Male-killing *Wolbachia* in two species of insect


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 (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.* 1995x), 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* 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 & Moquard 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.* 1997x). Male-killing behaviour is exhibited by a wide range of bacteria, including members of the Proteobacteria (Werren *et al.* 1986, 1994), the Flavobacteria...
(Hurst et al. 1997, 1999a), and the Mollicutes (Hackett et al. 1986; Hurst et al. 1999b). This has led to the view that male-killing behaviour is easily evolved (Hurst et al. 1997a), 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 F1 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 F1 crosses from the five matriline). 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 sulphanmethoxazole (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. 1999b), 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. (1999b). 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 (ftsZ, ftsZ1) that permits amplification of the ftsZ gene of all Wolbachia, but not other eubacteria (Werren et al. 1995). 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 ftsZ A-specific and B-specific primer pairs (Werren et al. 1995) (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 16SBr, 16SBt) (Werren et al. 1995), the ftsZ gene (primer pair ftsZBi, ftsZBr) (Werren et al. 1995), and the wsp gene (primer pair wsp81F, wsp91R) (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) F1 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 CI-J-1751, CI-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. 1995b).

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 proof-reading activity (Expand High Fidelity PCR system, Boehringer-Mannheim, Germany), plasmids being isolated using the Wizard Miniprep 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 (Swoford 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 uninterpreted 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 DNAMLc 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-Kishino-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 Glade of five Wolbachia strains containing the A. encedon and A. bipunctata male-killing strains. Within this Glade, 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 Tribolium 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 Glade. The ‘best’ tree had a log likelihood of -2934.68 and showed the male-killers to belong to different lineages, whereas the tree
male-killing behaviour. Phylogenetic analysis suggests that male-killing *Wolbachia* of the two different host species belong to two different lineages of 13-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 arthropodous species (Hurst *et al*. 1997a), suggesting it is possible to evolve this trait in species with differing reproductive biology. 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. The 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; Southamer 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*. 1995a; 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.

3. DISCUSSION

We can now state that in addition to inducing CI, feminization and parthenogenesis, *Wolbachia* has evolved
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% per lineage per 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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