***Wolbachia* and cytoplasmic incompatibility in mycophagous *Drosophila* and their relatives**

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Bacterial symbionts belonging to the genus *Wolbachia* are associated with postzygotic reproductive incompatibility in a number of insect species. Using polymerase chain reaction (PCR) amplification of bacterial gene sequences, strains from 10 species belonging to the closely related *quinaria*, *testacea* and *tripunctata* groups of *Drosophila* were screened for the presence of *Wolbachia* in their reproductive tissues. Those screened included the mycophagous species *D. falleni*, *D. recens*, *D. phalerata*, *D. testacea*, *D. neotestacea*, *D. orientacea*, *D. putrida* and *D. tripunctata*, and the nonmycophagous species *D. palustris* and *D. quinaria*. Two species, *D. recens* and *D. orientacea*, were found to be infected with *Wolbachia*. Subsequent tests of four additional strains of *D. recens* found all to be infected with the bacteria. It was established that these bacteria cause partial cytoplasmic incompatibility in *D. recens* by antibiotic curing followed by crosses between cured and uncured strains. Curing was confirmed by a PCR assay. Although most species of insects shown to be infected with *Wolbachia* are cosmopolitan and/or have undergone recent range expansion in association with human activity, *D. recens* and *D. orientacea* are endemic species with specialized ecological habits. Preliminary molecular phylogenetic analysis indicates that, among the species we examined, *D. quinaria* is most closely related to *D. recens*. To determine whether the bacteria are involved in reproductive isolation between these two species, reciprocal crosses were carried out between *D. quinaria* and both infected and uninfected (cured) strains of *D. recens*. Although these species did mate with each other, all interspecific crosses failed to yield hybrid progeny, indicating that the bacteria are not responsible for reproductive incompatibility between these species.

Keywords: cytoplasmic incompatibility, mycophagous *Drosophila*, *Wolbachia*.

Introduction

Wolbachia are a group of closely related bacteria that infect the reproductive tissues of various arthropods. These bacteria are transmitted through the egg cytoplasm and alter reproduction in their arthropod hosts in ways that promote the spread of the bacteria through host populations. So far, *Wolbachia* have been found to be associated with postzygotic reproductive incompatibility (termed cytoplasmic incompatibility or CI) (Barr, 1980; Breeuwer *et al.*, 1992; O'Neill *et al.*, 1992; Rousset *et al.*, 1992), parthenogenesis (Stouthamer *et al.*, 1993) and feminization of genetic males (Rousset *et al.*, 1992).

Cytoplasmic incompatibility, which was originally discovered in mosquitoes (Laven, 1957; Barr, 1980), is manifested as partial to complete embryonic death in crosses between certain strains. The factors responsible for CI were shown to be cytoplasmically (mater-

nally) inherited. Subsequent research in a number of different species has shown that CI is caused by intracellular bacteria found in the reproductive tissues.

Bacterially induced cytoplasmic incompatibility is of interest as a potentially rapid mechanism for development of reproductive isolation and subsequent speciation (Breeuwer & Werren, 1990; Coyne, 1992). Consistent with this possibility, bidirectional incompatibility has been found between geographical races of *Culex pipiens*, between different geographical isolates of *Drosophila simulans* (O'Neill & Karr, 1990) and between closely related species of *Nasonia* wasps (Breeuwer & Werren 1990; Breeuwer, 1993). To determine the possible role of these bacteria in facilitating speciation events, the extent of association of the bacteria with reproductive incompatibility between closely related species must be determined.

Demonstration of a definitive association between *Wolbachia* and CI includes showing that: (i) *Wolbachia* are present in at least one strain or species, (ii) antibiotic curing eliminates the *Wolbachia*, and (iii) subse-

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quent crosses between cured and uncured strains demonstrate unidirectional incompatibility. Although the precise mechanisms of CI are unknown, cytogenetic studies in the parasitic wasp *Nasonia vitripennis* indicate that CI is caused by improper condensation and fragmentation of the paternal chromosomes in the first mitotic division of the fertilized egg (Ryan & Saul, 1968; Breeuwer & Werren, 1990; Reed & Werren, 1995). Similar abnormal mitoses have been observed in *Drosophila simulans* infections, indicating that the same mechanism may be operating in diverse species. In haplodiploid species, such as *N. vitripennis*, paternal genome loss typically results in production of male (haploid) offspring, whereas in diploidiploids it results in zygotic lethality.

Although *Wolbachia* are widespread in insects (Werren *et al.*, 1995; R. Giodano & H. M. Robertson, personal communication), occurring in 5–10 per cent of species examined, *Wolbachia*-associated cytoplasmic incompatibility has only been definitively demonstrated in a small number of species, including several Diptera (*Culex pipiens*, *Drosophila simulans* and *D. melanogaster*), two Coleoptera (*Tribolium confusum* and *Hypera postica*), one Homoptera (*Laedophax striatellus*), one Lepidoptera (*Ephestia cautella*) and three Hymenoptera (*Nasonia vitripennis*, *N. giraulti* and *N. longicornis*). Many of the species in which CI has been demonstrated have recently achieved cosmopolitan distributions and thus may not represent populations at evolutionary equilibrium. For example, *T. confusum* and *E. cautella* are associated with stored grain products. The cosmopolitan species *C. pipiens*, *D. melanogaster*, *D. simulans* and *N. vitripennis* have widespread distributions, apparently caused at least in part by recent range expansion as a result of human activities. *H. postica* and *L. striatellus* are insect pests associated with human agriculture.

Here we focus on the presence of *Wolbachia* and its role in CI in several endemic species belonging to the closely related *quinaria*, *testacea* and *tripunctata* groups of *Drosophila*. There is no evidence that the geographical ranges and breeding biology of these woodland species have been strongly altered by human activity. Each of these species is endemic to a particular geographical region in either North America or Eurasia.

Methods

Insect strains

Assays for the presence of *Wolbachia* were carried out in 10 species of *Drosophila* belonging to the closely related *quinaria*, *testacea* and *tripunctata* species

groups of the subgenus *Drosophila*. The species and the origin, including date of collection, of the strains used are as follows: *quinaria* group members *D. falleni* (Rochester, New York, 1992), *D. recens* (two strains from the White Mountains, New Hampshire, 1993; three strains from Rochester, 1992 (1) and 1993 (2)), *D. quinaria* (Rochester, 1993), *D. palustris* (Rochester, 1993) and *D. phalerata* (Leiden, Netherlands; Regensburg, Germany, 1990); *testacea* group members *D. testacea* (Regensburg, 1990), *D. neotestacea* (Rochester, 1992), *D. orientacea* (Sapporo, Japan, 1990) and *D. putrida* (Rochester, 1992); and *D. tripunctata* (Great Smoky Mountains, Tennessee, 1987) of the *tripunctata* group. These strains had been maintained in the laboratory at 21°C on Instant *Drosophila* Medium (Carolina Biological Supply) plus commercial *Agaricus bisporus* mushrooms. All of the species breed primarily on mushrooms in the wild, except *D. quinaria* and *D. palustris*, which breed on decaying skunk cabbages (*Symplocarpus foetidus*). *D. falleni*, *D. recens*, *D. quinaria*, *D. palustris*, *D. putrida*, *D. neotestacea* and *D. tripunctata* are endemic to North America. *D. phalerata* and *D. testacea* are known from Europe and continental Asia, and *D. orientacea* is endemic to Japan.

PCR assay

DNA from each strain was prepared from four to 10 ovaries of fecund females. Ovaries were dissected in sterile double-distilled deionized water on a sterile slide and then serially transferred to droplets of sterile H₂O prior to transfer into a 0.5 mL microfuge tube containing 0.3 mL sterile zirconium beads (Biospec Products, Bartlesville, OK), 200 µL Tris, 10 µL 20 per cent SDS and 200 µL phenol. Samples were shaken in a mini-Bead beater (Biospec Products) for 3 min. Phases were separated by 10 min spinning in a microcentrifuge. The DNA was then ethanol precipitated and resuspended in 20 µL sterile TE buffer (10 mM Tris (pH 8), 1 mM EDTA). Extreme care was taken throughout the procedure to avoid bacterial contamination. All solutions were filter sterilized (0.22 µm pore size). Control DNA samples were prepared using pupae of known infected and uninfected strains of the parasitoid wasp, *Nasonia vitripennis*.

Bacterial *ftsZ* DNA was amplified using PCR in a volume of 25 µL (1 µL DNA sample, 2.5 µL 10× buffer (Promega), 0.75 µL MgCl₂ (50 mM), 0.5 µL nucleotide mix (10 mM each), 0.350 µL 20 mM primer 1, 0.350 µL 20 mM primer 2, 0.25 µL *Taq* polymerase (Promega) and double-distilled H₂O was added to a final volume of 25 µL). Two primer pairs designed for amplification of *Wolbachia ftsZ* sequences were used,

ftsZf1 5'-GTTGTCGCAAATACCGATGC-3' and *ftsZr1* 5'-CTTAAGTAAGCTGGTATATC-3'. These yield an amplification product of 1035–1047 bp.

The PCR reaction mix was prepared in one batch and then added to each sample. The remainder was run as a control for contamination. PCR cycling conditions were one cycle (1 min at 94°C, 1 min at 55°C, 3 min at 72°C), 35 cycles (15 s at 94°C, 1 min at 55°C, 3 min at 72°C) and 1 cycle (15 s at 94°C, 1 min at 55°C and 10 min at 72°C) (Erincomp thermal cycler). After PCR, 1 µL of amplified reaction product was run on a 1 per cent agarose gel to determine presence and size of amplified DNA.

Cloning and sequencing

For cloning purposes, a 50 µL PCR reaction was used (doubling of solutions above) for amplification of the two known positives, *D. recens* and *D. orientacea*. PCR products were purified with the GeneClean kit (Bio 101, La Jolla, CA) and dissolved in 20 µL distilled deionized H₂O, and then directly cloned into a T-tailed M13mp18 derivative (W. Burke, unpublished data). Two clones each were sequenced for *D. recens* and *D. orientacea*. Two regions of ambiguity in the sequences caused by apparent secondary structure effects were resolved by additional sequencing reactions using the modified base analogue dITP.

Antibiotic curing

A strain of *D. recens* collected in 1993 in the White Mountains of New Hampshire was cured of its symbiotic *Wolbachia* as follows. Fresh *Agaricus bisporus* mushrooms were soaked to saturation in a solution of tetracycline (0.1, 0.25, 1.0 or 2.0 mg tetracycline per mL). These mushrooms were then placed in vials with 10 males and 10 females of *D. recens* at 22°C. Flies were allowed to oviposit on the mushrooms for 6 days. Tetracycline solution of the appropriate strength was added on the sixth day to the mushrooms to keep them saturated; at this point all of the cultures had first and second instar larvae actively feeding. Upon emergence of adults, flies were kept on instant plus mushroom medium without tetracycline. Treated strains were tested using the PCR assay to determine if they harbored detectable levels of *Wolbachia*.

Crossing experiments

The effect of the *Wolbachia* on cytoplasmic incompatibility was tested twice, once after two generations of maintenance on tetracycline-free medium, and once after three generations on this medium. Compatibility

was assayed by carrying out crosses between single males and single females. For each tetracycline concentration in which the larvae were initially grown (0.1, 0.25, 1.0, and 2.0 mg/mL), reciprocal crosses were carried out with flies of an untreated, *Wolbachia*-infected strain. In addition, crosses were carried out between treated males and treated females for each tetracycline treatment. Ten replicates of each cross were used. Adults were left in their culture vials for 14 days in the first trial and 7 days in the second.

Preliminary molecular phylogenetic analysis indicates that *D. quinaria* is more closely related to *D. recens* than any other *quinaria* group species we examined (G. Spicer, personal communication). To determine whether *Wolbachia* play a role in the reproductive isolation between these sympatric species, we carried out reciprocal crosses between *D. quinaria* and either *Wolbachia*-positive or *Wolbachia*-negative strains of *D. recens*. For each cross type, five males and five females were placed in each of three vials. These were watched for 5 h to determine if any copulation took place. The flies were left in the vials and the cultures were kept at 22°C. The cultures were thoroughly examined under a microscope (12×) to see if any viable larvae were produced in any of the cultures.

Statistical analysis

An effect of *Wolbachia* on cytoplasmic incompatibility would be manifest by inequalities in offspring production between the reciprocal crosses between *Wolbachia*-positive and *Wolbachia*-negative flies. For each tetracycline concentration, the numbers of offspring produced per pair were analysed for three cross types, where I (Infected) refers to the untreated *Wolbachia*-infected strain and T (Treated) refers to flies treated with tetracycline: T female×I male, I female×T male, and T female×T male. Crosses between treated males and females were included in the analysis to test the possibility that reduced offspring production in the T female×I male crosses was because of reduced fertility of T females, rather than of cytoplasmic incompatibility. An analysis of variance (PROC ANOVA, SAS Institute, 1990) was used to determine if there was heterogeneity among cross types in offspring production. If there was significant variation, then a Fisher's Least Significant Difference test (LSD) was carried out to determine which cross type(s) produced fewer offspring than the others.

Results

To screen for the presence of *Wolbachia* by PCR, primers were utilized that amplify the bacterial *ftsZ*

gene (Werren *et al.*, 1995). Sequencing of *ftsZ* product from over 25 different insect species has revealed that these primers are highly specific for *Wolbachia* (no other bacterial genera have been sequenced from amplification products using these primers) and that the primers amplify both of the two major subdivisions of *Wolbachia* (Werren *et al.*, 1995). Table 1 shows the results of attempts to amplify *Wolbachia ftsZ* product from ovaries of 10 species of *Drosophila*. Each strain was examined at least twice, with the flies used for DNA isolation in the different replicates having been reared separately. Two species, *D. recens* and *D. orientacea*, were found to be positive for *Wolbachia*. PCR reactions from both yielded an amplification product of the expected size, 1035–1047 bases.

Following the positive result from *D. recens*, four additional strains of this species were tested. Two of these had recently been collected from field populations in upstate New York and two from New Hampshire. All five strains of *D. recens* tested positive for *Wolbachia*, indicating that these bacteria are widespread and common in this species. Additional strains of *D. orientacea* were not available for analysis.

Amplification products from *D. recens* and *D. orientacea* were cloned into an M13 derivative and sequenced. A phylogenetic analysis utilizing these

sequences and a representative sample from 25 other species was performed (data not shown). The analysis was performed using the neighbour-joining algorithm following determination of Jukes & Cantor (1969) genetic distances. *Wolbachia* from both *D. recens* and *D. orientacea* belong to the Dm clade of the A subdivision of these bacteria and are thus referred to as Adm. The Adm 'strain' of *Wolbachia* is a subgroup that shows a high degree of sequence similarity but is found in a diverse range of insect hosts (including Diptera, Hymenoptera and Lepidoptera) (Werren *et al.*, 1995). This pattern indicates that this strain of *Wolbachia* has been undergoing considerable horizontal transmission between insects, accounting for the low amount of sequence divergence. This strain of *Wolbachia* is also found in other drosophilid species, including *D. melanogaster* and *D. simulans*.

The results of crosses between treated (descendants of tetracycline-treated larvae) and untreated flies are presented in Table 2. It should be noted that the treated strain was derived by antibiotic treatment two to three generations previously from the same infected strain used in these crosses. As observed in other known cases of cytoplasmic incompatibility in Diptera, crosses between infected males and cured females in *D. recens* yielded significantly fewer adult progeny than the reciprocal crosses. This effect was observed in both replicate trials, which were carried out in sequential generations. By the criterion of incompatibility, a single generation of larval development in mushrooms treated with 1.0, 2.0 and probably 0.25 mg of tetracycline per mL was sufficient to produce at least partial cytoplasmic incompatibility. PCR analysis also indicated that these strains were free of *Wolbachia*. Development in mushrooms soaked the lowest concentration (0.1 mg/mL) of tetracycline apparently was not effective in curing the flies of *Wolbachia*.

Table 2 also shows that the crosses between treated males and treated females produced offspring numbers comparable to the compatible crosses between infected females and treated males. This shows that the significantly lower offspring production in the crosses between infected males and treated females is not the result of reduced fertility of treated females.

In the interspecific crosses between *D. recens* (both cured and infected) and *D. quinaria*, which does not harbour *Wolbachia* (see Table 1), at least one interspecific mating was seen during the 5-h observation period for each of the following cross types: *D. quinaria* male $\times$ *D. recens* female (cured), *D. recens* male (cured) $\times$ *D. quinaria* female, and *D. recens* male (infected) $\times$ *D. quinaria* female. No larvae were observed in any of the cultures for any of the interspecific crosses. This indicates that there is complete

Table 1 Results of PCR amplification of *Wolbachia ftsZ* sequences in 10 species of *Drosophila*

Species	Strain	No. of replicates	<i>ftsZ</i> sequences†
<i>falleni</i>	Rochester	2	Absent
<i>quinaria</i>	Rochester	2	Absent
<i>recens</i>	Mendon 1992	3	Present
	NH-1	2‡	Present
	NH-2	2‡	Present
	Rochester-1	2‡	Present
	Rochester-2	2‡	Present
	Treated	2	Absent
<i>palustris</i>	Rochester	2	Absent
<i>phalerata</i>	Regensburg, Germany	2	Absent
	Leiden, Netherlands	2	Absent
<i>tripunctata</i>	Great Smoky Mtns, TN	2	Absent
<i>putrida</i>	Rochester	2	Absent
<i>neotestacea</i>	Rochester	3	Absent
<i>testacea</i>	Regensburg, Germany	3	Absent
<i>orientacea</i>	Sapporo, Japan	3	Present

† All replicates for each strain yielded identical results. Replicates represent independent isolations of DNA and PCR amplification, except for the cases marked ‡ which were separate amplifications from the same DNA isolation.

Table 2 Mean ($\pm$ SE) number of offspring produced in *Drosophila recens* crosses

Tetracycline treatment (mg/mL)		Cross type (female $\times$ male)*				Probability
		I $\times$ I	T $\times$ I	I $\times$ T	T $\times$ T	
Trial 1	0.1	58.1 $\pm$ 10.0	38.1 $\pm$ 14.6 ^a	36.3 $\pm$ 13.6 ^a	63.6 $\pm$ 13.9 ^a	0.28
	0.25	58.1 $\pm$ 10.0	26.8 $\pm$ 17.1 ^{a,b}	22.8 $\pm$ 9.0 ^a	71.6 $\pm$ 19.7 ^b	0.07
	1.0	58.1 $\pm$ 10.0	13.0 $\pm$ 10.0 ^a	58.7 $\pm$ 16.2 ^b	51.7 $\pm$ 7.3 ^b	0.024
	2.0	58.1 $\pm$ 10.0	3.6 $\pm$ 2.1 ^a	64.6 $\pm$ 20.5 ^b	42.8 $\pm$ 14.7 ^{a,b}	0.019
Trial 2	0.1	38.8 $\pm$ 12.2	34.3 $\pm$ 9.2 ^a	30.1 $\pm$ 11.4 ^a	53.6 $\pm$ 14.6 ^a	0.35
	0.25	38.8 $\pm$ 12.2	3.5 $\pm$ 1.6 ^a	49.9 $\pm$ 13.8 ^b	31.9 $\pm$ 8.8 ^b	0.007
	1.0	38.8 $\pm$ 12.2	1.7 $\pm$ 1.5 ^a	35.9 $\pm$ 6.3 ^b	29.0 $\pm$ 9.7 ^b	0.003
	2.0	38.8 $\pm$ 12.2	1.4 $\pm$ 0.9 ^a	22.7 $\pm$ 6.8 ^b	35.4 $\pm$ 10.4 ^b	0.008

T: flies descended from tetracycline-treated larvae; I: flies infected with *Wolbachia* (untreated).

Trial 1 was performed two generations after antibiotic treatment; trial 2 was carried out the following generation.

Probability values are those obtained by analysis of variance of offspring numbers within rows.

*Entries within rows marked with the same superscript (a or b) are not significantly different ($P < 0.05$) by a least significant difference (LSD) test (SAS Institute, 1990). I $\times$ I crosses were not included in ANOVA s or LSD tests because only one set of 10 crosses per trial was carried out.

post-mating reproductive isolation between these species and that curing *D. recens* of *Wolbachia* does not confer reproductive compatibility between this species and *D. quinaria*. The result indicates that *Wolbachia* are not involved in reproductive isolation between these species.

Discussion

Of the 10 species of *Drosophila* tested, two (*D. recens* and *D. orientacea*) were found to be positive for *Wolbachia*. All five strains of *D. recens* tested were found to be infected with *Wolbachia*. Thus, *Wolbachia* infection appears to be common in this species. Because only one strain of *D. orientacea* was available for screening, we cannot infer the prevalence of infection.

It should be emphasized that a negative PCR result does not establish that a species is uninfected. Some strains have been kept under laboratory rearing conditions for several years, and could have lost *Wolbachia* during this time. In addition, individual species may be polymorphic for *Wolbachia* infections in natural populations. For example, studies of *D. melanogaster* in Australia reveal polymorphisms for *Wolbachia* infections in several different populations (Hoffmann, 1988; Solignac *et al.*, 1994). Similarly, *D. simulans* is polymorphic for *Wolbachia*, although studies indicate that the infection is rapidly spreading through that species (Hoffmann *et al.*, 1986; Turelli & Hoffmann, 1991). Some theoretical studies predict that polymorphisms for cytoplasmic incompatibility *Wolbachia* infections

will often be transient, with most infections eventually spreading to fixation in the population (Caspari & Watson, 1959). However, this prediction is based on the assumption of complete maternal transmission of the infection to progeny. Polymorphism for infection is predicted under a variety of conditions (Rousset *et al.*, 1991; Stevens & Wicklow, 1992; Wade & Stevens, 1994; Turelli, 1995).

Several of the insects in which *Wolbachia* cause cytoplasmic incompatibility are cosmopolitan in distribution, having undergone dramatic range increases within historical times. These include *D. melanogaster*, *D. simulans*, *Culex pipiens*, *Tribolium confusum*, *Nasonia vitripennis* and *Ephestia cautella*. It is therefore possible that acquisition of *Wolbachia* infections is associated with range expansion or occupation of disturbed habitats. In contrast, *D. recens* is endemic to forests of northeastern North America (Wheeler, 1960). It breeds only in natural substrates, primarily mushrooms, and is not associated with humans. Similarly, *D. orientacea* is endemic to natural habitats in Japan. Our results with *D. recens* and *D. orientacea* argue that rapid range expansion and association with disturbed environments is not a requirement for acquisition of *Wolbachia*. It will be worthwhile to determine whether the incidence of *Wolbachia* infection is greater in cosmopolitan species than in those that have not undergone recent range expansions.

Although *Wolbachia* are typically transmitted vertically within the egg cytoplasm of their host species, it is clear from molecular phylogenetic studies that hori-

zontal transmission of these bacteria can occur, even between different orders of insects (O'Neill *et al.*, 1992; Stouthamer *et al.*, 1993; Werren *et al.*, 1995). The mechanisms of this horizontal transmission are unknown, although transmission via associated parasitoid insects has been postulated as one possibility. Werren *et al.* (1995) present phylogenetic evidence that such transfer has occurred between parasitic wasps of the genus *Nasonia* and their blowfly hosts, *Protophthora*. Recent experiments using microinjection techniques to move *Wolbachia* between insect species also show that *Wolbachia* strains can become established in different species and still cause incompatibility (Boyle *et al.*, 1993; Braig *et al.*, 1994; Chang & Wade, 1994). Presumably, occasional natural transmission of these bacteria across species boundaries can result in subsequent expansion of the infection and associated cytoplasmic incompatibility in the new host species.

The 10 drosophilid species screened for *Wolbachia* are interesting because, except for *D. quinaria* and *D. palustris*, they share a common ecology, the mushroom-feeding habit. Several of these mycophagous species, including *D. falleni*, *D. putrida*, *D. neotestacea* and *D. tripunctata*, are sympatric with *D. recens* over much of their range in eastern North America. Furthermore, these species are normally found associated on individual mushrooms (Jaenike & James, 1991): mushrooms yielding many individuals of *D. recens*, which is infected with *Wolbachia*, typically yield numerous individuals of the other species. Finally, the phylogenetic positions of the *Wolbachia* found in *D. recens* (and *D. orientacea*) indicate that these flies obtained their resident *Wolbachia* via horizontal transfer from other host taxa. Thus, there would appear to be ample ecological opportunity for horizontal spread of *Wolbachia* from *D. recens* to the other mycophagous drosophilids. It is interesting that *D. recens* harbours these bacteria while closely related and ecologically associated drosophilids apparently do not. Similarly, Hoffmann *et al.* (1994) found that many Australian populations of *D. melanogaster* are infected with CI *Wolbachia*, whereas microsympatric *D. simulans* populations appear to be uninfected. This pattern suggests that horizontal transfer is a rare event or is typically not mediated directly through a shared feeding substrate.

We postulate that the frequency of infected species results from a balance between rates of (i) horizontal transmission to uninfected species, (ii) loss of *Wolbachia* from infected species, and (iii) speciation and extinction of infected and uninfected species. That relatively few insects harbour *Wolbachia* (5–10 per cent) could be explained in a variety of ways. For instance, infected species may experience greater rates of extinc-

tion, although this could be offset by greater rates of speciation, as *Wolbachia* may increase speciation rates by providing an additional mechanism for reproductive isolation, namely cytoplasmic incompatibility. Mechanisms of loss in natural populations include the evolution of resistance (Turelli, 1995), reduced fitness of infected individuals, natural curing (Stevens & Wickow, 1992), and stochastic loss of bacteria within infected individuals.

It may be a coincidence, but the two species we found to be infected with *Wolbachia*, *D. recens* and *D. orientacea*, have the most northerly distributions of the 10 species we examined (Wheeler, 1960; Grimaldi *et al.*, 1992). It would be interesting to determine if an endemic species with a broad latitudinal range exhibited geographical variation in the prevalence of infection. It would also be worthwhile to determine in the laboratory if reductions in host fitness or rates of spontaneous loss of symbionts were temperature-dependent.

Evidence suggests that at least one strain of these bacteria (the Adm strain) is undergoing considerable recent horizontal transmission (Werren *et al.*, 1995). This conclusion is based on molecular phylogenetic data (Werren *et al.*, 1995) and the observation that this strain is currently sweeping through *D. simulans* (Turelli & Hoffmann, 1991); it may have been recently acquired by *D. melanogaster* as well (Hoffmann, 1988; Solignac *et al.*, 1994). *D. recens* and *D. orientacea* also harbour the Adm strain of *Wolbachia*. Because these two species, unlike *D. melanogaster* and *D. simulans*, are not associated with human-disturbed habitats, the finding that it is infected with the Adm strain of *Wolbachia* suggests that these bacteria are an integral part of natural as well as disturbed ecological communities. Analysis of the ecological and phylogenetic patterns of association of hosts and *Wolbachia* could provide useful insights into the primary mode of horizontal transmission of these bacteria.

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