Wolbachia segregation dynamics and levels of cytoplasmic incompatibility in *Drosophila sechellia*

S Charlat, P Bonnavion and H Merçot

Institut Jacques Monod, Laboratoire Dynamique du Génome et Evolution, CNRS-Universités Paris 6 & 7, 2 Place Jussieu 75251 Paris Cedex 05, France

In *Drosophila sechellia*, the endocellular bacterium *Wolbachia* induces cytoplasmic incompatibility (CI): in crosses involving infected males, a partial or complete embryonic mortality occurs unless the female bears the same *Wolbachia*. *D. sechellia* is known to harbour two *Wolbachia* variants, namely *wSh* and *wSn*, closely related to *wHa* and *wNo*, respectively, two strains infecting the populations of *D. simulans* from the Seychelles archipelago and New Caledonia. Strikingly, the two species show similar infection patterns: in *D. sechellia*, *wSh* can be present on its own or in double infection with *wSn*, but individuals carrying *wSn* only do not occur; in *D. simulans*, *wHa* can be present on its own or in double infection with *wNo*, but individuals carrying *wNo* only do not occur, or occur at very low frequency. Previous

experiments on *D. simulans* showed that lines singly infected by *wNo* can be obtained by segregation, and stably maintained. Here we investigate this issue in *D. sechellia* through an 18 generation experiment, and show that *wSn* and *wSh* singly infected lines can arise by segregation. Using singly infected lines obtained in this experiment, we estimate the CI intensities of *wSh* and *wSn* in *D. sechellia*, and compare these to the CI intensities of the same *Wolbachia* injected into *D. simulans*. Our results do not suggest any consistent effect of the host species on the CI induced by *wSh*. On the contrary, it seems that *wSn* expression is repressed by host factors in *D. sechellia*.

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Introduction

In *Drosophila sechellia*, as in many other Arthropod species, the endocellular bacterium *Wolbachia* induces an intriguing form of male sterility, known as cytoplasmic incompatibility (CI) (reviewed in Hoffmann and Turelli, 1997; Charlat *et al.*, 2002a). Males carrying the symbiont suffer a complete or partial loss of fertility when mated with uninfected females, but fertility is restored if the female harbours the same *Wolbachia* variant. Uninfected females thus suffer a fertility deficit relative to infected ones, the intensity of which depends on the frequency of infected males. The bacterium being transmitted by females only, through the egg cytoplasm, this highly deleterious effect on male hosts is not counter selected. On the contrary, infected females being fitter than uninfected ones, CI allows *Wolbachia* to invade uninfected host populations in a positive frequency-dependent manner and then maintain itself (Caspari and Watson, 1959; Turelli and Hoffmann, 1995). In fact, CI is only one of the various means by which *Wolbachia* manipulates its host's reproduction for its own benefit (O'Neill *et al.*, 1997; Stouthamer *et al.*, 1999). The molecular basis of CI is currently unknown, but a model proposes that two bacterial functions at least are likely to be involved: *mod* (for modification) would be a sort of

poison, affecting paternal chromosomes before *Wolbachia* is shed from maturing sperm and *resc* (for rescue) would be a sort of antidote, saving the embryo from death when expressed in infected eggs (Werren, 1997; Poinsot *et al.*, 2003).

D. sechellia is endemic from the Seychelles archipelago. It is known to carry two different *Wolbachia* strains: *wSh* and *wSn* (Rousset and Solignac, 1995; Bourtzis *et al.*, 1996). These two variants are distinguishable by several molecular markers (Rousset and Solignac, 1995; Werren *et al.*, 1995; Zhou *et al.*, 1998; Charlat *et al.*, 2002b). They belong to the two different main clades of Arthropods' *Wolbachia* (clades A and B), having diverged for about 60 million years. In laboratory strains, two different types of infected individuals can be found: those carrying *wSh* only (singly infected) and those carrying *wSh* and *wSn* (doubly infected). On the contrary individuals carrying *wSn* only are not observed (Rousset and Solignac, 1995).

D. simulans is very closely related to *D. sechellia* (Hey and Kliman, 1993; Kliman *et al.*, 2001). It is known to harbour several *Wolbachia* strains, among which are *wHa* and *wNo*, closely related to *wSh* and *wSn*, respectively (Rousset and Solignac, 1995; Werren *et al.*, 1995; Zhou *et al.*, 1998; Charlat *et al.*, 2002b). *wHa* and *wNo* infect the *D. simulans* populations from New Caledonia and the Seychelles archipelago, where the infection pattern strikingly parallels the situation of *D. sechellia*: some individuals harbour only *wHa*, while others carry both *wHa* and *wNo*, but individuals singly infected by *wNo* are absent or very rare (James *et al.*, 2002).

Experiments have shown that this pattern of infection in *D. simulans* is not because of the impossibility of *wNo*

Correspondence: S Charlat, Institut Jacques Monod, Laboratoire Dynamique du Génome et Evolution, CNRS-Universités Paris 6 & 7, 75251 Paris Cedex 05, France. E-mail: charlat@ijm.jussieu.fr

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lines being obtained by segregation from double infection, and subsequently maintained (Poinsot *et al*, 2000). Here we address the same issue in *D. sechellia*, through an 18-generation experiment, based on a similar procedure, and show that *wSh* as well as *wSn* singly infected lines can be obtained by segregation and stably maintained. Using *D. sechellia* singly infected lines as well as *D. simulans* lines artificially infected by *wSh* and *wSn* (Charlat *et al*, 2002b), we estimate CI levels in these two species. Our results do not suggest any consistent effect of the host species on the CI induced by *wSh*. By contrast, *wSn* expression seems to be repressed by host factors in *D. sechellia*.

Materials and methods

D. sechellia strains

S9 is a strain naturally infected by *wSh* (Bourtzis *et al*, 1996). *Dsech* is a strain infected by *wSh+wSn*, founded in the 1980s, originating from the Seychelles archipelago. *DsechTC* is an uninfected strain, obtained from *Dsech* by antibiotic treatment (Tetracycline).

Dse1 to *Dse14* are 14 lines used in the segregation experiment, each initiated using one single female from the *Dsech* strain. Among the singly infected lines obtained by segregation from these doubly infected lines (see below), three have been used in subsequent CI assays, which took place 30 generations after the end of the segregation experiment.

D. simulans strains

STC is an inbred uninfected strain obtained by antibiotic treatment (Tetracycline) from the *Seychelles* strain, naturally infected by *wHa+wNo*, derived from flies collected on Mahe island (Seychelles archipelago) in 1981.

ASh and *CSH* are isofemale lines, infected by *wSh*, obtained by cytoplasmic injection from *D. sechellia* into the STC strain (Charlat *et al*, 2002b). *ASn* and *BSn* are isofemale lines, infected by *wSn*, obtained by cytoplasmic injection from *D. sechellia* into the STC strain (Charlat *et al*, 2002b). CI assays involving *ASh*, *CSH*, *ASn* and *BSn* took place 40 generations after the injection into *D. simulans*.

Rearing conditions

In order to ensure optimal conditions for the maintenance of *Wolbachia* infections, host strains were maintained at 25°C, at low larval density, on axenic medium (David, 1962).

Segregation experiment

The present experiment followed a similar procedure to that of Poinsot *et al* (2000). At generation G0, one female from each of the 14 doubly infected lines (*Dsech-1* to *Dsech-14*, infected by *wSh+wSn*) was crossed with two *DsechTC* males, before its infection status was checked by PCR. CI does not occur in such crosses, since males are not infected. Consequently, the hatching probabilities of the eggs laid by infected mothers do not depend on their infection status. From each of the 14 crosses, one G1 female was picked randomly and backcrossed with two *DsechTC* males, before its infection status was determined by PCR. A similar procedure was applied for 18 generations, allowing the total bacterial load as well as the proportion of *wSh* vs *wSn* to vary randomly.

Wolbachia detection and identification

The detection of *Wolbachia* and the distinction between the different *Wolbachia* variants were made by PCR. DNA was obtained according to O'Neill *et al* (1992) and the *wsp* gene was amplified according to Zhou *et al* (1998). Primer specificity allows distinguishing *wSh* (primer 178F and 691R) from *wSn* (primers 183F and 691R).

Measurement of embryonic mortality

Embryonic mortality was measured in *D. simulans* and *D. sechellia* using individual crossings between males aged 3–4 days and females aged 4–7 days. Mating was observed, and crosses where copulation lasted for less than 15 min were discarded. Inseminated females were individually placed at 25°C, on axenic medium, coloured with neutral red. *D. simulans* females were left to lay for 48 h and *D. sechellia* females for 96 h, owing to their lower fecundity. Laying boxes were left for an additional 24 h at 25°C so that all viable embryos could hatch, and finally placed at 4°C until egg counting. Embryonic mortality was then determined as the percentage of unhatched eggs. Laying boxes with less than 20 eggs were discarded. In *D. sechellia*, average egg number was 46.9, ranging from 20 to 96. In *D. simulans*, average egg number was 86, ranging from 31 to 142. For crosses showing 0% hatching, a fertility test was performed by crossing each parent with individuals harbouring a compatible infection. This procedure allows to distinguish between crosses where CI is 100% and crosses involving intrinsically sterile individuals. Crosses with one sterile parent were discarded. Finally, the infection status of parents was checked by PCR.

CI intensity

CI intensity (or CI level) is defined here as the percentage of embryos that do not hatch because of CI, in crosses between infected males and uninfected females. CI intensity experiments thus involve crosses between infected males and uninfected females (strain *DsechTC* in *D. sechellia*, strain STC in *D. simulans*). In each experiment, control crosses, involving uninfected males, were also performed, in order to determine the control cross mortality (CCM). This allows the calculation of a corrected CI (CI_{corr}), taking into account the embryonic mortality not caused by CI. If EM stands for the observed embryonic mortality, $CI_{corr} = (EM - CCM) / (1 - CCM)$ (Poinsot *et al*, 1998).

Statistical analysis

All statistical tests were performed on SAS (1989, version 6.12) after root arcsine transformation.

Results

Segregation rates

The rate of segregation from doubly to singly infected cytoplasm was monitored through 18 generations, in conditions allowing the proportion of *wSh* and *wSn*, as well as the total bacterial load, to vary stochastically. The loss of *wSh* occurred in four lines out of 14 (*Dse5* at generation 4, *Dse6* and *Dse8* at generation 14, *Dse2* at generation 18). The loss of *wSn* occurred in only one line (*Dse14*, at generation 2). The complete loss of infection did not occur. At the end of the experiment, the respective proportions of doubly infected, *wSh* and

wSn lines were 64.3, 7.1 and 28.6%. In total, 230 crosses involved females still bearing *wSh*. Among these, *wSh* was lost in four crosses, making an overall loss rate of 1.7%. Similarly, *wSn* was lost in one cross out of 236 involving females still bearing this variant, making an overall loss rate of 0.42%. These two rates are not significantly different (Fisher's exact test: $P = 0.211$).

CI intensity in *D. sechellia* and *D. simulans*

The CI intensity of *wSh* was estimated in two *D. sechellia* lines: *S9* (naturally singly infected) and *Dse14-Sh* (obtained by segregation) and in two *D. simulans* lines: *ASh* and *CSH* (obtained by cytoplasmic injection (Charlat et al, 2002b)). The results are summarized in Table 1a. As expected, *wSh* was found to induce CI in all lines in both species: *t*-test comparisons with control crosses (Table 1c) are all significant, with $P < 0.001$ (not shown). The host effect was investigated by ANOVA (Table 2a). The *Line* factor was found significant ($P = 0.0028$). Indeed, important between-line variations occur in *D. simulans* ($CI_{\text{corr}} = 78.7$ and 50.0% for *ASh* and *CSH*, respectively), which are consistent with previous observations (Charlat et al, 2002b). Owing to this *Line* effect, the two species are not found to be significantly different, although the means are more than 20% apart ($CI_{\text{corr}} = 90\%$ in *D. sechellia* vs 67% in *D. simulans*). In other words, the between-species variation is not significantly higher than the between-line variation. Multiple comparisons, realised through the LSMEAN/TDIFF statement in SAS (1989), lead to similar conclusions: *S9* and *ASh*, although belonging to *D. sechellia* and *D. simulans*, respectively, do not differ significantly (not shown).

A similar experiment was carried out with *wSn*. CI intensity was estimated in two *D. sechellia* lines: *Dse5-Sn* and *Dse6-Sn* (obtained by segregation) and in two *D. simulans* lines: *ASn* and *BSn* (obtained by cytoplasmic injection (Charlat et al, 2002b)). The results are summarized in Table 1b. As expected, *wSn* was found to induce CI in all lines in both species: *t*-test comparisons with control crosses are all significant, with $P < 0.001$ (not

Table 2 CI intensity, ANOVAs

Source	df	Mean square	F	F denominator	Pr>F
(a) <i>wSh</i>					
Species	1	2.1308	4.10	Line	NS
Line	2	0.5211	6.40	Error	0.0028
Error	71	0.0814	—	—	—
(b) <i>wSn</i>					
Species	1	1.6577	25.15	Error	0.0001
Line	2	0.0524	0.80	Error	NS
Error	71	0.0659	—	—	—

Line is nested within *Species*.

shown). As shown in Table 2b, there does not seem to be any intraspecific variation in this case (*Line* factor nonsignificant). By contrast, the *Species* factor is found to be significant ($P = 0.0001$). Thus, *wSn* in *D. sechellia* induces a lower CI than in *D. simulans* (mean $CI_{\text{corr}} = 39\%$ vs 64%). Multiple comparisons lead to similar conclusions: within each species, the lines do not differ, but all interspecific comparisons are significant (not shown).

Discussion

Segregation rates

The rates of loss of *wSh* and *wSn* from initially doubly infected cytoplasm were followed through 18 generations, in conditions where the effect of CI (the selection of doubly infected embryos by doubly infected males) was relaxed. At every generation, infected females were backcrossed to uninfected males harbouring the same genomic background. The two types of singly infected cytoplasm were obtained and stably maintained. The transmission efficiencies of the two variants were not found to differ significantly. This experiment demonstrates that the absence of lines singly infected by *wSn* in *D. sechellia* is not because of a perfect transmission of this

Table 1 CI intensity in *D. sechellia* and *D. simulans*: descriptive statistics

Host species	Male	Female	n crosses	n eggs	EM (%)	CI_{corr} (%)	SE (%)
(a) <i>wSh</i>							
<i>D. sechellia</i>	<i>Dse14-Sh</i> (<i>wSh</i>)	<i>DsechTC</i>	19	857	92.8	90.4	2.58
<i>D. sechellia</i>	<i>S9</i> (<i>wSh</i>)	<i>DsechTC</i>	17	713	90.4	87.3	3.89
<i>D. simulans</i>	<i>ASh</i> (<i>wSh</i>)	<i>STC</i>	23	1978	82.9	78.7	3.82
<i>D. simulans</i>	<i>CSH</i> (<i>wSh</i>)	<i>STC</i>	16	1230	59.5	50.0	7.67
(b) <i>wSn</i>							
<i>D. sechellia</i>	<i>Dse5-Sn</i> (<i>wSn</i>)	<i>DsechTC</i>	16	908	49.8	34.1	6.72
<i>D. sechellia</i>	<i>Dse6-Sn</i> (<i>wSn</i>)	<i>DsechTC</i>	23	1255	55.7	41.7	5.33
<i>D. simulans</i>	<i>ASn</i> (<i>wSn</i>)	<i>STC</i>	19	1831	70.4	63.0	3.77
<i>D. simulans</i>	<i>BSn</i> (<i>wSn</i>)	<i>STC</i>	17	1667	72.4	66.0	3.85
(c) Control crosses							
<i>D. sechellia</i>	<i>DsechTC</i> (uninfected)	<i>DsechTC</i>	28	1097	24.9	—	4.11
<i>D. simulans</i>	<i>STC</i> (uninfected)	<i>STC</i>	33	2589	19.5	—	2.37

EM: raw embryonic mortality, CI_{corr} : corrected CI, SE: Standard Error.

variant, or to its lethality when present on its own. Interestingly, the complete loss of *Wolbachia* infection did not occur, suggesting that the total bacterial load did not decrease during the experiment. In other words, it seems that the emergence of singly infected cytoplasm was rather due here to stochastic variations in the proportions of the two variants than to a decrease of bacterial population size.

In a similar experiment realised in *D. simulans*, Poinso et al (2000) followed the segregation dynamics of *wHa* and *wNo* through 18 generations. Although the experiments were not performed together, a comparison can be made with the present results. The distribution of the various infection types at the end of the experiment with *D. simulans* can be considered: 33.3% doubly infected, 24.3% singly infected by *wHa*, 12.1% singly infected by *wNo* and 30.3% uninfected ($n=33$). These proportions contrast with those observed in *D. sechellia* (Fisher's exact test: $P=0.014$). In *D. simulans*, not only did singly infected lines of both types occur more frequently, but uninfected ones did as well. These results suggest that *wSh* and *wSn* in *D. sechellia* are more efficiently transmitted, than are *wHa* and *wNo* in *D. simulans*.

CI intensity in *D. sechellia*

The CI intensity of *wSh* and *wSn* was estimated in their natural host *D. sechellia*. Such estimations have been made previously for *wSh*, naturally present as a single infection (Rousset and Solignac, 1995; Bourtzis et al, 1996). In these two studies, the raw embryonic mortality in crosses between *wSh* males and uninfected females was, respectively, 71 and 70%. If one takes into account the control crossmortality (Poinso et al, 1998), the corrected CI levels are 62 and 66%, which is lower than what we observed (90.4% in *Dse14-Sh* and 87.3% in *S9*, the line used by Bourtzis et al, 1996). These differences are likely to be because of environmental factors, such as larval density, which are known to affect CI levels (Sinkins et al, 1995). Interestingly, we found that *wSh* expresses the same CI intensity in *Dse14-Sh* and *S9*, although the first line derives from the segregation experiment and the second is naturally singly infected. This is consistent with the view that natural singly infected lines derive from doubly infected ones through imperfect transmission.

Before our experiment, *D. sechellia* lines singly infected by *wSn* were not available. However, the CI intensity of *wSn* had been indirectly investigated by crossing doubly infected males with females bearing only *wSh* (Rousset and Solignac, 1995). In such crosses, the raw embryonic mortality was 35%. In this case, CI_{corr} is also 35% since the control crossmortality is 0%. This value is very close to the ones we observed in crosses involving males singly infected by *wSn* (34.1% in *Dse5-Sn* and 41.7% in *Dse6-Sn*), suggesting that the two variants in double infections do not affect each other (the intensity of one variant does not depend on the presence or absence of the other), consistent with several previous observations (Merçot et al, 1995; Rousset and Solignac, 1995; Rousset et al, 1999).

Based on our estimations of transmission efficiency and CI intensity, one can calculate the equilibrium frequencies expected under Hoffmann and Turelli's model (1997). Assuming (i) that neither *wSh* nor *wSn*

affect host fitness (apart from CI) and (ii) that the CI effects of the two strains are additive, the expected frequencies are the following: 99.17% doubly infected, 0.63% *wSh*, 0.19% *wSn* and 0.001% uninfected. Thus, *wSh* flies are expected to be three times more frequent than *wSn*, but both types of singly infected cytoplasms are expected to be rare. The very sparse data available on the frequencies of singly infected lines in the wild suggest that they might be more frequent (Rousset and Solignac, 1995). Two different factors might explain this discrepancy: our experimental conditions allow the expression of high CI levels and high transmission efficiency, both factors that increase the expected equilibrium frequencies of the double infection, and reduce the expected frequency of single infections. Gathering parameter estimates from the wild, together with additional data on the frequencies of the various infection types, might clarify this issue.

Evidence for a host effect

In order to investigate the possibility of host control on *wSh* and/or *wSn*, CI assays were realised in the foreign host *D. simulans*, using transinfected lines obtained by cytoplasmic injection (Charlat et al, 2002b).

Concerning *wSh*, the results do not suggest any variations between the two hosts. Within *D. simulans*, a significant difference was detected between lines. Previous results indicate that such differences do not remain stable over time, suggesting that they might be caused by uncontrolled environmental factors (Charlat et al, 2002b). Whatever the causal factor, this intraspecific variation in *D. simulans* lowers the statistical power of our comparison. Indeed, although the mean CI_{corr} in the two species is more than 20% apart, the difference is not significant. This highlights the importance of using more than one line to investigate such effects, in order to avoid erroneous conclusions.

On the contrary, *wSn* was shown to induce different CI levels in the two species. Consistent with previous observations, there does not seem to be any between-line variation for *wSn* within *D. simulans* (Charlat et al, 2002b). Thus, *wSn* expresses a higher CI in *D. simulans* than in its natural host (mean $CI_{corr}=39\%$ in *D. sechellia* vs 64% in *D. simulans*). Overall, it appears that the differences between *D. simulans* and *D. sechellia* regarding the CI intensity of *wSn* and *wNo* are mainly because of host effects, and not because of divergence between the two strains. Indeed, while the present results show that *wSn* induces a lower CI in *D. sechellia* than in *D. simulans*, previous experiments suggest that *wSn* and *wNo*, within *D. simulans*, induce similar levels of mortality (Charlat et al, 2002b). Under this view, one could predict that *wNo* and *wSn* should induce the same CI levels in *D. sechellia*. This remains to be investigated.

It is notable that the CI assays realised on *wSh* and *wSn* did not lead to identical conclusions regarding the host controls on CI levels: it appears that *wSn* only is repressed in *D. sechellia*. Indeed, if there might be a slight host effect for *wSh*, undetected in the present experiment, the tendency is rather that of a higher level of expression in *D. sechellia* than in *D. simulans*, suggesting that the host control observed here is *Wolbachia* strain-specific.

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