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Use of *Wolbachia* to drive nuclear transgenes through insect populations

Steven P. Sinkins[1*] and H. Charles J. Godfray[2]

[1]*Vector Research Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK*
[2]*NERC Centre for Population Biology, Department of Biological Sciences, Imperial College London, Silwood Park Campus, Ascot, Berkshire SL5 7PY, UK* (*c.godfray@imperial.ac.uk*)

Wolbachia is an inherited intracellular bacterium found in many insects of medical and economic importance. The ability of many strains to spread through populations using cytoplasmic incompatibility, involving sperm modification and rescue, provides a powerful mechanism for driving beneficial transgenes through insect populations, if such transgenes could be inserted into and expressed by *Wolbachia*. However, manipulating *Wolbachia* in this way has not yet been achieved. Here, we demonstrate theoretically an alternative mechanism whereby nuclear rather than cytoplasmic transgenes could be driven through populations, by linkage to a nuclear gene able to rescue modified sperm. The spread of a 'nuclear rescue construct' occurs as long as the *Wolbachia* show imperfect maternal transmission under natural conditions and/or imperfect rescue of modified sperm. The mechanism is most efficient when the target population is already infected with *Wolbachia* at high frequency, whether naturally or by the sequential release of *Wolbachia*-infected individuals and subsequently the nuclear rescue construct. The results provide a potentially powerful addition to the few insect transgene drive mechanisms that are available.

Keywords: cytoplasmic incompatibility; rescue; mosquito; *Wolbachia*

1. INTRODUCTION

There is increasing interest in novel forms of arthropod control in which transgenes beneficial to humans are spread through pest populations of economic or medical importance. With disease vectors, the intended function of the beneficial gene is typically to disrupt the transmission of pathogens. The prospects for this type of intervention have been boosted by the germline transformation of several mosquito species (Coates *et al.* 1998; Catteruccia *et al.* 2000; Grossman *et al.* 2001; Allen *et al.* 2001) and the demonstration that transgenes can be introduced that reduce or abolish the ability to transmit malaria or dengue (Olson *et al.* 1996; de Lara Capurro *et al.* 2000; Ito *et al.* 2002). If these advances are to be translated into control strategies, mechanisms for driving transgenes through field populations in a self-sustaining manner are essential, and this has been identified as a priority research area (Alphey *et al.* 2002). However, although several different nuclear drive mechanisms have been suggested, for example involving autonomous transposable elements, meiotic drive or homing endonucleases (Wood *et al.* 1977; Ribeiro & Kidwell 1994; Burt 2003), the practicalities of none of them have yet been demonstrated.

A further possible drive mechanism involves endosymbiotic bacteria in the genus *Wolbachia*. *Wolbachia* have been shown to be widespread in many taxa of insects, and to have a variety of different effects on their hosts' reproduction that allow them to spread through populations (O'Neill *et al.* 1997). The most common is cytoplasmic incompatibility (CI): in infected males sperm is modified such that it can no longer successfully fertilize uninfected eggs, which are thus at a disadvantage compared with infected eggs, which can be fertilized by sperm from any male. CI allows the maternally inherited *Wolbachia* to spread through uninfected populations, provided it is present initially at a frequency above a threshold determined by the fidelity of transmission, any negative fitness effects of the bacterium on the host and the strength of the cytoplasmic incompatibility (Turelli *et al.* 1992; Turelli 1994; Turelli & Hoffmann 1995). Spreading *Wolbachia* infections are not expected to have any effect on the frequency of nuclear genes, because successful mating between uninfected males and infected females allows continuous gene flow. In principle, a transgene could be inserted into and expressed by *Wolbachia*, which would then be introduced into a population to drive the novel gene to high frequencies or fixation (Turelli & Hoffmann 1999; Sinkins & O'Neill 2000).

There are, however, several practical difficulties associated with the use of *Wolbachia* as both a transgene drive mechanism and expression vehicle. *Wolbachia* genomic transformation has not yet been achieved, and suitable expression and secretion of the gene product to allow its anti-pathogen action pose considerable technical challenges. Furthermore, limiting transgene expression to specific tissues and times to minimize any associated fitness costs, for example using promoters specific to the salivary glands or to the midgut after a bloodmeal (Kokoza *et al.* 2000; Ito *et al.* 2002), would be much less straightforward if *Wolbachia* is used as the expression system rather than host nuclear transformation.

Here, we suggest an alternative strategy that could circumvent these difficulties. There is evidence that the sperm modification that occurs in the infected male and the 'rescue' effect seen in the infected embryo have a different functional basis in the bacterium: although most CI *Wolbachia* are able to both modify and rescue sperm, rare variants such as the *w*Cof strain occur that can rescue but

*Author for correspondence (ssinkins@liv.ac.uk).

not modify, designated mod$^-$resc$^+$ (Bourtzis *et al.* 1998; Merçot & Poinsot 1998; Veneti *et al.* 2003). At the moment, the molecular mechanism of the modification and rescue functions are not understood, but with the publication of the first *Wolbachia* whole-genome sequence (Wu *et al.* 2004), there is great optimism that this may be elucidated in the near future. Given the identification of the molecular basis of the rescue function, we explore whether it might be possible to drive a transgene through a host population already containing *Wolbachia* by linking it to a rescue-function gene (or genes) and inserting them onto a host chromosome. We will refer to this combination of genes inserted on the host chromosome as the nuclear rescue construct (NRC).

2. MATERIAL AND METHODS

A full model of the spread of a beneficial gene linked to a rescue gene on a host chromosome in a population of individuals, a certain fraction of which harbour *Wolbachia*, requires that the frequency of 18 separate classes of individuals are tracked. To simplify the problem we make two major assumptions. First we assume that the beneficial and rescue genes are perfectly linked. If the genes were directly adjacent in the construct, then recombination acting to produce two separated functional genes would be extremely rare, or alternatively the genes may be inserted in such a way as to make recombination impossible. Second, we assume haploid genetics so that individuals can be characterized by the presence or absence of the NRC. No possible target of this type of intervention is haploid, but work on related problems shows that haploid models capture the dynamics of more complex models for reasonable assumptions about the phenotypes of heterozygotes, such as no overdominance (Bulmer 1994). These simplifications allow the population to be divided into four classes based on the presence or absence of the NRC and *Wolbachia*.

Our model of the dynamics of *Wolbachia* closely follows those constructed to describe *Drosophila* (Turelli *et al.* 1992; Turelli 1994; Turelli & Hoffmann 1995), extended to take account of the presence of a rescue function on the nuclear chromosomes. For fitness, we shall use the subscripts $\varnothing$, W, C and 2 to refer to individuals with neither the NRC nor *Wolbachia* ($\varnothing$), with *Wolbachia* alone (W), with the NRC alone (C) or with both (2). The fitness of individuals in each class is $1 - f_\varnothing$, $1 - f_W$, $1 - f_C$ and $1 - f_2$ where we set $f_\varnothing = 0$ without loss of generality. We assume that the probability an egg can rescue the sperm of an infected male depends on (i) whether the egg contains *Wolbachia* and (ii) whether the mother carries the NRC (we assume maternal expression of the rescue function, since rescue occurs before karyogamy in the egg). Let this probability be defined by $1 - h_{i,j}$, where $i = \{\varnothing,C\}$ denotes whether the mother carries the NRC and $j = \{\varnothing,W\}$ indicates the infection status of the egg. Note that in their models, Turelli & Hoffmann (1995) assume $h_{\varnothing,W} = 0$, i.e. that *Wolbachia*-infected eggs show perfect rescue, but here we are also concerned with *Wolbachia*–host combinations that show imperfect rescue. In electronic Appendix A (available on The Royal Society's Publications Web site) we show how relaxing this assumption affects the threshold for *Wolbachia* spread and its equilibrium frequency. Finally, we assume that the frequency with which *Wolbachia* is transmitted to a female's offspring is $1 - \mu_W$ and $1 - \mu_2$ for the two relevant categories of individuals.

Unless stated otherwise, we make a series of biologically motivated assumptions to reduce the number of parameters. We assume that zygotes carrying *Wolbachia* produced by mothers with the NRC enjoy the better of the two rescue probabilities $h_{C,W} = \min(h_{C,\varnothing}, h_{\varnothing,W})$; that *Wolbachia* transmission is independent of the presence of the NRC, $\mu = \mu_W = \mu_2$; and that if both *Wolbachia* and the NRC have associated fitness costs, individuals with both suffer the larger of the two fitness penalties, $f_2 = \max(f_C, f_W)$. The number of progeny in each of the four classes that arise as a result of the 16 different mating combinations can be calculated (details in electronic Appendix A). Then, given the initial frequencies of the four types (and assuming no assortative mating among types), the dynamics can be iterated into the future. We can also derive some analytical conditions for invasion by assuming the *Wolbachia* is at a stable high equilibrium and calculating when a rare NRC can increase in frequency (see electronic Appendix A).

3. RESULTS AND DISCUSSION

(a) *Spread of a nuclear rescue construct*

An idealized *Wolbachia* infection would be transmitted to 100% of progeny ($\mu_W = 0$), have perfect rescue ($h_{\varnothing,W} = 0$) and would impose no fitness penalty on its host ($f_W = 0$). Such a bacterium will spread through an uninfected population from arbitrary low frequencies to fixation. An NRC of the type we have described cannot invade a population infected with *Wolbachia* with these idealized parameters and either has neutral dynamics, or would decrease in frequency if it imposes costs on its host.

Accurately estimating these three parameters for natural *Wolbachia* infections can be difficult but there is evidence from various systems that departures from the idealized conditions described above are common (Hoffmann & Turelli 1997). In *Drosophila simulans* for example, between 0% and *ca.* 50% of the progeny of individual infected wild-caught females were uninfected, with an average *ca.* 5% ($\mu_W = 0.05$; Turelli & Hoffmann 1995). Examples of imperfect self-rescue also exist, particularly when strains are inserted into novel hosts; thus a mosquito *Wolbachia* inserted into a *Drosophila* had $h_{\varnothing,W} \sim 0.1$ (Braig *et al.* 1994). Small fecundity costs could be detected in *D. simulans* in laboratory cages, but not in wild-caught females (Turelli & Hoffmann 1995), whereas a strain of *Wolbachia* designated *popcorn* reduced host lifespan in laboratory colonies of *Drosophila melanogaster* (Min & Benzer 1997) and thus might affect fitness in the field. Evidence for a small fitness benefit associated with *Wolbachia* has even been reported in female *Aedes* mosquitoes in the laboratory (Dobson *et al.* 2002). We explore whether an NRC can invade a *Wolbachia*-infected population with each of these departures from idealized parameters.

Consider first imperfect transmission ($\mu > 0$, $h_{\varnothing,\varnothing} = 1$; all other $f.$ and $h.,. = 0$): as long as the rate of non-transmission is low, and the *Wolbachia* population frequency exceeds the threshold value, the bacterium can invade and reach high frequencies (Hoffmann & Turelli 1997). However, such a population, where most individuals carry the bacteria, can always be invaded by the NRC, which increases to fixation and displaces the *Wolbachia* (figure 1). The reason for this is that individuals that carry the NRC never produce eggs that are unable to be

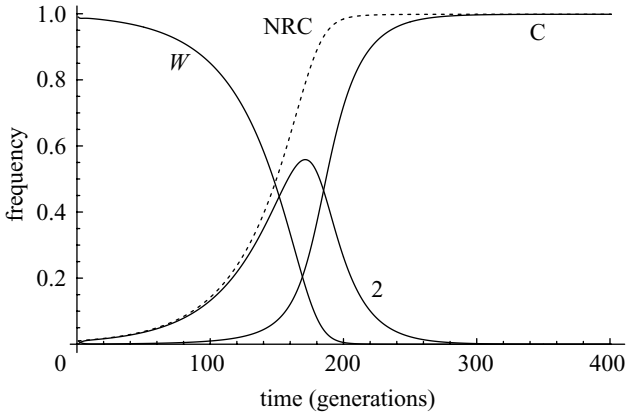

Figure 1. The spread of an NRC through a population fixed for *Wolbachia* with imperfect transmission. The three solid lines are the frequencies of individuals carrying *Wolbachia* alone ('*W*'), the NRC alone ('C') or both *Wolbachia* and the NRC ('2'). The dotted line labelled NRC is the frequency of individuals carrying the construct, with or without *Wolbachia*. Parameter values: $\mu = 0.05$, $h_{\varnothing,\varnothing} = 1$; all other *f.* and *h.*,. = 0; the simulation initiated with the starting frequencies of '*W*' and 'C' equal to 0.99 and 0.01, respectively.

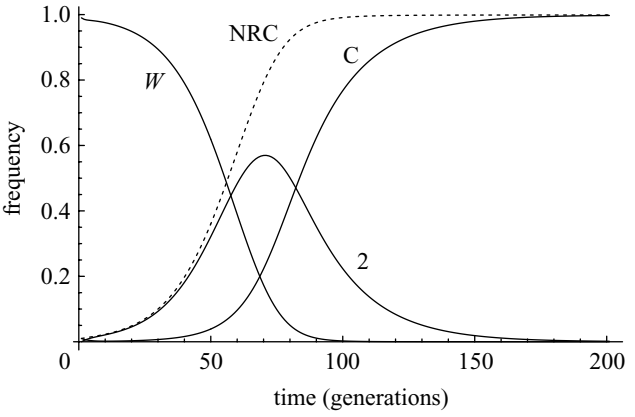

Figure 2. The spread of an NRC through a population fixed for a *Wolbachia* strain with imperfect rescue and transmission. Schema as in figure 1; parameter values: $\mu = 0.05$, $h_{\varnothing,W} = 0.1$, $h_{\varnothing,\varnothing} = 1$; all other *f.* and *h.*,. = 0; the simulation initiated with the starting frequencies of '*W*' and 'C' equal to 0.99 and 0.01, respectively.

fertilized by modified sperm (because unlike *Wolbachia* the NRC is present in all cells), nor suffer losses due to non-transmission, and hence the NRC is at an advantage compared with the '*W*' class. As the NRC spreads and modified sperm are able to be used by an increasing fraction of the population, the *Wolbachia* itself is no longer positively selected and begins to decline at a rate determined by the probability of non-transmission.

The NRC can still spread if it imposes some costs to its host (f_C). Invasion analysis (see electronic Appendix A) predicts the NRC will spread if

$$\mu > f_C - \frac{f_C^2}{1 + 2f_C},$$

which to first order accords with the intuitive expectation that the fitness cost of the NRC should be less than the cost of non-transmission to the *Wolbachia*, i.e. $\mu > f_C$ (the exact condition is more complicated as when rare most rescue constructs are in *Wolbachia*-infected hosts, whose fitness they affect).

Now consider a *Wolbachia* with imperfect self-rescue ($h_{\varnothing,W} > 0$, $h_{\varnothing,\varnothing} = 1$; all other μ., *f.* and *h.*,. = 0). In the absence of non-transmission and any fitness costs to carrying the bacteria, *Wolbachia* can always invade (Hoffmann & Turelli 1997) and this is not altered by the presence of imperfect self-rescue (see electronic Appendix A). The reason for this is that in the early generations, when most males are uninfected, infected females suffer very little cost associated with this parameter.

A population fixed for an imperfectly self-rescuing *Wolbachia* can be invaded by an NRC if the latter increases its bearer's fitness by ensuring that a greater proportion of its offspring can be fertilized by modified sperm. In this limiting case (μ., *f.* and *h.*,. $\neq h_{\varnothing,W} = 0$) it is the '2' class of individual with both *Wolbachia* and the NRC that goes to fixation, though this result is due to the lack of production of individuals without *Wolbachia* because transmission is perfect. If some non-transmission is also assumed, then the speed with which the rescue construct invades is enhanced and ultimately it drives the *Wolbachia* to extinction (figure 2).

Finally, assume that the *Wolbachia* reduces the fitness of its host ($f_W = f_2 > 0$; $h_{\varnothing,\varnothing} = 1$; all other μ., *f.* and *h.*,. = 0). The *Wolbachia* can spread provided it exceeds a threshold frequency equal to f_W, and it then goes to fixation (Hoffmann & Turelli 1997). But unlike the previous two cases, this population cannot be invaded by an NRC. The reason for this is that when rare, most NRC occurs in individuals that also carry *Wolbachia* and so also suffer the cost of carrying the bacteria. If individuals with only the NRC are introduced at high frequency, or if the *Wolbachia* shows imperfect rescue or transmission, then fecundity costs can increase the rate of spread of the NRC, though the effect is weak. Were the NRC in some way to mitigate the cost of carrying *Wolbachia* ($f_2 < f_W$), for example if the construct also included an antibacterial peptide, then invasion could occur, but we do not think this would be easy to achieve. In any case, fitness costs of *Wolbachia* infection seem to be very low or undetectable in most natural populations.

So far we have assumed that the NRC is released into a population where *Wolbachia* is already present at high population frequency, which is the case in several potential target species (e.g. various *Culex* and *Aedes* mosquito vectors of filariasis and dengue, tsetse flies and a variety of agricultural pests). In the case of uninfected target populations, most notably malaria-transmitting *Anopheles* mosquitoes, if *Wolbachia* infections can be successfully established in laboratory colonies then *Wolbachia* and the NRC could in theory be released at the same time. It is much more difficult to study this case analytically, but numerical studies indicate that where the *Wolbachia* is able to spread through an uninfected population (i.e. where the conditions of Turelli & Hoffmann (1995) apply) and the rescue construct is also able to invade by the mechanism that we have described, then both occur in sequence (figure 3). In other words, the presence of the NRC seems to have little effect on the spread of the *Wolbachia*. However, while the *Wolbachia* spreads, the frequency of the NRC may become very low, with a major risk in the real

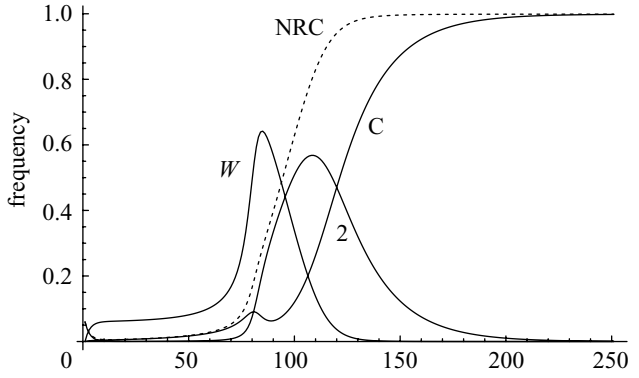

Figure 3. The spread of *Wolbachia* and an NRC through an uninfected population after simultaneous release of both elements. Parameter values as in figure 2 except that the starting frequencies of '∅' and '2' individuals are 0.94 and 0.06, respectively.

world of stochastic loss, and sequential rather than simultaneous release would normally appear to be the best strategy for uninfected target populations.

Thus, an NRC can invade a population fixed for *Wolbachia* if the latter has either imperfect transmission or imperfect rescue (if nuclear rescue is superior). As long as some non-transmission occurs, the NRC will displace the *Wolbachia*. If the rescue function engineered on the host chromosome is tightly linked to a disease-blocking transgene, then this will have been driven successfully through the population.

(b) *Fate of a nuclear rescue construct*

When the *Wolbachia* has been displaced from the population, the selective advantage of rescue disappears and the NRC is then either selectively neutral (when $f_C = 0$) or, perhaps more likely, selected against and lost ($f_C > 0$; figure 4*a*). When the costs are low ($f_C \approx 0$) the time taken for selection to remove first *Wolbachia,* and then the NRC, is likely to be sufficiently long that it would have little or no impact on a disease control campaign. The process could also be repeated by introducing a different strain of *Wolbachia* followed by compatible rescue constructs. The procedure may in addition be useful in accelerating the spread of a gene with a positive but low benefit to the host, such as a transgene that prevents a mosquito from being infected by costly *Plasmodium* parasites (Hogg & Hurd 1997), where spread would otherwise be too slow to be practical; in this case the relative costs and benefits of the construct under natural conditions would determine whether it was maintained indefinitely in the population.

There are at least two ways of maintaining the NRC indefinitely in the population in the face of a fitness cost ($f_C > 0$). First, the decline in *Wolbachia* could be prevented if the nuclear rescue function is dependent on the presence of *Wolbachia* ($h_{\varnothing,\varnothing} = h_{C,\varnothing} = 0$). With this assumption the NRC will spread as long as it improves the rescue function ($h_{C,W} > h_{\varnothing,W}$), but the *Wolbachia* is not displaced. The second solution is to introduce the modification as well as the rescue function onto the host chromosome (or if it proves that modification and rescue are actually controlled by the same gene, engineer its expression in both ovaries/embryos and testes). If such a construct is introduced into a population with *Wolbachia*

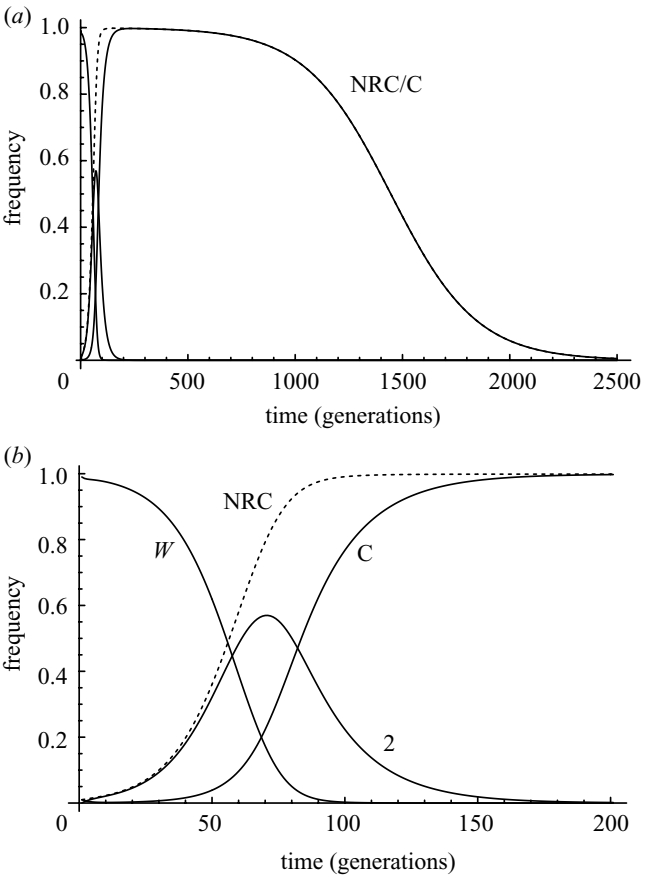

Figure 4. (*a*) The loss of a costly NRC after the disappearance of *Wolbachia*. Parameters and starting values as in figure 2 except $f_C = f_2 = 0.01$. The *Wolbachia* is lost and the frequency of 'C' individuals approaches 1 in the first 200 generations (with dynamics very similar to figure 2). In the absence of the *Wolbachia* the frequency of 'C' individuals carrying the NRC declines slowly. (*b*) The spread of an NRC that not only rescues a chromosome modified by *Wolbachia* but itself modifies any sperm produced by a male in which it is carried. We have assumed the same parameter values as in figure 4*a* with the NRC being more efficient at rescue than *Wolbachia*. However, now all sperm produced by '2', '*W*' and 'C' individuals are modified (in the same way) and require rescue. Despite the presence of a cost to the NRC ($f_C = f_2 = 0.01$), it is never lost after it has gone to fixation.

that shows either more imperfect transmission or an inferior rescue capability, then it will spread and go to permanent fixation (figure 4*b*), replacing the bacteria. Previous studies have explored the conditions under which a nuclear modification plus rescue construct alone could invade a *Wolbachia*-uninfected population (Sinkins *et al.* 1997; Turelli & Hoffmann 1999). The conditions are very restrictive: typically spread occurs only if the frequency of the construct exceeds a threshold of *ca.* 0.4. The reason why it is so much harder for a nuclear system to spread through an uninfected population is that nuclear rescue genes in females are passed to only 50% of the next generation whereas in males rescue genes modify their sperm so that during the early stages of spread they seldom find a receptive host. When *Wolbachia* itself is already present at high frequencies in the population then males with nuclear rescue genes do normally find receptive females.

This makes up for the reduced transmission in females compared with the maternally inherited *Wolbachia*.

(c) *Natural systems*

A fascinating recently discovered natural case of *Wolbachia*–host genetic exchange provides possible support for the drive strategies discussed here. Based on DNA sequencing studies, three *Wolbachia* strains were reported to coexist in a bean weevil, *Callosobruchus chinensis* (Kondo *et al.* 2002*a*), but surprisingly one showed Mendelian inheritance and was unaffected by antibiotics. Further investigation revealed that the PCR products arose from a stretch of the *Wolbachia* chromosome that had become incorporated into the beetle X chromosome (Kondo *et al.* 2002*b*). No actual *Wolbachia* strain that phylogenetically matches the X chromosome fragment has so far been found in these populations. The presence of the fragment at high frequencies (*ca.* 95% or more in all the populations examined) is difficult to explain.

We suggest that the fragment may have arisen by the translocation of a *Wolbachia* genomic fragment containing a functional rescue gene or genes, which would have then spread by the mechanism that we have described, resulting in the eventual loss of the *Wolbachia* strain from which the fragment was derived. The nuclear fragment appears to have no current rescue or modification functions based on crosses with lines containing either of the two *Wolbachia* strains present in these populations (Kondo *et al.* 2002*a*,*b*). However, after the nuclear fragment had reached fixation and the *Wolbachia* strain from which it was derived had been lost, there would have been no selection to retain the rescue function, which could have decayed through mutation accumulation. Once the rescue function had been lost, the host species would then be susceptible again to invasion by *Wolbachia*, or alternatively the strains currently present may have had an incompatible modification/rescue system. Further genetic characterization of the translocated fragment may be able to test these hypotheses.

An argument against this hypothesis is that endogenous bacterial promoters may not function in a eukaryotic chromosomal background. It is not known how easy it would be for a *Wolbachia* gene to be expressed after transfer to a eukaryotic genome—but there is good evidence that prokaryotic to eukaryotic lateral transfer of functional genes does occur (Andersson & Roger 2002). For the artificial construct used as a drive mechanism a suitable insect promoter would probably need to be used to express the rescue gene.

Theoretical studies have also been performed on the competition between the typical $mod^{+}resc^{+}$ *Wolbachia* and those rare variant strains that are able to rescue but not modify sperm, $mod^{-}resc^{+}$ (Prout 1994; Hurst & McVean 1996), which occur naturally (Bourtzis *et al.* 1998; Merçot & Poinsot 1998; Veneti *et al.* 2003). The assumption was made that mod^{-} strains would be likely to have lower fitness costs than mod^{+} strains. Under these conditions a mod^{-} strain would be expected to replace a mod^{+} strain, and then in the absence of any modified sperm in the population would itself disappear (or in the absence of costs show neutral dynamics). Clear parallels exist with our exploration of nuclear-based rescue.

Only very few possibilities for spreading transgenes in important pest species are currently available and all have disadvantages. As many alternatives as possible are desirable, and the availability of two or more mechanisms that could be used together would dramatically improve the chances of success of any genetic replacement programme. Our results suggest potential new avenues of research and provide further motivation for the isolation of the *Wolbachia* and host genes involved in CI. The various current sequencing projects that involve both *Wolbachia* and mosquito genomes should have a major impact on this goal.

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