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Wolbachia and genetic variability in the birdnest blowfly *Protocalliphora sialia*

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Abstract

***Wolbachia* are widespread cytoplasmically inherited bacteria that induce various reproductive alterations in host arthropods, including cytoplasmic incompatibility (CI), an incompatibility between sperm and egg that typically results in embryonic death. CI has been invoked as a possible mechanism for reproductive isolation and speciation in arthropods, by restricting gene flow and promoting maintenance (and evolution) of genetic divergence between populations. Here we investigate patterns of *Wolbachia* infection and nuclear and mitochondrial differentiation in geographical populations of the birdnest blowfly *Protocalliphora sialia*. Blowflies in western North America are infected with two A-group *Wolbachia*, with some individuals singly and others doubly infected. Individuals in eastern North America mostly show single infections with a B-group *Wolbachia*. Populations in the Midwest are polymorphic for infections and show A- or B-group infection. There is a low level of mitochondrial divergence and perfect concordance of mitochondrial haplotype with infection type, suggesting that two *Wolbachia*-associated selective sweeps of the mitochondrion have occurred in this species. Amplified fragment length polymorphism analysis of nuclear genetic variation shows genetic differentiation between the eastern–Midwestern and western populations. Both Midwestern and eastern flies infected with A-*Wolbachia* show eastern nuclear genetic profiles. Current results therefore suggest that *Wolbachia* has not acted as a major barrier to gene flow between western and eastern–Midwestern populations, although some genetic differentiation between A-*Wolbachia* infected and B-*Wolbachia* infected individuals in eastern–Midwestern populations cannot be ruled out.**

Keywords: gene flow, genetic variability, *Protocalliphora*, selective sweep, speciation, *Wolbachia*

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Introduction

Wolbachia are maternally transmitted alpha-proteobacteria found in the reproductive tissues of invertebrates (reviewed in Werren 1997a; Stouthamer *et al*. 1999). These bacteria cause a number of reproductive alterations in their hosts, including induction of thelytokous parthenogenesis, feminization of genetic males, male-killing and, most commonly, the induction of sperm–egg incompatibilities (termed cytoplasmic incompatibility). *Wolbachia* have attracted considerable interest, in part because of their potential role as agents of rapid speciation in arthropods through induction of cytoplasmic incompatibility (Laven 1959; Breeuwer & Werren 1990; Coyne 1992; Werren 1998; Bordenstein *et al*. 2001).

Cytoplasmic incompatibility (CI) is a *Wolbachia*-induced incompatibility of sperm and egg (see Hoffmann & Turelli 1997 for a review). There are two forms of incompatibility, unidirectional or bidirectional. When only one *Wolbachia* type is involved, CI is unidirectional: the cross between an uninfected female and an infected male produces few or no progeny, whereas the reciprocal cross is fertile (Breeuwer *et al*. 1992; O'Neill *et al*. 1992; Rousset *et al*. 1992). Bidirectional incompatibility occurs when a male and a female harbour different strains of *Wolbachia* that are mutually incompatible: crosses between individuals infected with different *Wolbachia* are incompatible in both directions (Breeuwer & Werren 1990; O'Neill & Karr 1990; Mercot *et al*. 1995; Perrot-Minnot *et al*. 1996; Werren 1997b). When

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populations of a species differ in infection status, the presence of *Wolbachia* could reduce gene flow and therefore promote genetic divergence and speciation (Werren 1998; Telschow *et al*. 2003a,b). Unidirectional CI alone is insufficient to suppress gene flow between populations because infected females are compatible with uninfected males. It will thus favour speciation only when associated with another isolation mechanism (Werren 1998; Shoemaker *et al*. 1999). In contrast, bidirectional CI can reduce or suppress gene flow between populations in both directions and may also select for premating isolation. Therefore, the presence of bidirectional CI between populations could allow evolution or maintenance of divergence between the populations, thus favouring speciation (Laven 1959, 1967; Powell 1982; Telschow *et al*. 2003a,b).

Interest in *Wolbachia*-associated speciation has increased due to the recent finding that 15–75% of insect species harbour these bacteria (Werren *et al*. 1995a; West *et al*. 1998; Jeyaprakash & Hoy 2000; Werren & Windsor 2000). However, whether *Wolbachia*-induced CI plays an important role in speciation in insects is still unknown (Hurst & Schilthuizen 1998; Werren 1998; Hurst & Werren 2001; Wade 2001). Theoretical studies are conflicting. Turelli (1994) has shown that selection often leads to the fixation of a single *Wolbachia* strain in the host population, which would not enhance speciation. However, Telschow *et al*. (2003a,b) have shown that bidirectional CI can increase genetic divergence between populations of the host species under a wide range of conditions, thus presumably also increasing the probability of speciation. Empirical studies are not conclusive either. There is growing evidence that many insect species harbour multiple strains of *Wolbachia* (see for example Laven 1967; Breeuwer *et al*. 1992; Mercot *et al*. 1995; Wenseleers *et al*. 1998; West *et al*. 1998; James & Ballard 2000; Malloch *et al*. 2000; Shoemaker *et al*. 2000), which is the prerequisite for *Wolbachia*-associated speciation by bidirectional cytoplasmic incompatibility. However, only a handful of examples are known in which *Wolbachia* are major contributors to reproductive incompatibility between closely related species (Giordano *et al*. 1997; Shoemaker *et al*. 1999; Bordenstein *et al*. 2001). The best documented of these examples occurs in the parasitoid wasps *Nasonia giraulti* and *N. longicornis*. A recent study by Bordenstein *et al*. (2001) has shown that *Wolbachia*-induced interspecies reproductive incompatibility has occurred in the early stages of speciation in this system, preceeding the evolution of other postmating isolating mechanisms.

Protocalliphora larvae are obligatory bloodsucking parasites for nestling birds, and are parasitized by *Nasonia* wasps. One of the commonest species of the genus, *P. sialia* is found throughout North America, and there are differences in adult and pupal morphology between eastern and western populations, suggesting possible genetic differentiation (Sabrosky *et al*. 1989; Whitworth unpublished). *Wolbachia* have been detected in *P. sialia*, although it is not yet known whether they induce CI or other phenotypes. Here we examine the potential association of *Wolbachia* with genetic differentiation between the eastern and western populations of *P. sialia*. Genetic, mitochondrial and *Wolbachia* variation is investigated in different geographical populations of *P. sialia*, to assess whether these bacteria are associated with genetic divergence. Although both different *Wolbachia* and genetic divergence is found between eastern and western populations, results from Midwestern populations suggest that *Wolbachia* are not maintaining genetic differentiation between geographical populations in this species.

Materials and methods

Sampling and DNA extraction

Our sample consisted of 60 *Protocalliphora sialia* individuals, which can be divided in three groups according to their geographical origin: east, Midwest or west (Table 1). Ten individuals from the related species *P. parorum* (Sabrosky *et al*. 1989) were also analysed. *P. parorum* is a blowfly parasite primarily of chickadees (Sabrosky *et al*. 1989). Blowfly larvae or pupae were collected from bird nests several days after fledging of the young birds. Collections were made either directly by the authors or by naturalists, during the summers 1999–2001 in the continental USA. Blowflies from bluebird and treeswallow nests were particularly collected because *P. sialia* frequently parasitizes these species (Sabrosky *et al*. 1989). In some cases, pupae and larvae were placed into individual test tubes and checked everyday for emerging adults. Emergent flies were placed into 100% ethanol, when possible with their pupal cases to facilitate species identification. To minimize screening of siblings, one individual per bird nest was subjected to molecular analysis. DNA of adult flies was extracted with QIAgen DNAeasy kit, as suggested by the manufacturer. The lower half of the abdomen of each fly was used for DNA extraction, as it contains the reproductive tissues in which *Wolbachia* is predominantly found. Extracted DNA was resuspended in 100 μL elution buffer.

Wolbachia *analysis*

A 454-bp fragment of the *wsp* gene (Braig *et al*. 1998) was amplified by polymerase chain reaction (PCR), initially from 10 randomly chosen *Protocalliphora* individuals. We used the general *wsp* primers designed by Braig *et al*. (1998) for *Wolbachia*: *wsp*81F (TGGTCCAATAAGTGATGAAGAAAC) and *wsp*691R (AAAAATTAAACGCTACTCCA). Thermocycle conditions were 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min 30 s, for a total of 35 cycles. PCR products

Table 1 *Protocalliphora sialia* and *P. parorum* individuals used in the study. The first four columns indicate the identification code, the state and location of provenance and the bird host species for each individual. The next four columns show the analysis results: the COI column indicates if the *COI* amplification product was cut or not by restriction enzyme *AccI* (see Materials and methods), whereas the three last columns shows the presence or absence of the three *Wolbachia* strains observed in *P. sialia* and *P. parorum* (see Materials and methods and Results)

ID	State	Location	Bird host*	COI	*w*A$_1$	*w*A$_2$	*w*B
P. parorum individuals							
00-69	CA	Kern County	Oak titmice	uncut	−	−	−
00-62	CA	Kern County	Pygmy nuthatch	uncut	+	−	−
00-143	CA	Kern County	Mountain chickadee	uncut	+	−	−
00-117	CA	Kern County	Mountain chickadee	uncut	+	−	−
00-151	CA	Kern County	Mountain chickadee	uncut	+	−	−
00-140	CA	Kern County	Pygmy nuthatch	uncut	+	−	−
00-119	CA	Kern County	Mountain chickadee	uncut	−	−	−
00-153	CO	Durango	Ash-throated flycatcher	uncut	−	−	+
00-152	MN	Sartell	Tree swallow	uncut	−	−	−
00-158	OR	Springfield	Mountain chickadee	cut	+	−	−
P. sialia individuals							
Western individuals							
00-164	BC	Osoyoos	Tree swallow	cut	+	+	−
Pen1 K6	BC	Penticton	Mountain bluebird	cut	+	+	
00-68	CA	Napa County	Tree swallow	cut	+	−	−
00-146	CO	Durango	Ash-throated flycatcher	uncut	−	−	−
00-175	ID	Clarton	Tree swallow	cut	+	+	−
00-134	MT	Philipsburg	Mountain bluebird	cut	−	−	−
00-122	MT	Philipsburg	Mountain bluebird	cut	+	+	−
00-108	MT	Philipsburg	Mountain bluebird	cut	+	+	−
00-111	MT	Philipsburg	Mountain bluebird	cut	+	+	−
00-101	MT	Philipsburg	Mountain bluebird	cut	+	+	−
00-104	MT	Philipsburg	Mountain bluebird	cut	+	+	−
00-114	MT	Philipsburg	Mountain bluebird	cut	+	+	−
00-155	OR	Springfield	Western bluebird	cut	+	−	−
UT53	UT			cut	+	−	−
UT20	UT			cut	+	−	−
Midwestern individuals							
00-128	MN	Sartell	Tree swallow	cut	+	−	−
00-172	MN	Saint Paul	Eastern bluebird	uncut	−	−	+
00-179	MN	Saint Paul	Eastern bluebird	cut	−	−	−
StP8621	MN	Saint Paul	Eastern bluebird	cut	+	−	−
StP8607	MN	Saint Paul	Eastern bluebird	cut	+	−	−
Maz WH15	WI	Mazomanie	Eastern bluebird	cut	+	−	−
Maz WH11	WI	Mazomanie	Eastern bluebird	cut	+	+	−
00-52	WI	Dane County	Eastern bluebird	cut	+	+	−
00-38	WI	Dane County	Eastern bluebird	cut	+	+	−
00-29	WI	Dane County	Eastern bluebird	cut	+	−	−
00-33	WI	Dane County	Eastern bluebird	cut	+	−	−
00-42	WI	Dane County	Eastern bluebird	cut	+	−	−
00-60	WI	Dane County	Eastern bluebird	cut	+	−	−
00-41	WI	Dane County	Eastern bluebird	cut	+	−	−
00-54	WI	Dane County	Eastern bluebird	cut	+	+	−
BlE U-13	WI	Black Earth	Eastern bluebird	uncut	−	−	+
BlE U-7	WI	Black Earth	Eastern bluebird	uncut	−	−	−
BlE U-15	WI	Black Earth	Eastern bluebird	cut	+	−	−
00-180	WI	Black Earth	Eastern bluebird	cut	+	+	−
Eastern individuals							
00-163	NJ	Sussex	Tree swallow	uncut	−	−	+
Ash59	NY	Ashford	Eastern bluebird	uncut	−	−	+
Brew1	NY	Brewerton	Eastern bluebird	uncut	−	−	+
Brew9	NY	Brewerton	Eastern bluebird	uncut	−	−	+

Table 1 *Continued*

ID	State	Location	Bird host*	COI	*w*A$_1$	*w*A$_2$	*w*B
Con226	NY	Concord	Eastern bluebird	uncut	−	−	+
Con22	NY	Concord	Eastern bluebird	uncut	−	−	+
EOt35	NY	East Otto	Eastern bluebird	uncut	−	−	+
EOt33	NY	East Otto	Eastern bluebird	uncut	−	−	−
MenPR4	NY	Mendon Ponds	Eastern bluebird	uncut	−	−	+
Nat2K	NY	Rochester	Eastern bluebird	uncut	−	−	+
Nat145	NY	Rochester	Eastern bluebird	uncut	−	−	−
NY701	NY			cut	+	−	−
NY16A	NY	Rush	House sparrow	cut	+	−	−
NY46	NY			uncut	−	−	+
NYBlen	NY	Blenheim		uncut	−	−	+
00-187	OH		Eastern bluebird	cut	+	−	−
00-189	OH		Eastern bluebird	uncut	−	−	+
00-43	PA	Chester Springs	Eastern bluebird	uncut	−	−	-
Ham38	PA	Hammond Lake	Eastern bluebird	uncut	−	−	+
Lit19	PA	Little Pine St. Pk.	Tree swallow	uncut	−	−	+
Lit2a	PA	Little Pine St. Pk.	House wren	uncut	−	−	+
PA702	PA			uncut	−	−	+
00-46	VA	Roanoke	Eastern bluebird	uncut	−	−	+
00-24	VA	Roanoke	Eastern bluebird	cut	+	−	−
Roa5	VA	Roanoke	Eastern bluebird	uncut	−	−	+
Roa21	VA	Roanoke	Eastern bluebird	uncut	−	−	+

*Bird host species: eastern bluebird, *Sialia sialis*; mountain bluebird, *Sialia currucoides*; western bluebird, *Sialia mexicana*; mountain chickadee, *Parus gambeli*; ash-throated flycatcher, *Myiarchus cinerascens*; pygmy nuthatch, *Sitta pygmaea*; house sparrow, *Passer domesticus*; tree swallow, *Tachycineta bicolour*; oak titmice, *Baeolophus inornatus*; house wren, *Troglodytes aedon*.

were purified and then sequenced with an ABI 377 automatic sequencer (Perkin–Elmer). The *wsp* sequences of the first 10 individuals revealed the presence of three different *Wolbachia* strains in our sample. Two bacteria belong to *Wolbachia*-A group (Werren *et al*. 1995b), hereafter called *w*A1 and *w*A2. The third one is a B group *Wolbachia* (Werren *et al*. 1995b), hereafter called *w*B, previously described by Werren & Bartos (2001). The *wsp* sequences of the three *Wolbachia* strains were proofread and aligned manually to a representative sample of previously determined insect *Wolbachia wsp* sequenced retrieved from GenBank.

Two primers specific of each A group *Wolbachia* of *Protocalliphora* were designed: *wsp*306F GGTATTACCTATAAGAAAGAC and *wsp*166F GGTATTACCTATAAGAAAGAC. Primers are numbered based on the *w*RI *wsp* gene sequence (GenBank Accession no. AF020070), as suggested by Zhou *et al*. (1998). We confirmed that when used with the general primer *wsp*691R, *wsp*306F and *wsp*166F amplify *w*A1 and *w*A2 in a selective manner. Subsequently, the *Wolbachia* infection status of each individual of our sample was determined by specific PCR. For each individual, two PCRs were performed with A group-specific primers (*wsp*136F and *wsp*691R) and B group-specific primers (*wsp*81F and *wsp*522R) (Zhou *et al*. 1998). Individuals that yielded an amplification with the A group primers were then tested with the *w*A1- and *w*A2-specific primers. All thermocycle conditions were as described above. Sequences have been deposited in GenBank under Accession nos AY188686 and AY188687.

Mitochondrial analysis

A 392-bp fragment of the Cytochrome Oxidase I gene was amplified by PCR using the following conserved primer pair: 5′-GC(A/T)AC(A/T)AC(A/G)TAATA(G/T)GTATCATG-3′ and 5′-CAACATTTATTTTTT-3′ (developed by Ted Schultz, see Gadau *et al*. 1999). Thermocycle conditions were 95 °C for 1 min, 47 °C for 1 min and 72 °C for 1 min 30 s, for a total of 35 cycles. Purified template DNA was sequenced with an ABI 377 automatic sequencer (Perkin–Elmer). COI sequencing of the first 10 individuals revealed that all A-infected *P. sialia* individuals present the same haplotype, whereas all B-infected individuals show another haplotype, differing by three substitutions. For all subsequent infected *P. sialia* individuals, the mitochondrial haplotype was thus determined by PCR followed by digestion with restriction enzyme *Acc*I, whose recognition sequence is present in the COI of A-infected individuals only. For uninfected *P. sialia*, as well as for *P. parorum* individuals, the mitochondrial haplotype was determined by sequencing. All COI sequences were proofread and aligned manually. A COI sequence of *Chrysomya semimetallica* (GenBank

Accession no. AF295562), a close relative of the *Protocalliphora* genus (Wells & Sperling 2001), was used as an outgroup (see below).

To determine whether COI was evolving under neutrality in *P. sialia*, we used the *H* statistic designed by Fay & Wu (2000) to specifically detect selective sweeps. This statistic measures an excess of high compared with intermediate frequency variants. It is the difference between two estimators of $\theta = N\mu$, the neutral mutation parameter of the population. $H = \theta_\pi - \theta_H$, where θ_π is the average number of pairwise differences in the sample (Tajima 1983) and θ_H is an estimate based on the frequency of derived variants (Fay & Wu 2000). *H* is negative when there is an excess of high frequency-derived alleles relative to the expectations of the standard neutral model. The derived variants were identified by using *P. falcozi* as an outgroup. To determine the probability of the observed values of the *H* statistics under neutrality, we ran 10 000 coalescent simulations (Hudson 1990) of a panmictic population using the ALLELIX program of S. Mousset (http://www.snv.jussieu.fr/mousset/).

AFLP analysis

The nuclear structure of *Protocalliphora* was analysed using the amplified fragment length polymorphism technique (AFLP; Vos *et al*. 1995). To perform the procedure, we used a subsample of 23 individuals, chosen to represent the whole geographical distribution of the *P. sialia* individuals in our sample. AFLP procedures were performed with a Perkin–Elmer kit, following the manufacturer's recommendations. Briefly, for each individual, 5.5 μL of genomic DNA was double-digested with *Eco*RI and *Mse*I. DNA fragments were ligated with *Eco*RI and *Mse*I adapters, generating template DNA for PCR amplification. A preselective amplification was performed using two primers complementary to the adapters and the restriction site sequences, in the following conditions: 94 °C for 1 min, 56 °C for 1 min 30 s and 72 °C for 2 min, for a total of 35 cycles. Next, a selective PCR was performed with primers similar to the preselective amplification primers but with three additional bases at the 3′-end. A total of five primers combinations (with the following selective bases: E-ACT and M-CAC, E-AAC and M-CAG, E-AA and M-CTA, E-ACA and M-CTC, E-ACC and M-CTG) was used to study 23 *Protocalliphora* individuals. PCR products were run on an ABI Prism 377 DNA Sequencer using a 5% acryl/bisacryl long ranger gel. Gels were analysed using GENESCAN 3.0 software packages (ABI). Each lane was tracked manually using GENESCAN 3.0.

Phylogenetic analyses

Phylogeny for *wsp* was generated using neighbour-joining analysis of the sequence data in PAUP* 4.0b10 (Swofford 2002). Maximum likelihood was used to calculate distances between taxa. We used likelihood ratio tests (Huelsenbeck & Rannala 1997) to determine which model of DNA sequence evolution is the most appropriate for the *wsp* data. We used MODELTEST 3.06 (Posada & Crandall 1998) to test hierarchically the effect of unequal base frequencies, different rates between transitions and transversions, different rates between all substitutions and rate variation over nucleotide sites. The model that best fit the *wsp* dataset included unequal base frequencies and a transition/transversion rate of 2.18, i.e. a HKY85 model (Hasegawa *et al*. 1985). There was also significant rate heterogeneity among sites (gamma distribution shape parameter of 0.3445). These parameter values were used to calculate the distance matrix for neighbour-joining analysis on 1000 bootstraps replicates.

Genetic relationships among COI mitochondrial sequences were summarized using a statistical parsimony cladogram (Templeton *et al*. 1992). The cladogram was constructed using TCS 1.13 (Clement *et al*. 2000). TCS uses statistical parsimony to generate an unrooted cladogram based on a pairwise matrix of absolute differences among haplotypes. TCS was run with a 95% limit of parsimony.

Nuclear genetic relationships among *P. sialia* and *P. parorum* individuals were summarized using a neighbour-joining analysis of the AFLP data with PAUP* 4.0b10 (Swofford 2002). We did not use character-based analyses as the presence of recombination in intraspecific data sets is misinterpreted as homoplasy by parsimony or maximum likelihood methods (Posada & Crandall 2002). The character matrix of presence or absence of bands produced by the AFLP procedure was converted into a distance matrix using Nei & Li (1979) and Upholt (1977) distances. The reliability of the tree obtained was examined using 1000 bootstrap replicates.

Results

Wolbachia *infections in* Protocalliphora sialia

Three different *Wolbachia* strains, called *w*A1, *w*A2 and *w*B, were found in our *Protocalliphora* samples. Strains *w*A1 and *w*B were present both in *P. sialia* and *P. parorum*, whereas *w*A2 was found only in *P. sialia*. Six different *w*A1 were sequenced from *P. sialia* and three from *P. parorum*, and there were no base differences between them over the 454 bp region (including the hypervariable region). Similarly the four *P. sialia* and one *P. parorum* B-*Wolbachia* were identical in sequence, as were the two *w*A2 sequenced from *P. sialia*.

Phylogenetic analysis (neigbour-joining on a distance matrix calculated by maximum likelihood) was performed using the *wsp* gene places *w*A1 and *w*A2 in two divergent clades of *Wolbachia* A group. Interestingly, *w*A2 is

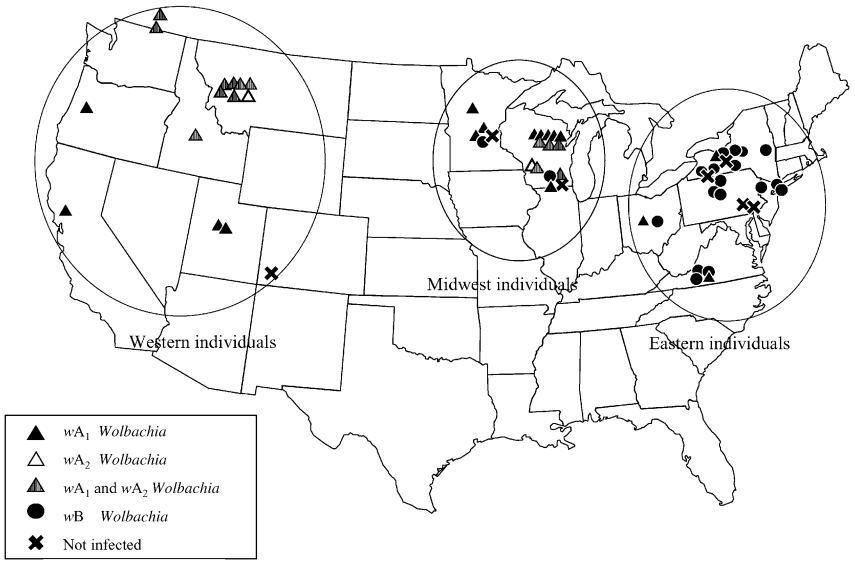

Fig. 1 *Wolbachia* infection status of *Protocalliphora sialia* individuals. Individuals infected with wA_1, wA_1 and wA_2, wA_2 or *w*B *Wolbachia* strains are respectively represented by an open triangle, a hatched triangle and a filled triangle and a circle. Uninfected individuals are symbolized by a cross.

relatively close to the A group *Wolbachia* found in *Nasonia girault*i, one of the wasps that parasitizes *P. sialia*. In contrast, *w*A1 is unrelated to the *Wolbachia* strains found in *Nasonia.* The B-group bacterium *w*B is also relatively far from the B group *Wolbachia* of the three *Nasonia* species. However, previous studies have shown that the *wsp* gene of the *P. sialia* B-group bacterium is the result of a recombination event between two different B *Wolbachia* (for details see Werren & Bartos 2001). The two A-group *Wolbachia* show 19.4% synonymous differences (Nei and Gojobori distance; Nei & Gojobori 1986), giving an estimated divergence time of 11.8–21.6 Myr depending on whether the synonymous substitution rate estimates for endosymbiotic (*Buchnera*) or free-living (*Escherichia coli*, *Salmonella*) bacteria are used (Clark *et al*. 1999; Ochman *et al*. 1999). As mentioned, recombination has been detected in *Wolbachia* (Jiggins *et al*. 2001; Werren & Bartos 2001), and therefore the phylogenetic relationships of single genes cannot be used to infer phylogenetic relationships among the bacteria. However, amplification using A- and B-specific primers for 16*S* and *fts*Z (Werren & Windsor 2000) and *wsp*-specific primers for the two A group bacteria found in *P. sialia* confirm the presence of three different bacterial types in this system.

The infection status of the 76 *Protocalliphora* individuals of our sample is shown in Table 1. Among the 60 *P. sialia* individuals that were screened for *Wolbachia*, 54 (90%) were positive for the bacteria. Of the 54 positive individuals, 14 were double-infected with *w*A1 and *w*A2, 17 were infected with *w*A1 alone, 2 with *w*A2 alone, and 21 were singly infected with *w*B. Interestingly, the related species *P. parorum* shared two of the same *Wolbachia* types (*w*A1 and *w*B), identical in *wsp* sequence to those found in *P. sialia*. Other *P. parorum* individuals were uninfected. Figure 1 presents the geographical pattern of infections observed in *P. sialia*.

The localization of the three *Wolbachia* strains observed in *P. sialia* is not random. In the eastern part of the USA, most individuals are infected with *w*B. Only a few individuals are infected with *w*A1 and none with *w*A2. In contrast, only *w*A1 and *w*A2 are present in western USA, with some individuals (mainly northerly samples) have double infections (*w*A1 + *w*A2) and others single infections with *w*A1. The Midwest populations appeared to be transitional between the east and west. Individuals are mostly doubly infected with *w*A1 + *w*A2, or singly infected with *w*A1, but a few individuals had *w*B bacteria, single *w*A2 infections or were uninfected. In the Midwest, infection status is variable even at single locations. At each location for which we have multiple samples, several *Wolbachia* infection states are observed. For example, the four individuals from Black Earth, WI, had four different infection types (no infection, *w*A1, *w*A1 + wA_2 and *w*B). Therefore, there appears to be considerable mixing of infection types in Midwestern populations. Among the *w*A2 infected *P. sialia* in our sample, 87.5% were also infected with *w*A1. The reverse did not occur, as among the 31 *w*A1-infected *P. sialia*, only 45.2% were also infected with *w*A2. Finally, no triple infected (*w*A1 + *w*A2 + *w*B) *P. sialia* were found, and *w*B was always found in single infections.

Wolbachia *and mitochondrial DNA variation*

Analysis of mitochondrial DNA (mtDNA) variation can be revealing in population studies of *Wolbachia*, because mitochondria and *Wolbachia* are both maternally transmitted and therefore co-segregate (Rousset & Solignac 1995; Hoffmann & Turelli 1997). The mitochondrial haplotypes

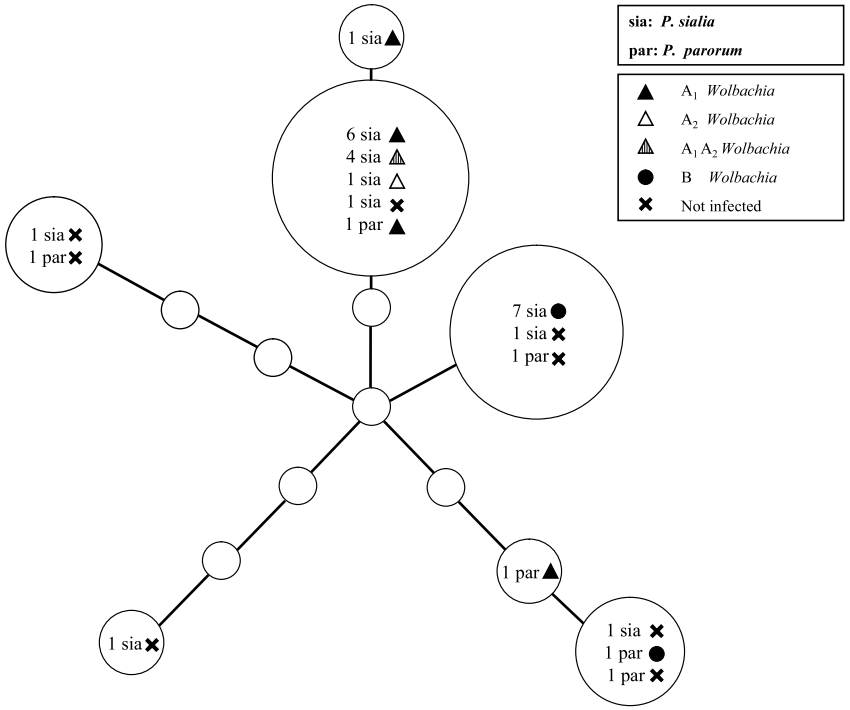

Fig. 2 Network of *Protocalliphora sialia* and *P. parorum* COI haplotypes. The size of the circles is proportional to the number of individuals found with that haplotype. Empty circles indicate haplotypes not found in the sample. The *Wolbachia* infection status of each individual is shown on the tree. Individuals infected with wA_1, wA_1 and wA_2, wA_2 or *w*B *Wolbachia* strains are respectively represented by an open triangle, a hatched triangle and a filled triangle and a circle. Uninfected individuals are symbolized by a cross.

found in *P. sialia* and *P. parorum*, and the estimated phylogenetic relationships between these haplotypes, are shown in Fig. 2, together with the *Wolbachia* infection status of each individual. The first noticeable characteristic of the cladogram is that *P. sialia* and *P. parorum* COI sequences do not show any tendency to cluster by species. Indeed, all but one *P. sialia* haplotypes have an identical *P. parorum* haplotype and correspondingly, all but one *P. parorum* haplotypes have an equivalent *P. sialia*. This indicates either introgression between the two species, or maintenance of an 'ancient' mitochondrial–*Wolbachia* polymorphism that existed prior to divergence of the species. There is a relatively low level of COI variation among the sequences of the two *Protocalliphora* species of our sample. Among and between species, we observed a total of only 12 polymorphic sites (11 synonymous changes and 1 replacement change) in the 392 bp fragment of COI. The maximum divergence observed between a pair of sequences is seven synonymous substitutions, which translates into a divergence of 8.7% at synonymous sites using the Nei & Gojobori (1986) distance. The two frequent haplotypes present in *w*A- and *w*B-infected individuals are closely related. They differ only by three synonymous substitutions, which correspond to a divergence of 3.6% at synonymous sites. Assuming similar rates of mitochondrial synonymous evolution as found in *Drosophila* (Tamura 1992), this would give a divergence time between mitochondria from *w*A- and *w*B-infected individuals of ≈ 300 000 years.

Among the infected *P. sialia* individuals, the mtDNA haplotype was determined by sequencing or digestion with restriction enzyme *Acc*I. The results of the digestion are indicated in Table 1. The most striking result is the perfect concordance between the *Wolbachia* type and the mtDNA haplotype among infected *P. sialia* individuals. The 31 individuals infected by *w*A1, *w*A2 or both, all present the same mitochondrial haplotype, which is recognized by restriction enzyme *Acc*I, whereas the 21 individuals infected by *w*B all have another haplotype (Fig. 2 and Table 1). In contrast, uninfected *P. sialia* have variable mtDNA hapotypes: two uninfected individual from Midwest show mtDNA haplotypes identical to the one of the *w*A- and *w*B-infected *P. sialia*, whereas three other uninfected individuals from eastern and western USA have unique haplotypes. Among the 10 *P. parorum* individuals of our sample, we found a total of 5 haplotypes. There seems to be no association between the *Wolbachia* infection and the mitochondrial haplotype in *P. parorum*, although this may be due to the small number of individuals analysed and would need further confirmation.

To determine whether COI was evolving under neutrality in *P. sialia*, we used the *H* statistics designed by Fay & Wu (2000) to detect selective sweeps. We found $H = -10.77$, which indicates a large excess of derived variants. Comparing this value with the distribution of *H* under neutrality generated with coalescent simulation shows the probability of getting such a negative value of *H* is < 0.001. The observed pattern of mtDNA variation of *P. sialia* is thus highly unlikely under neutrality, which suggests that a selective sweep occurred. Mitochondrial selective sweeps are expected to occur as a consequence of invasion and sweeps by

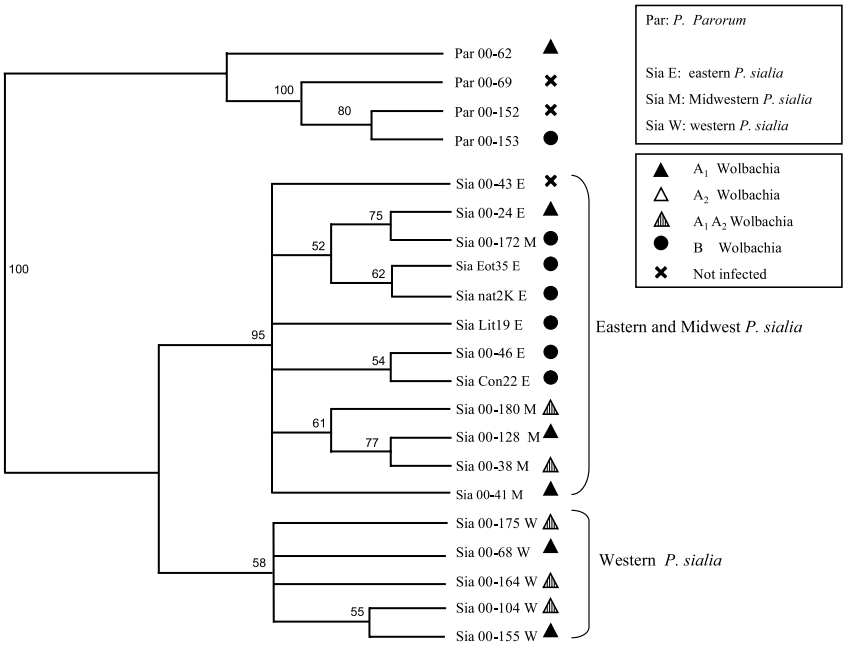

Fig. 3 Nuclear genetic structure of *Protocalliphora sialia* and *P. parorum* based upon AFLP data. The tree was generated by neighbour-joining using Nei and Li's distance. Bootstrap values are shown as percentages of 1000 replicates at each node only if they are 50% or greater. Nodes with < 50% bootstrap support are shown collapsed. The *Wolbachia* infection status of each individual is shown on the tree. Individuals infected with wA_1, wA_1 and wA_2, wA_2 or *w*B *Wolbachia* strains are respectively represented by an open triangle, a hatched triangle and a filled triangle and a circle. Uninfected individuals are symbolized by a cross. *P. sialia* individuals from east, Midwest and west are respectively represented by E, M and W.

Wolbachia, because both genomes are transmitted cytoplasmically (Rousset & Solignac 1995; Hoffmann & Turelli 1997).

Wolbachia *and nuclear DNA variation*

The mitochondrial and *Wolbachia* data show geographical differentiation between western and eastern *P. sialia* with possible admixture in the Midwest. We therefore analysed nuclear genetic variation by AFLP to determine whether the geographical populations were differentiated and whether *Wolbachia* type was associated with nuclear genetic variation in regions of co-occurrence of the different *Wolbachia* types. AFLP was also used to determine the nuclear genetic structure of *P. sialia* and resolve its relationship with *P. parorum*. The 5 primer pair combinations generated a total of 119 fragments, of which 96 were present in *P. sialia*. The neighbour-joining tree shown in Fig. 3 was constructed from the AFLP data with PAUP 4.0b10 (Swofford 2002) using Nei and Li's distance (Nei & Li 1986). A tree built with Upholt's distance (Upholt 1977) had a very similar topology (not shown). The pattern observed in the nuclear tree is very different from that of the mitochondrial tree: the AFLP tree shows three distinct clusters, corresponding to *P. parorum* and the two geographical races of *P. sialia*. Bootstrap values for clustering of *P. sialia* and *P. parorum* are 100%. Among *P. sialia* individuals, two well-separated groups are present (Fig. 3). The first, hereafter called east/Midwest group, contains the individuals from the eastern part of the USA and the Midwest and is supported by a bootstrap value of 95%. The second group is composed of the individuals from the western part of the USA and is supported by a bootstrap value of 58%.

Although *w*A1 and *w*A2 co-occur with the western *Protocalliphora* genotypes in the west, and *w*B co-occurs with the eastern genotype in the east, there is no evident association of *Wolbachia* with genetic divergence in *P. sialia* in Midwestern populations. Individuals from the Midwest that are *w*A infected do not cluster with the *w*A-infected western individuals, but with the infected eastern individuals. None of the AFLP bands is specific of *w*A- or *w*B-infected flies. The main structuring among *P. sialia* seems, therefore, to be of geographical origin and not caused by *Wolbachia*. There is a hint that three of four *w*A-infected individuals from the Midwest cluster with each other; however, the bootstrap values for this association are not high (61%, see Fig. 3). Overall, there is no clear association between AFLP variation and *Wolbachia* infection. There also appears to be little geographical structuring among the east/Midwest populations. Some of the most closely related individuals come from distant locations (for example individuals 00-24 from Minnesota and 00-172 from Virginia), whereas individuals from the same location can have more divergent nuclear DNA (for example individuals 00-38 and 00-41, both from Dane County, WI). This absence of marked structure within the east and Midwest suggests that *Protocalliphora* flies have high dispersal abilities.

Discussion

Wolbachia are known to be transmitted within species exclusively or almost exclusively through the egg cytoplasm and, as a result, these bacteria show intraspecific associations with mitochondrial haplotypes (Turelli *et al*. 1992; Rousset & Solignac 1995). However, these bacteria are also

distributed widely in arthropods and clearly undergo transfer between host species (O'Neill *et al*. 1992; Werren *et al*. 1995a,b). There are two basic mechanisms for interspecific movement of *Wolbachia*, interspecies hybridization and horizontal (infectious) transmission. If the bacterium is introduced by interspecies hybridization (e.g. matings between closely related species), then the mitochondrial haplotype from the infected species can sweep through the newly infected species along with *Wolbachia*. Such interspecific mitochondrial sweeps have likely occurred during *Wolbachia* infection in the *Drosophila simulans* complex (Rousset & Solignac 1995), in the *D. yakuba* complex (Lachaise *et al*. 2000) and in *Solenopsis invicta* and *S. richteri* (Shoemaker *et al*. 2000). By contrast, when *Wolbachia* comes from a phylogenetically distant species by infectious transfer, then the mitochondria of the origin species will not be introgressed into the newly infected species. Below we interpret the patterns of mitochondrial, nuclear and *Wolbachia* variation and further discuss implications of these findings to *Wolbachia*'s possible role in speciation.

Pattern of Wolbachia *infection in* Protocalliphora

An explanation for the pattern of *Wolbachia* infection in *Protocalliphora* needs to account for several observations. First, *P. sialia* and *P. parorum* share two *Wolbachia* strains. Second, the mtDNA haplotypes of *P. sialia* and *P. parorum* do not cluster by species but there is a perfect concordance between the *Wolbachia* type and the mtDNA haplotype among infected *P. sialia* individuals. In some cases, there are identical haplotypes in both species. Third, there is more mitochondrial haplotype variation among uninfected *P. sialia* than among infected individuals. Finally, the strains *w*A2 seem to be associated with *w*A1 and both are associated with the same mtDNA haplotype.

There are two general explanations for the presence of two common *Wolbachia* strains (and associated mitochondrial haplotypes) in *P. sialia* and *P. parorum*, either: (i) maintenance of an ancestral infection polymorphism that existed prior to divergence of the two species; or (ii) movement of *Wolbachia* between the species after their speciation event by interspecies hybridization or infectious transfer. In *P. sialia*, our sequence and restriction data reveal complete concordance between mtDNA haplotype and *Wolbachia* infection type (*w*A or *w*B). Furthermore, the observed pattern of mtDNA variation is highly unlikely under neutrality, as indicated by the strongly negative value of the *H* statistics. This suggests that (at least) two *Wolbachia*-associated sweeps of mtDNA occurred in *P. sialia*. These two sweeps are probably recent as, though the mitochondria of Diptera have a high substitution rate (Moriyama & Powell 1997), we observed almost no mitochondrial polymorphism among infected *P. sialia* individuals (only one wA_1-infected individual has a haplotype differing by one substitution from the others). The paucity of mitochondrial variation within *Wolbachia* infection types could reflect a recent acquisition of these bacterial or a more ancient acquisition with subsequent additional selective sweeps of *Wolbachia* and associated mitochondria (Charlat *et al*. 2001). However, further support of a relatively recent acquisition of *Wolbachia* in *P. sialia* comes from the presence of greater mitochondrial diversity among uninfected individuals than among infected individuals. Some uninfected haplotypes are identical to those found in *P. parorum* and others are identical to those present in *w*A- and *w*B-infected individuals. These observations can best be explained by presence of ancestral mitochondrial diversity among the uninfected subpopulation and conversion of infected haplotypes to uninfected haplotypes due to incomplete transmission of the bacteria (see Fig. 2). Incomplete vertical transmission of *Wolbachia* quickly leads to the elimination of ancestral uninfected haplotypes and conversion of mitochondrial haplotypes among uninfecteds to that of infected haplotypes (Johnston & Hurst 1996). Such a pattern, for example, has been observed in the butterfly *Acraea encedana* (Jiggins 2003). In contrast, the greater mitochondrial variation among uninfected *P. sialia* argues against maintenance of an ancestral infection polymorphism and for relatively recent acquisition. However, we cannot unequivocally rule out the former possibility without additional data.

The pattern observed for the *w*A2 *Wolbachia* in *P. sialia* suggests a relatively recent horizontal (infectious) transfer event of this bacterium; *w*A2 is almost always present with *w*A1 in double infections and individuals infected with *w*A1, *w*A2 or *w*A1 + *w*A2 show the same mtDNA haplotype. Furthermore, *w*A2 has so far not been found in *P. parorum*. Therefore, data are consistent with a horizontal transfer from another insect species, a pattern commonly found in *Wolbachia* (Werren *et al*. 1995a; Zhou *et al*. 1998). Interestingly, *w*A2 is quite similar to the *Wolbachia* strain present in *Nasonia giraulti*, one of the wasps that parasitize *P. sialia*. It is thus tempting to speculate that the *w*A2 strain of *P. sialia* might have originated from *N. giraulti* by transmission between the parasitoid and its host. However, even though the two bacteria are close in the phylogenetic tree, there is 3.73% divergence at synonymous sites between *w*A2 and the A-group *Wolbachia* of *N. giraulti*: 3.73% of divergence corresponds to a long time interval of 2.3–4.1 Ma, depending upon the silent site rate used (Clark *et al*. 1999; Ochman *et al*. 1999). This relatively old date does not seem compatible with the postulated recent transfer of *w*A2 from *N. giraulti* into *P. sialia*. Therefore, we are uncertain as to the source of the *w*A2 transfer.

Wolbachia *and speciation in* P. sialia

There is interest in the possibility that *Wolbachia*-induced CI can serve as a barrier to gene flow, and therefore

promote speciation (Breeuwer & Werren 1990; Hurst & Schilthuizen 1998; Werren 1998; Bordenstein *et al*. 2001; Wade 2001). We do not know whether *Wolbachia* cause CI in *P. sialia*. However, CI is the most common *Wolbachia* phenotype observed in insects, and an absence of skewed adult sex ratios in this species (Whitworth, unpublished data) argues against male-killing or feminizing *Wolbachia* in this system. Regardless of the phenotype, we can ask the simple question of whether *Wolbachia* infection differences are associated with genetic differentiation in this insect. Our current data are not consistent with the view that *Wolbachia* has maintained genetic differentiation between western and eastern forms of *P. sialia*. The most parsimonious interpretation of our data is that *w*A1 and *w*A2 *Wolbachia* have moved from western into eastern–Midwestern populations, bringing with them the western mitochondrial haplotype and leaving behind the western nuclear genotype. In other words, rather than providing a barrier to nuclear gene flow, the western *Wolbachia* have 'jumped into' the nuclear genetic background of the eastern–Midwestern *P. sialia*. Presumably, the *w*A1 and *w*A2 *Wolbachia* have some combination of higher CI level or higher transmission rate that provides an advantage in competition with the *w*B *Wolbachia* found in the east (Turelli 1994).

An alternative hypothesis to that proposed above is that the eastern–Midwestern populations were historically polymorphic for all three *Wolbachia*, rather than acquiring their wA *Wolbachia* from western populations. Under this scenario, *P. sialia* individuals infected with different *Wolbachia* strains may be partially reproductively isolated due to CI, but insufficient time has transpired for genetic differentiation to be detected by the AFLP method used here. Furthermore, it should be pointed out that the small sample sizes in this study would not allow detection of quantitative differences in some loci between individuals infected with different *Wolbachia* types in Midwestern populations.

Telschow *et al*. (2003b) investigated the effects of *Wolbachia* on genetic divergence between populations. They modelled a situation of migration between two populations with different bidirectionally incompatible *Wolbachia* and differential selection at a nuclear locus. They found that, under a variety of conditions of selection, migration and CI, differences in *Wolbachia* types are maintained between the populations, resulting in significantly higher divergence at the selected locus than in the absence of *Wolbachia*. However, when migration rate exceeds a certain threshold, the *Wolbachi*a from one population will replace the other, and genetic differentiation between the populations will not be maintained. Typically, the bacterium with higher CI and/or higher vertical transmission replaces the alternative type, with nuclear gene flow occurring in the opposite direction (Turelli 1994; Telschow *et al*. 2003a). This scenario is consistent with our observation of 'western' *w*A1 and *w*A2 *Wolbachia* in Midwestern populations but with the 'eastern' nuclear genotype, and predicts that the *w*B bacterium will be weaker in transmission or CI.

A further caveat is necessary. Telschow *et al*. (2003b) found that below the threshold migration rate, *Wolbachia* differences between the populations are maintained. In this situation, homogenization between the two populations occurs at neutral or very weakly selected loci, but genetic divergence is maintained at loci subject to moderate to strong divergent selection (e.g. $s > 10^{-3}$) in the two populations. Owing to insufficient sample sizes and number of nuclear markers, we would not be able to detect such a pattern in our current analysis. We therefore cannot rule out the possibility of some genetic differentiation between the different infection types in Midwestern populations, and there is even a suggestion of this in the AFLP phylogeny (Fig. 3). Furthermore, the observation that 'western' pupal and adult morphologies occur at low frequency in Midwestern and eastern populations (Whitworth, unpublished) also suggests the possibility that some differences may be maintained, although it remains to be established whether these are associated with infection status. Clearly, more detailed studies are needed to determine whether some genetic differentiation between A- and B-infected individuals occurs in Midwestern populations.

In summary, our results are most consistent with the view that *w*A1 and *w*A2 are replacing *w*B in Midwestern populations. This interpretation is supported by the AFLP data, which indicate that *w*A1- and *w*A2-infected individuals in the Midwest are genetically similar to eastern and Midwestern flies infected with *w*B. However, we are not yet able to rule out lineage sorting in Midwestern populations of an infection polymorphism, nor some genetic differentiation based on infection type. Further sampling is needed to resolve these issues. In addition, crossing experiments will be required to determine if the *Wolbachia* of *P. sialia* cause bidirectional CI. Based on the observations, it is presumed that *w*A1 and *w*A2 have a stronger CI type (or higher transmission) than *w*B. Owing to the levels of *Wolbachia* polymorphism observed in the Midwest, we would predict considerable CI in those populations. However, some studies have shown much lower levels of CI in field populations than predicted by CI levels measured in laboratory strains (Hoffmann *et al*. 1998). Levels of CI in natural populations, therefore, need to be measured. Further study is also needed to determine whether there is some genetic differentiation associated with infection status in Midwestern populations or whether they are completely admixed, and whether mating occurs independently of infection status in these populations.

Finally, it is worth pointing out that an interesting pattern is emerging. In the parasitic wasp genus *Nasoni*a, we observed

different *Wolbachia* infections between recently diverged species in eastern (*N. gira*ulti) and western (*N. longicornis*) North America (Bordenstein *et al*. 2001; Werren & Bartos 2001). In *P. sialia*, the preferred blowfly hosts of *Nasonia*, we have shown infection type differences between eastern and western populations. J. Jaenike (personal communication) observed similar differences in infection status among mushroom feeding *Drosophila*. These observations suggest that this geographical pattern may be common. Therefore, populations and closely related species of insects in eastern and western North America could provide an natural laboratory for measuring rates of acquisition of different *Wolbachia* in geographically separated populations, and for quantifying how frequently *Wolbachia* are associated with reproductive incompatibility between geographical populations and recently evolved species.

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