Wolbachia Infections and the Expression of Cytoplasmic Incompatibility in *Drosophila sechellia* and *D. mauritiana*

Rosanna Giordano,* Scott L. O'Neill† and Hugh M. Robertson*

**Department of Entomology, University of Illinois, Urbana, Illinois 61801 and †Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510*

Manuscript received November 28, 1994
Accepted for publication May 6, 1995

ABSTRACT

Various stocks of *Drosophila mauritiana* and *D. sechellia* were found to be infected with Wolbachia, a Rickettsia-like bacterium that is known to cause cytoplasmic incompatibility and other reproductive abnormalities in arthropods. Testing for the expression of cytoplasmic incompatibility in these two species showed partial incompatibility in *D. sechellia* but no expression of incompatibility in *D. mauritiana*. To determine whether absence of cytoplasmic incompatibility in *D. mauritiana* was due to either the bacterial or host genome, we transferred bacteria from *D. mauritiana* into an uninfected strain of *D. simulans*, a host species known to express high levels of incompatibility with endogenous Wolbachia. We also performed the reciprocal transfer of the natural *D. simulans* Riverside infection into a tetracycline-treated stock of *D. mauritiana*. In each case, the ability to express incompatibility was unaltered by the different host genetic background. These experiments indicate that in *D. simulans* and *D. mauritiana* expression of the cytoplasmic incompatibility phenotype is determined by the bacterial strain and that *D. mauritiana* harbors a neutral strain of Wolbachia.

CYTOPLASMIC incompatibility is typically expressed when a male insect that is infected with Wolbachia mates with a noninfected female. The number of surviving progeny from such a cross is greatly reduced, while any other combinations of matings are fully fertile. This phenomenon has been described from a number of insect species spanning several orders (*e.g.*, LAVEN 1951; GOTTLIEB 1972; YEN and BARR 1974; NODA 1984; HSIAO and HSIAO 1985; WADE and STEVENS 1985; CONNER and SAUL 1986; HOFFMANN *et al.* 1986). The occurrence of this phenomenon has been determined to be caused by Rickettsia-like bacteria, first described in *Culex pipiens*, and named *Wolbachia pipientis* (HERTIG 1936). In some species of Hymenoptera, eggs from incompatible crosses result in haploid male progeny due to abortive karyogamy (RYAN and SAUL 1968; BREEUWER and WERREN 1990; REED and WERREN 1995). In addition, Wolbachia have also been implicated in causing parthenogenesis in certain parasitic wasps (STOUTHAMER *et al.* 1990), and overriding chromosomal sex determination in the terrestrial isopods *Armadillidium vulgare*, *Chaetophiloscia elongata*, and *Porcellionides pruinosus* (JUCHAULT *et al.* 1994). Removal of the bacteria by treatment with the antibiotic tetracycline, or in some cases, exposure to high temperatures, restores fertility (TRPIS *et al.* 1981; WRIGHT and BARR 1981; RICHARDSON *et al.* 1987). In some species it has been shown that the degree of cytoplasmic incompatibility decreases as the male ages (SINGH *et al.* 1976; HOFFMANN *et al.* 1986), possibly as a result of eggs being fertilized with sperm developed in spermatocysts that are devoid of bacteria (BRESSAC and ROUSSET 1993). In contrast to the pattern of unidirectional incompatibility described above, in some species, matings between infected males and females of different strains of the same species can sometimes result in bidirectional incompatibility (YEN and BARR 1973; O'NEILL and KARR 1990; MONTCHAMP-MOREAU *et al.* 1991). While the precise mechanism of cytoplasmic incompatibility is unkown, it is likely that bidirectional incompatibility is due to infection with different Wolbachia strains (ROUSSET *et al.* 1992a; BREEUWER and WERREN 1993; BRAIG *et al.* 1994).

Sequencing the 16S rRNA gene of this bacterium has permitted the placement of Wolbachia within the alpha-Proteobacteria as well as allowing a preliminary look at the phylogenetic relationship of Wolbachia strains found in different insect hosts (O'NEILL *et al.* 1992; ROUSSET *et al.* 1992a; STOUTHAMER *et al.* 1993). While concordance can be seen in a phylogeny of aphid symbionts (*Buchnera aphidicola* complex) based on the 16S rRNA gene and a phylogeny of the host based on morphological characters (MORAN *et al.* 1993), a similar analysis with Wolbachia 16S rRNA sequences and their hosts does not show such concordance (O'NEILL *et al.* 1992; MORAN and BAUMANN 1994). This contrast indicates that while this lineage of aphids was possibly infected by symbionts only once, events leading to the infection of insects harboring Wolbachia have probably been more frequent and horizontal in nature. With the development of the polymerase chain reaction (PCR)

Corresponding author: Rosanna Giordano, Department of Entomology, 505 South Goodwin, University of Illinois, Urbana, Illinois 61801. E-mail: giordano@uiuc.edu

it has been possible to discern rapidly the presence and frequency of infection of this bacterium in arthropods.

In recent years it has been demonstrated that two members of the *Drosophila melanogaster* species complex, *D. melanogaster* and *D. simulans*, are sometimes infected with Wolbachia and express partial-to-extreme cytoplasmic incompatibility. Different populations of *D. simulans* have been shown to be infected with different strains of Wolbachia, for example the Riverside strain from Riverside, CA, is bidirectionally incompatible with the Hawaiian and Seychelles strains (O'NEILL and KARR 1990; MONTCHAMP-MOREAU *et al.* 1991). Infected populations and stocks of *D. melanogaster* have been found that show partial to no cytoplasmic incompatibility (HOFFMANN 1988; HOLDEN *et al.* 1993; BOURTZIS *et al.* 1994; HOFFMANN *et al.* 1994; SOLIGNAC *et al.* 1994). In this paper, we report that two other members of the melanogaster species complex, *D. sechellia* and *D. mauritiana*, are also infected with Wolbachia. Characterization of incompatibility expression in these two species shows that *D. sechellia* expresses the phenotype while *D. mauritiana* does not. The relative contributions of host and bacterial genome to these phenotypic differences have been explored by utilizing microinjection techniques to transfer these symbionts between different hosts, as well as assessing the relative density of bacteria found in naturally infected and *trans*-infected strains.

MATERIALS AND METHODS

Stocks: Sources and identification of the stocks of *D. sechellia* and *D. mauritiana* used to determine the prevalence of Wolbachia in these two species are listed in Table 1. Origins of the Drosophila stocks used in the crosses below are as follows. *D. mauritiana:* synthetic is a fusion of several isofemale lines and was obtained from J. COYNE. The *D. mauritiana* G and MS series stocks were originally collected on the island of Mauritius by A. FUKATAMI in 1979 and 1987, respectively. Some of the stocks from the G series were sent to the Bowling Green stock center by T. WATANABE. We obtained some of the G and the MS series stocks directly from Dr. WATANABE as well as the Bowling Green stock center. The *D. sechellia* stock used in the crosses was kindly provided by J. DAVID. The Riverside and Watsonville strains of *D. simulans* were supplied by M. TURELLI (abbreviated as DSR and DSW). *D. simulans* Hawaii was obtained from T. LYTTLE (abbreviated as DSH). Flies were cultured in bottles at room temperature (~23–25°) on standard agar molasses diet and maintained by mass transfer. Tetracycline-treated strains were established by placing 0.025% by weight of tetracycline in melted and cooled Drosophila media just before solidification. Flies were left to lay eggs for 3 days before being removed. Strains were treated for two generations and tested for infection status with PCR utilizing primers 99F and 994R, which are specific for the 16S rRNA of Wolbachia (O'NEILL *et al.* 1992).

Testing for presence of cytoplasmic incompatibility: Flies used in matings were reared in vials at a density of 20–30 larvae per vial. Upon emergence they were isolated as virgin males and females, kept at a density of about 20 individuals per vial, and crossed when 3–5 days old. Matings to test for the presence of incompatibility were set up in bottles upturned on agar/molasses caps, which were replaced daily to monitor the number of eggs laid. Hatching rates were scored 28 hr after egg collection. All matings were conducted with one female and two males with the exception of *D. sechellia* where, due to the low fecundity of this species, three females and three males were set up for the first experiment and two females and two males were used in experiment 2 (Table 2). The number of replicates done for each of the crosses is listed in their respective tables.

We also tested whether presence of a Wolbachia infection in *D. mauritiana* affected the fecundity of this species. Flies for this experiment were set up as described above and eggs were collected for seven consecutive days.

***Trans*-infection of isofemale lines:** Injected stocks were derived by direct egg cytoplasm microinjection from donor to recipient strain (BOYLE *et al.* 1993). Donor flies between the age of 2–10 days old were set up in an upturned bottle fitted with an agar/molasses cap and dabbed with yeast paste. Caps were changed during the day, approximately every hour for 2 days, to stimulate egg production. Embryos to be used as donors were collected every hour and dechorionated for 1 min in a 50% solution of commercial hypochlorite. Dechorionated embryos were glued to coverslips with rubber cement to facilitate microinjection. Only embryos prior to pole cell formation (cycle 10) were used as either donors or recipients. Cytoplasm was removed from the posterior end of the donor embryos with a needle of borosilicate glass capillary tube. The cytoplasm collected from donors was injected into the posterior end of recipient eggs prepared as above but partially desiccated to allow volume for the injected material. Eggs were removed to a agar/molasses cap and the larvae that survived transferred upon emergence to vials of cornmeal-molasses medium. Isofemale lines were established with the emergent females as follows: seven lines of *D. simulans* Watsonville transinfected with bacteria from *D. simulans* Riverside, abbreviated as DSW(DSR); three lines of *D. simulans* Watsonville transinfected with bacteria from *D. mauritiana* [DSW(Mau)]; and 14 lines of *D. mauritiana* transinfected with bacteria from *D. simulans* Riverside [Mau(DSR)]. Females were mated either with males from injected eggs or to males from the original stock of uninfected recipient flies. Two individuals from the F_1 progeny were tested by PCR for infection using the 16S rRNA Wolbachia specific primers mentioned previously. Presence of infection was also confirmed by staining embryos with the fluorescent DNA-intercalating dye 4,6-diamino-2-phenylindole, (DAPI) (O'NEILL and KARR 1990). Of the seven DSW(DSR) isofemale lines, three were infected; of the three DSW(Mau) lines, one was infected; and of the 14 Mau(DSR) lines, five were infected. An infected line was chosen randomly, used to conduct crosses, and rechecked for its infection status prior to crossing.

Phylogenetic relationships of the Wolbachia: To determine the phylogenetic positions of the bacteria in *D. mauritiana* and *D. sechellia* a 1450-bp fragment of the 16S rRNA gene was sequenced. PCR products were generated using the 16S rRNA primer pairs 21F and 994R, and 99F and 1492R [see O'NEILL *et al.* (1992) for 21F, 99F and 994R; primer 1492R is 5'GGTTACCTTGTTACGACTT, *Escherichia coli* positions 1510–1492 reverse, courtesy of C. WOESE]. A contiguous sequence was assembled from two overlapping clones for *D. sechellia* and one clone for *D. mauritiana*. The fragment was sequenced in one direction. PCR products were purified in 0.75% NuSieve gels (FMC), cloned into pcDNAII (InVitrogen), and sequenced using the Sanger dideoxy termination method as described in ROBERTSON and MACLEOD (1993). The 16S rRNA sequences for *D. sechellia* and *D. mauritiana* have been deposited in GenBank and can be retrieved with accession numbers U17059 and U17060, respectively.

The new sequences were manually added to the original alignment of O'NEILL *et al.* (1992) using the data editor in

TABLE 1

Results from screening of stocks of *Drosophila mauritiana* and *D. sechellia*

Stock name or number	Infection status	Source	Stock name or number	Infection status	Source
D. mauritiana					
0241.0	+	A	2317	+	E
wl	−	B	2318	−	E
vermillion	−	B	2516	+	E
net, cy, cn, bw	−	B	G20	+	F
ww, bg	−	B	G23	+	F
white I	+	A	G76	−	F
L1	−	A	G102	−	F
RLwl	−	B	G197	+	F
Synthetic	+	C	G284	−	F
72 David	+	C	MS2	−	F
wPeach Hartl	+	C	MS4	−	F
w B. Green	+	C	MS5	−	F
105 David	+	C	MS8	−	F
Bowling Green	+	C	MS9	−	F
CL236	+	D	MS10	+	F
Les Galets	−	D	MS12	+	F
Petite Riviere	−	D	MS13	−	F
Lig 21	−	D	MS15	−	F
David	−	D	MS16	+	F
152	+	D	MS17	−	F
75	−	D	MS18	+	F
G20	+	A	MS19	+	F
G23	−	A	MS20	+	F
G29	−	A	MS30	−	F
G35	−	A	MS31	+	F
G72	−	A	MS34	−	F
G76	+	A	MS38	−	F
G93	+	A	MS42	+	F
Cambridge	−	A	MS48	+	F
wf	−	A			
w	−	A	*D. sechellia*		
zlf	−	A	"	+	A
zl	−	A	"	−	G
y plum	−	A	cn	+	E
bw cn	−	A	22	−	E
curved wing	−	A	81	−	E
w j e	−	A	24	+	E
pn "upt" loz	+	A	4	+	E
br. orange eyes	−	A	21	+	E
Robertson	+	D	pur	+	E
w (Coyne)	+	D	Robertson	+	E
2252	+	E	David	+	H
2278	+	E			
2282	+	E			
2308	+	E			

Code for sources of stocks listed above: A, PHYLLIS OSTER, Mid-American Drosophila Stock Center, Bowling Green, OH; B, RICHARD LYMAN, North Carolina State U., Raleigh, NC; C, JERRY COYNE, University of Chicago, Chicago, IL; D, CATHY LAURIE, Duke University, Durham, NC; E, KATHY MATTHEWS, Bloomington Stock Center, Bloomington, IN; F, TAKAO WATANABE, National Institute of Genetics, Mishima, Japan; G, JOHN ROOTE, Cambridge University, Cambridge, UK; and H, JEAN DAVID, CNRS, Gif sur Yvette, France.

PAUP 3.1.1 (SWOFFORD 1993). The Wolbachia sequences used are listed here followed by their GenBank accession number: *Trichogramma cordubensis* (LO2883), *T. deion* (CA strain, LO2886), *T. deion* (SD strain, LO2888), *T. pretiosum* (LO2885), *Muscidifurax uniraptor* (LO2882) (STOUTHAMER *et al.*1992); *Nasonia giraulti* (M84689), *N. vitripennis* (M84686), *N. longicornis* (M84691 and M84692), (BREEUWER *et al.* 1992); *D. simulans* (Noumea strain, X64267) (ROUSSET

TABLE 2

Percent egg hatch from *D. sechellia*

♀	♂	*N*	Number of eggs	Egg hatch (%)
a. Crosses with 3–5-day-old virgin males and females				
D. sechellia T	*D. sechellia*	20	1339	26.8 ± 3.2
D. sechellia T	*D. sechellia* T	20	1195	96.0 ± 0.7
D. sechellia	*D. sechellia* T	20	805	96.2 ± 0.9
D. sechellia	*D. sechellia*	19	1053	94.5 ± 2.1
b. Crosses with 19–20-day-old males and 4–5-day-old virgin females				
D. sechellia T	*D. sechellia*	15	311	72.7 ± 5.9
D. sechellia T	*D. sechellia* T	14	193	87.8 ± 2.8
D. sechellia	*D. sechellia* T	11	202	93.5 ± 2.3
D. sechellia	*D. sechellia*	10	250	87.4 ± 5.9

Crosses are between individuals that were naturally infected and a tetracycline-treated (T) stock of the same strain. Pairs of crosses are compared using the Mann-Whitney U test. There was no significant difference between any pair of crosses except for the first, which was $P < 0.0001$. Egg-hatch values are means ± SE. Crosses in b use a subset of males from a.

et al. 1992a); *Armadillidium vulgare* (X65669) (ROUSSET *et al.* 1992b); *Adalia bipunctata* (U4163), the male-killing strain (WERREN *et al.* 1994); *Rhinocyllus conicus* (M85267) (CAMPBELL *et al.* 1992); *Tribolium confusum* (X62247), *Hypera postica* (X62248), *Aedes albopictus* (X61767), *D. simulans* (Riverside, CA, strain, X61770), *D. simulans* (Hawaii strain, X61769), *Ephestia cautella* (X61771), (O'NEILL *et al.* 1992); *Anaplasma marginale* (M60313) (WEISBURG *et al.* 1991); *Ehrlichia canis* (M73226), *E. phagocytophila* (M73220) (ANDERSON *et al.* 1991); *Cowdria ruminantium* (X61659) (DAME *et al.* 1992); *Rickettsia prowazekii* (M21789) (WEISBURG *et al.* 1989). The final alignment consists of 1522 positions. Positions 1–64 and 1473–1522 were excluded from the phylogenetic analysis because data for these positions is missing for most taxa. Phylogenetic analysis was done using the heuristic algorithm of PAUP 3.1.1. Six heuristic searches were performed with random addition of sequences and tree-bisection-reconnection branch swapping. The tree was rooted using *R. prowazekii* as outgroup.

Density of Wolbachia in embryos: Embryos were collected from 4–5-day-old flies at 1-hr intervals, dechorionated in a 50% commercial hypochlorite solution, fixed in 1:1 heptane/methanol, and stained for 15 min in 1 μg/ml of DAPI (Boyle *et al.* 1993). Young embryos (precycle 10) used to determine bacterial density were mounted on slides with a bridged coverslip, in 70% glycerol and 0.35% n-propyl gallate as mounting medium and viewed with epifluorescence microscopy. The anterior portion of the eggs was photographed with a 40× objective, focusing in a single plane just beneath the vitelline membrane. Numbers of bacteria were counted on photographs in an area of 2.3 × 3.4 cm. These relative bacterial counts were square root transformed because the group variances were proportional to the means, and then analyzed by means of an ANOVA followed by a multiple comparison with the *a posteriori* Tukey test (ZAR 1984).

RESULTS

Wolbachia infections are present in both *D. mauritiana* and *D. sechellia* stocks: We screened 74 stocks of *D. mauritiana* and 10 stocks of *D. sechellia* for the presence of Wolbachia by diagnostic PCR. Positive PCR reactions were obtained from some stocks of both species with 45 and 70% infected, respectively (Table 1). The infection status for some of the G series stocks of *D. mauritiana* obtained from the Bowling Green stock center was not consistent with the results from those stocks obtained directly from T. WATANABE, who sent them to the stock center. Stock G23 from Bowling Green was not infected, but that from T. WATANABE was, while stock G76 from Bowling Green was infected and the corresponding stock from T. WATANABE was not. It is possible that the infection may have been lost from the uninfected strains. The overlapping *D. mauritiana* stocks between the T. WATANABE collection and that of the Bowling Green stock Center were treated as independent stocks as a result of the inconsistency in their infection profile and the length of time they had been separated.

A phylogenetic analysis including the sequences of the 16S rRNA from Wolbachia from *D. mauritiana* and *D. sechellia* resulted in 98 most parsimonious trees 829 steps long (Figure 1). In all most parsimonious trees, the Wolbachia from *D. simulans* Hawaii and *D. sechellia* form a clade. The relationships of the Wolbachia from *D. mauritiana* cannot be unambiguously resolved, but all trees indicate that its relationship to the endosymbiont in *D. sechellia* is distant (Figure 1). However, although there are 359 informative sites when all taxa are considered, only 81 inform the relationship of the 22 Wolbachia strains, thus the detailed relationships of the Wolbachia cannot be resolved with confidence.

Expression of cytoplasmic incompatibility: To test whether the infected strains of *D. mauritiana* and *D. sechellia* were capable of inducing the incompatibility phenotype, we cured representative stocks from each species with tetracycline, tested them via PCR and DAPI staining, and crossed them with their parental infected strains.

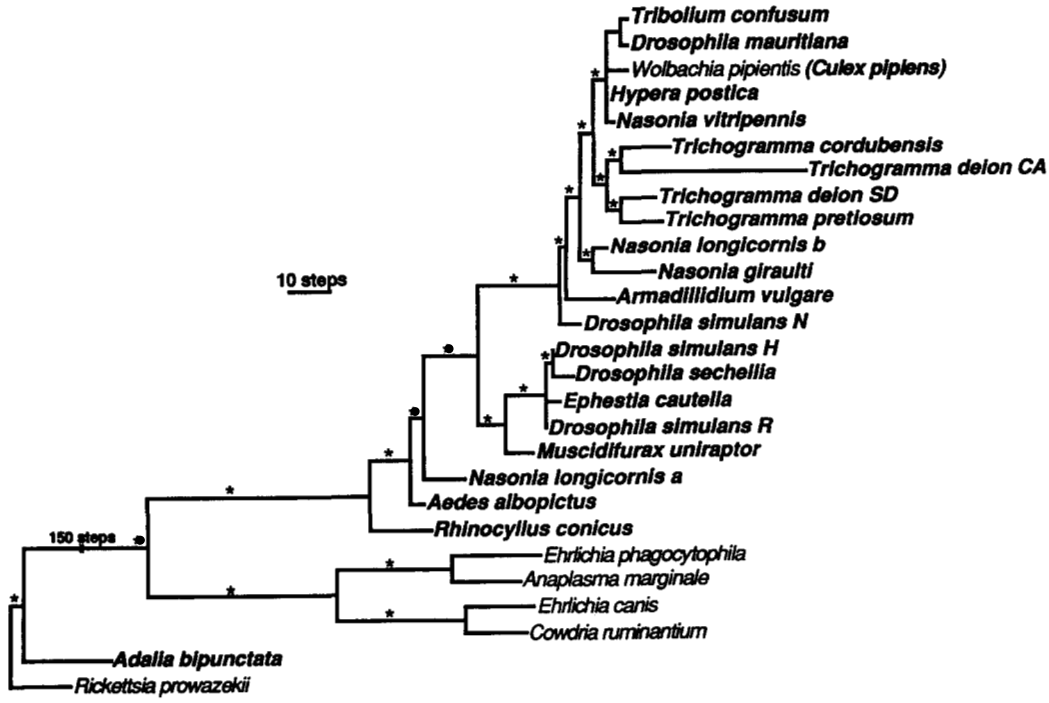

FIGURE 1.—One of 36 most parsimonious trees 853 steps long (retention index = 0.837, CI excluding uniformative characters = 0.726) showing phylogenetic relationships between Wolbachia from diverse arthropod hosts (hosts indicated by italics). *Rickettsia prowazekii* was used as the outgroup. *, branches supported by a strict consensus tree. Names of the arthropod hosts are in bold.

Typically, cytoplasmic incompatibility is expressed when infected males are mated to uninfected females. The reciprocal cross is usually fully compatible. When young 4–5-day-old virgin *D. sechellia* males and females of differing infection status were mated (Table 2), the potentially incompatible cross of infected males mated with uninfected females showed a significant reduction in egg hatch (26.8 ± 3.2%, n = 20; mean ± SE) when compared to the control cross between uninfected flies (96.0 ± 0.7%, n = 20; Mann-Whitney U test, $P < 0.0001$). When a subset of the infected and uninfected males were allowed to age 19–20 days and then mated to 4-to-5-day-old virgin females, the degree of incompatibility was drastically reduced and no significant decline in hatching was observed (72.7 ± 5.9%, n = 15; 87.8 ± 2.8%, n = 14; Table 2, experiment b).

In contrast, when similar crosses were performed with *D. mauritiana* strains quite different results were obtained. Table 3 shows the mean percent egg hatch from crossing two different infected strains of *D. mauritiana* with their respective tetracycline-treated stocks. Surprisingly, no significant difference in mean percent egg hatch was seen when crossing infected and uninfected males of either the *D. mauritiana* Synthetic (87.6 ± 2.7%, n = 36; 94.0 ± 1.1%, n = 36) or *D. mauritiana* MS18 strain (75.7 ± 4.8%, n = 23; 84.5 ± 3.9%, n = 19; Table 3, a and b) to tetracycline-cured females of the same strains. The lack of any discernible incompatibility expression raises questions as to how these bacteria are able to maintain themselves within the host population. To test whether presence of the bacteria affects the fecundity of *D. mauritiana*, a comparison of the number of eggs laid by treated and untreated flies of the Synthetic strain was conducted. During a 7-day period, infected females produced a mean of 153.3 ± 6.1 eggs (n = 68). Uninfected females produced a mean of 164.2 ± 6.9 (n = 60). A one-tailed t-test showed no significant difference between the mean of number of eggs laid by uninfected or infected flies ($t = -1.37$, d.f. = 126, $P = 0.17$).

Relative contributions of host and bacteria to incompatibility expression: To examine the relative contributions of the host to the observed differences in incompatibility expression between different species, we used microinjection techniques to transfer different Wolbachia strains into a common *D. simulans* genetic background. The naturally uninfected *D. simulans* Watsonville (DSW) strain was used to establish two stocks, one injected with cytoplasm from *D. simulans* Riverside (DSR), an infected strain which produces strong incompatibility, and the other with *D. mauritiana* Synthetic (Mau). The resulting *trans*-infected Drosophila strains are denoted as DSW(DSR) and DSW(Mau). Successful transfer of the Wolbachia infection in each case was monitored by PCR and DAPI staining. Once *trans*-infected lines were established, test crosses were performed with the uninfected DSW parental line as well as between the two *trans*-infected lines (Table 4). Crosses between DSW(DSR) males and DSW females resulted in greatly reduced egg hatch when compared

TABLE 3

Percent egg hatch from two different strains of *D. mauritiana*

♀	♂	*N*	Number of eggs	Egg hatch (%)
		a. *D. mauritiana* Synthetic		
D. mauritiana T	× *D. mauritiana*	36	3261	87.6 ± 2.7
D. mauritiana T	× *D. mauritiana* T	39	3902	94.0 ± 1.1
D. mauritiana	× *D. mauritiana* T	35	2679	85.8 ± 3.1
D. mauritiana	× *D. mauritiana*	35	3062	78.2 ± 4.6
		b. *D. mauritiana* MS18		
D. mauritiana MS18 T	× *D. mauritiana* MS18	23	1755	75.7 ± 4.8
D. mauritiana MS18 T	× *D. mauritiana* MS18 T	19	1098	84.5 ± 3.9
D. mauritiana MS18	× *D. mauritiana* MS18 T	22	1512	85.1 ± 2.6
D. mauritiana MS18	× *D. mauritiana* MS18	19	1512	79.0 ± 4.0

Comparison of each pair of crosses using the Mann-Whitney U test. None of the pairs showed any significant difference. Egg-hatch values are means ± SE.

with DSW control crosses (comparison c *vs.* d, Table 4). The degree of incompatibility expression was equivalent to what is typically seen in standard DSR incompatible crosses (HOFFMANN *et al.* 1986). However, the equivalent cross using the *trans*-infected DSW(Mau) flies showed no significant reduction in egg hatch (comparison e *vs.* d, Table 4). In addition, when males of the stock DSW(Mau) were crossed with DSR and DSH females (comparisons l *vs.* o and m *vs.* n in Table 4), no incompatibility was seen. However, reciprocal crosses of DSW(DSR), DSR, and DSH males crossed with DSW(Mau) females (comparisons h *vs.* g, i *vs.* g, and j *vs.* g in Table 4) all showed strong incompatibility. This unidirectional pattern is characteristic of what is observed when infected and uninfected strains are crossed, and in this case the DSW(Mau) strain behaved like an uninfected strain.

To determine if *D. mauritiana* is capable of expressing cytoplasmic incompatibility a tetracycline-treated stock of *D. mauritiana*, whose infection status was verified using PCR and DAPI staining, was *trans*-infected with Wolbachia from the strong incompatibility line of DSR. Cured *D. mauritiana* Synthetic flies were injected with cytoplasm from DSR, crossed with females of the original tetracycline-treated stock, and when compared with the cured strain, showed strong cytoplasmic incompatibility expression (comparison f *vs.* g, Table 5). In addition, when *trans*-infected males were crossed with

TABLE 4

Percent egg hatch from crosses using DSW injected with the cytoplasm of DSR and Mau

♀	♂	Cross	*N*	Number of eggs	Egg hatch (%)	Comparison	Significance
DSW(DSR)	× DSW(DSR)	a	21	4243	88.3 ± 3.4		
DSW(DSR)	× DSW	b	23	4516	88.3 ± 2.7	b *vs.* a	NS
DSW	× DSW(DSR)	c	18	3596	8.6 ± 1.3	c *vs.* d	$P < 0.0001$
DSW	× DSW	d	22	5187	94.8 ± 1.4		
DSW	× DSW(Mau)	e	19	4039	94.1 ± 0.8	e *vs.* d	NS
DSW(Mau)	× DSW	f	22	4467	88.1 ± 2.3	f *vs.* g	NS
DSW(Mau)	× DSW(Mau)	g	21	4155	85.3 ± 2.1		
DSW(Mau)	× DSW(DSR)	h	23	4399	12.7 ± 1.5	h *vs.* g	$P < 0.0001$
DSW(Mau)	× DSR	i	21	3804	11.8 ± 1.5	i *vs.* g	$P < 0.0001$
DSW(Mau)	× DSH	j	23	4819	11.4 ± 2.6	j *vs.* g	$P < 0.0001$
DSW(DSR)	× DSW(Mau)	k	22	4463	88.1 ± 2.3	k *vs.* a	NS
DSR	× DSW(Mau)	l	21	3469	93.2 ± 2.1	l *vs.* o	NS
DSH	× DSW(Mau)	m	22	3195	89.96 ± 3.0	m *vs.* n	NS
DSH	× DSH	n	19	2935	92.70 ± 3.5		
DSR	× DSR	o	20	3010	91.79 ± 3.1	o *vs.* l	NS
DSH	× DSR	p	18	2796	7.39 ± 1.1		
DSR	× DSH	q	19	2650	4.36 ± 0.7		

The nomenclature is as follows: *e.g.*, DSW(DSR) indicates a strain of DSW injected with the bacteria from DSR. DSW, *D. simulans* Watsonville; DSR, *D. Simulans* Riverside; Mau, *D. mauritiana*. Comparison of each pair of crosses using the Mann-Whitney U test. Egg-hatch values are means ± SE.

TABLE 5

Percent egg hatch from *D. mauritiana* Synthetic injected with DSR

♀	♂	Cross	*N*	Number of eggs	Egg hatch (%)	Comparison	Significance
D. mauritiana	× *D. mauritiana*	a	18	3044	67.5 ± 5.9	a *vs.* b	$P < 0.0001$
D. mauritiana	× *D. mauritiana* T(DSR)	b	21	3726	1.9 ± 0.6		
D. mauritiana T (DSR)	× *D. mauritiana*	c	22	3721	61.4 ± 5.6		
D. mauritiana T (DSR)	× *D. mauritiana* T (DSR)	d	22	4051	79.9 ± 3.3	d *vs.* c	NS
D. mauritiana T (DSR)	× *D. mauritiana* T	e	21	4123	72.4 ± 4.2	e *vs.* d	NS
D. mauritiana T	× *D. mauritiana* T (DSR)	f	20	3371	2.5 ± 0.9	f *vs.* g	$P < 0.0001$
D. mauritiana T	× *D. mauritiana* T	g	17	2933	78.9 ± 4.2		

D. mauritiana T (DSR) indicates a strain that was treated with tetracycline and injected with cytoplasm from DSR. Crosses were done two generations after injection. Comparison of each pair of crosses using the Mann-Whitney U test. Egg-hatch values are means ± SE.

naturally infected *D. mauritiana* females, strong incompatibility expression was also seen (comparison a *vs.* b), but not in the reciprocal cross (comparison a *vs.* c, Table 5). Whether males from *D. mauritiana* Tet (DSR) strain were crossed with female *D. mauritiana* that were naturally infected or uninfected, a similar decreased egg hatch resulted. Therefore, it again appeared that the naturally infected *D. mauritiana* flies behaved phenotypically as if uninfected. Furthermore, the *trans*-infected *D. mauritiana* Tet (DSR) isofemale line used in the experiments reported in Table 5 was kept in the laboratory for ~11 generations and when crossed again yielded similar results (Table 6).

Bacterial densities: BOYLE *et al.* (1993), BREEUWER and WERREN (1993), and ROUSSET and DE STORDEUR (1994) have suggested that bacterial density affects the expression of cytoplasmic incompatibility. Therefore we examined the bacterial density of some of the strains used in the crosses above to determine if lack of expression of cytoplasmic incompatibility in *D. mauritiana* correlates with low levels of bacteria. Table 7 shows that the highest densities of bacteria are in embryos of DSR, DSW(DSR), Mau(DSR), and DSW(Mau) in descending order. The lowest densities of bacteria were found in DSH, *D. sechellia*, and *D. mauritiana* MS18. Tukey's multiple comparison test (ZAR 1984) indicates that there is no significant difference in the following comparisons: DSR and DSW(DSR), Mau(DSR) and DSW(Mau), and DSW(Mau) compared with Mau MS18, *D. sechellia*, and DSH, as well as DSH and *D. sechellia*, DSH and Mau MS18, and *D. sechellia* and *D. mauritiana* MS18. All other comparisons were highly significant, with the exception of the Mau(DSR) and DSH comparison, which had a probability value of 0.05 (Table 7).

DISCUSSION

We have determined that infections of Wolbachia are widespread in stocks of *D. mauritiana* and *D. sechellia*. Phylogenetic analysis of 16S rRNA sequences shows that these bacteria cluster with the other known Wolbachia. The data from the 16S rRNA gene lack resolution because of the small number of informative characters and cannot be used to establish a definite relationship between Wolbachia found in different species of insects, however preliminary indications are that the Wolbachia from the two sister species *D. mauritiana* and *D. sechellia* are more closely related to Wolbachia strains in distantly related species of insects than they are to each other. Taken together with their differences in expression of cytoplasmic incompatibility we conclude that the Wolbachia infecting these two sibling species are distinct strains of the nominal species *Wolbachia pipientis*.

Test crosses between infected and tetracycline-cured strains of *D. sechellia* show that the Wolbachia in this species is capable of inducing partial cytoplasmic incompatibility. The number of unhatched eggs in incompatible crosses in *D. sechellia* appears intermediate be-

TABLE 6

Percent egg hatch from *D. mauritiana* T Synthetic injected with DSR

♀	♂	*N*	Number of egg	Egg hatch (%)	
D. mauritiana T	× *D. mauritiana* T	18	1210	96.0 ± 0.7	$P < 0.0001$
D. mauritiana T	× *D. mauritiana* T (DSR)	19	1435	0.6 ± 0.6	
D. mauritiana T (DSR)	× *D. mauritiana* T (DSR)	20	1619	91.4 ± 3.5	NS
D. mauritiana T (DSR)	× *D. mauritiana* T	19	1339	92.1 ± 3.0	

Crosses with the same stock used in Table 5. Comparison of each pair of crosses using the Mann-Whitney U test. Egg hatch values, taken ~11 generations after injection, are means ± SE.

TABLE 7

Means of relative bacterial densities in embryos of naturally infected and *trans*-infected *Drosophila* strains, followed by an ANOVA of square root transformed means and the *a posteriori* Tukey test

	N	Mean
DSR	21	121.0 ± 8.1
DSW(DSR)	24	117.2 ± 6.6
Mau(DSR)	16	74.9 ± 6.0
DSW(Mau)	12	67.7 ± 7.1
DSH	20	49.6 ± 4.1
Seche	35	45.4 ± 3.3
Mau Ms18	14	42.2 ± 5.5

	DF	Sum of Squares	Mean Square	F	*P*
Strains	6	473.880	79.980	35.360	<0.0001
Residual	135	301.532	2.234		

Strain A	Strain B	$\sqrt{\bar{x}_A + 3/8} - \sqrt{\bar{x}_B + 3/8}$	Observed Q Span 7	*P*
DSR	Mau MS18	4.4	12.3	<0.001
DSR	Seche	4.2	14.5	<0.001
DSR	DSH	3.9	11.9	<0.001
DSR	DSW(Mau)	2.7	7.2	<0.001
DSR	Mau(DSR)	2.3	6.6	<0.001
DSR	DSW(DSR)	0.1	0.5	NS
DSW(DSR)	Mau MS18	4.3	12.1	<0.001
DSW(DSR)	Seche	4.0	14.5	<0.001
DSW(DSR)	DSH	3.7	11.8	<0.001
DSW(DSR)	DSW(Mau)	2.5	6.9	<0.001
DSW(DSR)	Mau(DSR)	2.1	6.3	<0.001
Mau(DSR)	Mau MS18	2.1	5.5	<0.005
Mau(DSR)	Seche	1.9	5.9	<0.001
Mau(DSR)	DSH	1.6	4.5	<0.05
Mau(DSR)	DSW(Mau)	0.4	1.0	NS
DSW(Mau)	Mau MS18	1.7	4.1	NS
DSW(Mau)	Seche	1.4	4.2	NS
DSW(Mau)	DSH	1.1	3.0	NS
DSH	Mau MS18	0.5	1.4	NS
DSH	Seche	0.3	1.0	NS
Seche	Mau MS18	0.2	0.7	NS

tween the strong expression of incompatibility typical of naturally infected *D. simulans* strains (HOFFMANN *et al.* 1986; O'NEILL and KARR 1990; MONTCHAMP-MOREAU *et al.* 1991) and the relatively weak incompatibility described in *D. melanogaster* (HOFFMANN 1988; HOLDEN *et al.* 1993; HOFFMANN *et al.* 1994; SOLIGNAC *et al.* 1994). As in other Wolbachia-mediated incompatibility systems, penetrance of this phenotype in *D. sechellia* is greatly attenuated in aged males. This aging effect is consistent with what has been previously described in *D. simulans* (HOFFMANN *et al.* 1986, 1990) and seems likely to be due to sperm that mature in spermatocysts devoid of bacteria (BRESSAC and ROUSSET 1993).

We did not find expression of cytoplasmic incompatibility in two different strains of naturally infected *D. mauritiana* (Table 3) despite the presence of Wolbachia in embryos at density levels not significantly different from those observed in *D. sechellia* and DSH. To investigate the degree of influence of the host and bacterial genome on the expression of cytoplasmic incompatibility in *D. simulans* and *D. mauritiana*, we first transferred bacteria from DSR into DSW where they induced cytoplasmic incompatibility in this naturally uninfected strain. In previous experiments of this kind, Wolbachia were transferred back into tetracycline-cured stocks of the originating infected strains (BOYLE *et al.* 1993). We therefore conclude that DSW is not resistant to infection with Wolbachia and is permissive for the expression of cytoplasmic incompatibility. When DSW was then infected with Wolbachia from *D. mauritiana*, however, we saw no expression of incompatibility, indicating that these bacteria are not capable of inducing cytoplasmic incompatibility either in their natural or in this particular transinfected host. In a reciprocal experiment, introduction of DSR bacteria into tetracycline-cured *D. mauritiana* resulted in strains that were capable of inducing strong incompatibility. Together these results indicate that the genetic and cytoplasmic environments of *D.*

simulans and *D. mauritiana* are similar and permissive with respect to the expression of cytoplasmic incompatibility but that the *D. mauritiana* bacteria cannot cause cytoplasmic incompatibility in either species of Drosophila. In addition, the strain of Wolbachia from *D. mauritiana* is unable to rescue the phenotype in females, as demonstrated in the incompatible cross of DSW females *trans*-infected with *D. mauritiana* bacteria mated to DSW males *trans*-infected with DSR bacteria (Table 4). As such, the *D. mauritiana* infection appears to be neutral with regard to expression of cytoplasmic incompatibility, suggesting the presence of bacterial genes responsible for the expression of incompatibility. The existence of a neutral variant will facilitate the isolation of such genes.

BOYLE *et al.* (1993) suggested that in *D. melanogaster*, a threshold infection density is needed for expression of incompatibility, and BREEUWER and WERREN (1993) have also shown that low bacterial densities can reduce the expression of incompatibility in wasps. We therefore examined the strains used herein for density of Wolbachia. Our data (Table 7) indicate that although density levels do differ between some of the strains, the absence of cytoplasmic incompatibility and rescue in strains with the *D. mauritiana* Wolbachia cannot entirely be explained by low density. The density of bacteria in the original *D. mauritiana* strain is relatively low, and the density of these Wolbachia in DSW is about half that of the DSR Wolbachia in DSW. While these differences might contribute to lowered expression of cytoplasmic incompatibility by the bacteria native to *D. mauritiana*, other comparisons indicate that it is unlikely that such a simple relationship to density is the cause of the absence of cytoplasmic incompatibility in this strain of Wolbachia. For example, the Wolbachia strain in DSH shows strong incompatibility with levels of bacterial density comparable to those found in *D. mauritiana* and DSR (Mau). Conversely, when the DSR Wolbachia were introduced into *D. mauritiana*, their density was considerably lower than in DSR, yet they caused strong cytoplasmic incompatibility. Furthermore, there is no significant difference in bacterial density between Mau (DSR), which shows high levels of incompatibility, and DSW(Mau), which does not. An alternative method for examining densities that is unfortunately not quantitative, but would be more directly relevant to levels of cytoplasmic incompatibility, is staining of spermatocysts (BRESSAC and ROUSSET 1993). When examined this way, *D. mauritiana* MS18, *D. sechellia*, and *D. simulans* Hawaii again show low levels of infection, while Mau (DSR) and DSR are highly infected and are indistinguishable (R. GIORDANO, unpublished results). Thus again the *D. mauritiana* infection has densities comparable to the DSH infection, yet does not cause cytoplasmic incompatibility. While these comparisons inevitably involve different strains of bacteria in different host species and strains, it seems clear that low density of infection by the Wolbachia strain in *D. mauritiana* cannot alone explain our results. We therefore conclude that the Wolbachia from *D. mauritiana* are genetically distinct from those that can cause cytoplasmic incompatibility, perhaps in having lost whatever genetic factors are responsible for the expression and rescue of cytoplasmic incompatibility.

The absence of incompatibility in *D. mauritiana* was consistently seen in Synthetic and MS18 strains, and has been observed independently (F. ROUSSET, personal communication). Infections that do not cause cytoplasmic incompatibility have also been reported from *D. melanogaster* lab stocks (HOLDEN *et al.* 1993), while recently wild-caught strains of this species usually show weak incompatibility (HOFFMANN 1988; HOLDEN *et al.* 1993; BOURTZIS *et al.* 1994; HOFFMANN *et al.* 1994; but see SOLIGNAC *et al.* 1994 for a strain showing considerable cytoplasmic incompatibility). Absence of incompatibility in infected flies has also recently been observed with field populations of *D. simulans* in Australia, USA, and Ecuador (A. A. HOFFMANN and M. TURELLI, personal communication). The lack of an incompatibility phenotype raises the question of how these infections initially spread and how they are retained within populations. This observation is particularly puzzling because Wolbachia infections in *D. simulans* are known to confer a slight fitness cost to the host as well as being imperfectly transovarially transmitted (HOFFMANN *et al.* 1990; STEVENS and WADE 1990). Therefore, without the action of cytoplasmic incompatibility and from our current understanding of how this agent interacts with its host, it would be expected that these infections would be unable either to invade a host population or to maintain themselves. One possibility that might explain how the infections initially spread into the species is that the presently neutral bacteria "hitchhiked" into the population during a cytoplasmic sweep induced by an additional superinfecting Wolbachia strain, which has subsequently been lost. These neutral infections could maintain themselves in a population if they are actually beneficial to the host that carries them. We performed experiments that examined total egg production from infected and uninfected individuals of *D. mauritiana* but could not detect any significant difference in the number of eggs produced, indicating that the bacteria seem to have no detectable effect on fecundity in this species. Another possibility is that Wolbachia are retained as a result of their close association with the spindle apparatus of nuclei during division (CALLAINI *et al.* 1994). This association would ensure their partitioning to all cells and most importantly the germline. We know that the infection has been present in wild populations of *D. mauritiana* at least from 1979 to 1987 because collections made by A. FUKATAMI in both of those years show the presence of infected lines. The lack of detectable deleterious fitness effects together with possible high rates of transovarial transmis-

sion may mean that the infection is being lost quite slowly or not at all. Another possibility is that selection pressures on the symbiont have favored an attenuated form of the bacteria (PROUT 1994; TURELLI 1995). Clearly additional data relating to rates of horizontal and vertical transmission are needed to understand better the dynamics of these infections.

Our finding that expression of incompatibility in *D. simulans* or lack thereof in *D. mauritiana* is a consequence of the bacterial strain that infects them does not concur with the findings of BOYLE *et al.* (1993), who concluded that in *D. melanogaster* host factors mediate the expression of cytoplasmic incompatibility. It is possible that due to their closer phylogenetic relationship (*e.g.*, SCHLÖTTERER *et al.* 1994), the effect of a given Wolbachia strain would be similar in *D. simulans* and *D. mauritiana* which together with *D. sechellia* form a sister group and are distinct from the more distantly related *D. melanogaster*. TURELLI (1995) has suggested that levels of incompatibility could vary from one species to another as a result of differences in fidelity of bacterial trasmission and in costs associated with infection, while HOFFMANN *et al.* (1994) conclude that the present relationship between *D. melanogaster* and its symbiont is a result of selection on the nuclear genome of the host. Thus it seems that differences exist in the manner in which strains of *D. melanogaster*, *D. simulans* and *D. mauritiana* interact with Wolbachia, which may in part reflect the length of time they have been associated.

We thank L. BOYLE and D. LAMPE for help with microinjections; M. SHARKEY, K. ZUMPANO and P. WHITE for technical assistance; A. W. GHENT and A. MANNELLI for discussions on statistics; and F. SOTO-ADAMES, J. NARDI, J. WERREN and an anonymous reviewer for comments on the manuscript. Our appreciation to the individuals who sent the Drosophila stocks: J. COYNE, J. DAVID, C. LAURIE, R. LYMAN, T. LYTTLE, T. WATANABE, as well as P. OSTER, J. ROOTE and K. MATTHEWS at the Bowling Green, Cambridge and Indiana University Drosophila stock centers. Sequencing employed the facilities of the Center for Molecular Phylogeny at the University of Illinois. This work was supported by USDA grant 91-37302-6766, National Institute of Allergy and Infectious Disease grant AI-34355, and funds from the Research Board at the University of Illinois.

LITERATURE CITED

ANDERSON, B. E., J. E. DAWSON, D. C. JONES and K. H. WILSON, 1991 *Ehrlichia chaffeensis* a new species associated with human ehrlichiosis. J. Clin. Microbiology **29:** 2838–2842.

BOURTZIS, K., A. NIRGIANAKI, P. ONYANGO and C. SAVAKIS, 1994 A prokaryotic dnaA sequence in *Drosophila melanogaster*: *Wolbachia* infection and cytoplasmic ncompatibility among laboratory strains. Insect Mol. Biol. **3:** 131–142.

BOYLE, L., S. L. O'NEILL, H. M. ROBERTSON and T. L. KARR, 1993 Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. Science **260:** 1796–1799.

BRAIG, H. R., H. GUZMAN, R. B. TESH and S. L. O'NEILL, 1994 Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. Nature **367:** 453–455.

BRESSAC, C., and F. ROUSSET, 1993 The reproductive incompatibility system in *Drosophila simulans*: DAPI-staining analysis of the *Wolbachia* symbiont in sperm cysts. J. Invertebr. Pathol. **61:** 226–230.

BREEUWER, J. A. J., and J. H. WERREN, 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature **346:** 558–560.

BREEUWER, J. A. J., and J. H. WERREN, 1993 Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. Genetics **135:** 565–574.

BREEUWER, J. A. J., R. STOUTHAMER, S. M. BARNS, D. A. PELLETIER, W. G. WEISBURG *et al.*, 1992 Phylogeny of cytoplasmic incompatibility micro-organisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. Insect Mol. Biol. **1:** 25–36.

CALLAINI, G., M. G. RIPARBELLI and R. DALLAI, 1994 The distribution of cytoplasmic bacteria in the early Drosophila embryo is mediated by astral microtubules. J. Cell Sci. **107:** 673–682.

CAMPBELL, B. C., T. S. B. RAGG and C. TURNER, 1992 Phylogeny of symbiotic bacteria of four weevil species (Curculionidae: Coleoptera) based on analysis of ribosomal DNA. Insect Biochem. Mol. Biol. **22:** 415–421.

CONNER, G. W., and G. B. SAUL, 1986 Aquisition of incompatibility by inbred wild type stocks of *Mormoniella*. J. Hered. **77:** 211–213.

DAME, J. B., S. M. MAHAN and C. A. YOWELL, 1992 Phylogenetic relationship of *Cowdria ruminantium*, agent of heartwater, to *Anaplasma marginale* and other members of the order Rickettsiales determined on the basis of 16S rRNA sequence. Int. J. Syst. Bacteriol. **42:** 270–274.

GOTTLIEB, F. J., 1972 A cytoplasmic symbiont of *Ephestia kuehniella*: location and morphology. J. Invertebr. Pathol. **20:** 351–355.

HERTIG, M., 1936 The Rickettsia, *Wolbachia pipientis* and associated inclusions of the mosquito, *Culex pipiens*. Parasitology **28:** 453–490.

HOFFMANN, A. A., 1988 Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. Entomol. Exp. Appl. **48:** 61–67.

HOFFMANN, A. A., M. TURELLI and G. M. SIMMONS, 1986 Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution **40:** 692–701.

HOFFMANN, A. A., M. TURELLI and L. G. HARSHMAN, 1990 Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. Genetics **126:** 933–948.

HOFFMANN, A. A., D. J. CLANCY and E. MERTON, 1994 Cytoplasmic incompatibility in Australian populations of *Drosophila melanogaster*. Genetics **136:** 993–999.

HOLDEN, P. R., P. JONES and J. F. Y. BROOKFIELD, 1993 Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster*. Genet. Res. Camb. **62:** 23–29.

HSIAO, C., and T. H. HSIAO, 1985 Rickettsia as the cause of cytoplasmic incompatibility in the Alfalfa Weevil, *Hypera postica*. J. Invertebr. Pathol. **45:** 244–246.

JUCHAULT, P., M. FRELON, D. BOUCHON and T. RIGAUD, 1994 New evidence for feminizing bacteria in terrestrial isopods: evolutionary implications. C. R. Acad. Sci. Ser. III Sci. Vie **317:** 225–230.

LAVEN, H., 1951 Crossing experiments with *Culex* strains. Evolution **5:** 370–375.

MONTCHAMP-MOREAU, C., J. F. FERVEUR and M. JACQUES, 1991 Geographic distribution and Inheritance of three cytoplasmic incompatibility types in *Drosophila simulans*. Genetics **129:** 399–407.

MORAN, N. A., and P. BAUMANN, 1994 Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends Ecol. Evol. **9:** 15–20.

MORAN, N. A., M. A. MUNSON, P. BAUMANN and H. ISHIKAWA, 1993 A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. R. Soc. London. B Biol. Sci. **253:** 125–129.

NODA, H., 1984 Cytoplasmic incompatibility in allopatric filed populations of the small brown planthopper, *Laodelphax striatellus*, in Japan. Entomol. Exp. Appl. **35:** 263–267.

O'NEILL, S. L., and T. L. KARR, 1990 Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature **348:** 178–180.

O'NEILL, S. L., R. GIORDANO, A. M. E. COLBERT, T. L. KARR and H. M. ROBERTSON, 1992 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA **89:** 2699–2702.

PROUT, T., 1994 Some evolutionary possibilities for a microbe that causes incompatibility in its host. Evolution **48:** 909–911.

REED, K. M., and J. H. WERREN, 1995 Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. Mol. Reprod. Dev. **40:** 408–418.

RICHARDSON, P. M., W. P. HOLMES and G. B. SAUL II, 1987 The effect of

Tetracycline on nonreciprocal cross incompatibility in *Mormoniella* (*Nasonia*) *vitripennis*. J. Invertebr. Pathol. **50:** 176–183.

ROBERTSON, H. M., and E. G. MACLEOD, 1993 Five major subfamilies of mariner transposable elements in insects, including the Mediterranean fruit fly, and related arthropods. Insect Mol. Biol. **2:** 125–139.

ROUSSET, F., and E. DE STORDEUR, 1994 Properties of *Drosophila simulans* strains experimentally infected by different clones of the bacterium *Wolbachia*. Heredity **72:** 325–331.

ROUSSET, F., D. VAUTRIN and M. SOLIGNAC, 1992a Molecular identification of *Wolbachia*, the agent of cytoplasmic incompatibility in *Drosophila simulans*, and variability in relation with host mitochondrial types. Proc. R. Soc. Lond. B Biol. Sci. **247:** 163–168.

ROUSSET, F., D. BOUCHON, B. PINTUREAU, P. JUCHAULT and M. SOLIGNAC, 1992b *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. B Biol. Sci. **250:** 91–98.

RYAN, S. L., and G. B. SAUL II, 1968 Post fertilization effect of incompatibility factors in *Mormoniella*. Mol. Gen. Genet. **103:** 29–36.

SCHLÖTTERER, C., M. HAUSER, A. VON HAESELER and D. TAUTZ, 1994 Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. Mol. Biol. Evol. **11:** 513–522.

SINGH, K. R. P., C. F. CURTIS and B. S. KRISHNAMURTY, 1976 Partial loss of cytoplasmic incompatibility with age in males of *Culex fatigans*. Ann. Trop. Med. Parasitol. **70:** 463–466.

SOLIGNAC, M., D. VAUTRIN and F. ROUSSET, 1994 Widespread occurrence of the proteobacteria *Wolbachia* and partial cytoplasmic incompatibility in *Drosophila melanogaster*. C. R. Acad. Sci. Ser. III Sci. Vie **317:** 461–470.

STEVENS, L., and M. J. WADE, 1990 Cytoplasmically inherited reproductive incompatibility in Tribolium flour beetles: the rate of spread and effect on population size. Genetics **124:** 367–372.

STOUTHAMER, R., R. F. LUCK and W. D. HAMILTON, 1990 Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. Proc. Natl. Acad. Sci. USA **87:** 2424–2427.

STOUTHAMER, R., J. A. J. BREEUWER, R. F. LUCK and J. H. WERREN, 1993 Molecular identification of microorganisms associated with parthenogenesis. Nature **361:** 66–68.

SWOFFORD, D. L., 1993 *PAUP: phylogenetic analysis using parsimony*, Version 3.1.1. Illinois Natural History Survey. Champaign, IL.

TRPIS, M., J. B. PERRONE, M. REISSIG and K. L. PARKER, 1981 Control of cytoplamic incompatibility in the *Aedes scutellaris* complex. J. Hered. **72:** 313–317.

TURELLI, M., 1995 Evolution of incompatibility-inducing microbes and their hosts. Evolution. (In press).

WADE, M. J., and L. STEVENS, 1985 Microorganism mediated reproductive isolation in flour beetles (Genus *Tribolium*). Science **227:** 527–528.

WEISBURG, W. G., M. E. DOBSON, J. E. SAMUEL, G. A. DASCH, L. P. MALLAVIA *et al.*, 1989 Phylogenetic diversity of the Rickettsia. J. Bacteriol. **171:** 4202–4206.

WEISBURG, W. G., S. M. BARNS, D. A. PELLETIER and D. J. LANE, 1991 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:** 697–703.

WERREN, J. H, G. D. D. HURST, W. ZHANG, J. A. J. BREEUWER, R. STOUTHAMER *et al.*, 1994 Rickettsial relative associated with male killing in the Ladybird Beetle (*Adalia bipunctata*). J. Bacteriol. **176:** 388–394.

WRIGHT, J. H., and A. R. BARR, 1981 *Wolbachia* and the normal and incompatible eggs of *Aedes polynensiensis*. J. Invertebr. Pathol. **38:** 409–418.

YEN, J. H., and A. R. BARR, 1973 The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. J. Invertebr. Pathol. **22:** 242–250.

YEN, J. H., and A. R. BARR, 1974 Incompatibility in *Culex pipiens*, pp. 97–118 in *The Use of Genetics in Insect Control*, edited by R. PAL and M. J. WHITTEN. Elsevier, Amsterdam, Netherlands.

ZAR, J. H., 1984 *Biostatistical Analysis*, Ed. 2. Prentice Hall, NJ.

Communicating editor: R. D. DENELL