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WOLBACHIA AND THE EVOLUTION OF REPRODUCTIVE ISOLATION BETWEEN *DROSOPHILA RECENS* AND *DROSOPHILA SUBQUINARIA*

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Abstract.—Endosymbiotic bacteria of the genus *Wolbachia* are widespread among insects and in many cases cause cytoplasmic incompatibility in crosses between infected males and uninfected females. Such findings have been used to argue that *Wolbachia* have played an important role in insect speciation. Theoretical models, however, indicate that *Wolbachia* alone are unlikely to lead to stable reproductive isolation between two formerly conspecific populations. Here we analyze the components of reproductive isolation between *Drosophila recens*, which is infected with *Wolbachia*, and its uninfected sister species *Drosophila subquinaria*. Laboratory pairings demonstrated that gene flow via matings between *D. recens* females and *D. subquinaria* males is hindered by behavioral isolation. Matings readily occurred in the reciprocal cross (*D. quinaria* females × *D. recens* males), but very few viable progeny were produced. The production of viable hybrids via this route was restored by antibiotic curing of *D. recens* of their *Wolbachia* symbionts, indicating that hybrid offspring production is greatly reduced by cytoplasmic incompatibility in the crosses involving infected *D. recens* males. Thus, behavioral isolation and *Wolbachia*-induced cytoplasmic incompatibility act as complementary asymmetrical isolating mechanisms between these two species. In accordance with Haldane's rule, hybrid females were fertile, whereas hybrid males invariably were sterile. Levels of mtDNA variation in *D. recens* are much lower than in either *D. subquinaria* or *D. falleni*, neither of which is infected with *Wolbachia*. The low haplotype diversity in *D. recens* is likely due to an mtDNA sweep associated with the spread of *Wolbachia*. Nevertheless, the existence of several mtDNA haplotypes in this species indicates that *Wolbachia* have been present as a potential isolating mechanism for a substantial period of evolutionary time. Finally, we argue that although *Wolbachia* by themselves are unlikely to bring about speciation, they can increase the rate of speciation in insects.

Key words.—Behavioral isolation, cytoplasmic incompatibility, *Drosophila*, gene flow, Haldane's rule, mtDNA, nucleotide diversity, reproductive isolation, speciation, *Wolbachia*.

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Wolbachia are maternally-transmitted alpha-proteobacteria that infect the reproductive tissues of arthropods and whose transmission is enhanced by a variety of mechanisms, including cytoplasmic incompatibility, thelytokous parthenogenesis, feminization of genetic males, and increased mating success of infected males via sperm competition (Breeuwer et al. 1992; O'Neill et al. 1992; Beard et al. 1993; Stouthamer et al. 1993; Sinkins et al. 1995; Wade and Chang 1995; for recent reviews, see Werren et al. 1995a,b; Werren and O'Neill 1997; Werren 1997a,b). The most commonly described phenotypic effect of *Wolbachia* in *Drosophila* is unidirectional cytoplasmic incompatibility (CI), which occurs when an uninfected female mates with an infected male (Hoffmann et al. 1986; Breeuwer and Werren 1990). Such matings generally result in few or no progeny because of abortive karyogamy—the condensation of paternal chromosomes in the developing embryo (Breeuwer and Werren 1990; Callaini et al. 1996; Lassy and Karr 1996). Because all other matings result in normal progeny numbers, the net effect of CI is to reduce offspring production of uninfected females compared to infected females (Hoffmann et al. 1986; Turelli 1994; Hoffmann and Turelli 1998). This reproductive advantage to infected females results in the spread of *Wolbachia* through a population (Caspari and Watson 1959; Fine 1978; Turelli and Hoffmann 1991; Turelli 1994).

Wolbachia also are known to cause bidirectional incompatibility in several arthropod species (Breeuwer and Werren 1990; O'Neill and Karr 1990). Bidirectional incompatibility occurs because of the presence of more than one strain of *Wolbachia* within a species or between hybridizing species and is expressed when individuals carrying different strains mate (Breeuwer and Werren 1990; O'Neill and Karr 1990; Clancy and Hoffmann 1996; Perrot-Minnot et al. 1996; Werren 1997a).

Recently, there has been considerable interest in these microbes because of their potential role in the speciation process (Werren 1997b). In particular, cytoplasmic incompatibility induced by *Wolbachia* may be important in preventing gene flow between incipient species in two ways. First, bidirectional CI may directly prevent introgression between populations or species harboring different strains of *Wolbachia* (Werren 1997b). Such bidirectional CI has been demonstrated between the two wasps *Nasonia vitripennis* and *N. giraulti* (Breeuwer and Werren 1990). Second, *Wolbachia*-induced CI may contribute to reproductive isolation by serving as one of several reproductive isolating mechanisms, which together prevent or greatly restrict interspecific gene flow (Werren 1997b).

In the present paper we examine the potential role of *Wolbachia* in reproductive isolation by this latter mechanism, that is, by acting as one of several barriers to gene flow between two species of *Drosophila* flies: *D. recens*, which is infected with *Wolbachia*, and *D. subquinaria*, which is not. Both of these species breed primarily in mushrooms in the cooler regions of North America. *Drosophila recens* inhabits much of the northeastern United States, but has been found as far west as North Dakota. Most records of *D. subquinaria*

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come from the Pacific Northwest and Rocky Mountain states. However, this species has been found as far east as Sault Ste. Marie, Ontario (Wheeler 1960). Thus, the two species may be sympatric over a broad range across the northern United States and southern Canada. The overlapping geographical ranges and the use by both species of mushrooms as mating and oviposition sites suggest that breeding individuals of these species probably encounter each other frequently in natural populations. Because molecular data indicate that *D. recens* and *D. subquinaria* have diverged recently (G. Spicer, pers. comm.), they are appropriate for examining the role of *Wolbachia*-induced unidirectional CI in contributing to reproductive isolation between species. Below we present data from interspecific crossing experiments that support the hypothesis that unidirectional CI can act as one of a set of isolating mechanisms between species.

Because *Wolbachia* are maternally transmitted via the cytoplasm, all maternally inherited organelles, including mitochondria, that were associated with the initially infected female will spread by hitchhiking in association with a successful *Wolbachia* strain (Turelli and Hoffmann 1991, 1995; Solignac et al. 1994; Turelli 1994; Hoffmann and Turelli 1998). Thus, the particular mtDNA haplotype (and mutational derivatives) associated with such a strain will sweep through the population as the *Wolbachia* infection spreads (Caspari and Watson 1959; Fine 1978; Turelli and Hoffmann 1991; Prout 1994; Turelli 1994). This prediction can be tested by comparing levels of mtDNA variation between infected and uninfected populations or closely related species (Turelli and Hoffmann 1991; Turelli 1994; Ballard et al. 1996; Hoffmann and Turelli 1998). Here we contrast haplotype diversity in *D. recens* with that in *D. subquinaria* and another ecologically similar, uninfected species from the *quinaria* group, *D. falleni*.

Finally, because a *Wolbachia*-associated sweep of a given mtDNA haplotype essentially resets the molecular clock, sequence variation among the extant mtDNA haplotypes represents mutations that have arisen since the initial *Wolbachia* sweep. The data we present on mtDNA diversity within *D. recens* indicates that *Wolbachia* have been associated with this species over substantial evolutionary time periods and is not the result of a recent invasion, as in *D. simulans* (Turelli and Hoffmann 1991).

MATERIALS AND METHODS

Reproductive Isolation

The culture of *D. subquinaria* was established from 10 male and 10 female flies collected in Seattle, Washington, in the summer of 1995 by R. Huey. The *D. recens* strain was established from a previously inseminated wild-caught female collected in 1993 in the White Mountains of New Hampshire. These flies had been maintained in the laboratory at 22°C, using three vials per strain, which were intercrossed each generation to reduce inbreeding. The White Mountain strain of *D. recens* was found by polymerase chain reaction (PCR) assay to be infected with *Wolbachia* (Werren and Jaenike 1995). An uninfected strain of *D. recens* was obtained in 1994 by rearing larvae of the White Mountain strain on tetracycline-treated mushrooms, as described in Werren and Jaenike (1995). CI was evident in crosses between females of the uninfected strain and males of the infected strain (Werren and Jaenike 1995). The two strains of *D. recens*, designated *recens*^{W} (infected with *Wolbachia*) and *recens*^{O} (cured of *Wolbachia*), were used in the crosses described below.

In March 1996, newly emerged adults of *D. recens*^{W} and *D. subquinaria* were collected as virgins and separated by sex. They were then aged for one week to allow reproductive maturation. Individual males and females were then placed together (without anesthetization) in a vial containing Instant Drosophila Medium (Carolina Biological Supply) plus a small piece of *Agaricus bisporus* mushroom. The vials were small (1.2 cm × 2.5 cm) and the males quickly found the females and initiated courtship. These pairs were observed for three hours and scored for whether they mated within that period. All crosses were done simultaneously.

To assess the importance of *Wolbachia* in causing postmating reproductive isolation between these two species, reciprocal crosses were set up between *D. subquinaria* and either *D. recens*^{W} or *D. recens*^{O} in November 1995. A total of 20 single-pair crosses were set up for each of the four types of crosses. As in the behavioral isolation experiment, the flies had been aged as virgins for one week before putting them together for mating. The flies were allowed to remain together to improve the chances of mating. All emergent offspring were sexed and counted.

To determine whether factors besides behavioral isolation and cytoplasmic incompatibility might affect gene flow between *D. recens* and *D. subquinaria*, we examined the fertility of the hybrid progeny resulting from the interspecific crosses described in the previous section. Upon emergence, hybrid progeny were separated by sex and aged for one week. They were then backcrossed in single-pair matings to either of the parental species. The number of backcross progeny produced is used as a measure of hybrid fertility.

Although hybrid individuals have a complete chromosome set from each parental species (except the sex chromosomes), progeny resulting from backcrosses to either parental species do not, and this could lead to hybrid breakdown. To test this possibility, the male and female progeny obtained from the backcrosses described above were crossed to either *D. recens* or *D. subquinaria*, and offspring production determined. Twenty single-pair crosses were set up between all combinations of parental species and backcross type.

Wolbachia *and Mitochondrial DNA Variation*

Additional samples of *D. subquinaria* and *D. recens* were collected from several sites in the United States and Canada for screening of *Wolbachia* and mtDNA variation (Table 1). All female *D. recens* that we collected were returned to the laboratory and set up as isofemale lines in vials containing Instant Drosophila Medium plus a small piece of *A. bisporus* mushroom. We also collected and screened individuals of *D. falleni* at the eastern U.S. sites (Table 1). We included this latter species because, like *D. recens* and *D. subquinaria*, it is a member of the *quinaria* species group and because its geographical distribution and breeding site use are similar to those of *D. recens*. Thus, in terms of similarity in ecology and effective population size, *D. falleni* is an appropriate

TABLE 1. Collecting localities and sample sizes for *Drosophila falleni, D. recens,* and *D. subquinaria.* Collection periods indicated in superscripts: 1, June 1995; 2, August 1995; 3, June through August 1995; 4, June 1997.

Site	Species (*n*)		
	D. falleni	*D. recens*	*D. subquinaria*
Mendon Ponds Park, Monroe County, New York (MPP)	30[1]	46[3]	
Big Moose Lake, Herkimer County, New York (BML)	30[1]	31[3]	
Brunswick Pines, Cumberland County, Maine (BP)	30[2]	28[2]	
Peachland, British Columbia (PEK)			34[4]
Port Hardy, British Columbia (PH)			32[4]
Seattle, Washington (SW)			6[4]

standard against which to measure mtDNA variation in *D. recens*, whereas *D. subquinaria* is the best phylogenetic control. *Wolbachia* infections have not been found in *D. falleni* (Werren and Jaenike 1995).

DNA isolation methods followed those of Ross and Shoemaker (1997). DNA from each fly was screened for the presence of *Wolbachia* via the PCR using the primers W1620sF and W1620sR (Werren et al. 1995a,b; Perrot-Minnot et al. 1996; primer sequences available from J. Werren, University of Rochester). These primers specifically and reliably amplify only *Wolbachia* DNA; DNA from the closely related genus *Ehrlichia* are not amplified (J. Werren, unpubl. data). A single individual fly was screened from each *D. recens* isofemale line for the presence of *Wolbachia*. Up to six additional flies (including the wild-caught female) were screened from lines testing negative for *Wolbachia* to increase our confidence that these lines indeed were uninfected.

PCRs were performed in 25-μL volumes containing 10× buffer (GibcoBRL), 0.1 mM of each dNTP, 1.5 mM of magnesium chloride, 25pM of each primer, 1–3 μL of template DNA, and 1.5 units of thermostable (*Taq*) DNA polymerase (Perrot-Minnot et al. 1996). Amplifications were carried out in a thermocycler programmed as follows: 1 min at 94°C for one cycle; 30 sec at 94°C, 1 min at 60°C, and 1 min at 72°C for 35 cycles; 5 min at 72°C for one terminal cycle. Approximately 10 μL of the amplified PCR product was electrophoresed in 2% agarose gels. Bands were visualized by staining gels with ethidium bromide under UV illumination; stained gels were photographed using a camera with 667 Polaroid film.

Total genomic DNA isolated from the same individuals of *D. recens, D. subquinaria,* and *D. falleni* used to screen for *Wolbachia* also was used for assessing mtDNA variation in each species (sample sizes are given in Table 1; Shoemaker and Jaenike 1997). A 4-kb portion of the mitochondrial DNA (mtDNA) genome (including the A + T-rich noncoding region) was PCR amplified using the two conserved primers C1-N-2191 (Nancy) and SR-J-14612 (12sair; for primer sequences, see Simon et al. 1994) and subsequently digested with a number of restriction enzymes. Detailed methods of the PCR reactions, PCR profiles, restriction enzyme digestion procedures, and electrophoresis and scoring of digestion products are given in Shoemaker and Jaenike (1997).

The minimum spanning unrooted network of the most parsimonious genealogical relationships among haplotypes within each species was constructed using the program MINSPNET (Excoffier et al. 1992). We also estimated the average pairwise percent sequence divergence (*d*) between haplotypes for each species using the maximum-likelihood method of Nei and Tajima (1983). Mitochondrial DNA nucleotide diversity within and among populations of all three species (π) was estimated and used to construct a tree of the relationships of all populations as implemented in the program NUCLEODIV (Holsinger and Mason-Gamer 1996).

TABLE 2. Mating frequency of intra- and interspecific crosses between *Drosophila recens and D. subquinaria.*

Cross		Mated within 3 h	Did not mate
Female	Male		
D. subquinaria	*D. recens*	32	23
D. recens	*D. subquinaria*	11	39
D. recens	*D. recens*	38	12
D. subquinaria	*D. subquinaria*	42	8

RESULTS

Reproductive Isolation

The numbers of pairs of flies that mated within the 3-h observation period are shown in Table 2. An analysis of the within-species pairings (*D. recens* female × *D. recens* male versus *D. subquinaria* female × *D. subquinaria* male) does not reveal a significant difference between the species in the probability of copulation ($\chi^2 = 0.56$, df = 1, $P = 0.45$). However, in crosses between species, the probability of copulation in pairings between *D. subquinaria* females and *D. recens* males and was about 3.5 times greater than in the reciprocal direction ($\chi^2 = 12.72$, df = 1, $P < 0.001$).

The mean number of emergent offspring per interspecific cross type is shown in Table 3. All four types of crosses yielded viable hybrid progeny of both sexes. The effect of *Wolbachia* on hybrid viability can be seen by comparing the mean number of progeny sired by *Wolbachia*-infected *D. recens*^{W} males with the number sired by uninfected *D. recens*^{O} males when crossed to *D. subquinaria* females. Considering all crosses, including those yielding no progeny, the mean number of offspring was over 10 times greater in crosses with the uninfected *D. recens*^{O} males than in crosses involving *Wolbachia*-infected *D. recens*^{W} males ($F = 18.62$, df = 1, $P < 0.0001$). For crosses in which mating must have occurred, as evidenced by the appearance of larval progeny, the uninfected *D. recens*^{O} males sired nearly seven times as many offspring as the *D. recens*^{W} males ($F = 10.56$, df = 1, $P = 0.0037$). This latter comparison is likely to underestimate

TABLE 3. Mean number of offspring (± standard error) produced in crosses between *Drosophila recens* and *D. subquinaria*. Sample sizes (number of crosses) indicated in parentheses.

Cross		All crosses	Excluding crosses yielding no offspring
Female	Male		
D. subquinaria	*D. recens*^{W}	3.3 ± 1.7 (20)	6.6 ± 3.2 (10)
D. subquinaria	*D. recens*^{O}	36.1 ± 7.4 (20)	45.1 ± 7.7 (16)
D. recens^W	*D. subquinaria*	13.4 ± 8.1 (20)	53.6 ± 26.6 (5)
D. recens^O	*D. subquinaria*	11.2 ± 5.0 (20)	44.6 ± 10.4 (5)

the degree of cytoplasmic incompatibility because crosses yielding no progeny are excluded. Thus, *Wolbachia* infection clearly has a major effect on offspring production in crosses between *D. recens* males and *D. subquinaria* females.

In crosses between *D. subquinaria* males and either *D. recens*^{W} or *D. recens*^{O} females, there was no significant difference in offspring production, whether one includes all crosses ($F = 0.06$, df = 1, $P = 0.81$) or just those in which mating must have occurred ($F = 0.10$, df = 1, $P = 0.76$). Thus, the infection status of the female had no discernible effect on hybrid offspring production.

Crosses between *D. subquinaria* males and *D. recens* females yielded more offspring than *D. recens*^{W} male × *D. subquinaria* female crosses, but fewer than crosses between *D. recens*^{O} males and *D. subquinaria* females. However, because of the behavioral isolation in *D. subquinaria* male × *D. recens* female pairings, many of these crosses yielded no progeny. When crosses yielding no progeny were excluded from the analysis, the mean offspring production in crosses between *D. subquinaria* males and *D. recens* females was not significantly different from the number produced in crosses between uninfected *D. recens*^{O} males and *D. subquinaria* females. These results show that the presence of *Wolbachia* in males of *D. recens* is a major determinant of hybrid viability in crosses between *D. recens* and *D. subquinaria*.

The mean number of progeny produced in single-pair crosses between *D. recens–D. subquinaria* hybrids and either parental species are shown in Table 4. Not one of the hybrid males sired a single offspring, regardless of the cross type. Dissections of hybrid males revealed that they invariably lacked motile sperm. Hybrid females produced substantial numbers of progeny when backcrossed to males of either parental species, the only exception being crosses between *D. recens* males and hybrid females produced from *D. subquinaria* female × *D. recens* male crosses. Note especially that offspring production was 17 times greater in crosses between *D. recens* males and *Wolbachia* infected hybrid females than in crosses between genetically similar flies involving females that were not infected with *Wolbachia* (Table 4).

The mean number of offspring produced in crosses between either *D. recens* or *D. subquinaria* and backcross progeny are shown in Table 5. It is readily apparent that the female offspring of backcrosses are far more fertile than the males, both in terms of mean number of offspring produced (49.2 vs. 3.4) and the number yielding any offspring at all (54/80 vs. 3/80). The high fertility of the female backcross progeny indicates that there is little if any hybrid breakdown to prevent gene flow between these two species. The low fertility of the corresponding males is probably a manifestation of Haldane's rule, as observed in the F_1 hybrids.

Wolbachia *and Mitochondrial DNA Variation*

Of 105 lines of *D. recens* that were screened, 103 were infected with *Wolbachia*. Both uninfected strains were collected from Brunswick Pines, Cumberland County, Maine. Lack of infection in these two strains was confirmed by screening multiple individuals (as well as the original wild-caught female) and, for one strain, crossing females to infected males. These crosses yielded low offspring numbers, as would be expected if the females were not infected with *Wolbachia*. None of the wild-caught strains of *D. falleni* ($n = 90$) nor *D. subquinaria* ($n = 72$) tested positive for the presence of *Wolbachia*.

TABLE 4. Mean number of offspring (± standard error) produced in crosses between *Drosophila recens–D. subquinaria* hybrids and either parental species. Sample sizes (number of crosses) indicated in parentheses. Hybrids used in crosses are identified as female parent–male parent.

Backcross		Number of offspring
Female	Male	
D. recens–D. subquinaria	*D. recens–D. subquinaria*	0 (11)
D. subquinaria–D. recens	*D. subquinaria–D. recens*	0 (11)
D. recens	*D. recens–D. subquinaria*	0 (20)
D. recens	*D. subquinaria–D. recens*	0 (20)
D. subquinaria	*D. recens–D. subquinaria*	0 (10)*
D. subquinaria	*D. subquinaria–D. recens*	0 (20)
D. recens–D. subquinaria	*D. recens*	97.3 ± 16.8 (10)
D. subquinaria–D. recens	*D. recens*	5.7 ± 3.3 (15)*
D. recens–D. subquinaria	*D. subquinaria*	62.0 ± 8.7 (20)
D. subquinaria–D. recens	*D. subquinaria*	53.8 ± 6.8 (15)

* Crosses between *Wolbachia*-infected males and uninfected females.

TABLE 5. Offspring production in crosses between the progeny of backcrosses and either parental species (n = 20 crosses per category).

Female	Male	Mean number of offspring (± SE)	Number fertile (of 20)
D. recens	backcross to *D. recens* male	0	0
D. subquinaria	backcross to *D. recens* male	0	0
D. recens	backcross to *D. subquinaria* male	13.6 ± 8.8	3
D. subquinaria	backcross to *D. subquinaria* male	0	0
backcross to *D. recens* male	*D. recens*	50.6 ± 6.1	17
backcross to *D. subquinaria* male	*D. recens*	42.7 ± 9.9	10
backcross to *D. recens* male	*D. subquinaria*	53.1 ± 9.5	14
backcross to *D. subquinaria* male	*D. subquinaria*	51.2 ± 9.8	13

The total numbers of mtDNA haplotypes found in *D. recens, D. falleni*, and *D. subquinaria* and the estimated phylogenetic relationships among these haplotypes are shown in Figure 1. We found only nine distinct haplotypes in *D. recens* compared to 31 in *D. falleni* and 34 in *D. subquinaria*. Only two of the nine haplotypes within *D. recens* were found in more than a single individual. In contrast, in both *D. falleni* and *D. subquinaria*, between 20% and 30% of the different haplotypes were found in several populations. Finally, the eight rare haplotypes within *D. recens* differ by a only one or two restriction sites from the single common haplotype 1, whereas the numbers of restriction site differences among the most divergent haplotypes are much greater in *D. falleni* (eight) and *D. subquinaria* (15; Fig. 1). The mtDNA network and the abundance of haplotype 1 suggest that this haplotype was associated with the initial sweep of *Wolbachia* through *D. recens* and that the other haplotypes in this species arose subsequently.

We found consistently lower mtDNA variation in all three populations of *D. recens* compared to populations of *D. falleni* and *D. subquinaria* (Fig. 2). Mitochondrial DNA nucleotide diversity estimates within populations (π) range from 0.27% to 0.59% in *D. falleni* and from 0.44% to 0.99% in *D. subquinaria*, but only from 0.06% to 0.12% in *D. recens* (Fig. 2). The difference in mtDNA nucleotide diversity is even

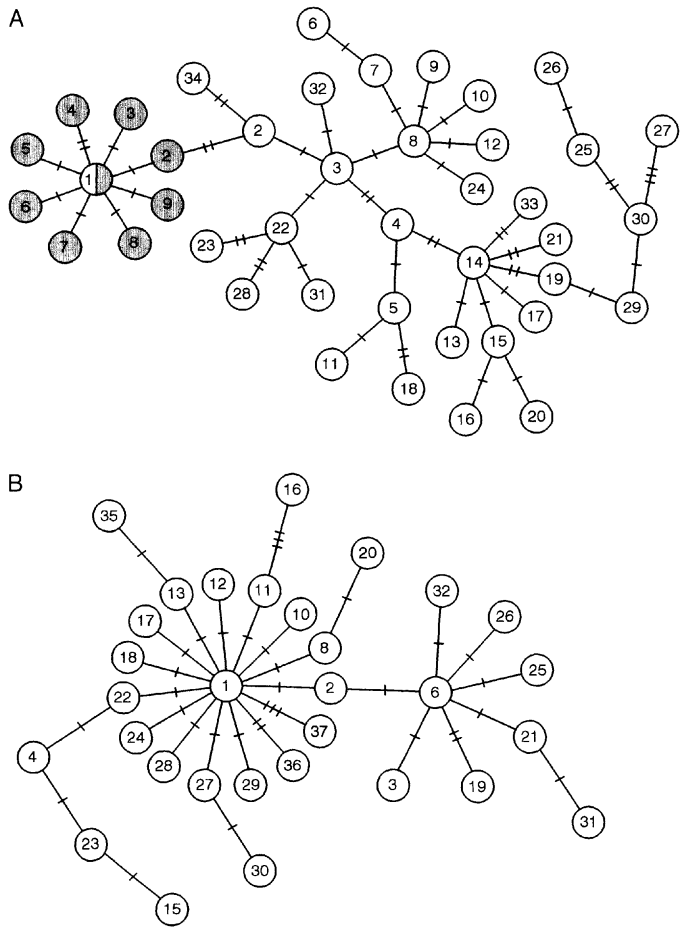

FIG. 1. Unrooted minimum spanning parsimony networks among haplotypes found within *Drosophila recens* and *D. subquinaria* (A), and *D. falleni* (B). Haplotypes found within *D. recens* are indicated by shading, whereas those found in *D. subquinaria* are unshaded (A). Numbers refer to haplotype variants *within* each species and do not identify identical haplotypes across species. With the exception of haplotype 1 of *D. recens* and *D. subquinaria*, no haplotypes were shared across species. Restriction site differences between connected haplotypes are indicated by ticks on connecting lines.

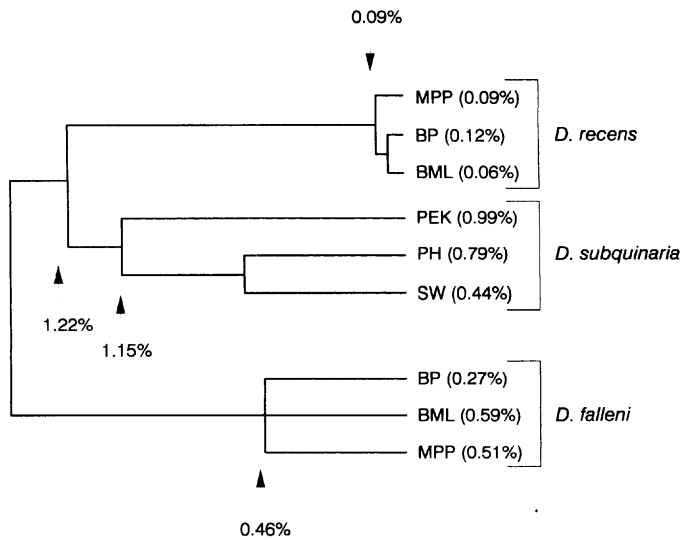

FIG. 2. Estimates of nucleotide diversity (π; Nei and Tajima 1983) within populations of *Drosophila recens, D. subquinaria*, and *D. falleni*. Population abbreviations correspond to those in Table 1. Within-population estimates of nucleotide diversity are indicated in parentheses. The values next to arrows indicate the overall estimates of nucleotide diversity within each of the three species, as well as within the *D. recens–D. subquinaria* clade. Branch lengths are proportional to the estimates of p among populations.

greater at the species level (Fig. 2), with the diversity of *D. recens* being one-fifth that of *D. falleni* and less than one-tenth that of *D. subquinaria*.

DISCUSSION

Because *Wolbachia* are known to occur in a substantial fraction of insect species that have been examined (reviewed in Werren and O'Neill 1997) and because *Wolbachia* infections can lead to CI in crosses between infected and uninfected individuals, it has been argued that these bacterial endosymbionts may be an important isolating mechanism between populations and thus play a major role in insect speciation (Fine 1978; Werren 1997b). In contrast, Caspari and Watson (1959) and Turelli (1994) have shown theoretically that selection leads to the fixation of a single *Wolbachia* variant within host populations. Accordingly, one would not expect to find stable coexistence of infected and uninfected subpopulations of hosts or subpopulations infected with different *Wolbachia* strains if these subpopulations are connected by considerable levels of gene flow. Turelli's (1994) findings therefore suggest that *Wolbachia* by themselves might not be important agents in the process of speciation. We will argue that our findings suggest a means by which *Wolbachia* can contribute to the evolution of reproductive isolation between populations and thus play a supporting role in the process of speciation.

Our laboratory studies reveal that several pre- and postmating barriers bring about reproductive isolation between *D. recens* and *D. subquinaria*. First, our behavioral assays showed that although interspecific matings occurred in both directions, *D. recens* females were much less likely to accept *D. subquinaria* males than *D. subquinaria* females were to accept *D. recens* males. Thus, premating behavioral isolation is asymmetric between the two species and represents an initial one-way barrier to interspecific gene flow.

These two species also are isolated by at least two postmating mechanisms. A comparison of crosses between infected versus uninfected males of *D. recens* and *D. subquinaria* females revealed that *Wolbachia* reduced the production of hybrid progeny by a factor of seven to 11 (Table 3). Such *Wolbachia*-induced CI has been documented in several species of insects (Werren 1997a). Second, in accordance with Haldane's rule (Haldane 1922; Coyne and Orr 1989), we found that hybrid males invariably were sterile, whereas hybrid females were fertile and could produce numerous offspring in backcrosses to either parental species (Table 4). The fertility of hybrid females, as well as that of the female progeny resulting from backcrosses to either parental species, indicates that gene flow can occur between these two species.

Although hybrid male sterility was observed in both reciprocal crosses, the other two isolating mechanisms were strongly asymmetric and operated in opposite directions. Consequently, behavioral isolation and *Wolbachia*-induced CI may act in a complementary fashion to reduce the production of hybrid progeny in areas where *D. recens* and *D. subquinaria* are sympatric.

This pattern of asymmetric premating and postmating isolating mechanisms operating in different directions runs counter to expectations of a reinforcement process, although the theory of reinforcement is complex (Butlin 1995). Specifically, one might predict that the females involved in incompatible interspecific crosses (*D. subquinaria*) should be more discriminating against heterospecific males than females involved in compatible crosses (*D. recens*). Our studies reveal the opposite pattern, with *D. subquinaria* females being less discriminating than those of *D. recens*. Such complementary asymmetrical isolating mechanisms also have been found in reciprocal crosses between *Drosophila mojavensis* and *D. arizonae* (Markow and Hocutt 1998), and we have preliminary evidence for a similar pair of asymmetrical isolating mechanisms (i.e., behavior and CI) operating between *D. testacea* and *D. orientacea*.

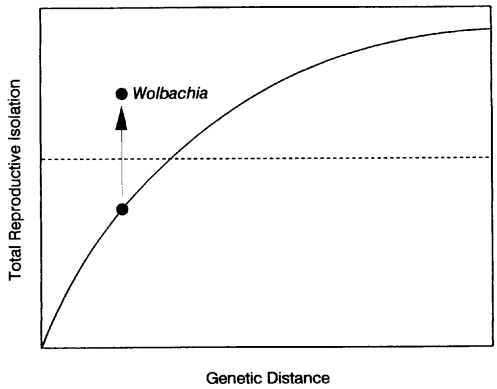

FIG. 3. Model of the role of *Wolbachia* in accelerating reproductive isolation between incipient species. Solid line illustrates how total reproductive isolation between populations increases through time (Coyne and Orr 1997). Once isolation is greater than that indicated by the dashed line, fusion upon secondary contact becomes unlikely. Increased reproductive isolation resulting from *Wolbachia*-induced cytoplasmic incompatibility may decrease the amount of time required to prevent fusion upon secondary contact.

The existence of such complementary asymmetric isolating mechanisms is consistent with selection acting at a higher hierarchical level (Muller 1942). Specifically, if both pre- and postmating isolating mechanisms operated in only one direction, then gene flow via pairings in the reciprocal direction would result in fusion of the two incipient species. Species isolated by complementary asymmetric mechanisms may be more likely to persist and retain their integrity.

We envision the role of *Wolbachia* and unidirectional CI in the evolution of reproductive isolation between species as follows. If populations of a given species become geographically isolated, their independent evolution will result in a gradual increase in reproductive isolation between them. Coyne and Orr (1989, 1997) show that, for allopatric taxa of *Drosophila*, both pre- and postmating isolation increase in proportion to the genetic distance between these taxa. Thus, reproductive isolation appears to increase steadily with time (see Fig. 3). If two populations require a certain level of reproductive isolation to prevent fusion upon secondary contact (dashed line in Fig. 3), then there may be a minimum period of geographic isolation necessary for speciation to occur. Now suppose that while they are geographically separated one population becomes infected with *Wolbachia*, which will result in unidirectional CI with the other population upon secondary contact. Such a *Wolbachia* infection

should accelerate the rate at which total reproductive isolation evolves and, therefore, reduce the time required before secondary contact can occur without fusion (Fig. 3). Thus, *Wolbachia* may contribute to speciation in insects by decreasing the fraction of vicariance events that result in fusion upon secondary contact. The importance of *Wolbachia* in the evolution of reproductive isolation depends on the rate at which isolated populations become infected with a new strain of CI-inducing *Wolbachia* relative to the rate at which other isolating mechanisms evolve. The high degree of genetic similarity of *Wolbachia* strains across many unrelated insect species (Werren et al. 1995b) suggests that infection and horizontal transmission is a fairly common and ongoing process.

Wolbachia would have to have been present in *D. recens* before the two species came into secondary contact to have contributed to the persistence of *D. recens* and *D. subquinaria* as separate species. Our data on the frequency of *Wolbachia* infection and mtDNA variation in *D. recens* and *D. subquinaria* indicate that *Wolbachia* invaded *D. recens* in the distant evolutionary past and not within recent historical times, as it has in *D. simulans* (Turelli and Hoffmann 1991). First, all but two of the wild-caught *D. recens* we examined were infected with *Wolbachia*. These two lines probably lacked *Wolbachia* as a result of secondary loss of infection, which may occur through incomplete maternal transmission. Although our laboratory studies reveal that 100% of the offspring of infected *D. recens* females are infected with *Wolbachia* (unpubl. data), maternal transmission may be less effective in the field (Turelli and Hoffmann 1995). That our two uninfected strains of *D. recens* harbored mtDNA haplotypes typical of infected flies (haplotype 1) is consistent with secondary loss through incomplete maternal transmission. Thus, we conclude that *Wolbachia* effectively have already swept to fixation within *D. recens*.

None of the *D. subquinaria* in our samples were infected with *Wolbachia*, suggesting that *Wolbachia* invaded *D. recens* some time after the two lineages split. We cannot completely rule out the possibility of infection of the common ancestor of the two species followed by subsequent loss of *Wolbachia* from *D. subquinaria*, a process that is at least theoretically feasible (Hurst and McVean 1996; Clancy and Hoffmann 1997). However, our mtDNA data cast doubt on this scenario. If *Wolbachia* had infected the common ancestor of *D. recens* and *D. subquinaria*, with *D. subquinaria* subsequently losing *Wolbachia*, then one would expect *D. recens* to exhibit greater mtDNA haplotype diversity than *D. subquinaria*. Instead, however, mtDNA nucleotide divergence in *D. subquinaria* was an order of magnitude greater than that in *D. recens*, which is the expected pattern for a postdivergence infection of *D. recens* by *Wolbachia*.

It is possible that *D. recens* has lower mtDNA diversity than *D. subquinaria* or *D. falleni* because of differences in effective population size and is not the result of a selective sweep associated with *Wolbachia*. If this were the case, then the diversity of nuclear genes in *D. recens* should also be low. However, the level of nuclear variation in *D. recens* is similar to that found in *D. falleni*, a species with a similar geographical range and breeding site use (mushrooms; Shoemaker and Jaenike 1997). These findings indicate that the reduced mtDNA variation in *D. recens* is specific to this genome and is not due to this species having an especially small effective population size. Therefore, we conclude that the low mtDNA diversity in *D. recens* is the genetic legacy of a *Wolbachia*-associated mitochondrial sweep in this species. Ballard et al. (1996) reported a similar disparity between levels of mitochondrial and nuclear diversity in *D. simulans* and attributed this to an mtDNA sweep caused by *Wolbachia*.

The common *D. recens* haplotype at the center of the starlike network may represent the ancestral haplotype associated with the *Wolbachia* infection, the others having arisen subsequently via mutation of an already infected lineage (Fig. 1). Such a starlike network of mtDNA haplotypes and the lack of more divergent haplotypes also strongly argues against paternal transmission of *Wolbachia* as a significant source of mtDNA variation in *D. recens*. This is because paternal transmission would result in capture of a random sample of mtDNA haplotypes from a diverse network, such as that seen in *D. subquinaria*, and not a set of very closely related haplotypes.

Our measures of mtDNA nucleotide diversity within and between *D. recens* and *D. subquinaria* allow us to estimate the time of divergence of these two species, as well as the time of invasion and spread of *Wolbachia* within *D. recens* or at least the most recent such invasion. Assuming 1% sequence divergence per million years (2% between lineages; Brower 1994; Guillemaud et al. 1997), we estimate that *D. recens* and *D. subquinaria* diverged approximately 0.6 million years ago. Divergence among mtDNA haplotypes within *D. recens* suggests that *Wolbachia* may have invaded this species about 50,000 years ago. (A new coalescent model is being developed to provide a more reliable estimate of when this *Wolbachia* sweep occurred [Tavare, Shoemaker, and Jaenike, unpubl. data].) Although 50,000 years is much less than the estimated divergence time between *D. recens* and *D. subquinaria*, it is nonetheless possible that *Wolbachia* invaded *D. recens* before the two species came into secondary contact, thus contributing to the reproductive isolation that prevented fusion of the two populations. Because these species occur primarily in the cooler, more northern regions of North America—*D. recens* in the east and *D. subquinaria* in the west—they may have come into secondary contact after the retreat of the Wisconsin glacier about 10,000 years ago. In any case, our data on mtDNA variation within *D. recens* indicate that *Wolbachia* have served as an isolating mechanism between these species for a substantial period of evolutionary time. Although *Wolbachia*-induced CI by itself may not be sufficient to prevent gene flow between *D. recens* and *D. subquinaria*, it could play an important role in preventing their fusion by acting in concert with behavioral isolation and hybrid male sterility.

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