported that most individuals of *D. simulans* collected in New Caledonia are doubly infected, with a few being singly infected with *w*Ha (Merçot et al. 1995). No naturally occurring fly line with the *si*I haplotype and a single *w*No bacterial infection has yet been described, but the existence of such lines cannot be ruled out. Additional collections from New Caledonia are required.

Collections from North and South America, Southern and Western Africa, and Europe have the *si*II haplotype (Montchamp-Moreau et al. 1991; Ballard and Kreitman 1994; Rand et al. 1994) and, if infected, most carry the *w*Ri strain of bacteria (Hoffmann and Turelli 1988; Turelli and Hoffmann 1991). Incompatibility induced by the *w*Ri strain is similar to that induced by the *w*Ha strain because infected males are only compatible with females that carry the same strain of *Wolbachia*. It is curious that populations are polytypic for infection (Fig. 4). *Wolbachia* infections that cause CI are expected to increase in polytypic populations (Caspari and Watson 1959) if fitness is not reduced in infected individuals or the environment does not diminish the expression of CI

or the frequency of infection. A few mechanisms have been described that may explain the maintenance of the polytypism. Imperfect transmission of *Wolbachia* from mother to offspring has been described in field populations, and environmental curing (such as antibiotic treatment) has been observed in the laboratory (Hoffmann et al. 1986, 1990; Clancy and Hoffmann 1998). Male experience (age, mating history) may decrease the expression of incompatibility, and carrying a strain may concur reduced fitness on the host. Hoffmann et al. (1990) found that incompatibility levels diminish with male age, and Karr et al. (1998) found this was exaggerated when the male had mated multiply. In addition, Clancy and Hoffmann (1998) found reduction in the expression of CI between *w*Ri-infected males and uninfected females when male larvae were fed moderate levels of tetracycline, when the males were reared or aged at high temperatures. It is possible that the magnitude of these effects may differ between strains, explaining different frequencies of *Wolbachia* in populations that carry different strains. It is also possible that the mainland has a more variable environment than the Pacific islands, where *w*Ha is found. If bacterial infections are influenced by the environment, this may lead to lower frequencies of *w*Ri than *w*Ha.

The *si*II mitochondrial haplotype is found in Madagascar and Reunion. In these populations, flies with the *si*IIB haplotype were infected with the *w*Au strain at a frequency of 56%, whereas all individuals with the *si*IIA haplotype were uninfected. A single uninfected *si*II fly was collected on Reunion. Males that carry *w*Au bacteria have low expression of CI. It is a challenge to explain how a bacterial infection that does not induce CI in the host can exist at intermediate frequencies. Perhaps these strains caused incompatibility in the past. An expressing strain has been shown to sweep through populations to around a frequency of 95% in three years (Turelli and Hoffmann 1991). Hoffmann and Turelli (1997) discussed how a non-CI-expressing *Wolbachia* can be maintained by a balance between incomplete transmission of the bacteria and enhanced fitness of carriers. One explanation is that the *w*Au strain that infects flies in the Indian Ocean induces a specific fitness advantage in different ways from the well-studied *w*Ri strain. An alternative is that *w*Au is subject to random drift and the frequency is not stable.

There are two enigmatic populations from North and South America. Lines of *D. simulans* from Atacames, Ecuador, and Lantana, Florida, are infected with *Wolbachia,* but express low levels of incompatibility (Turelli and Hoffmann 1995; Ballard et al. 1996). Other *D. simulans* populations in North and South America carry the high-CI-expressing *w*Ri strain of *Wolbachia* when infected. The Ecuador lines are no longer available, so we do not know what bacterial strain they carried. However, we have recently obtained *wsp* sequence from the Florida lines studied by Ballard et al. (1996) and found that they carry the *w*Au strain of *Wolbachia.* We do not know if this is an introduction of the strain or if this population is a refuge from an ancestral population. A comprehensive worldwide survey of *Wolbachia* strains would be informative.

Turelli (1994) modeled interactions between CI-inducing parasites and hosts. He predicted that, whether selection is acting on the host or bacterial genome, reduced incompatibility will be favored. In these models, a rare, nonexpressing bacterial strain can invade a population in which a nonexpressing bacteria already exists if females infected with the new bacteria either have greater fecundity or if transmission of the bacteria is more effective than the pre-existing strain. Other fitness parameters may be involved in the invasion of a nonexpressing strain of *Wolbachia.* Hoffmann et al. (1994) demonstrated that Australian lines infected with the *w*Au strain produced more eggs than uninfected lines; however, this difference was not significant. Hariri et al. (1998) found a line of *Sphyracephala beccarii* (a stalk-eyed fly) in which males infected with *Wolbachia* did not express CI, but had higher fertility.

The *si*III haplotype occurs in Madagascar and Reunion, and 32% these lines were infected with the *w*Ma strain of *Wolbachia.* Males carrying *w*Ma were compatible with females harboring any other strain of *Wolbachia,* but showed puzzling results when mated with uninfected females. Males from four replicate lines carrying the *w*Ma strain of bacteria were mated with females from three uninfected lines. Of these, four crosses were compatible, five were equivocal, and three were incompatible. These results could be caused by variable density of *Wolbachia* in *w*Ma males. An alternative explanation is that *w*Ma is, in fact, a complex of distinct strains.

It is intriguing that each mitochondrial haplotype has a distinct set of bacterial infections. Flies with the *si*I mtDNA haplotype are infected with *w*Ha and rarely doubly infected with *w*Ha and *w*No. In this study *si*IIA flies harbor *w*Ri, whereas *si*IIB carry *w*Au. In flies with *si*III mtDNA, we only observe the *w*Ma strain of *Wolbachia.* H. Merçot (pers. comm.) reports that flies infected with *w*Ki carry the *si*III haplotype. Turelli and Hoffmann (1995) suggest that *Wolbachia* strains may not be tightly linked to mitochondrial haplotype because of the potential for paternal leakage, horizontal transfer, immigration, or line contamination. In this study, we observe that a monophyletic clade of the *si*IIB haplotype is infected with the *w*Au strain from flies from the Indian Ocean. It will be interesting to see if these subtypes will be consistently associated with the bacteria strains in other populations.

We place the worldwide differences in frequencies of *Wolbachia* infection into perspective with the expression of cytoplasmic incompatibility. In populations that carry either *w*Ha (or multiply infected with *w*Ha/*w*No) or *w*Ri, in which males have high expression of incompatibility, the frequency of infection is also high. Populations that have strains of *Wolbachia* with reduced expression of CI (*w*Ma or *w*Au) have low frequencies of infection. Future studies should investigate if the expression of CI between strains is correlated with bacterial densities in the host and if there are differential fitness effects of the various bacteria strains in the hosts.

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