

Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*

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Inherited bacteria that kill male hosts early in their development are known from five insect orders. We ask to what extent the incidence of male-killers might be restricted by the rate at which new host–parasite interactions arise, by testing whether multiple male-killers have invaded a single host species. In Uganda, the butterflies *Acraea encedon* and *A. encedana* are both infected by the same strain of male-killing *Wolbachia* and there was no evidence of variation within the population. In Tanzanian *A. encedon* however, two phylogenetically distinct strains of male-killing *Wolbachia* were found within the same population. If this pattern of male-killer polymorphism is found to be general across infected species, it suggests that new male-killing infections arise frequently on an evolutionary time scale. Whether this polymorphism is stable, and what forces may be maintaining it, are unknown.

Keywords: *Acraea encedana*, *Acraea encedon*, Lepidoptera, male-killing, sex ratio, *Wolbachia*.

Introduction

Maternally inherited genes and the rest of the genome are under conflicting selection pressures regarding the sex ratio of their offspring. Natural selection favours maternally inherited genes if they cause the production of daughters only, whereas, all else being equal, the autosomal genes are selected to produce equal numbers of sons and daughters (Fisher, 1930; Hamilton, 1967). Maternally inherited symbionts have evolved several strategies to increase the number or fecundity of daughters, one of the most widespread of which is to kill males early in development. These bacteria spread by kin selection, because male death benefits the female survivors who will pass the bacteria to their offspring (Hurst *et al.*, 1997). Their spread will have direct evolutionary consequences for the host by selecting for suppressors of the transmission and action of the bacteria, and indirect effects associated with the sex ratio bias they produce. In the butterflies *A. encedon* and *A. encedana* the sex ratio distortion can be so extreme as

to cause sex-role reversal in the host species (Jiggins *et al.*, 2000a; Randerson *et al.*, 2000a).

There appear to be few genetic constraints to the evolution of male-killing. Male-killers are known from five insect orders with varied sex determination mechanisms, suggesting that they are not restricted to particular host genetic systems. Similarly, the male-killing bacteria themselves are surprisingly diverse, the phenotype having independently evolved at least six times across the Eubacteria (Hurst *et al.*, 1999a). Therefore, relative to other forms of bacterial sex ratio distortion, the trait of male-killing is easily evolved by bacteria.

In the absence of genetic constraints, the incidence of male-killers will depend upon which hosts have ecologies that permit their spread and the rate at which male-killers arise in these hosts. Records of male-killers are mostly confined to host species with strong sibling interactions, thus confirming the importance of host ecology in determining the incidence of male-killers. We ask whether the incidence of male-killers is also restricted by the rate at which new host–parasite interactions arise.

We can test whether new interactions occur frequently during evolution by examining species where a male-killer is known, and seeing if more than one element is present, as these are known permissive species. In *Adalia bipunctata* this approach showed the presence of male-killers from three bacterial genera, suggesting a high rate

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of invasion by novel male-killers (Hurst *et al.*, 1999a; Hurst *et al.*, 1999b). However, this example may prove to be exceptional, as a smaller sample of another ladybird, *Harmonia axyridis*, contained only one male-killer (Majerus *et al.*, 1999). Further data are clearly necessary.

We aim to test these ideas in a second insect order by investigating in more detail the butterflies *A. encedon* and *A. encedana*, which are known to be host to male-killing *Wolbachia* bacteria (Jiggins *et al.*, 1998, 2000b; Hurst *et al.*, 1999a). We address three questions. First, how many times has the butterfly *A. encedon* been invaded by male-killers? Second, are multiple male-killers maintained within a population and do the causes of male-killing vary between populations? Finally, do any new male-killing agents identified in *A. encedon* also occur in *A. encedana*?

Methods

Identification of male-killing lines

We used male-killing lines previously identified from Ugandan populations of *A. encedon* (Jiggins *et al.*, 1998) and *A. encedana* (Jiggins *et al.*, 2000b) and reared additional lines of *A. encedon* from a population in Tanzania, where distorted sex ratios were recorded in 1974 (Owen & Smith, 1991). Tanzanian butterflies were collected from swampy areas (mostly within a patch of approximately 200 m × 100 m) on the University of Dar es Salaam campus in June 1999. Following oviposition on the host plant *Commelina* sp., the eggs and newly emerged larvae were counted to give hatch rates. In order to increase the data set, additional egg clutches were obtained from the F₁ generation, their hatch rates recorded, and then they were discarded. The larvae were transported to Cambridge, UK and reared on *Commelina benghalensis* in a greenhouse. When the adults emerged, some were mated in order to rear a second generation. Male-killer matriline lines were identified on the basis of their low egg hatch rates combined with female-biased sex ratios.

Are all male-killing lines infected by *Wolbachia*?

We had previously shown that nine male-killer lines of *A. encedon* from Uganda were all infected with *Wolbachia* (Hurst *et al.*, 1999a). We then looked for different male-killers in *A. encedon* by testing the Tanzanian and further Ugandan male-killer lines for *Wolbachia*. DNA was extracted from the ovaries of female butterflies using a method modified from Walsh *et al.* (1991). A small amount of ovary tissue was incubated at 56°C with Chelex 100 ion exchange resin (Bio-Rad), dithiothreitol and proteinase-K, then boiled and the

supernatant used directly for PCR. We tested for the presence of *Wolbachia* using the PCR primers *wsp*81F and *wsp*691R, which amplify the bacterium's *wsp* gene (Zhou *et al.*, 1998). The extractions that tested negative for *Wolbachia* were then amplified using primers for the ITS1 region in insects (Hillis & Dixon, 1991) to check that the DNA extraction had been successful.

Are all *Wolbachia* infections the same?

The *wsp* gene was amplified as above from Tanzanian and additional Ugandan male-killer matriline lines and the PCR product purified using Microcon-50 Microconcentrators (Amicon Ltd). One strand was sequenced in totality direct from the PCR product using one of the PCR primers. Sequencing was performed by cycle sequencing using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer), visualizing the results on an ABI 384 automated sequencer (Perkin Elmer). When a new sequence was identified, the second PCR primer was used to sequence the gene in the opposite direction.

In order to increase our sample size and the geographical range covered, we also sequenced the *wsp* gene from infected wild females of unknown sex ratio type from Ghana, Zimbabwe (Victoria Falls) and Tanzania (Dar campus). Having identified a novel *Wolbachia* in the Tanzanian population of *A. encedon*, we then screened a larger Ugandan sample for this bacterium. The restriction enzyme *EcoRV* cuts the *wsp* sequence of the new strain (T) but not the previously identified male-killer (strain U). Therefore, we amplified the *wsp* gene from additional Ugandan male-killer lines and digested them with *EcoRV*, alongside controls of known T and U sequences.

Antibiotic curing of lines infected with novel *Wolbachia*

In order to check that newly identified *Wolbachia* strains found in male-killer lines were causing the male-killing trait, we fed caterpillars from these lines on leaves painted on one side with 1% w/v tetracycline hydrochloride. The resulting butterflies were mated and their offspring's sex ratios recorded. The offspring were then tested with the *wsp* primer to check whether the antibiotics had cured them of the *Wolbachia* infection.

The phylogeny of *Wolbachia*

The newly isolated *wsp* sequence from *A. encedon* T was added to the previous alignment of Schulenburg *et al.* (2000), excluding some highly similar sequences from *Trichogramma* spp. to enhance the speed of tree reconstruction. We excluded regions of the gene that are

impossible to align because they are highly variable and contain insertions and deletions. Phylogenetic analysis was performed with the program PAUP*, written by David L. Swofford, using the maximum likelihood criterion and the general time reversible substitution model with gamma-distributed substitution rate heterogeneity across sites (Swofford *et al.*, 1996). Tree reconstruction was based on branch-swapping by nearest-neighbour interchanges. The robustness of the inferred topology was assessed by performing 100 nonparametric bootstrap replicates using the same settings as above. In addition, the hypothesis of a single origin of male-killing *Wolbachia* from *A. encedon* was tested with the Kishino–Hasegawa test (Kishino & Hasegawa, 1989). For this purpose, phylogenetic trees were inferred as above, with the topological constraint of exclusive monophyly for these two taxa. The resulting likelihood score was then compared to that of the optimal tree, inferred without such constraints. Schulenburg *et al.* (2000) give further details of the methods and substitution model used.

Results

Are all male-killing lines infected by *Wolbachia* bacteria?

The sample of *A. encedon* collected in Tanzania contained 54% females ($n = 35$) and 11 of the 19 females were infected with *Wolbachia*. Eggs were obtained from 13 of these females; eight infected females produced only daughters (Table 1) and had low hatch rates (Fig. 1),

Table 1 The inheritance of sex ratio from Tanzanian *Acraea encedon* arranged in maternal lines. The sex ratios of the normal sex ratio broods were homogeneous (including the antibiotic-cured brood: $\chi^2 = 6.1$; d.f. = 10; NS) and did not differ from a 1:1 sex ratio (summed data: 462 males: 477 females)

Sex ratio	Parental		F ₁	
	Male	Female	Male	Female
All-female	0	45	0	30
All-female	0	10	0	69
All-female	0	18	0	51
All-female	0	11	0	33
All-female	0	28	0	31
All-female	0	27		
All-female	0	18	0	64
All-female	0	17		
Normal	22	16	23	24
Normal	59	54	36	33
Normal	22	27		
Normal	30	30		
Normal	38	33	22	18

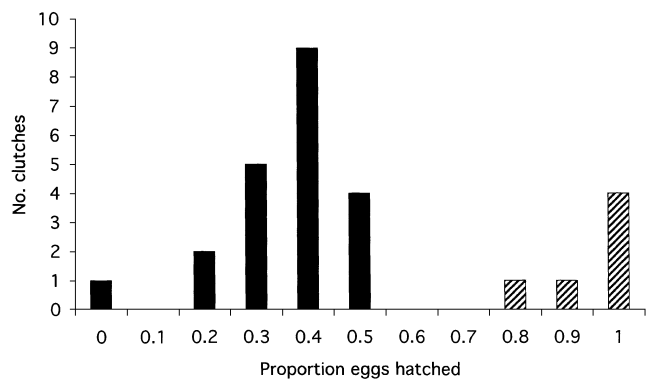


Fig. 1 Hatch rates of all-female (filled) and normal sex ratio (hatched) Tanzanian *A. encedon* broods. All-female broods have lower hatch rates than normal sex ratio broods (Mann–Whitney *U*-test: $n_1 = 21$, $n_2 = 6$; $U = 20$; $P < 0.05$).

indicating the presence of a male-killer. Five uninfected females produced normal sex ratios. Twenty-one Ugandan male-killer lines were all infected with *Wolbachia* (12 from this study, nine from Hurst *et al.*, 1999a).

Are all *Wolbachia* infections the same?

Two different *Wolbachia wsp* sequences were isolated from the 11 infected Tanzanian *A. encedon* females. Four females were infected with strain U, which was identical to the Ugandan male-killer (Hurst *et al.*, 1999a). The remaining seven females were infected with strain T, which differs from strain U by one 3-bp insertion and approximately 100 nucleotide substitutions (6%; precise alignment of some homologous sites was difficult due to the position of the insertion being uncertain). In contrast, there was no variation in the *Wolbachia wsp* gene isolated from *A. encedon* collected in Uganda ($n = 7$), Ghana ($n = 1$) or Zimbabwe ($n = 2$), with all these infections being strain U. Similarly, the Ugandan population of *A. encedana* ($n = 22$) was only infected with strain U. The *wsp* PCR product from a further 13 Ugandan specimens of *A. encedon* did not cut with *EcoRV*, which cuts strain T but not strain U.

Phylogenetic analysis of *wsp* gene sequences strongly suggested that these two strains fall into different clades within the B-group *Wolbachia* (bootstrap support > 50 ; Fig. 2). This is confirmed by the results of the Kishino–Hasegawa test, which reject the hypothesis of exclusive monophyly of these strains ($\Delta l = 159.016$; $SD = 16.968$; $T = 9.372$; $P < 0.0001$).

Is the new *Wolbachia* strain killing males?

Two broods were reared from lines infected with the newly identified *Wolbachia* strain that had been fed on antibiotics. The resulting sex ratios were 85 males: 85

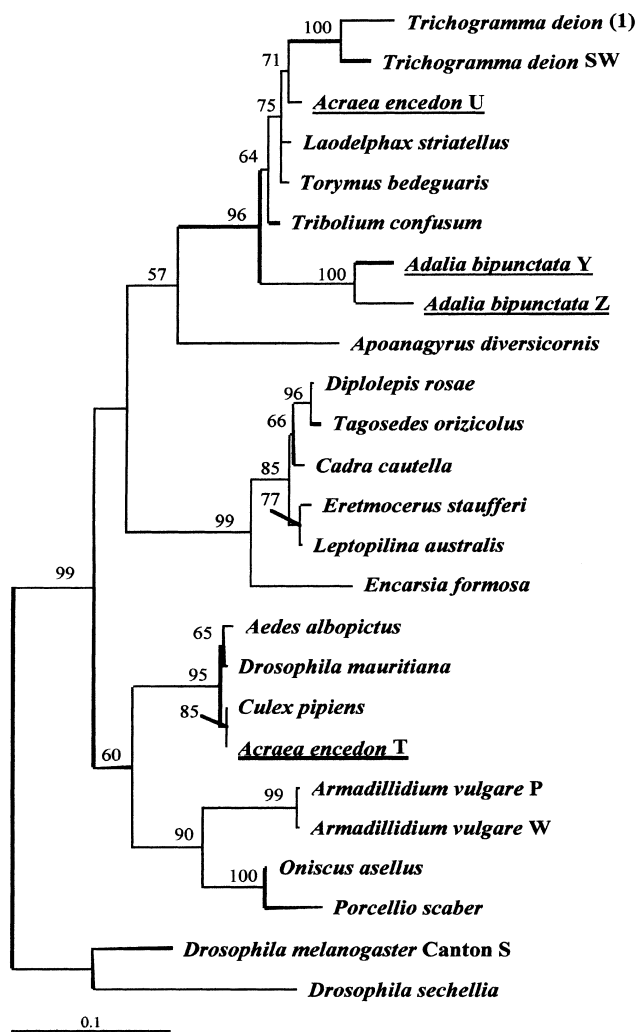


Fig. 2 Maximum likelihood phylogeny of the B-group *Wolbachia* *wsp* gene. The labels refer to the host species and male-killing strains are underlined. The tree is rooted with *Drosophila melanogaster*, strain Canton S, and *D. sechellia*. The figure shows one of three topologies with identical likelihoods ($L = -3031.922$) that differ only in the position of the *L. striatellus* and *T. bedeguaris* relative to *A. encedon* U, and the two *Trichogramma* species. The results of 100 bootstrap replicates are shown above the branches.

females and 1 male: 0 females. As a control these broods were reared alongside the F_1 generation shown in Table 1, which were treated identically, except for the presence of antibiotics. Antibiotic treatment did not alter the sex ratio of two normal sex ratio lines (33 males: 39 females and 92 males: 118 females).

Discussion

From this study we can conclude that two different male-killing *Wolbachia* strains occur sympatrically in

the Tanzanian population of *A. encedon*. In contrast, we found no evidence of male-killer polymorphisms in Ugandan populations of either *A. encedon* or *A. encedana*. Similarly, there was no evidence of male-killers from other bacterial groups in either butterfly. Within the T and U strains there was no sequence variation, suggesting that relatively recent invasions or selective sweeps have occurred.

The other insect in which a diversity of male-killers within a population has been found is the two-spot ladybird, *Adalia bipunctata*. In this species, from one to four different male-killers occur within single populations (Hurst *et al.*, 1999b; Majerus *et al.*, 2000). These male-killers belong to three bacterial genera, *Spiroplasma*, *Rickettsia* and two strains of *Wolbachia*. In contrast to the two *Wolbachia* in *A. encedon*, the two *Wolbachia* strains in *A. bipunctata* form a monophyletic group. The only other species where the diversity of male-killers has been investigated is another ladybird, *Harmonia axyridis*, where 19 male-killer lines were all infected with *Spiroplasma* bacteria. However, gene sequence data were not obtained from all these lines to test whether these bacteria were monophyletic within the genus *Spiroplasma* (Majerus *et al.*, 1999).

Wolbachia that cause cytoplasmic incompatibility and induce parthenogenesis are known, on an evolutionary time scale, to frequently move between host species (Werren *et al.*, 1995; Schilthuizen & Stouthamer, 1997). Our observations suggest that this may be true for all *Wolbachia* phenotypes, and we predict that further cases of multiple male-killer strains within species will be discovered.

The two male-killers were found within the same population. Multiple strains of *Wolbachia* that cause cytoplasmic incompatibility (CI) commonly coexist within a host population, with two or more bacterial strains infecting single individuals (Rousset & Solignac, 1995). This is expected from classical models of cytoplasmic incompatibility, which predict that a second bacterial strain can invade an infected host population provided that it is of a different crossing type and initially arises in a female infected with the original strain (Hoffmann & Turelli, 1997). In contrast, simple models of sex-ratio-distorting *Wolbachia* do not predict the presence of multiple bacterial strains within a single population. As predicted, the parthenogenesis-inducing *Wolbachia* from a single population of *Trichogramma kaykai* were all found to fall into a monophyletic group (Schilthuizen *et al.*, 1998). Currently no data exist on the diversity of feminizing *Wolbachia* within populations.

The cause of male-killer polymorphisms is a matter for speculation. It is possible that we have observed a transient polymorphism, as one male-killer replaces the other. Alternatively, the two male-killers may be

favoured in different populations by environmental or host genetic factors, and we may have observed mixing between these populations. However, if the polymorphism is stably maintained within the population, then this situation cannot be explained by simple models of male-killer dynamics (Randerson *et al.*, 2000b). The incorporation into these models of costly autosomal resistance genes, each specific to a particular male-killer, provides a means by which polymorphisms can be maintained (Randerson *et al.*, 2000b). This closely resembles the better characterized situation in plants, where both male-sterile cytotypes and autosomal loci that restore male fertility in the presence of specific cytotypes may be highly polymorphic (Van Damme & Van Delden, 1982; Laughnan & Gabay-Laughlan, 1983).

The known male-killing *Wolbachia* fall into three clades on our phylogeny of the bacterium's surface protein gene (*wsp*), and we were able to reject the hypothesis that the two *A. encedon* sequences are monophyletic. Assuming that the male-killing phenotype is encoded by bacterial and not host genes, this suggests that either male-killing has evolved multiply or that *Wolbachia* lineages recombine, meaning that our phylogeny does not reflect the evolutionary history of the male-killing phenotype (see discussion in Schulenburg *et al.*, 2000). This can only be resolved by identifying the gene(s) that cause the male-killing trait itself.

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