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Male-killing *Wolbachia* in two species of insect

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The inherited bacterium *Wolbachia* spreads through the manipulation of host reproduction, and has been suggested to be an important factor in arthropod evolution, from host speciation to the evolution of sex-determination systems. Past work has shown that members of this group may produce cytoplasmic incompatibility, feminize genetically male hosts, and induce host parthenogenesis. Here, we report an expansion of the range of reproductive manipulations produced by members of this clade, recording *Wolbachia* strains that kill male hosts during embryogenesis in two host species, the ladybird *Adalia bipunctata*, and the butterfly *Acraea encedon*. Both male-killing bacteria belong to the B group of *Wolbachia*. However, phylogenetic analyses were unable to resolve whether the bacteria in the two species are monophyletic, or represent independent origins of male-killing among the B-group *Wolbachia*. We also found significant divergence within the *wsp* gene of *Wolbachia* strains found in different *A. bipunctata* individuals, suggesting this host species contains two *Wolbachia* strains, diverged in *wsp* sequence but monophyletic. Our observations reinforce the notion that *Wolbachia* may be an important agent driving arthropod evolution, and corroborates previous suggestions that male-killing behaviour is easily evolved by invertebrate symbionts.

Keywords: *Wolbachia*; inherited bacteria; Coccinellidae; Nymphalidae; selfish genetic element

1. INTRODUCTION

Wolbachia, a genus of maternally inherited bacteria that live inside the cells of many arthropods, have attracted much attention because of their ability to manipulate the reproductive biology of their hosts. Three main classes of manipulation have been described. Most commonly observed is cytoplasmic incompatibility (CI), where (in the simplest case in a diploid) infected males only produce zygotes with normal viability when mated to a female infected with the same strain of *Wolbachia*; zygotes formed following matings to either uninfected females, or to females infected with a different strain of *Wolbachia*, show high mortality (Laven 1951; Yen & Barr 1973; for the full complexity of CI systems see Hoffmann & Turelli (1997)). In Crustacea, feminization is also observed in addition to CI. Here, *Wolbachia* converts genetically male individuals to female development (Rousset *et al*. 1992; Bouchon *et al*. 1998). In Hymenoptera, as well as producing CI, *Wolbachia* strains are responsible for the induction of thelytokous parthenogenesis (Stouthamer *et al*. 1993). In this case, the chromosome complement of unfertilized eggs is doubled, converting them from male to female development (Stouthamer & Kazmer 1994). In contrast to these arthropod cases, *Wolbachia* is also common in filarial nematodes, where it is thought to be a beneficial symbiont (Bandi *et al*. 1998).

The frequent incidence of *Wolbachia* (Werren *et al*. 1995*a*), and their ability to manipulate host reproduction, have led to their being proposed as important agents in the evolution of arthropod hosts (Werren 1997). *Wolbachia* that induce changes in host sex or sexuality, for instance, are considered to be important in the evolution of host reproductive and sex-determination systems. This is exemplified by some populations of the pill woodlouse, *Armadillidium vulgare*, where the sex-determination system of the host is based on the presence–absence of *Wolbachia*, rather than the presence–absence of sex chromosomes (Juchault & Mocquard 1993; Rigaud 1997).

The importance of *Wolbachia* in arthropod evolution is likely to relate to the range of hosts in which it occurs and the variety of manipulations it achieves. In this latter respect, search for male-killing strains is timely. Inherited bacteria that kill male hosts during early development are known in a diverse range of insect species (Hurst *et al*. 1997*a*). Male-killing behaviour is exhibited by a wide range of bacteria, including members of the Proteobacteria (Werren *et al*. 1986, 1994), the Flavobacteria

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(Hurst *et al*. 1997*b*, 1999*a*), and the Mollicutes (Hackett *et al*. 1986; Hurst *et al*. 1999*b*). This has led to the view that male-killing behaviour is easily evolved (Hurst *et al*. 1997*a*), which raises the likelihood of *Wolbachia*, the most common inherited bacterium, having also evolved male-killing behaviour. Here, we demonstrate an association between *Wolbachia* presence and male-killing in two species, the two-spot ladybird *Adalia bipunctata*, and the butterfly *Acraea encedon*. Using the DNA sequence of the *wsp* gene, we examine the phylogenetic position of these *Wolbachia* to determine whether they represented one or two transitions to male-killing behaviour within the *Wolbachia* clade, and also the level of divergence within *Wolbachia* found within a host population.

2. MATERIALS, METHODS AND RESULTS

(a) *Male-killing strains and inheritance*

Lines bearing inherited male-killing elements were isolated from *A. bipunctata* (Coleoptera: Coccinellidae) from Moscow, Russia, and *A. encedon* (Lepidoptera: Nymphalidae) from Kampala, Uganda. Evidence of male-killing was based on the combined basis of presence of a female-biased sex ratio and low egg-hatch rate.

Details of the inheritance of the male-killing trait in *A. encedon* are given in Jiggins *et al*. (1998). Previous studies suggested the production of female-biased sex ratios was associated with meiotic drive. However, Jiggins *et al*. (1998) showed the bias to be associated with high egg mortality, and to be curable by treatment of the female parent with antibiotics. It is thus a case of infection with male-killing bacteria. Eighty-six per cent of wild females in this species bear the male-killing trait, which is maternally inherited, with very high transmission efficiency (no loss of infection in 18 F_1 crosses, no males produced in 48 families from females bearing the male-killer).

The male-killing trait in *A. bipunctata* from Moscow is similar to that previously recorded in Cambridge (Hurst *et al*. 1992). Five male-killer-infected lines from Moscow were examined. The male-killer-infected females produced eggs with half the normal hatch rate, and families that were either all- or near all-female broods (mean 2.8% males; $n = 17$ parental and F_1 crosses from the five matrilines). In contrast to male-killing systems in some other ladybird beetles (Hurst *et al.* 1996), the sons produced by infected Moscow females are produced sporadically through her life, with no obvious increase or decrease in frequency with maternal age. The vertical transmission efficiency of the trait from infected individuals is high, with just one female derived from a sex ratio-biased family having been recorded exhibiting the 'normal' phenotype (eggs with high hatch rate, production of a family with a normal sex ratio) from a total of 13 crosses performed with females from such families. The trait is maternally inherited (it has now been maintained through four generations of outcrossing), and not apparently affected by the derivation of the male partner.

The microbial nature of the trait was confirmed by testing sensitivity to antibiotics and microinjection experiments. In brief, sex ratio and egg-hatch rate returned to normal following treatment of male-killer-infected females with the antibiotics rifampicin (Merck) and sulphamethoxazole (Merck) (three repeats). The trait could be artificially transmitted into uninfected lines through injection of a macerate of infected tissue into uninfected pupae (lag of four weeks before breeding, three repeats, using the methodology of Gotoh (1982)).

(b) *Test for known male-killers in* Adalia bipunctata

Genomic DNA was prepared from ovaries of one individual from each of the five *A. bipunctata* lines from Moscow, and from nine *A. encedon* lines bearing male-killers, using proteinase K digestion followed by extraction through phenol-chloroform, followed by chloroform, and ethanol precipitation. The *A. bipunctata* lines were tested for the presence of the previously established *A. bipunctata* male-killing bacteria, a *Rickettsia* (Werren *et al*. 1994) and a *Spiroplasma* (Hurst *et al*. 1999*b*), using diagnostic PCR amplifications. In the case of the *Rickettsia*, PCR was based on a primer pair that amplifies the gene encoding the 17 kDa outer-membrane protein (Williams *et al*. 1992). In the case of the *Spiroplasma*, primers that amplify the 16S rDNA of members of the Mollicute family were employed (Van Kuppeveld *et al*. 1992). For details of primers and amplification profiles, see Hurst *et al*. (1999*b*). Both reactions failed to produce amplification products with the test specimens, but produced successful amplification with known positive controls, indicating absence of these bacteria from test specimens.

(c) *Association of* Wolbachia *with the male-killing trait*

A. bipunctata and *A. encedon* lines bearing male-killers were then tested for the presence of *Wolbachia* using *Wolbachia*-specific PCR. Initially, we tested the male-killing strains for *Wolbachia* using a primer pair (ftsZf1, ftsZr1) that permits amplification of the *fts*Z gene of all *Wolbachia,* but not other eubacteria (Werren *et al*. 1995*b*). *Wolbachia* was found in each of the *A. bipunctata* and *A. encedon* lines exhibiting male-killing. Thereafter, the group affiliation of the *Wolbachia* was determined using the *fts*Z A-specific and B-specific primer pairs (Werren *et al*. 1995*b*) (A-specific, ftsZAdf, ftsZAdr; B-specific, ftsZBf, ftsZBr). In each case, the PCR reactions amplified DNA when using the B-group specific primers, but not in PCRs using those specific to the A group.

We then tested the correlation between *Wolbachia* presence and the male-killing trait across infected and uninfected lineages of both species. We used three different PCR assays, based on *Wolbachia* 16S rDNA (primer pair 16SBf, 16SBr) (Werren *et al*. 1995*b*), the *fts*Z gene (primer pair ftsZBf, ftsZBr) (Werren *et al*. 1995*b*), and the *wsp* gene (primer pair wsp81F, wsp691R) (Zhou *et al*. 1998). Amplification was attempted on genomic DNA template derived from (i) individuals from each of the known infected lines (five *A. bipunctata*, nine *A. encedon*, as above); (ii) individuals from known uninfected lines (ten *A. bipunctata* lines, all from Moscow; five *A. encedon* lines); (iii) individuals from male-killing lines that had been cured by administration of antibiotics (two *A. bipunctata* lines; five *A. encedon* lines) (see Hurst *et al*. (1992) and Jiggins *et al*. (1998) for methodology); (iv) F_1 individuals from normal lines that had developed the male-killing trait following microinjection of infected homogenate (two *A. bipunctata* lines); and (v) individuals from *A. bipunctata* lines known to bear *Spiroplasma* or *Rickettsia* male-killers.

A control for failure of PCR due to poor template quality was carried out using a PCR which amplifies part of the COI gene of mtDNA from insects (primer pair Cl-J-1751, Cl-N-2191) (Simon *et al*. 1994).

In each of the three amplifications, *Wolbachia*-specific PCR products of appropriate size were produced when using the template derived from the test male-killer lines (five *A. bipunctata*, nine *A. encedon*) and from artificially infected individuals (*A. bipunctata*), but not when using the template from naturally

uninfected or antibiotic-cured *A. bipunctata* or *A. encedon*. *Wolbachia* were also not found in *A. bipunctata* lines known to be infected with male-killing *Spiroplasma* or *Rickettsia*. Templates that failed in amplification with *Wolbachia*-specific primers were successfully amplified in PCR using the control COI primers, indicating that failure in the *Wolbachia*-specific amplifications was not caused by poor DNA quality.

(d) *Testing the uniqueness of the association between* Wolbachia *and the trait*

To test whether *Wolbachia* was the only bacterium associated with the trait, we performed a PCR using primers that amplify the 16S rDNA of eubacteria in general (primer pair 27f, 1495r) (Weisburg *et al*. 1991). This was performed on genomic DNA from three out of the five naturally infected *Wolbachia*-bearing *A. bipunctata* lines and five *A. encedon* lines. The product was purified using Microcon-50 Microconcentrators (Amicon Ltd), and ligated into pGEM T-vector (Promega). The resulting plasmids were transformed into *E. coli* DH5α, the *E. coli* being grown for less than 1 h before plating to prevent duplication before plating. Colonies were then picked, and regrown on new LB-carbenicillin plates, to separate colonies from the unligated PCR product and untransformed plasmid contaminating the original plate surface. The regrown colonies were tested by PCR assay for the presence of insert DNA (using pUC general primers). Those bearing an insert were then tested for the presence of *Wolbachia* 16S rDNA (using the *Wolbachia*-specific primers 16SBf and 16SBr; Werren *et al*. 1995*b*).

For *A. bipunctata*, 60 colonies bearing inserts were picked from each of three male-killing lines, and all 180 of these were positive for the presence of the *Wolbachia* 16S rDNA insert. Negative controls (water blank, blue colonies) failed to amplify with *Wolbachia*-specific primers. For *A. encedon*, 42 clones bearing inserts were obtained from the five male-killer-infected lines. Of these, 39 were positive for the *Wolbachia* 16S rDNA insert. Thus, it can be concluded that the 16S rDNA amplified in the PCR employing general eubacterial primers is nearly all of *Wolbachia* origin, both for *A. bipunctata* and *A. encedon*.

(e) *Phylogenetic affiliation of male-killing* Wolbachia

We examined the phylogenetic affiliation of the *A. bipunctata* and *A. encedon* male-killing *Wolbachia* using the sequence of the *wsp* gene. This was amplified and cloned as before from an individual of each of the five infected *A. bipunctata* lines, and two of the infected *A. encedon* lines, using polymerase with proofreading activity (Expand High Fidelity PCR system, Boehringer-Mannheim, Germany), plasmids being isolated using the Wizard Minipreps DNA purification system (Promega). DNA sequences were obtained via cycle sequencing using the ABI PRISM Dye Terminator cycle-sequencing ready-reaction kit (Perkin Elmer), visualizing results on an ABI 384 automated sequencer (Perkin Elmer). Sequences were obtained completely through both strands with the help of pUC/M13 forward and reverse primers and the original PCR primers.

For each host specimen (five *A. bipunctata*, two *A. encedon*), three clones were sequenced. A majority rule consensus sequence was generated for *Wolbachia* from each of the host specimens to account for PCR errors (total, seven sequences). In *A. encedon*, the *wsp* sequences were identical for the *Wolbachia* from both host specimens. In contrast, there was inter-host individual variation in *wsp* sequence of the *Wolbachia* in *A. bipunctata*. The five lines gave rise to two sequences, one *wsp* sequence being common to two of the strains, the other to the remaining three. Interestingly, these two sequences differed from each other in 38 nucleotides (6.92% divergence, uncorrected for multiple hits).

The three sequences (one from *A. encedon*, two from *A. bipunctata*: EMBL accession numbers AJ130714–AJ130716) were aligned to published sequences of B-group *Wolbachia* and two outgroup A-group *Wolbachia*, taking into account coding structure. Phylogenetic analysis was then performed using maximum parsimony and maximum likelihood methods. Maximum parsimony analysis, as implemented in PAUP v. 3.1 (Swofford 1991), was based on a heuristic search using the nearest-neighbour interchanges branch-swapping algorithm and the two included A-group *Wolbachia* as outgroup taxa. Robustness of resulting tree topologies was tested via bootstrapping, using the same settings and 500 bootstrap replicates. Maximum parsimony analysis was repeated three times, using different weighting schemes for indels. Indels were either treated as missing data, with each uninterrupted gap of the sequence alignment being given a total weight of one, or they were treated on a codon-by-codon basis, each codon deletion or insertion being given a weight of one or zero, respectively.

Maximum likelihood analysis was performed with the help of DNAML of PHYLIP, v. 3.57c (Felsenstein 1995). Analysis was repeated with the default options, but different transition-to-transversion rates (1.0, 2.0, 5.0, and 10.0), and, in each case, a randomized input order of sequences. In addition, we used parameter estimates for the transition-to-transversion rate and for gamma-rate heterogeneity categories. These parameters were inferred from the data set using the program PUZZLE, v. 4.0 (Strimmer & von Haesler 1996), assuming the Hasegawa–Kishono–Yamo (HKY) model of sequence evolution (Hasegawa *et al*. 1985) and consideration of eight gamma-rate heterogeneity categories.

Phylogenetic analysis clearly confirmed that all the isolated *Wolbachia* were members of the B group. Maximum parsimony analysis clearly shows the two different *Wolbachia* strains from *A. bipunctata* to be monophyletic. Further, they indicate that the *A. encedon* and *A. bipunctata* male-killing *Wolbachia* belong to different lineages, although bootstrap support for the bifurcations separating the male-killing *Wolbachia* of the two different host species was below 70% (figure 1).

These conclusions were generally corroborated by maximum likelihood analysis. The tree with the highest log likelihood was obtained when rate heterogeneity among sites was taken into account and estimated parameter values were used. The only difference between this tree and that found through parsimony was in the clade of five *Wolbachia* strains containing the *A. encedon* and *A. bipunctata* male-killing strains. Within this clade, maximum likelihood analysis placed the *Wolbachia* of *Tribolium confusum* and *A. bipunctata* in a monophyletic group, with the *Wolbachia* of *A. encedon* being most closely related to that of *Trichogramma deion* and thereafter that of *Laodelphax striatellus*.

The above suggests that the *wsp* DNA sequence data contains insufficient unambiguous phylogenetic information to fully resolve the relationships of these five taxa. This was confirmed by the results of a Kishino–Hasegawa test (Kishino & Hasegawa 1989) on the significance of log likelihood differences between alternative tree topologies, as implemented in PUZZLE. Under the HKY model of sequence evolution (Hasegawa *et al*. 1985), with eight gamma-rate heterogeneity categories, we compared 12 tree topologies, which differed only in the branching order within the above-mentioned clade. The 'best' tree had a log likelihood of −2934.68 and showed the male-killers to belong to different lineages, whereas the tree

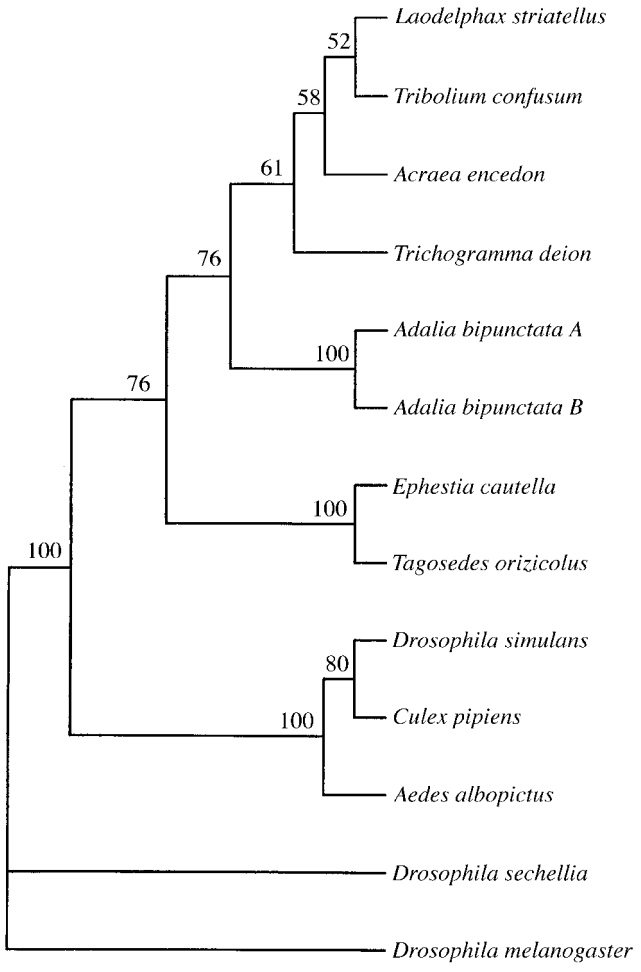

Figure 1. 50% majority-rule consensus tree of maximum parsimony-based bootstrap analysis on *wsp* DNA sequence data of B-group *Wolbachia*, with A-group *Wolbachia* of *Drosophila sechellia* and *D. melanogaster* as outgroups (program PAUP; heuristic search, branch swapping, nearest-neighbour interchanges). Each codon deletion was given a weight of one. Bootstrap values are indicated above the branches. *Wolbachia* strains are represented by the names of their host species. Suffix letters for *A. bipunctata* refer to the two different *Wolbachia* strains. GenBank accession numbers of included published sequences are as follows: AF020059, AF020060, AF020061, AF020065, AF020069, AF020073, AF020074, AF020076, AF020080, AF020083, AF020084, AF020085.

topology with the highest log likelihood for male-killer monophyly had a log likelihood of −2943.28. This difference is not significant at the 5% level according to the Kishino–Hasegawa test.

Although the relationship between the *A. encedon* and *A. bipunctata Wolbachia* is not resolved by analysis of the *wsp* sequence, the *fts*Z gene of the *A. encedon Wolbachia* does contain a nine base pair indel absent in *A. bipunctata Wolbachia*, but present in B-group *Wolbachia* from *Gryllus pennsylvanicus* and *Tribolium confusum*. This indel, being rarely found within the B group, again suggests that the *A. bipunctata* and *A. encedon* male-killing *Wolbachia* represent independent evolutions of male-killing within the *Wolbachia* clade. The issue, however, still remains to be resolved.

3. DISCUSSION

We can now state that in addition to inducing CI, feminization and parthenogenesis, *Wolbachia* has evolved male-killing behaviour. Phylogenetic analysis suggests that male-killing *Wolbachia* of the two different host species belong to two different lineages of B-type *Wolbachia*, with *A. bipunctata* additionally being host to two different but closely related strains. The sequence data do not contain sufficient phylogenetic information for us to reject firmly the hypothesis of male-killer monophyly. However, it is clear from the diversity of male-killing agents outside the *Wolbachia* clade, and the fact that at least three male-killers are found in one species, that male-killing is a widely evolved phenomenon. This would suggest that it is an easily evolved phenomenon, making it likely that further study will reveal male-killing to have evolved more than once within the clade *Wolbachia*.

Our studies also suggest that male-killing associated with *Wolbachia* will be widely found among insect hosts. Male-killing bacteria have so far been recorded in five different orders of insect, including arrhenotokous species (Hurst *et al*. 1997*a*), suggesting it is possible to evolve this trait in species with differing reproductive biologies. Within this study, we have observed *Wolbachia* associated with male-killing in one species that is a male-heterogametic coleopteran and one which is female-heterogametic lepidopteran. Thus, we can conclude that *Wolbachia* can be a male-killing agent in hosts of significantly differing sex-determination systems. This pattern contrasts with feminization and parthenogenesis induction, which for mechanistic reasons are thought to be restricted to hosts with certain sex-determination systems (Rigaud 1997; Stouthamer 1997). We thus predict male-killing *Wolbachia* will be more taxonomically widespread than both feminizing and parthenogenesis-inducing *Wolbachia*.

If, as we suspect, mutation to male-killing behaviour may happen freely across *Wolbachia* clades in a variety of hosts, then the incidence of male-killing *Wolbachia* will be dictated by whether male-killing mutants spread. Only certain host species are permissive to the spread of male-killers. Such spread occurs when there is (i) sibling cannibalism; or (ii) competition between siblings for food; or (iii) where female fitness is reduced in the field by disadvantageous inbreeding (Hurst 1991; Skinner 1985; Werren 1987). Under these conditions, the death of males enhances the survivorship or fecundity of their infected sisters.

Given that many hosts are ecologically permissive to the spread of male-killers, we thus expect male-killing *Wolbachia* to be common. In this context, it is at first surprising that male-killing *Wolbachia* have not been uncovered previously. However, male-killing *Wolbachia* would only be likely to be revealed in studies such as ours, which are designed to establish the causal agent underlying a previously found male-killing phenotype. It is unlikely that male-killing *Wolbachia* would be found from examining the phenotype produced by *Wolbachia* found in mass screenings. This is because these screenings generally try to maximize species coverage, using, therefore, just one or two individuals per species as tokens of infection (Werren *et al*. 1995*a*; West *et al*. 1998). Given that male-killers most commonly exist at intermediate prevalence in infected (between 5 and 25% of female individuals, although note Jiggins *et al* (1998); Majerus *et al* 1998), and CI-inducing *Wolbachia* exist at high prevalence in both sexes, these screens will pick up CI-inducing *Wolbachia* much more frequently than they will pick up male-killing infections.

The other notable feature within our data is the level of DNA sequence divergence between the *wsp* genes of male-killing *Wolbachia* in different *A. bipunctata* individuals (6.92% divergence, uncorrected for multiple hits). The *wsp* gene evolves with a substitution rate around ten times that of 16S rDNA (Zhou *et al*. 1998), the latter evolving with a substitution rate of 1–2% lineage^{-1} 50 Myr^{-1} (Moran *et al*. 1993). This strongly suggests that the two 'strains', isolated from different host individuals, diverged more than one million years before present, although rate heterogeneity in the evolution of the *wsp* gene makes accurate estimation impossible. Given that they are monophyletic, they may represent a single origin within the species, with subsequent divergence over time. However, we cannot at present rule out the possibility that the two strains did not diverge within *A. bipunctata*, but in fact represent dual invasion of *A. bipunctata* following independent horizontal transmission events.

The biology of male-killing by *Wolbachia* has not been discussed here. Three main questions arise. First, does male-killing involve manipulation of host chromosomes, as is found for CI (Breeuwer & Werren 1990) and parthenogenesis induction (Stouthamer & Kazmer 1994), or is it mechanistically separate (as is suspected for feminization)? Second, is male death induced in similar ways in all hosts, with *Wolbachia* responding to the same cue of host sex, or are there a variety of ways in which hosts are killed, and a variety of cues used to detect sex? Third, are transitions from male-killing behaviour to CI as likely as transitions in the opposite direction? These questions are left for future research.

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REFERENCES

Bandi, C., Anderson, T. J. C., Genchi, C. & Blaxter, M. L. 1998 Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. Lond.* B **265**, 2407–2413.

Bouchon, D., Rigaud, T. & Juchault, P. 1998 Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. Lond.* B **265**, 1081–1090.

Breeuwer, J. A. J. & Werren, J. H. 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346**, 558–560.

Felsenstein, J. 1995 *PHYLIP: phylogenetic inference package v. 3.57c*. Department of Genetics, University of Washington, Seattle, USA.

Gotoh, T. 1982 Experimental transfer of abnormal 'sex-ratio' in the lady-bird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). *Appl. Entomol. Zool.* **17**, 319–324.

Hackett, K. J., Lynn, D. E., Williamson, D. L., Ginsberg, A. S. & Whitcomb, R. F. 1986 Cultivation of the *Drosophila* spiroplasma. *Science* **232**, 1253–1255.

Hasegawa, M., Kishino, H. & Yano, K. 1985 Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**, 160–174.

Hoffmann, A. A. & Turelli, M. 1997 Cytoplasmic incompatibility in insects. In *Influential passengers: inherited microorganisms and arthropod reproduction* (ed. S. L. O'Neill, A. A. Hoffmann & J. H. Werren), pp. 42–80. Oxford University Press.

Hurst, L. D. 1991 The incidences and evolution of cytoplasmic male killers. *Proc. R. Soc. Lond.* B **244**, 91–99.

Hurst, G. D. D., Majerus, M. E. N. & Walker, L. E. 1992 Cytoplasmic male killing elements in *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). *Heredity* **69**, 84–91.

Hurst, G. D. D., Hammarton, T. C., Obrycki, J. J., Majerus, T. M. O., Walker, L. E., Bertrand, D. & Majerus, M. E. N. 1996 Male-killing bacteria in a fifth ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Heredity* **77**, 177–185.

Hurst, G. D. D., Hurst, L. D. & Majerus, M. E. N. 1997*a* Cytoplasmic sex ratio distorters. In *Influential passengers: microbes and invertebrate reproduction* (ed. S. L. O'Neill, A. A. Hoffmann & J. H. Werren), pp. 125–154. Oxford University Press.

Hurst, G. D. D., Hammarton, T. C., Majerus, T. M. O., Bertrand, D., Bandi, C. & Majerus, M. E. N. 1997*b* Close relationship of the inherited parasite of the ladybird, *Coleomegilla maculata*, to *Blattabacterium*, the beneficial symbiont of the cockroach. *Genet. Res.* **70**, 1–6.

Hurst, G. D. D., Bandi, C., Sacchi, L., Cochrane, A., Bertrand, D., Karaca, I. & Majerus, M. E. N. 1999*a* *Adonia variegata* (Coleoptera: Coccinellidae) bears maternally inherited Flavobacteria that kill males only. *Parasitology* **118**, 125–134.

Hurst, G. D. D., Schulenburg, J. H. G. v. d., Majerus, T. M. O., Bertrand, D., Zakharov, I. A., Baungaard, J., Volkl, W., Stouthamer, R. & Majerus, M. E. N. 1999*b* Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Mol. Biol.* **8**, 133–139.

Jiggins, F. M., Hurst, G. D. D. & Majerus, M. E. N. 1998 Sex ratio distortion in *Acraea encedon* (Lepidoptera: Nymphalidae) is caused by a male-killing bacterium. *Heredity* **81**, 87–91.

Juchault, P. & Mocquard, J. P. 1993 Transfer of a parasitic sex factor to the nuclear genome of the host: a hypothesis on the evolution of sex-determination mechanisms in the terrestrial isopod *Armadillidium vulgare* Latr. *J. Evol. Biol.* **6**, 511–528.

Kishino, H. & Hasegawa, M. 1989 Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* **29**, 170–179.

Laven, H. 1951 Crossing experiments with *Culex* strains. *Evolution* **5**, 370–375.

Majerus, T. M. O., Majerus, M. E. N., Knowles, B., Wheeler, J., Bertrand, D., Kuznetsov, V. N., Ueno, H. & Hurst, G. D. D. 1998 Extreme variation in the prevalence of inherited male-killing microorganisms between three populations of *Harmonia axyridis* (Coleoptera: Coccinellidae). *Heredity* **81**, 683–691.

Moran, N. A., Munson, M. A., Baumann, P. & Ishikawa, H. 1993 A molecular clock in endosymbiotic bacteria is callibrated using the insect hosts. *Proc. R. Soc. Lond.* B **253**, 167–171.

Rigaud, T. 1997 Inherited microorganisms and sex determination of arthropod hosts. In *Influential passengers: inherited microorganisms and arthropod reproduction* (ed. S. L. O'Neill, A. A. Hoffmann & J. H. Werren), pp. 81–102. Oxford University Press.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. & Solignac, M. 1992 *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. Lond.* B **250**, 91–98.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. 1994 Evolution, weighting and phylogenetic utility

of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* **87**, 651–701.

Skinner, S. W. 1985 Son-killer: a third extrachromosomal factor affecting sex ratios in the parasitoid wasp *Nasonia vitripennis*. *Genetics* **109**, 745–754.

Stouthamer, R. 1997 *Wolbachia*-induced parthenogenesis. In *Influential passengers: inherited microorganisms and invertebrate reproduction* (ed. S. L. O'Neill, A. A. Hoffmann & J. H. Werren), pp. 102–124. Oxford University Press.

Stouthamer, R. & Kazmer, D. 1994 Cytogenetics of microbe-associated parthenogenesis and its consequence for gene flow in *Trichogramma* wasps. *Heredity* **73**, 317–327.

Stouthamer, R., Breeuwer, J. A. J., Luck, R. F. & Werren, J. H. 1993 Molecular identification of organisms associated with parthenogenesis. *Nature* **361**, 66–68.

Strimmer, K. & von Haesler, A. 1996 Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13**, 964–969.

Swofford, D. L. 1991 *PAUP: phylogenetic analysis using parsimony v. 3.0s*. Natural History Museum, University of Arizona, USA.

Van Kuppeveld, F. J. M., Van der Logt, H. T. M., Angulo, A. F., Van Zoest, M. J., Quint, W. G. V., Niesters, H. G. M., Galama, J. M. D. & Melchers, W. J. G. 1992 Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl. Environ. Microbiol.* **58**, 2606–2615.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 1991 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**, 697–703.

Werren, J. H. 1987 The coevolution of autosomal and cytoplasmic sex ratio factors. *J. Theor. Biol.* **124**, 317–334.

Werren, J. H. 1997 Biology of *Wolbachia*. *A. Rev. Entomol.* **42**, 587–609.

Werren, J. H., Skinner, S. W. & Huger, A. M. 1986 Male-killing bacteria in a parasitic wasp. *Science* **231**, 990–992.

Werren, J. H., Hurst, G. D. D., Zhang, W., Breeuwer, J. A. J., Stouthamer, R. & Majerus, M. E. N. 1994 Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). *J. Bacteriol.* **176**, 388–394.

Werren, J. H., Windsor, D. & Guo, L. 1995*a* Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond.* B **262**, 197–204.

Werren, J. H., Zhang, W. & Guo, L. R. 1995*b* Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc. R. Soc. Lond.* B **261**, 55–71.

West, S. A., Cook, J. M., Werren, J. H. & Godfray, H. C. J. 1998 *Wolbachia* in two insect host–parasitoid communities. *Mol. Ecol.* **7**, 1457–1465.

Williams, S. G., Sacci, J. B. J., Schriefer, M. E., Andersen, E. M., Fujioka, K. K., Sorvillo, F. J., Barr, A. R. & Azad, A. F. 1992 Typhus and typhus-like rickettsiae associated with opposums and their fleas in Los Angeles. *J. Clin. Microbiol.* **30**, 1758–1762.

Yen, J. H. & Barr, A. R. 1973 The etiologic agent of cytoplasmic incompatibility in *Culex pipiens*. *J. Invert. Pathol.* **22**, 242–250.

Zhou, W., Rousset, F. & O'Neill, S. 1998 Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond.* B **265**, 509–515.