How many species are infected with *Wolbachia*? Cryptic sex ratio distorters revealed to be common by intensive sampling

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Inherited bacterial symbionts from the genus *Wolbachia* have attracted much attention by virtue of their ability to manipulate the reproduction of their arthropod hosts. The potential importance of these bacteria has been underlined by surveys, which have estimated that 17% of insect species are infected. We examined whether these surveys have systematically underestimated the proportion of infected species through failing to detect the low-prevalence infections that are expected when *Wolbachia* distorts the sex ratio. We estimated the proportion of species infected with *Wolbachia* within *Acraea* butterflies by testing large collections of each species for infection. Seven out of 24 species of *Acraea* were infected with *Wolbachia*. Four of these were infected with *Wolbachia* at high prevalence, a figure compatible with previous broad-scale surveys, whilst three carried low-prevalence infections that would have had a very low likelihood of being detected by previous sampling methods. This led us to conclude that sex-ratio-distorting *Wolbachia* may be common in insects that have an ecology and/or genetics that permit the invasion of these parasites and that previous surveys may have seriously underestimated the proportion of species that are infected.

Keywords: *Acraea*; Lepidoptera; *Wolbachia*; male killing; sex ratio; cytoplasmic incompatibility

1. INTRODUCTION

Wolbachia (Rickettsiales) is a genus of bacterial symbionts known from arthropods and nematode worms that is maternally transmitted through the cytoplasm of eggs. This pattern of transmission has led them to evolve a number of strategies that increase the number, survival or fecundity of daughters produced by infected female hosts. *Wolbachia* probably plays an unknown beneficial role in the host's metabolism in nematode worms (Bandi *et al.* 1999). However, most infections in arthropods are thought to have spread by manipulating the reproduction of their hosts. These manipulations fall into two classes. Sex-ratio-distorting strains increase the production of daughters at the expense of sons by killing males, feminizing genetic males or inducing parthenogenesis (Rousset *et al.* 1992; Stouthamer *et al.* 1993; Hurst *et al.* 1999). Other strains induce cytoplasmic incompatibility, which (in singly infected diploid hosts) causes a reduction in the viability of offspring in crosses between infected males and uninfected females (O'Neill *et al.* 1992). This harms the uninfected females and, therefore, increases the relative fitness of the infected cytoplasm.

It has been suggested that *Wolbachia* may be an important force driving evolutionary change in arthropods. This view is in part based upon the observation that infection is extremely common, with surveys across a taxonomically diverse range of insects detecting *Wolbachia* in 17% of Panamanian, 19% of North American and 22% of British (Lepidoptera and Hymenoptera) species (Werren *et al.* 1995; West *et al.* 1998; Werren & Windsor 2000).

However, as recognized at the time (Werren *et al.* 1995), all these studies included only small samples of each host species and were therefore unlikely to detect the majority of sex-ratio-distorting strains, which commonly occur at low prevalence. The prevalence of *Wolbachia* and, hence, the likelihood of detection depends in part on the reproductive phenotype it induces. Strains that cause cytoplasmic incompatibility typically occur at a high prevalence, often near fixation. Parthenogenesis inducers and feminizers both range from a fairly low prevalence to fixation in females (Rigaud 1997; Stouthamer 1997; Bouchon *et al.* 1998). However, male killers typically occur in 1–30% of females (Hurst & Jiggins 2000), although much higher prevalences have been known (Jiggins *et al.* 2000a).

The aim of this study was to estimate the degree to which previous surveys have underscored the proportion of species that are infected with *Wolbachia* by missing low-prevalence infections. Initially, we tested large collections of females from a number of host species for *Wolbachia*. We then tested males in species where infected females were detected. From these data we predicted that *Wolbachia* present at high prevalence in both sexes would cause cytoplasmic incompatibility, while lower prevalence infections of females would be sex ratio distorters. Finally, we tested this prediction by determining the phenotype of one infection in each class (high prevalence in both sexes and low prevalence in females). We chose to investigate a single genus of insects, the *Acraea* butterflies (Nymphalidae), which are already known to contain a pair of sibling species that are both infected with male-killing *Wolbachia* (these species were excluded from this analysis) (Jiggins *et al.* 1998, 2000a; Hurst *et al.* 1999).

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2. METHODS

(a) *Collection of butterflies*

Samples of more than ten female individuals were collected from a variety of species of *Acraea* butterflies between February 1998 and July 1999 in southern Uganda (around Kampala and in Mabira Forest near Jinja). The specimens were identified, sexed and stored in absolute ethanol at 4 °C.

(b) *Detection of Wolbachia*

Ovary segments were taken from females of each species of *Acraea*. Segments from up to five individual females of the same species were pooled and tested for the presence of *Wolbachia*. In brief, DNA was extracted from pools of ovary tissue using conventional phenol–chloroform extraction (Sambrook *et al.* 1989). *Wolbachia* was detected using the polymerase chain reaction (PCR) primers *wsp81f* and *wsp691r*, which amplify a surface protein gene of the bacterium (Zhou *et al.* 1998). Prior to the addition of template DNA, we irradiated the PCR reactions with 150 mJ of ultraviolet light in a Stratagene UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) in order to cross-link any contaminant DNA and reduce the risk of false positives. The conditions on a Techne Omnigene thermal cycler (Techne Omnigene, Cambridge, UK) were 95 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s and, finally, 10 min at 72 °C. In order to check that the DNA extractions had been successful, we used PCR with primers that amplify the ITS1 region in all insects (Hillis & Dixon 1991). All PCR reactions were run alongside positive controls and water blanks.

When a pool of females tested positive, we then tested the individual females in that pool (using the remaining ovary tissue) and males of the same species. This allowed us to determine both the prevalence of the infection and whether it was restricted to females.

If any DNA pool from a species was found to bear *Wolbachia*, DNA was then prepared from individual males and females of that species and tested for *Wolbachia*. This procedure both replicated the initial positive result and gave an estimate of the prevalence in males and females. DNA was extracted from single specimens using a method modified from Walsh *et al.* (1991). A small piece of abdomen tissue was digested for 1 h at 56 °C with 5% w/v Chelex 100 ion exchange resin (Sigma, Poole, UK) in 200 µl of 33 mM dithiothreitol with 20 µg of proteinase K, boiled and the supernatant used directly as the PCR template. The extractions were tested for *Wolbachia* as above.

(c) *Confirmation of cytoplasmic incompatibility*

The butterfly *Acraea acerata* was tested for cytoplasmic incompatibility. Clutches of wild eggs were collected from sweet-potato fields (*Ipomoea batatas*: Convolvulaceae) and allowed to hatch. Each clutch was then split into two groups. The first group was fed on leaves dipped in 1% w/v tetracycline hydrochloride during the first and second larval instars (seven or more days) and the second group was fed on untreated leaves. Treated and untreated males and females were then crossed in all four possible combinations. All crosses were between non-sibs. The hatch rates of the resulting egg clutches were then recorded.

(d) *Confirmation of sex ratio distortion*

In order to test whether *Wolbachia* distorts the sex ratio in *Acraea eponina*, egg batches were collected in the Kampala area from March to June 1998. The larvae were reared to adulthood in plastic jars on the larval host plant *Triumfetta rhomboidea*

(Tiliaceae) in order to determine the sex ratio of these broods. The temperature at which the larvae were reared was not controlled. The inheritance of the sex ratio was assessed in the F₁ and F₂ generations by crossing females from clutches with different sex ratios with either wild males or males from broods containing males.

The association between *Wolbachia* and any distortion of the sex ratio was investigated in two ways. First, the infection status of the broods was determined by *post hoc* PCR testing for *Wolbachia* in the F₁ adults, as in §2(b). Second, the larvae were fed on leaves dipped in 1% w/v tetracycline hydrochloride during the late larval instars (seven or more days). The sex ratio of offspring produced by treated females was then recorded.

3. RESULTS

(a) *Proportion of species infected*

The *Wolbachia* infections we detected fell broadly into two classes (table 1). First, there are infections that occur at high prevalence in both sexes, which were found in *A. acerata*, *Acraea alcinoe*, *Acraea pharsallus* and *Acraea althoffi*. The second class is low-prevalence infections that are only found in females, which were identified in *Acraea peneleos*, *A. eponina* and *Acraea macarista*.

Therefore, 16.7% ($n=24$) of the species were infected with high-prevalence infections in both sexes. In calculating the proportion of species with low-prevalence infections of females, it was first necessary to exclude those taxa where both sexes were infected anyway, as such infections preclude the identification of low-prevalence infections in females. Then, of the remaining species, 15% ($n=20$) were infected. This estimate was conservative as it did not account for the limited sample size in many species.

(b) *Cytoplasmic incompatibility*

Table 2 shows that antibiotic treatment of *A. acerata* females made them incompatible with untreated males. While no eggs hatched in this cross, the other crosses all had similar egg hatch rates. This pattern is consistent with the hypothesis that *Wolbachia* causes cytoplasmic incompatibility in this host.

(c) *Sex ratio distortion*

Sixteen per cent ($n=93$) of female *A. eponina* produced strongly female-biased sex ratios (table 3) (normal broods not shown). This trait was imperfectly maternally transmitted, with some females producing a biased sex ratio, but their daughters producing a 'normal' one (table 3). The daughters of 'normal' females always produced both sons and daughters (data not shown). Seven female-biased matrilineal lines tested positive for *Wolbachia*, while six normal lines all proved to be uninfected.

Treatment with antibiotics caused all-female lines to revert to a near 1:1 sex ratio ($n=14$ crosses and mean proportion of males = 0.50). Two controls were performed. In the first, untreated siblings of the 'cured' females, which were reared alongside the treated larvae, mostly continued to produce only daughters (see the F₁ generation in table 3). In the second control, antibiotic treatment of 'normal' females did not alter the sex ratio they produced ($n=8$ crosses and mean proportion of males = 0.51).

Table 1. *The species, number and sex of butterflies that tested positive and negative for infection with Wolbachia*

species	females		males	
	infected	uninfected	infected	uninfected
<i>Acraea aganice</i>	0	60	—	—
<i>Acraea acerata</i>	51	0	20	0
<i>Acraea servona</i>	0	44	—	—
<i>Acraea peneleos</i>	2	38	0	20
<i>Acraea consanguinia</i>	0	39	—	—
<i>Acraea pseudoegina</i>	0	35	—	—
<i>Acraea perenna</i>	0	30	—	—
<i>Acraea lycoa</i>	0	30	—	—
<i>Acraea egina</i>	0	29	—	—
<i>Acraea jodutta</i>	0	28	—	—
<i>Acraea vivianna</i>	0	24	—	—
<i>Acraea orineta</i>	0	24	—	—
<i>Acraea macaria</i>	0	24	—	—
<i>Acraea bonasai</i>	0	23	—	—
<i>Acraea alcinoe</i>	22	0	4	0
<i>Acraea pharsallus</i>	21	0	5	1
<i>Acraea eponina</i>	5	15	0	19
<i>Acraea zetes</i>	0	20	—	—
<i>Acraea neobule</i>	0	18	—	—
<i>Acraea macarista</i>	3	11	0	15
<i>Acraea althoffi</i>	12	0	11	0
<i>Acraea epaea</i>	0	12	—	—
<i>Acraea orestia</i>	0	12	—	—
<i>Acraea poggei</i>	0	11	—	—

Table 2. *The effect of antibiotic treatment of either or both parents on the hatch rate of A. acerata eggs*

(The number of egg clutches in each class is given in parentheses. The mean number of eggs in each clutch was 94.)

	infected male	cured male
infected female	0.56 (<i>n</i> = 20)	0.69 (<i>n</i> = 6)
cured female	0.00 (<i>n</i> = 26)	0.54 (<i>n</i> = 20)

4. DISCUSSION

We surveyed *Acraea* butterflies intensively for the presence of *Wolbachia* in order to ascertain the degree to which previous studies may have underestimated the proportion of species infected with *Wolbachia* by failing to detect low-prevalence infections. We found four species (17%) that bore high-prevalence infections in our sample of 24 species. These infections are likely to cause cytoplasmic incompatibility, as was demonstrated for one species, *A. acerata*. This level of infection was similar to that of previous surveys. We revealed the presence of three further infections, which were only present in a minority of females, by intensive surveying within a species. This sex bias in prevalence, together with the breeding data from *A. eponina*, suggested that these were sex-ratio-distorting strains. It is highly likely that these infections would have been missed by previous surveys, which only took one or two individuals (sometimes only males) and we suggest that these surveys will have systematically underscored the frequency of *Wolbachia* infection.

Table 3. *Strongly female-biased broods and the inheritance of the sex ratio in A. eponina*

(Broods are arranged in maternal lines.)

parental		F ₁		F ₂	
male	female	male	female	male	female
0	9	—	—	—	—
3	43	0	17	—	—
—	—	0	18	—	—
—	—	0	20	—	—
—	—	1	69	—	—
—	—	0	64	—	—
—	—	0	9	—	—
4	15	0	14	—	—
—	—	4	3	—	—
2	25	0	49	—	—
—	—	29	19	—	—
0	12	0	31	—	—
1	16	—	—	—	—
0	80	0	11	0	61
—	—	0	2	5	53
—	—	0	37	—	—
0	25	0	28	3	102
—	—	0	45	8	8
—	—	0	71	0	73
—	—	—	—	0	61
—	—	—	—	0	47
—	—	—	—	0	87
—	—	—	—	0	117
—	—	—	—	0	36
0	11	—	—	—	—
2	23	—	—	—	—
3	22	—	—	—	—
0	45	—	—	—	—
0	104	—	—	—	—
0	34	—	—	—	—
3	47	—	—	—	—

We therefore concluded that the true proportion of insect species infected with *Wolbachia* will be significantly higher than the current estimate of 17–22% of all species. The extent to which the levels of infection were underestimated will have depended on two factors. First, to what extent did our sampling underestimate the proportion of species infected with sex-ratio-distorting strains? Second, to what extent were the *Acraea* data typical of other insect taxa?

Our estimate of the proportion of species infected with sex-ratio-distorting *Wolbachia* in *Acraea* was likely to have been conservative due to our small sample sizes. This problem will have been particularly acute if some *Wolbachia* had prevalences considerably lower than the 5–25% range that we observed. A number of records of insect populations where only *ca.* 1% of females are infected with a male-killing bacterium do exist and all of these cases are in *Drosophila* where large sample sizes are possible (Williamson & Poulson 1979).

Is it likely that further studies will find similar patterns in other taxonomic groups of insects? On the basis of our experiments on *A. eponina* and our knowledge of the taxonomic distribution of sex ratio distorters, it is probable that the low-prevalence *Wolbachia* we observed were male killers (note that curing a feminizer with antibiotics would

cause females to produce sons only) (Kageyama *et al.* 1998). Male-killing bacteria are known from five insect orders and are therefore probably not restricted to certain insect groups. Instead their distribution is determined by the ecology of their hosts: male killers spread where there are antagonistic sibling interactions or cannibalism of the dead males (Hurst & Majerus 1993). The ecology of the butterflies in this study is in many respects very diverse as most of the species eat unrelated host plants with varied growth forms (small annuals to trees) and live in varied habitats (rainforest to arid grassland). However, they all lay their eggs in clutches and eat plants. Therefore, it is possible that our data may prove to be typical of phytophagous insects that lay their eggs in clutches, although our prior knowledge that two *Acraea* species (which were excluded from this study) were hosts to male killers requires that further independent surveys are undertaken.

Recently, Jeyaprakash & Hoy (2000) suggested that *Taq* DNA polymerase often fails to amplify *Wolbachia* genes and that this has caused the incidence of *Wolbachia* to be underestimated. They found a greater number of infections being detected when a mixture of the enzymes *Taq* and *Pwo* was used for PCR. We do not think that this problem affected our results as our laboratory has been routinely using both enzyme regimes for amplifying *Wolbachia* genes for some years and has never detected any tendency for the mixture of enzymes to detect infections missed by *Taq* alone.

We concluded that sex-ratio-distorting *Wolbachia* are likely to be common in many insect taxa and, therefore, may be an important force in insect evolution, for instance driving change in sex-determining mechanisms or causing sex role reversal in their hosts (Jiggins *et al.* 2000c). This is particularly significant as we only looked for sex-ratio-distorting *Wolbachia*, while the majority of male killers that have been identified belong to other bacterial genera (Hurst *et al.* 1999), including a male-killing *Spiroplasma* that was identified in another Nymphalid butterfly (Jiggins *et al.* 2000b).

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