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Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria

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Abstract

Male-killing bacteria, which are inherited through the female line and kill male progeny only, are known from five different orders of insect. Our knowledge of the incidence of these elements has stemmed from discovery of their phenotype in different species. Our estimate of the frequency with which insects have been invaded by these elements therefore depends on each observation of the male-killing phenotype within a species being associated with a single micro-organism. We here record an example of a single insect species being infected with two taxonomically distinct male-killing bacteria. Western European populations of the two-spot ladybird, *Adalia bipunctata*, have previously been shown to bear a male-killing *Rickettsia*. However, we here show that the majority of the male-killing lines tested from Central and Eastern Europe do not bear this bacterium. Rather, 16S rDNA sequence analysis suggests male-killing is associated with a member of the genus *Spiroplasma*. We discuss this conclusion in relation to the evolutionary genetics of male-killing bacteria, and the evolution of male-killing behaviour in the eubacteria.

Keywords: *Spiroplasma*, symbiont, inherited parasite, selfish genetic elements, *Rickettsia*, Coccinellidae.

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Introduction

Selfish genetic elements, complexes of co-inherited genes which distort the normal pattern of inheritance of their host such that their own transmission is enhanced, were first recorded by Gershenson (1928). Since then a variety of different types of elements have been recorded in eukaryotes. Despite this variety, these elements have often be considered interesting curios, their general rarity making them unlikely to be widely important in evolution. Recent studies of meiotic drive genes (Merçot *et al.*, 1995; Jaenike, 1996) and *Wolbachia*, the intracellular bacterium that alters its host's reproductive biology (Breeuwer *et al.*, 1992; O'Neill *et al.*, 1992; Rousset *et al.*, 1992; Stouthamer *et al.*, 1993; Werren *et al.*, 1995), have suggested that certain types of element may be more common than previously supposed. This has led to the suggestion that they therefore could be of greater importance than previously imagined (Hurst & Hurst, 1996).

Our studies have concentrated on establishing the frequency with which insects are infected with male-killing bacteria. There is an increasing appreciation that infection with male-killing bacteria may be very common in certain groups. In the coccinellid beetles, where sibling egg consumption is considered to make male-killing behaviour highly selected (Hurst & Majerus, 1993), there are five species known to be infected with male-killing bacteria (Lus, 1947; Shull, 1948; Matsuka *et al.*, 1975; Niijima & Nakajima, 1981; Hurst *et al.*, 1996), and random survey suggest around 30% of aphidophagous species are infected (Hurst *et al.*, 1997a).

The suggestion that species in groups like the coccinellid beetles have been frequently invaded by male-killing bacteria would be corroborated by an observation of multiple infections within a species. We have been investigating a potential case of dual invasion in the two-spot ladybird beetle *Adalia bipunctata*. Male-killing in *A. bipunctata* was first observed in Russian populations (Lus, 1947). It was then recorded 50 years later in UK populations (Hurst *et al.*, 1992, 1993). Male-killing in the UK population, and in a single

line from Leiden, Holland, was found to be associated with a member of the genus *Rickettsia* (Werren *et al.*, 1994; Balayeva *et al.*, 1995; Hurst *et al.*, 1996). However, study of a male-killing line from St Petersburg, Russia, found no evidence for the presence of this *Rickettsia* (Zakharov *et al.*, 1996). This suggested the presence of two different male-killing bacteria in the species. If this is the case, then estimates of the frequency of invasion with male-killing bacteria based on incidences of the male-killing phenotype alone will be an underestimate of the true frequency with which male-killing bacteria have invaded populations. This study is a reassessment of male-killing in *A. bipunctata* to confirm or refute the proposition that male-killing is polycausal in this species. We examined a range of populations of this species for male-killing bacteria, and here report on the causal agents found.

Results

Isolation of male-killer lines, test for Rickettsia *presence*

The male-killing trait was observed in fourteen of 115 wild-collected females, deriving from five populations (Table 1). PCR test for *Rickettsia* presence, using the primer pair R1 and R2 amplifying a 434 bp portion of the gene encoding the 17 kDa antigen (Williams *et al.*, 1992), proved positive with four of the fourteen specimens (three from Denmark, one from Berlin). PCR involving template from the other ten lines never produced the expected amplification product (two from Bielefeld, one from Berlin, five from Bayreuth and two from St Petersburg) (Fig. 1).

Table 1. Presence of male-killing lines in five different populations of *Adalia bipunctata*.

Population	Lines assayed	Male-killing lines
Bielefeld (West Germany)	26	2
Bayreuth (South Germany)	23	5
Berlin (East Germany)	16	2
Ribe (South West Denmark)	43	3
St Petersburg (Russia)	7	2

Identification of putative associated organism

A sequence of 1440 bp was obtained from a St Petersburg individual using the primers 27f and 1495r that amplify the 16S rDNA block from a broad spectrum of Eubacteria (EMBL accession no. AJ006775). This showed a high degree of similarity to members of the Mollicutes, the closest sequence match being to members of the genus *Spiroplasma*. Phylogenetic analysis using parsimony showed the organism to be most closely related to *Spiroplasma ixodetis*, a member of the genus borne by ticks (Tully *et al.*, 1995) (Fig. 2). *Spiroplasma ixodetis* previously formed a 'subgroup' on its own, and (like the majority of the genus) is not known to be associated with male-lethality (Weisburg *et al.*, 1989). The tree topology was robust, receiving strong support from bootstrap resampling. Further, the topology was maintained when the analysis was performed including regions containing insertions and deletions, and was also found using Maximum Likelihood estimation. A parsimony analysis suggests that it represents a second evolution of male-killing within the genus *Spiroplasma*, independent from that of the *D. willistoni* male-killing *Spiroplasma*

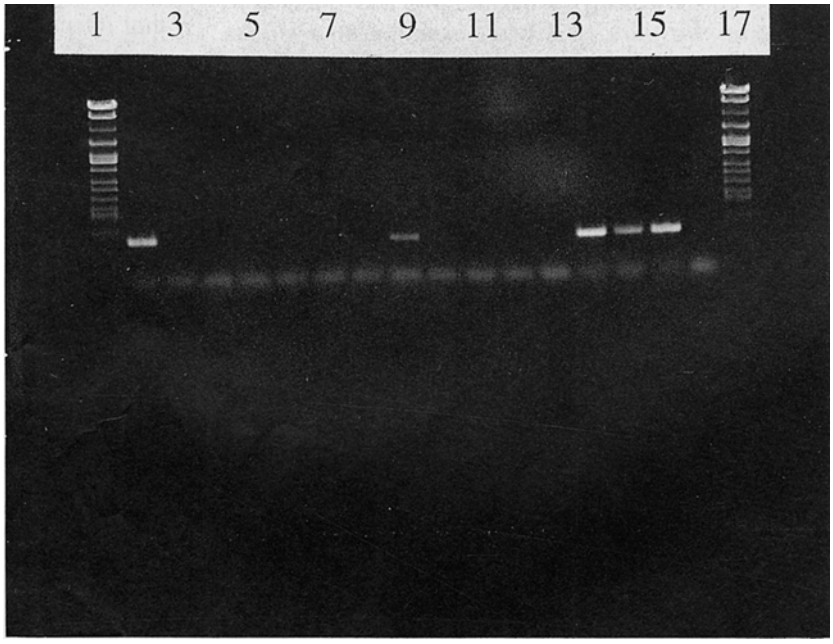

Figure 1. Test for *Rickettsia* presence in fourteen male-killing lines. An amplification product of 434 bp is expected in the presence of *Rickettsia* genomic DNA. Lane 1, λ *Hin*dIII/*Eco*RI/*Bam*HI ladder; lane 2: amplification product with Cambridge, *Rickettsia*-infected *A. bipunctata* genomic DNA as template (positive control), lanes 3–7: product with template derived from the five different Bayreuth lines; lanes 8–9: product with template derived from the two Berlin lines; lanes 10–11: product with template derived from the two Bielefeld lines; lanes 12–13: product with template derived from the two St Petersburg lines; lanes 14–16: product with template derived from the three Danish lines; lane 17: control run of PCR cocktail.

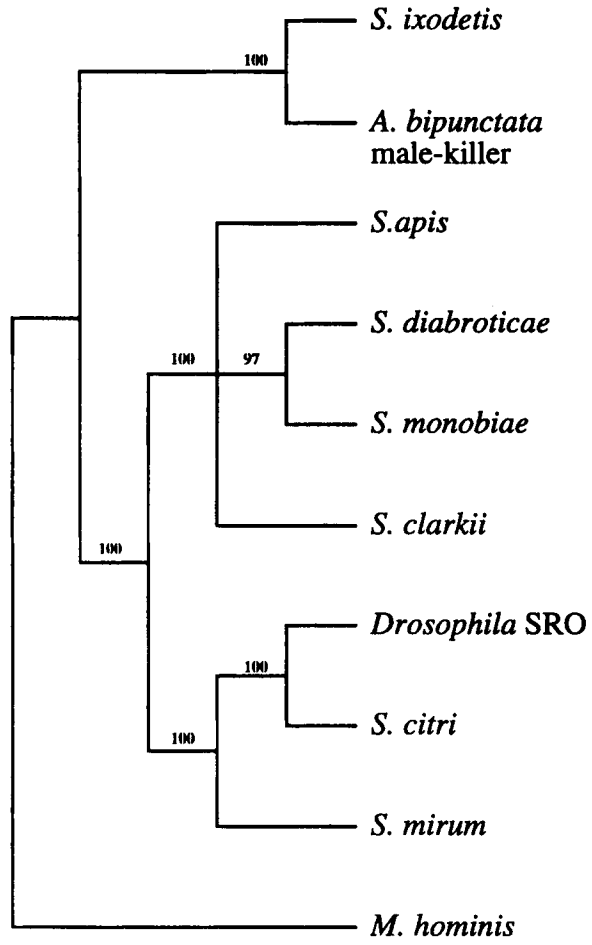

Figure 2. The phylogenetic position of the organism found in a St Petersburg male-killing line of *A. bipunctata*, as ascertained from 16S rDNA sequence analysis. Sequences of 16S rDNA from various members of the genus *Spiroplasma* and *Mycoplasma hominis* (outgroup) were manually aligned taking into account secondary structure, regions containing insertions or deletions omitted, and the phylogenetic relationships estimated through Maximum Parsimony, using the branch-and-bound algorithm of PAUP V3.1. The robustness of the tree was estimated through bootstrap resampling (branch-and-bound, 100 replicates), the values of which are indicated on each of the nodes. *Drosophila* SRO means *Drosophila* sex ratio organism, i.e. the male-killing *Spiroplasma* in *D. willistoni*.

(otherwise known as the *Drosophila* SRO: Hackett *et al.*, 1986). Nucleotide divergences (Table 2), using Moran *et al.*'s (1993) 16S rDNA clock, suggest a divergence time from the *Drosophila* SRO between 205 and 410 Ma BP. The sequence similarity of the 16S rDNA blocks of *S. ixodetis* and the *Spiroplasma* from *A. bipunctata* (98.8% identity) suggests a fairly recent divergence of these two lineages (15–30 Ma BP).

Testing association of Spiroplasma *with the male-killing trait*

The association of the *Spiroplasma* with the trait was confirmed by use of Mollicute-specific PCR (primers Haln1 and MGSO) (Van Kuppeveld *et al.*, 1992). Three tests of the hypothesis were performed:

(a) Male-killer lines not infected with the *Rickettsia* (see above) should produce a product with the Mollicute-specific PCR, and the product should on direct sequencing be found to be that of the *Spiroplasma*.

This reaction successfully produced the 429 bp amplification product with template from each of the ten SR lines that failed to amplify with the *Rickettsia*-specific amplification, but conversely produced no product with the four templates that did amplify in the *Rickettsia*-specific PCR (Fig. 3). On direct single-stranded sequencing, the products obtained were found to be identical in sequence ($\pm$ two bases) to that reported above (mean coverage of sequence, 250 bp).

(b) Individuals from lines that do not show the male-killing trait should not produce amplification products with the Mollicute-specific PCR.

Amplification was attempted with template DNA from representatives from each of sixteen lines that were normal with respect to sex ratio (three from Denmark, four from Bielefeld, four from Bayreuth, four from Berlin and one from St Petersburg). Two templates from (a) were used as a positive control against failure of the PCR. No template from the sixteen uninfected lines tested produced amplification product with

Table 2. Nucleotide divergence between the 16S rDNA sequences of the genus *Spiroplasma*, calculated using Kimura's two-parameter model.

	1	2	3	4	5	6	7	8
1. *S. ixodetis*								
2. *A. bipunctata* male-killer	0.0124							
3. *S. apis*	0.1470	0.1547						
4. *S. diabroticae*	0.1435	0.1480	0.0288					
5. *S. monobiae*	0.1483	0.1526	0.0375	0.0142				
6. *S. clarkii*	0.1510	0.1578	0.0456	0.0421	0.0388			
7. *Drosophila* SRO	0.1621	0.1642	0.1119	0.1116	0.1119	0.1403		
8. *S. citri*	0.1661	0.1648	0.1208	0.1203	0.1126	0.1324	0.0251	
9. *S. mirum*	0.1487	0.1560	0.1170	0.1186	0.1108	0.1215	0.0510	0.0507

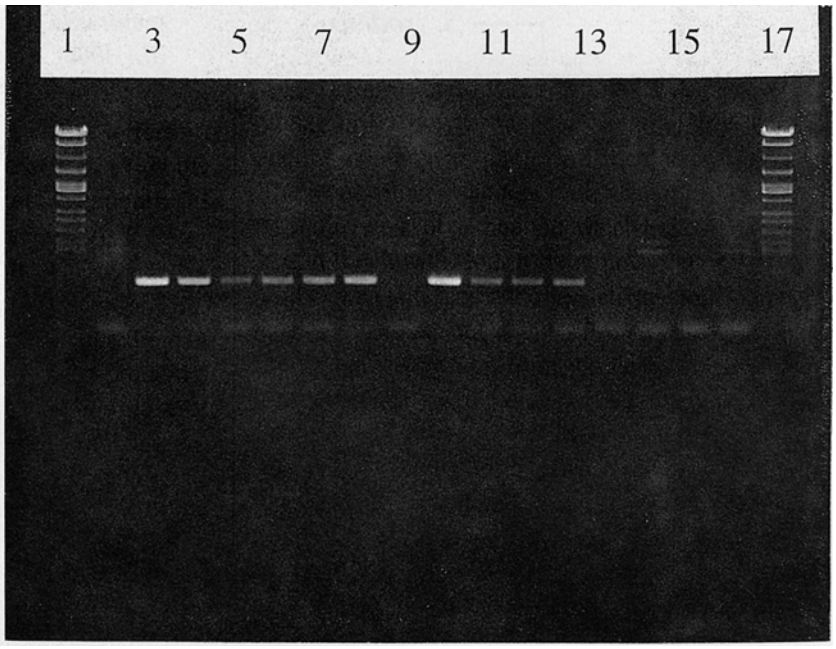

Figure 3. Test for Mollicute presence in fourteen male-killing lines. An amplification product of 429 bp is expected in the presence of Mollicute genomic DNA. Lane 1, λ *Hin*dIII/*Eco*RI/*Bam*HI ladder; lane 2: amplification product with Cambridge, *Rickettsia*-infected *A. bipunctata* genomic DNA as template (negative control); lanes 3–7: product with template derived from the five different Bayreuth lines; lanes 8–9: product with template derived from the two Berlin lines; lanes 10–11: product with template derived from the two Bielefeld lines; lanes 12–13: product with template derived from the two St Petersburg lines; lanes 14–16: product with template derived from the three Danish lines; lane 17: control run of PCR cocktail.

the Mollicute-specific PCR (not shown), although these samples were capable of amplification with primers for the COI gene of the insect mtDNA.

(c) Individuals from lines that originally bore the male-killer, but have lost the trait (either through antibiotic treatment, or naturally), should not produce an amplification product. Individuals that derive from lines which did not originally show the male-killing trait, but those that have acquired the trait through microinjection, should.

Template derived from lines where the trait had successfully been artificially transferred proved positive, whereas those where it was not successful proved negative, as did material following control transfers with uninfected homogenate (data not shown).

Discussion

This study reveals that two phylogenetically distinct male-killing bacteria currently exist in *Adalia bipunctata*. Females from the UK, from Leiden (Holland) and from Ribe (Denmark) bear a *Rickettsia* that kills males (Werren *et al.*, 1994, and this study). Females from St Petersburg, Bielefeld and Bayreuth bear a *Spiroplasma* that kills males. Females from Berlin may bear either type of infection. Thus what appeared as a single phenotype is clearly polycausal. Our previous estimate of the frequency with which male-killing bacteria have spread through coccinellid beetle populations, based on incidences of the male-killing phenotype amongst species, must be regarded as an underestimate.

How can we account for the existence of a dual infection in this species? As yet we can only address this question theoretically. It might be argued that the case of *A. bipunctata* is an oddity. Two male-killing symbionts cannot coexist at equilibrium in a genetically and environmentally monotonous population; the male-killer with the lowest impact on female lifetime fecundity and the highest transmission efficiency eliminates the alternate cytotype. Thus it is possible that dual infection in *A. bipunctata* is transient. We may be observing the spread of a recently introduced 'more-superior' male-killer, which is progressively spreading away from its source, eliminating the other male-killer as it does so.

Explanations for the existence of two male-killers need not rely on a 'recent invasion' however. If we take the data at face value, the UK and Danish populations comprise only *Rickettsia*-infected individuals, the Bielefeld, Bayreuth and St Petersburg ones only *Spiroplasma*-infected individuals, and the Berlin population has both *Spiroplasma*- and *Rickettsia*-infected individuals. If the critical parameters in the dynamics of male-killers (transmission efficiency and benefit to the female host) vary over space, it is then possible for different male-killers to predominate in different locations. We can imagine that the *Rickettsia* could generate more efficient transmission or lower direct costs to its hosts on the Dutch/British/Danish genetic and environmental background than the *Spiroplasma*, and the *Spiroplasma*, in contrast, is a more 'efficient' symbiont on the Eastern and Central European genetic and environmental backgrounds. Populations

bearing both infections would be found at the contact point between these different areas.

However, the evidence for such broad-scale partitioning of the infections is weak. With samples of infected individuals within a location being small (maximum of five, in Bayreuth) it is hard to say with certainty that any particular population is pure for a particular infection. Polymorphism within populations could be explained by the presence of specific alleles that resist the transmission of particular symbionts to the next generation, and that are costly in the absence of the parasite. In such matching allele models, parasite polymorphism can be maintained.

Clearly, elucidation of the population biology of this dual infection requires further study. In the first place, effort must be placed into determining whether particular populations are in fact monomorphic for particular infections. After this, analysis of any host genetic variation affecting parasite transmission efficiency may be timely.

This example also adds to the number of independent evolutions of male-killing behaviour within the Eubacteria. There are now five known independent evolutions of male-killing behaviour, including records from the alpha and gamma groups of Proteobacteria (Werren *et al.*, 1986, 1994), the Flavobacteria (Hurst *et al.*, 1997b) and Mollicutes of the genus *Spiroplasma* (Hackett *et al.*, 1986; this study). We would predict the discovery of further elements in future studies. Given the frequency of *Spiroplasma*–insect interactions, it should perhaps be no surprise that the genus *Spiroplasma* contains more than one male-killing agent, and it is certainly likely that more examples exist in this group.

Experimental procedures

Sampling and isolation of male-killer infected strains, test for Rickettsia *presence*

Adalia bipunctata samples were collected from St Petersburg (Russia), Berlin, Bayreuth and Bielefeld (all Germany) and Ribe (Denmark), which were then bred in the laboratory, with checks being made on the sex ratio and egg hatch-rate of broods laid by the ladybird pairs. Larvae from broods with low egg hatch-rate (< 70% eggs hatching in a sample of > 50 eggs) were reared to maturity and sexed. Where females produced all, or nearly all, female broods, the inheritance of the trait was tested to confirm their status as bearing a male-killing microorganism.

The status of male-killer infected lines with respect to infection by *Rickettsia* was tested via PCR-analysis using *Rickettsia*-specific primers. Briefly, genomic DNA was extracted from infected beetles: ovaries were dissected out in sterile distilled water, rinsed in sterile water, ground in 250 μl of digestion buffer (80 mM EDTA, 1% SDS, 160 mM sucrose, 100 mM Tris HCl pH 8.0) and incubated overnight at 37°C in the presence of 20 μg proteinase K. The DNA was then shaken with an equal volume of phenol–chloroform, once with chloroform, and thereafter ethanol precipitated, washed with 70% ethanol, and resuspended in 100 μl of sterile distilled water.

PCR was used to attempt amplification of 434 bases of the 17 kDa antigen gene common to members of the *typhi* group of the genus *Rickettsia* using the primers R1 (5′-GCTCTTGCAACTTCTATGTT-3′) and R2 (5′-CATTGTTCGTCAGGTTGGCG-3′) described by Williams *et al.* (1992). A volume of 1 μl of a tenfold dilution of the genomic DNA to be tested was added to a buffered reaction mix (NH_4 buffer: Bioline) containing 2.5 mM $MgCl_2$, 1 unit of Taq polymerase (Bioline), 0.2 mM of each nucleotide (Pharmacia), 20 pmol of each primer and sterile distilled water to give a final volume of 50 μl. PCR cycling conditions in a Hybaid OmniGene machine were one cycle of 1 min at 94°C; 35 cycles of 15 s at 92°C, 1 min at 56°C, and 1 min at 72°C; one cycle of 5 min at 72°C. Amplification controls were run for the PCR cocktail, for template DNA from known *Rickettsia*-infected insects, and for test individuals. PCR products were visualized using UV light following electrophoresis on a 1% agarose gel containing ethidium bromide.

Identification of putative associated organism

An individual from one of the St Petersburg lines in which the *Rickettsia*-specific PCR failed was analysed for the presence of other bacteria through general amplification of the 16S rDNA block of Eubacteria. Primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTACGA-3′), derived from Weisburg *et al.* (1991), were used in PCR reactions constituted as above. Cycle conditions on a Hybaid Omnigene machine were: one cycle of 2 min at 94°C; 35 cycles of 15 s at 94°C, 30 s at 55°C, 3 min at 72°C; one cycle 20 min at 72°C. The resultant PCR products were run out on a 1% gel against a size standard. The DNA was purified from the agarose block using Prep-a-Gene (Bio-Rad), and directly sequenced using dye-labelled terminators in a cycle-sequencing reaction, the products being visualized on an ABI 384 automated sequencing machine. Through the use of primers internal to the 16S rDNA block, the whole unit was sequenced through both strands.

The eubacterial group to which our putative bacterium was affiliated was ascertained by a BLAST search (Altschul *et al.*, 1990). The sequence was then manually aligned (taking secondary structure into account) to a variety of 16S rDNA sequences from members of this group, and phylogenetic analysis performed using PAUP (Swofford, 1993), excluding regions containing multiple insertions and deletions, and using an outgroup from the nearest known related group. The robustness of the tree topology was tested through bootstrap resampling. Topology was also estimated through the Maximum Likelihood algorithm DNAML of PHYLIP (Felsenstein, 1993). Nucleotide divergences were calculated using Kimura's two-parameter model.

Testing association of putative organism with male-killing trait

The hypothesis that this sequence represented the causal bacterium was tested through the application of a PCR-amplification specific to the 16S rDNA block of this group to a range of specimens of different infection status. The primer MGSO of Van Kuppeveld *et al.* (1992) (5′-TGCACCATCTGTCACTCTGT-

TAACCTC-3') has been shown to produce amplification products, in association with a general forward primer, only in the presence of a member of the Mollicutes (*Mycoplasma*, *Spiroplasma*, *Acholeplasma*, *Ureaplasma*). Amplification was attempted with the general forward primer HaIn1 (5'-GCTCAACCCCTAACCGCC-3'), with cycle conditions one cycle of 2 min at 94°C; 35 cycles of 15 s at 94°C, 60 s at 55°C, 30 s at 72°C; one cycle of 20 min at 72°C. A product of 429 bp is expected in the presence of the Mollicute identified above. In this test, samples that fail in amplification are only taken as valid 'fails' if their ability to be amplified using general insect primers had been confirmed. The quality of the insect genomic DNA extractions was tested through attempting amplification with a pair of primers that amplify the *COI* gene of coleopteran mtDNA (Howland & Hewitt, 1995).

The amplification test was carried out on naturally infected and naturally uninfected individuals. It was also performed on individuals from antibiotic-cured lines, and individuals from naturally uninfected lines that had been heritably transformed to bearing the male-killer through microinjection.

Antibiotic-cured individuals were created by treatment with tetracycline over a period of two generations as described in Hurst *et al.* (1992). The treated females recovered both in terms of sex ratio produced and egg hatch-rate. The daughters of these (also treated with antibiotics) did not display the trait. The F2 generation were tested for Mollicute presence in the PCR test.

Artificially infected individuals were created via microinjection, following Gotoh (1982). In brief, three females bearing the non-*Rickettsia* male-killer were homogenized in 1 ml 0.9% saline on ice. The homogenate was gently spun, and a small quantity of supernatant injected into the dorsal surface of a pupa from a normal line, away from the midline. This pupa was reared, and discarded if male. If female, the individual was crossed after 2 weeks, and the sex ratio and egg hatch rate recorded. Heritability of the trait was assessed, and a line regarded as successfully artificially infected only if the F1 also showed the male-killing phenotype. F2 individuals were used in tests.

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