Heredity (2002) 88, 182–189
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Dynamics of double and single *Wolbachia* infections in *Drosophila simulans* from New Caledonia

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The bacterial symbiont *Wolbachia* can cause cytoplasmic incompatibility in *Drosophila simulans* flies: if an infected male mates with an uninfected female, or a female with a different strain of *Wolbachia*, there can be a dramatic reduction in the number of viable eggs produced. Here we explore the dynamics associated with double and single *Wolbachia* infections in New Caledonia. Doubly infected females were compatible with all males in the population, explaining the high proportion of doubly infected flies. In this study, males that carry only *w*Ha or *w*No infections showed reduced incompatibility when mated to uninfected females, compared with previous reports. These data suggest that either the DNA of these bacterial isolates have diverged from those previously collected, or the genetic background of the host has lead to a reduction in the phenotype of incompatibility. Mitochondrial sequence polymorphism at two sites within the host genome was assayed to investigate population structure related to infection types. There was no correlation between sequence polymorphism and infection type suggesting that double infections are the stable type, with singly infected and uninfected flies arising from stochastic segregation of bacterial strains. Finally, we discuss the nomenclature of *Wolbachia* strain designation.
Heredity (2002) **88,** 182–189. DOI: 10.1038/sj/hdy/6800025

Keywords: *Drosophila simulans*; *Wolbachia*; New Caledonia; cytoplasmic incompatibility; strain concept

Introduction

Wolbachia pipientis is a common symbiont of *Drosophila simulans*. *Wolbachia* may cause a number of reproductive abnormalities, including cytoplasmic incompatibility (Yen and Barr, 1971; Hoffmann *et al*, 1986). In *D. simulans*, incompatibility may occur when an infected male mates with an uninfected female, or a female with a different strain of *Wolbachia*. In such a cross, there may be a dramatic reduction in the number of viable eggs produced. The reciprocal cross usually yields normal numbers of progeny, unless the parents harbor different strains of *Wolbachia*. The aim of this study is to explore the dynamics of a host population from New Caledonia with uninfected, singly infected, and doubly infected individuals.

D. simulans is an excellent model system for investigating the host and symbiont population genetics. *D. simulans* is a cosmopolitan species that may harbor five strains of *Wolbachia* (*w*Ha, *w*No, *w*Ri, *w*Au, and *w*Ma). A sixth strain, *w*Ki, has been described from Tanzania, but is homosequential with *w*Ma at both the 16S rDNA and *wsp* loci and is likely the same. In this study, we refer to *w*Ma instead of *w*Ki because *w*Ma has nomenclatural precedence. In *D. simulans*, mitochondrial variation is structured into three monophyletic haplotypic groups designated *si*I, *si*II, and *si*III (Baba-Aïssa *et al*, 1988; Solignac and Monnerot, 1986). Furthermore, mitochondrial DNA (mtDNA) appears to be non-randomly associated with the bacterial strains (Montchamp-Moreau *et al*, 1991; Rousset and Solignac, 1995; James and Ballard, 2000). The *w*Ha and *w*No strains have only been detected in individuals with the *si*I haplotype, *w*Ri and *w*Au in flies with *si*II haplotypes, and *w*Ma in *si*III individuals. The goal of this study is to focus on the dynamics of flies that carry the *si*I haplotype and their *w*Ha and/or *w*No strains of *Wolbachia*.

It has been demonstrated both theoretically (Caspari and Watson, 1959) and empirically (Turelli and Hoffmann, 1991) that *Wolbachia* infections that cause incompatibility spread once they reach a threshold frequency. As a *Wolbachia* infection sweeps through a population, other cytoplasmic factors hitchhike with bacterial transmission. For example, Nigro and Prout (1990) started two sets of *D. simulans* population cages carrying two mitochondrial types (C and P), with one of the types at a frequency of 20% in one set and 80% in the other. The C type occurred in a host infected with *Wolbachia* whereas the P type was associated with an uninfected host. In all cages, there was a rapid increase in the frequency of the C type as the infection became predominant under unidirectional incompatibility. In the mosquito *Aedes albopictus*, changes in mtDNA frequencies have also been associated with a spreading *Wolbachia* infection in the laboratory (Kambhampati *et al*, 1992).

Wolbachia also causes shifts in mtDNA variation in natural populations. Incompatibility in *Drosophila* was first discovered in crosses between a predominantly infected population of *D. simulans* near Riverside, California, with various uninfected populations in northern and

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Received 22 June 2001; accepted 31 October 2001

central California (Hoffmann *et al*, 1986). Initially, the infection was limited to sites south of the Tehachapi transverse range that separates the Los Angeles basin from the Central Valley. However, infected flies became increasingly common in the Central Valley after 1988 and a rapid spread north was observed from 1989 until 1994 (Turelli and Hoffmann, 1991; Turelli *et al*, 1992; Hoffmann and Turelli, 1997). As the infection swept through populations, the mtDNA variant initially associated with the infected *D. simulans* increased in frequency. Extending earlier work on incompatibility (Caspari and Watson, 1959), Turelli and colleagues (Turelli *et al*, 1992; Hoffmann and Turelli, 1997) developed a model with intrapopulation dynamics for the joint frequency of incompatibility types and mtDNA genotypes.

The existence of double infections in New Caledonia are somewhat of a mystery as the *w*No infection type is not reported to elicit high incompatibility. To the north and east of New Caledonia, *w*No is not found. Populations in Hawaii and Tahiti have very high frequencies of single infections with *w*Ha individuals (O'Neill *et al*, 1992; Rousset *et al*, 1992; Turelli and Hoffmann, 1995). To the southwest, Australian populations are infected with *w*Au (Hoffmann *et al*, 1996). The only other known locality where double infections have been reported is in the Seychelles (Merçot *et al*, 1995b; Merçot and Poinsot, 1998).

Here we investigate empirically why these two strains coexist in a seemingly stable frequency in New Caledonia. Are they acting synergistically, independently, or is *w*No parasitizing *w*Ha? To address these questions, we assayed infection types in a population known to harbor double infections, identified their incompatibility phenotypes, and sequenced two polymorphic regions in the mitochondrial genome of their host. One notable finding is that *w*Ha does not always induce strong incompatibility, as previously observed. Furthermore, infection status did not correlate with any detectable mitochondrial substructure within the *si*I haplotype. These data suggest that the population has reached an equilibrium where singly infected or uninfected flies arise through stochastic segregation from doubly infected mothers. We discuss these results and link them back to an inclusive strain concept for *Wolbachia*.

Materials and methods

Drosophila lines and molecular classification of *Wolbachia*

Fifty-five isofemale lines of *D. simulans* were established from flies collected in Nouméa, New Caledonia, on 29 and 31 December 1999. DNA was isolated from these lines within 1.5 months of them being established in the laboratory. To determine the *Wolbachia* infection status of these lines we employed a strain specific PCR assay that generated an amplicon of specific size for each *Wolbachia* strain. We sequenced select lines to confirm the lines were infected with the expected *Wolbachia* strains.

DNA from all fly lines was extracted using the Puregene™ kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's protocol designated 'DNA isolation from fixed tissue'. Final DNA concentrations of fly extracts were determined using a GeneQuant™ spectrophotometer (Pharmacia, Alamedia, CA, USA). The control lines used in the molecular classification of *Wolbachia* strains and mitochondrial haplotypes have been described in detail elsewhere (Ballard, 2000a, b; James and Ballard, 2000). NC48 is infected with both *w*Ha and *w*No. TT01 carries the *w*Ha infection, and RU07 carries the *w*Ma infection (which differs from *w*No by 1 bp in the 16S rDNA but is homosequential to *w*No at the *Wolbachia* major surface protein [*wsp*] locus).

To assay for presence or absence of *Wolbachia* infection, *Wolbachia* 16S rDNA was PCR amplified following a modified protocol of O'Neill *et al* (1992). The thermal profile was shortened to 30 cycles, and the denaturation and annealing steps were run for 15 s each instead of 1 min. Any uninfected result was checked by running the same extraction and an independent extract of three flies from the isofemale line with primers that amplify a region of the *wsp* locus (Zhou *et al*, 1998).

To survey the population for specific *Wolbachia* strains, a multiplex PCR reaction that amplifies a region of the *wsp* locus was designed. The forward primer 81F of Zhou *et al* (1998) and newly designed reverse primers 463R (5′-TACCATTTTGACTACTCACAGCG-3′) and 635R (5′-GATCTCTTTAGTAGCTGATAC-3′) were used. The 81F primer anneals to both *w*Ha and *w*No sequence. With our protocol, 487R amplifies a 427 bp product from *w*Ha and 658R amplifies a 570 bp product from *w*No (Figure 1). The 10 μl PCR reactions consisted of 10 ng template DNA, 1 μl 81F, 1 μl 658R, 0.35 μl 487R (all primers at 10 ng/μl), 1 μl of 8 mM dNTP, 4.625 μl ddH$_2$O, 1 μl 10× PCR buffer with 25 mM MgCl^{2+}, and 0.25 units *Taq* poly-

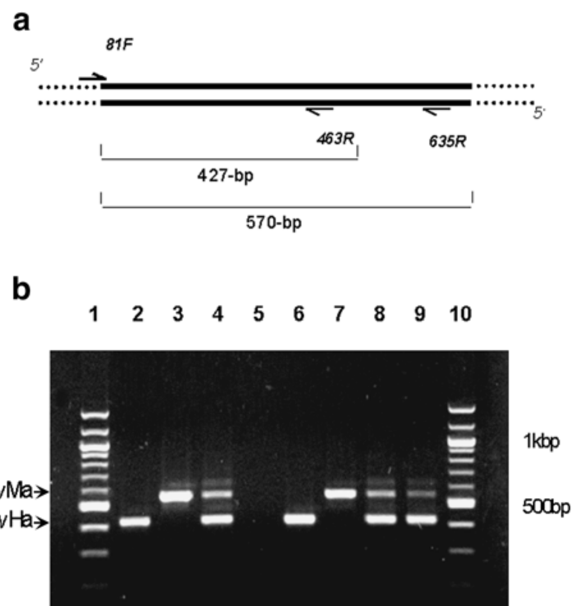

Figure 1 Detection of *Wolbachia* infections in *D. simulans*. (**a**) Schematic diagram of PCR amplification of surface binding protein gene from *Wolbachia* (*wsp*). Primers 81F and 635R amplify a 570 bp fragment specific for *w*No; primers 81F and 463R produce a 427 bp fragment specific for strain *w*Ha. Two fragments are produced from doubly infected individuals. (**b**) PCR amplicons from three controls and five experimental lines on a 1.5% agarose gel. Lane 1: 100-bp DNA ladder; lane 2: TT01 (*w*Ha control); lane 3: RU07 (*w*No or *w*Ma control); lane 4: NC48 (*w*Ha/*w*No doubly infected control); lane 5: NC103 (uninfected); lane 6: NC112 (*w*Ha-infected); lane 7: NC117 (*w*Ma-infected); lane 8: NC102 (doubly infected); lane 9: NC125 (doubly infected); lane 10: 100-bp DNA ladder.

merase (Roche, Nutley, NJ, USA). The PCR profile was 35 cycles at 94°C for 30 s, 52°C for 15 s, and 72°C for 1 min. PCR amplicons were electrophoresed on a 1.5% agarose gel stained with ethidium bromide to visualize the size of the products. If the fly line was identified as infected with only one strain, the PCR was repeated with single pairs of primers for positive and negative verification of infections.

We also sequenced portions of the 16S rDNA and *wsp* loci for lines that we included in the incompatibility assays as described in James and Ballard (2000). The NC112 line was infected with *w*Ha, with 16S rDNA sequence identical to GenBank accession number X61769 (O'Neill *et al*, 1992) and *wsp* sequence identical to accession number AF020068 (Braig *et al*, 1998). The NC117 is infected with *w*No, with 16S rDNA identical to number AF312372 (James and Ballard, 2000) and *wsp* number AF020074 (Zhou *et al*, 1998). The *wsp* sequences of each strain present in doubly infected flies from New Caledonia have previously been shown to be identical to those in the singly infected lines (James and Ballard, 2000).

Cytoplasmic incompatibility

Collection of doubly infected, singly infected, and uninfected lines from New Caledonia provided a unique opportunity to study the dynamics between symbiont strains collected from a single population. Most previous intrapopulational studies have focused on the dynamics between flies infected with a single strain of *Wolbachia* and uninfected flies (Hoffmann and Turelli, 1988; Turelli and Hoffmann, 1991, 1995; Turelli *et al*, 1992). We also determined if the incompatibility phenotype associated with singly infected *w*No flies was the same as experimentally constructed lines (Merçot *et al*, 1995b; Merçot and Poinsot, 1998).

Four lines from New Caledonia were chosen for incompatibility phenotype analyses. NC102 is doubly infected with *w*Ha and *w*No, NC112 carries only *w*Ha, NC117 carries only *w*No, and NC103 is uninfected. However, we do not have replicate lines within each *Wolbachia* infection status, and we cannot distinguish if the results presented here are specific to the lines used here or a general result for the whole population.

Our cytoplasmic incompatibility assay technique is described in James and Ballard (2000). Briefly, larvae were raised at constant temperature and density, and virgin adults were collected within a 12-h period. They were aged 3 days and then pairs were introduced in vials and left 24 h to mate. *D. simulans* isofemale lines were shown to mate at random (Ballard *et al*, in press) and we do not expect any bias in mating success among lines. Females were isolated and then placed in fresh vials for three 24-h periods. The vials from the first day were discarded, while all eggs laid on the latter two were counted within 8 h of the transfer. Between 26 and 36 h after the transfer, the number of eggs left unhatched was counted. The expression of cytoplasmic incompatibility was quantified as the number of eggs left unhatched in the second counting period divided by the total number of eggs laid. We counted between 13 and 20 pairs per cross (Table 1). Each of these 281 crosses laid, on average, 92 (±2 s.e.) eggs. The uninfected flies employed as controls in this study were collected in the field free of *Wolbachia* infection. An alternative design would be to tetracycline treat infected flies to cure them of infection (O'Neill and Karr, 1990). A disadvantage of using tetracycline treated lines is that the antibiotic may influence the fitness of the flies.

We arbitrarily assign compatibility as less than 30% eggs unhatched, incompatibility as greater than 70% eggs unhatched and partial incompatibility as any intermediate percentage. No median compatibility was greater than 8%, no incompatibility less than 97%, and partial incompatibility ranged from 30 to 42%. We present medians rather than means, because these incompatibility data are not normally distributed.

The Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal and Rohlf, 1995, pp 446–447) was used to test the model of incompatibility levels between each bacterial infection status. We sequentially removed the bacterial infection types that have the most complex incompatibility patterns and re-test the data to determine what effects remain significant (after James and Ballard, 2000).

mtDNA sequencing

If any infection type (single or double) in New Caledonia was in the process of increasing in frequency, the linked mtDNA genotype may also be expected increase in parallel, yielding a pattern of strong correlation between haplotype and infection type (Turelli *et al*, 1992). In this study, we were interested in testing whether the distinct *Wolbachia* strains collected in New Caledonia were associated with a specific mtDNA genotype. To investigate this question, we sequenced a region containing a variable length AT repeat that occurs in an intervening sequence between ND3 and alanine tRNA gene sequences, and a single nucleotide polymorphism (SNP) at site 5545 in an intervening sequence between COIII and the glycine tRNA (Ballard, 2000a). Although only *si*I flies have been collected from New Caledonia (Merçot *et al*, 1995b), sequencing this region would also determine if *si*II or *si*III flies were also collected. No *si*II or *si*III flies were found.

Based on GenBank accession number AF200834 (a complete mitochondrial sequence of the *si*I haplotype used as a control in this manuscript), the 3′ end of the forward primer (5′ ATTGACATTTGTTGATGTAGTTT 3′) aligns to position number 5471, and the reverse primer (5′ TGAATATTCAATACTTTTTGAATG 3′) to base 6035. The 50 μl PCR reactions consisted of 4 μl template DNA (10 ng/μl), 2 μl of each primer (25 ng/μl), 5 μl of 8 mM dNTP, 31.8 μl ddH2O, 5 μl of 10× PCR buffer with 25 mM $MgCl^{2+}$, and 0.2 μl Taq polymerase. The PCR profile included 35 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min.

To visualize amplification products, 4 μl of the PCR product was run on a 1.5% agarose gel stained with ethidium bromide. The remaining PCR product was precipitated with 23 μl 7.5 M ammonium acetate and 69 μl cold 100% ethanol. Precipitates were washed with 200 μl cold 70% ethanol, dried, and re-suspended with 25 μl water. Purified PCR products were then quantified using a GeneQuant™ spectrophotometer (Pharmacia) prior to sequencing.

Sequencing reactions were carried out using 30–35 ng purified PCR product, 25 ng of primer, 4 μl 1:2 TRR mix from an ABI Prism Big Dye™ Terminator Cycle Sequencing Kit, and brought to 10 μl with ddH_2O. We collected double stranded sequence for all samples.

Table 1 Median eggs unhatched between fly lines with different bacterial strains (sample size; 25th and 75th quantiles). Each value represents the female labels from the top mated with the males to the left

Male fly line	*Bacteria strain*	*Female fly line*			
		NC103 *w*-	*NC112* *w*Ha	*NC117* *w*No	*NC102* *w*Ha + *w*No
NC103	*w*-	0.00 (19; –0.00, +0.01)	0.01 (18; –0.00, +0.03)	0.01 (16; –0.00, +0.05)	0.00 (19; –0.00, +0.08)
NC112	*w*Ha	0.33 (17; –0.23, +0.80)	0.04 (15; –0.01, +0.04)	1.00 (15; –0.96, +1.00)	0.05 (18; –0.05, +1.00)
NC117	*w*No	0.08 (19; –0.02, +0.16)	0.30 (15; –0.03, +1.00)	0.04 (15; –0.02, +0.24)	0.02 (20; –0.00, +0.24)
NC102	*w*Ha + *w*No	0.97 (18; –0.93; +0.99)	0.34 (17; –0.08, +0.62)	0.42 (20; –0.23, +0.59)	0.00 (20; –0.00, +0.02)

Results

Drosophila lines and *Wolbachia* infections

Of 55 isofemale lines from New Caledonia, 47 were doubly infected, four were singly infected with *w*Ha and three were singly infected with *w*No. One line was identified as uninfected by the initial screen and confirmed by further analyses.

Cytoplasmic incompatibility

The expression of incompatibility (Table 1) is shown schematically in Figure 2. All lines are self compatible (range of medians of proportion eggs unhatched is 0.00–0.04), and uninfected males can successfully reproduce with females that carry any bacteria (0.00–0.01). Doubly infected males are incompatible with uninfected females (median proportion eggs unhatched is 0.97) and partially incompatible with females that carry either *w*Ha (0.34) or *w*No (0.42) singly. Males with *w*Ha are compatible with females doubly infected with *Wolbachia* (0.05). They are incompatible with females that carry *w*No (1.00), and partially incompatible with uninfected females (0.33). Males that carry *w*No are partially incompatible with *w*Ha-infected females (0.30) and exhibit low, but significant, incompatibility when crossed with uninfected females (0.02).

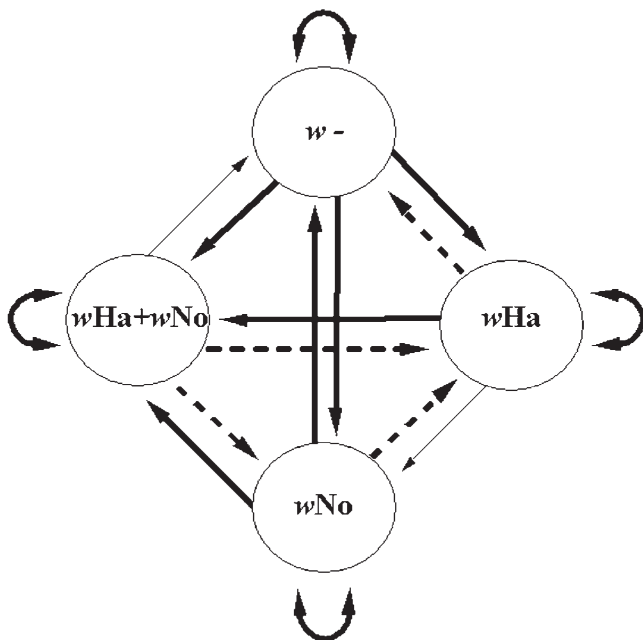

Figure 2 Schematic of cytoplasmic incompatibility between *D. simulans* lines that either carry the *Wolbachia* strain *w*Ha, *w*No, both, or are uninfected. Arrows go from males to females. The thickness of the line represents the level of gene flow. Thin lines represent incompatibility, thick lines represent compatibility, and dashed lines represent variable expression of incompatibility.

When all lines are considered, there are significant incompatibility differences between males and females, and there is a significant interaction between the two (Table 2a). As a consequence of this result we removed doubly infected lines. Doubly infected males are known to elicit strong incompatibility, while females are expected to be compatible with all the males in this study (Merçot *et al*, 1995b; Merçot and Poinsot, 1998). Table 2b shows that there are significant incompatibility differences between the remaining singly infected and uninfected males and there is a significant male-by-female

Table 2 Kruskal-Wallis tests of cytoplasmic incompatibility in males and females from different lines

Source	*df*	*SS*	*H*	*P*
(a) All lines				
Male	3	360461.6	65.52	<0.001
Female	3	210300.5	32.97	<0.001
Male × Female	9	281910.0	44.20	<0.001
Error	278	919656.9		
(b) Comparison of three fly lines (NC103 (*w*-), NC112 (*w*Ha) and NC117 (*w*No))				
Male	2	79744.0	44.65	<0.001
Female	2	2004.4	1.12	NS
Male × Female	4	42599.2	23.85	<0.001
Error	146	260760.0		
(c) Comparison of two fly lines (NC103 (*w*-) and NC117 (*w*No))				
Male	1	4322.7	11.87	<0.001
Female	1	7.8	0.02	NS
Male × Female	1	268.7	0.76	NS
Error	66	24045.5		

NS = not significantly different.

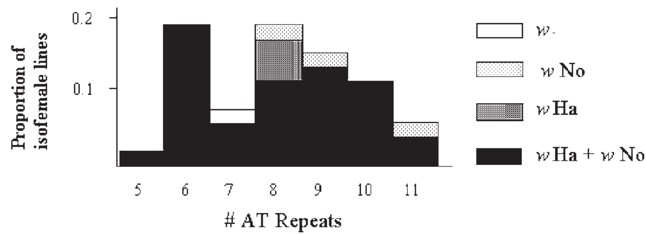

Figure 3 The number of AT repeats sequenced from 55 lines collected in New Caledonia. The repeat region occurs in an intervening spacer region between COIII, and the glycine tRNA coding sequence.

interaction effect. Singly infected and uninfected females do not differ significantly in their incompatibility with singly infected or uninfected males. We then removed the singly infected *w*Ha line because this *Wolbachia* strain is known to elicit incompatibility (O'Neill and Karr, 1990; James and Ballard, 2000). Table 2c shows a significant male effect between *w*No infected males and uninfected females.

mtDNA

The number of AT repeats in the intervening sequence between ND3 and the alanine tRNA ranges from 5 to 11 (Figure 3). Doubly infected flies carry 5–11 repeats, singly infected *w*No carrying lines carry 8, 9, or 11 repeats and the uninfected line carries 7 repeats. The four singly infected *w*Ha lines all carry 8 repeats as previously reported by Ballard (2000a). Nine of these lines were shown to be heteroplasmic for repeat number. To test whether *w*Ha infection is directly linked with 8 AT repeats we assayed 18 *w*Ha-infected Hawaiian lines: one carried 6 AT repeats, one carried 7 repeats, 12 carried 8 repeats, two carried 9 repeats, and two carried 10 repeats.

To further explore genetic substructure of flies carrying the *w*Ha from New Caledonia, we assayed the SNP at position 5545. Three lines had an A at this site and one a G. The polymorphism at this site was not associated with any infection type in any detectable manner. We suggest these data indicate that *Wolbachia* infection status is not linked with a specific mtDNA genotype in this population.

Conclusions

Wolbachia

This is the first study to report on the collection of doubly infected, singly infected and uninfected flies from a single population. Forty-seven isofemale lines were doubly infected (85.5%; 95% confidence intervals (CI) calculated directly from the binomial distribution are (73.4 to 93.5%), four were singly infected with *w*Ha (7.2%; CI 2.0 to 17.6%), three were singly infected with *w*No (5.5%; CI 1.1 to 15.2%), and one was uninfected (1.8%; CI 0.0 to 2.4%). Double infections and single *w*Ha infections have previously been reported from New Caledonia but their relative frequencies have not been determined (Merçot *et al*, 1995b; Merçot and Poinsot, 1998). Doubly infected females are compatible with all males regardless of their infection status. Singly infected *w*No and uninfected *si*I lines have both been produced by stochastic loss of the bacterial strains in the laboratory (Merçot *et al*, 1995a; Merçot and Poinsot, 1998), but this is the first report of these types collected from nature.

Incompatibility assays indicate that the two single infections (*w*Ha and *w*No) from New Caledonia are not independent of each other. The incompatibility is higher when a singly infected male is crossed with a female harboring the other strain rather than with an uninfected female. These data suggest that both male and female components contribute to the phenotypic expression of incompatibility in this system. Males harboring *w*Ha show strong incompatibility when crossed to *w*No females (1.00) but intermediate incompatibility with uninfected females (0.33). Likewise, males infected with the *w*No strain show intermediate incompatibility with *w*Ha females (0.30) but low incompatibility with uninfected females (0.08).

Our results have important implications for studying the dynamics of *Wolbachia* infections in *D. simulans*. In this study, we employ a median and the 25th and 75th quantiles because the incompatibility data are not normally distributed. Most previous studies have presented incompatibility as a mean and standard error. To facilitate direct comparisons with previous studies we compare means and standard errors in this paragraph. We observed intermediate incompatibility when males infected with *w*Ha were crossed with uninfected females (54% ± 8). Poinsot and Merçot (2001) also report low incompatibility in *w*Ha infected males from New Caledonia (57.7%). In contrast, previous reports revealed strong incompatibly caused by *w*Ha infected males from Hawaii and Tahiti when crossed with uninfected females (greater than 95%, O'Neill and Karr, 1990; Merçot *et al*, 1995b; James and Ballard, 2000). Also, we find low (22% ± 8) but significant incompatibility between *w*No males and uninfected females. In previous studies, males carrying *w*No were incompatible (78–84%; Merçot *et al*, 1995b) or partially incompatible (56%; Merçot and Poinsot, 1998) with uninfected females. Consistent with previous reports, we observed that males carrying *w*No are compatible with doubly infected females (7% ± 3) and partially incompatible with females carrying *w*Ha (43% ± 1; Merçot *et al*, 1995b; Merçot and Poinsot, 1998).

Assuming that the differences in incompatibility are not an artifact of the methodologies employed to conduct the assays, there are at least three explanations for the observed results. First, the recently collected *Wolbachia* isolates from New Caledonia may have diverged from previous collections. Sequencing additional loci may help test this hypothesis. Second, the genetic background of the host may have diverged from the other lines tested. The phenotype of incompatibility and segregation of *Wolbachia* is dependent on the genetic background of the host line (Boyle *et al*, 1993; Poinsot *et al*, 2000). The latter alternative may be addressed by microinjecting, or backcrossing, the *w*Ha and *w*No isolates from New Caledonia into standard hosts to standardize for host genetic background. Microinjection will permit the separation of host *vs* symbiont effects, as well as the interaction between the two. Backcrossing maintains the maternal cytotype such that the *Wolbachia*-mitochondrial interaction is maintained. Third, the *w*No strain of *Wolbachia* may exhibit varying levels of incompatibility. James and Ballard (2000) previously reported that *w*Ma infected males are heterogeneous in their expression of incompatibility. The *w*No and *w*Ma isolates are identical in the region of the

wsp locus sequenced, and differ by a single substitution in the 16S rDNA. As a consequence it is possible that *w*No and *w*Ma are not distinct strains but sequence variants of the same strain. Resolution of this issue is not just taxonomic but also has important implications for the evolution of *Wolbachia* in *D. simulans*. Determination of strain status in *Wolbachia* is not simple and we discuss this nomenclatural issue more generally below.

One step to resolve these alternatives is to compare recently collected isolates with 'type' bacterial strains and fly lines. An informal meeting of biologists at *Wolbachia* 2000 (an international *Wolbachia* meeting in Crete, Greece) identified 'type' *D. simulans*/*Wolbachia* complexes (Table 3). These lines and *Wolbachia* sequence variants are available from the laboratories of A. A. Hoffmann (La Trobe University, Australia), H Merçot (Jacques Monod, France) and the authors.

Mitochondria

Two *Wolbachia* strains infect the *si*II haplotype. James and Ballard (2000) observed that *w*Ri infected flies were always associated with *si*IIA mtDNA and *w*Au flies the *si*IIB mtDNA. In this study, all lines had the *si*I haplotype but *Wolbachia* infection status was not correlated with a specific mtDNA genotype and we hypothesize that singly infected flies arose through stochastic loss of one bacterial strain from a doubly infected mother, as has been predicted theoretically (Frank, 1998). Uninfected flies may have arisen from the loss of infection from either doubly or singly infected flies. In this study, about 7.2% of lines were *w*Ha, 5.5% *w*No, and 1.8% uninfected. We do not have estimates on the loss of infection in the field, however, Poinsot *et al* (2000) studied the segregation rate from doubly infected mothers to singly infected and uninfected progeny in the laboratory. On the basis of backcrossing studies in the laboratory, they estimated that doubly infected females could produce 3.5% *w*Ha singly infected, 1.8% *w*No singly infected and 0.8% uninfected eggs. Perrot-Minnot *et al* (1996) also observed stochastic loss of *Wolbachia* double infections after an artificially long diapause in *Nasonia* lines.

In future studies we will endeavor to reconstruct the movement of *Wolbachia* strains and mtDNA genotypes around the world. This study gives our first glimpse into the movement of the *si*I mtDNA haplotype and the *Wolbachia* strains it harbors. It is likely that the *D. melanogaster* subgroup diverged in East Africa (Lachaise *et al*, 1986) and the simulans clade in the islands of East Africa. Doubly infected *si*I flies have only been collected from the Seychelles and New Caledonia suggesting that the flies traveled from the Seychelles to New Caledonia. However, additional collections between these islands is needed. New Caledonia may be the source of the *w*Ha infection that has spread to other Pacific Islands. Lines collected in Hawaii carried a subset of the bacterial strains (only *w*Ha).

Nomenclature

Currently, there is no established nomenclatural system and a variety of criteria have been employed to define a strain of *Wolbachia*. In *D. simulans*, strains have been designated on the basis of incompatibility phenotype (Hoffmann *et al*, 1986; Merçot *et al*, 1995b; O'Neill and Karr, 1990), 16S rDNA sequence variation as little as a single base pair change (O'Neill *et al*, 1992; Rousset *et al*, 1992; Hoffmann *et al*, 1996; James and Ballard, 2000), and host collection locality (Merçot and Poinsot, 1998). Lincoln *et al* (1998) define a 'strain' as 'a group of individuals with common physiological traits and presumed common ancestry; an infraspecific group having characteristic properties'. 'Presumed common ancestry' can be determined independently of the host by studying DNA sequence variation. In contrast, 'common physiological traits' and 'characteristic properties' are best studied in the context of a specific host genetic background.

One methodology to test 'presumed common ancestry' (Lincoln *et al*, 1998) from *Wolbachia* isolates is to construct phylogenetic hypotheses from DNA sequence data, and identify strains that are reciprocally monophyletic. The phylogenetic method is independent of host genotype but it is not always clear how selection and recombination (Schulenburg *et al*, 2000; Jiggins *et al*, 2001; Werren and Bartos, 2001) influence phylogenetic reconstruction (Slowinski and Page, 1999; Ballard, 2000c). As a consequence great care must be taken when defining a strain phylogenetically. An alternative would be to construct an arbitrary level of sequence divergence at a specific locus. However, arbitrary rules of phenetic divergence may not be biologically meaningful (Ballard *et al*, in press).

Wolbachia have a variety of 'physiological traits' (Lincoln *et al*, 1998) that are likely to be dependent on the host-genetic background. These include density in the host (Breeuwer and Werren, 1993), segregation rate (Hoffmann and Turelli, 1988; Hoffmann *et al*, 1990), and their influence on specific life history traits (Hoffmann and Turelli, 1997). We suggest that effects of *Wolbachia* on each of these traits should be studied in standardized host genetic backgrounds.

Table 3 Lines of *D. simulans* and the 'type' *Wolbachia* sequence variants defined by 16s rDNA and *Wolbachia surface protein* coding sequences

Fly Line	*MtDNA*	*Wolbachia*	*Collection Site*	*Reference*
DSW	*si*II	*w*-	Watsonville, CA, USA	Hoffmann *et al*, 1986
DSR	*si*II	*w*Ri	Riverside, CA, USA	Hoffmann *et al*, 1986
NC48	*si*I	*w*Ha + *w*No	Nouméa, New Caledonia	Rousset and Solignac, 1995
Hawaii	*si*I	*w*Ha	Honolulu, HA, USA	O'Neill and Karr, 1990
N7No	*si*I	*w*No	Nouméa, New Caledonia	Merçot and Poinsot, 1998
Coffs	*si*II	*w*Au	Coffs Harbour, Australia	Hoffmann *et al*, 1996
MD199[a]	*si*III	*w*Ma	Joffreville, Madagascar	James and Ballard, 2000

[a]As the original line had been lost, this fly line has been designated the replacement because it was collected in the same locality. A sixth variant of *Wolbachia* has been reported in the literature (*w*Ki). This variant is likely to be identical to *w*Ma and is not included in this list (Herve Merçot and Sylvain Charlat, personal communication).

A complementary method to define a strain is 'an infraspecific group having characteristic properties' (Lincoln *et al*, 1998). One characteristic property of *Wolbachia* in *D. simulans* is its incompatibility phenotype. Although strains may induce strong, weak, or intermediate incompatibility, these simple phenotypic definitions are confounded by differences in host genetic background, which can greatly affect the bacterial expression of incompatibility (Poinsot and Merçot, 2001). This plasticity of phenotypic expression makes us wary of using the level of incompatibility in the definition of a strain until both host genetic background and *Wolbachia* density are controlled. Phenotypic definitions may become especially labile under certain environmental conditions, such as larval rearing conditions (Sinkins *et al*, 1995), heat shock or multiple matings (Hoffmann *et al*, 1986; Snook *et al*, 2000).

Here we investigate the effects of single and double *Wolbachia* infections on expression of incompatibility and mtDNA divergence of *D. simulans* from New Caledonia. Doubly infected females were compatible with all males in the population, explaining the high proportion of doubly infected flies. Males infected with *w*Ha from New Caledonia showed reduced incompatibility when mated to uninfected females compared to males from Hawaii or Tahiti. Also, males carrying *w*No had reduced incompatibility from studies previously reported. These data suggest that the DNA of these bacterial isolates may have diverged from those previously collected, the genetic background of the host has lead to a reduction in the phenotype of incompatibility, and/or *w*No infected males, like *w*Ma infected males, are heterogeneous in their expression of incompatibility. There was no association between mtDNA sequence polymorphism and infection type suggesting that single and uninfected flies arise from stochastic loss of bacteria strains. These points stimulate us to contemplate the factors that should be considered when designating *Wolbachia* strains.

Acknowledgements

We are grateful to Ary A Hoffman, Hervé Merçot, and Sylvain Charlat for productive discussions leading to the designation of the *Wolbachia* type strains. We also thank Sylvain Charlat for sending us an unpublished manuscript. Kirrie Ballard helped collect flies in Nouméa. All sequencing was done at the Pritzker Laboratory of Molecular Systematics and Evolution, The Field Museum, Chicago Illinois, USA. Funds were provided by National Science Foundation Grant No. DEB-9702824 and the Field Museum Marshall Field Fund.

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