Molecular Ecology (2002) **11**, 1275–1283

Recent changes in phenotype and patterns of host specialization in *Wolbachia* bacteria

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Abstract

***Wolbachia* are a genus of bacterial symbionts that are known to manipulate the reproduction of their arthropod hosts, both by distorting the host sex ratio and by inducing cytoplasmic incompatibility. Previous work has suggested that some *Wolbachia* clades specialize in particular host taxa, but others are diverse. Furthermore, the frequency with which related strains change in phenotype is unknown. We have examined these issues for *Wolbachia* bacteria from *Acraea* butterflies, where different interactions are known in different host species. We found that bacteria from *Acraea* butterflies mostly cluster together in several different clades on the bacterial phylogeny, implying specialization of particular strains on these host taxa. We also observed that bacterial strains with different phenotypic effects on their hosts commonly shared identical gene sequences at two different loci. This suggests both that the phenotypes of the strains have changed recently between sex ratio distortion and cytoplasmic incompatibility, and that host specialization is not related to the bacterial phenotype, as suggested from previous data. We also analysed published data from other arthropod taxa, and found that the *Wolbachia* infections of the majority of arthropod genera tend to cluster together on the bacterial phylogeny. Therefore, we conclude that *Wolbachia* is most likely to move horizontally between closely related hosts, perhaps because of a combination of shared vectors for transmission and physiological specialization of the bacteria on those hosts.**

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

Received 10 September 2001; revision received 13 February 2002; accepted 10 April 2002

Introduction

Wolbachia (Rickettsiaceae) is a genus of intracellular bacteria that are common throughout the arthropods (Werren *et al*. 1995a). Living within the host's cell cytoplasm, they are transmitted through the egg from mother to daughter. This has resulted in some strains evolving to distort the sex ratio towards females, by feminizing genetic males, inducing parthenogenesis, or killing male embryos (Rousset *et al*. 1992; Stouthamer *et al*. 1993; Hurst *et al*. 1999). Other strains have spread through arthropod populations by inducing cytoplasmic incompatibility, which (in singly infected diploids) reduces the viability of offspring in crosses between infected males and uninfected females (O'Neill *et al*. 1992). This harms uninfected females, and therefore increases the relative number of daughters produced by the infected females in the population.

Early analyses suggested that *Wolbachia* typically shows very little phylogenetic congruence with its host. Closely related *Wolbachia* strains are observed in phylogenetically disparate hosts, and related hosts (such as species of *Drosophila* and *Phyllonoryter*) bear distantly related *Wolbachia* strains (West *et al*. 1998; Zhou *et al*. 1998). In contrast to this rule, there is evidence of specialization by some *Wolbachia* clades on particular host taxa. There are monophyletic clades of parthenogenesis inducing *Wolbachia* in *Trichogramma* wasp species, and a similar clade of feminizing strains in different species of isopod (Schilthuizen & Stouthamer 1997; Bouchon *et al*. 1998; Cordaux *et al*. 2001). Interestingly, both of these hosts are ones where *Wolbachia*

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causes distortion of sex ratio or sexuality, suggesting that the ability to distort the sex ratio may constrain the ability to transmit successfully to distantly related host taxa, or make transmission to closely related hosts more likely.

Wolbachia is also known to have several phenotypes. Whilst the dispersion of these phenotypes over the phylogeny suggests that the phenotype changes frequently (Werren *et al*. 1995b), there are insufficient data from closely related *Wolbachia* with which to assess this rate. There are also problems in interpreting previous work in this area, with the relatedness of strains assessed purely from single gene sequences. The observation that *Wolbachia* recombine, and that the phylogeny of different genes isolated from the same strains are incongruent, require this area to be re-examined, as an apparent change in phenotype of the bacterium may reflect a recombination event involving the gene used in reconstructing the phylogeny (Jiggins *et al*. 2001c; Werren & Bartos 2001).

The *Wolbachia* bacteria in *Acraea* butterflies represent a perfect group in which to study both host specialization and the frequency of changes in bacterial phenotype. This genus of butterflies is host to a variety of *Wolbachia* that either induce cytoplasmic incompatibility or distort sex ratios (Jiggins *et al*. 2001a). Knowledge of the bacterial phenotypic effects comes from two sources. The phenotype of six bacterial strains from four host species is known directly from breeding experiments (Jiggins *et al*. 1998, 2000, 2001a). The phenotype of the remaining strains has been inferred from the distribution of the bacteria in their hosts: low-prevalence infections present in females, but not males, are assumed to distort the sex ratio, whilst high-prevalence infections of both sexes are thought to induce cytoplasmic incompatibility (Jiggins *et al*. 2001a). Crossing experiments found that this approach successfully predicted the bacterial phenotype (Jiggins *et al*. 2001a). In this paper, we examine whether these strains represent a case of specialization upon a host group, as found for the feminizing and parthenogenesis-inducing *Wolbachia*. We then ask how frequently these bacterial strains have changed in phenotype, by examining the degree to which related strains share a common phenotype.

We resolve these issues by sequencing three genes, *wsp*, *groE* and *ftsZ* (Werren *et al*. 1995b; Masui *et al*. 1997; Zhou *et al*. 1998). This reduces the confounding effects of recombination, as if all three genes from two bacterial strains are similar, then it is likely that the majority of the genome of the two bacteria is closely related.

Materials and methods

Wolbachia *strains*

Breeding experiments have identified male-killing *Wolbachia* in *Acraea encedana* and two strains (U and T) in *A. encedon*, which occur in the same population but never 'double-infect' the same host individual (Hurst *et al*. 1999; Jiggins *et al*. 1998, 2000, 2001b). A third sex ratio-distorting strain is found in *A. eponina*, although in this case it is not known if the bacterium is killing males (Jiggins *et al*. 2001a). Additionally, low prevalence infections of females, which are assumed to distort the sex ratio, have been found in *A. peneleos* and *A. macarista* (Jiggins *et al*. 2001a).

Breeding experiments found that *A. acerata* carries a *Wolbachia* infection that causes cytoplasmic incompatibility (Jiggins *et al*. 2001a). Additionally, cytoplasmic incompatibility is thought to be the phenotype of high-prevalence infections found in both sexes of *A. alcinoe*, *A. althoffi*, *A. pentapolis*, *A. pharsallus* and *A. equatoria* (Jiggins 2000; Jiggins *et al*. 2001a). All the species in this study were collected from southern Uganda, with the exception of *A. encedon* strain T and *A. equatoria* which were collected from Tanzania and South America, respectively.

Gene sequences

Eight of the sequences used have been obtained during previous work (Hurst *et al*. 1999; Jiggins *et al*. 2000, 2001c), and the remaining 33 were sequenced for this study. Three *Wolbachia* gene regions, *wsp*, *groE* and *ftsZ*, were amplified and sequenced from two individuals per host species. These regions encode a surface protein (*wsp*), a cell cycle gene (*ftsZ*) and two heat shock proteins together with a noncoding intergene region (*groE*). Where a species infected with cytoplasmic incompatibility-inducing (CI) *Wolbachia* was found to carry two different *Wolbachia* strains, then at least one of the individuals sequenced was a male. This guards against the possibility that the species was infected with both CI and sex ratio-distorting (SR) bacteria.

Polymerase chain reaction (PCR) used polymerase with proof-reading activity (Expand High Fidelity PCR system: Boehringer-Mannheim). The *wsp* gene was amplified using the primers *wsp*81f and *wsp*691r (Zhou *et al*. 1998), the *ftsZ* gene was amplified using the primers *ftsZ*Bf and *ftsZ*Br (Werren *et al*. 1995b) and the *groE* gene amplification used the primers *groE*fl and *groE*rl (Masui *et al*. 1997). The PCR products were then purified using Microcon 50 Microconcentrator columns (Amicon Ltd).

In the case of the *wsp* gene, the PCR product was sequenced directly using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer) and the results were visualized on an ABI 384 automated sequencer (Perkin Elmer). The presence of more than one *wsp* sequence in a single insect was detected by the presence of 'double peaks' in the sequence. In addition, the *wsp* PCR product was digested with the restriction enzymes *Rca*I and *Hin*dIII, each of which cuts about half of the sequences obtained. The presence of restriction fragment

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length polymorphisms provided an additional means of identifying double-infected taxa.

In order to sequence both the *ftsZ* gene (all strains), and the *wsp* gene in multiply infected taxa, the PCR product was cloned and multiple clones were sequenced. The PCR product was purified as above, and then ligated into a T-tailed pGEM vector (Promega). The vector was then transformed into *Escherichia coli* DH5α, the cells being grown for only 45 min prior to plating to minimize any duplication of the clones. The cells were then plated out on Luria (LA)-agar containing Carbenicillin, isopropyl-beta-D-thiogalactoside (IPTG) and X-Gal, and grown at 37 °C overnight. Blue colonies represent bacteria containing the vector with no insert, while white colonies bear an insert. In order to confirm further that the white colonies carry both vector and insert, they were assayed using M13 PCR primers. Positive colonies were then grown and the plasmids were purified with a Wizard Miniprep DNA purification system (Promega). The inserts were sequenced using the M13 plasmid primers and primers designed internal to the *ftsZ* gene. At least four clones were sequenced of each gene sequence from each butterfly species. Double infections were detected from the *ftsZ* gene both by the presence of clones containing different *ftsZ* sequences, and by looking for restriction fragment length polymorphisms when the PCR product was digested with *Eco*RV.

The *groE* gene was sequenced directly from the PCR product, and double infections were detected from 'double peaks' in the sequence. These PCR product were then digested with the restriction endonuclease *Xmn*1, which cuts only one of the two sequences observed. The digested fragments and uncut PCR product were then separated on an agarose gel and sequenced directly.

Pairwise distances

In order to test for host specialization, the mean pairwise genetic distance between *Wolbachia* sequences isolated from the same host genera was compared to the mean pairwise distance between randomly selected sequences. This analysis was only conducted on the *ftsZ* and *wsp* genes for which large published data sets exist.

The *Acraea* sequences were aligned with published *wsp* and *ftsZ* sequences using Clustal W. In many cases there were multiple published sequences from the same host species, which ranged from being identical to highly divergent. So as to avoid making an arbitrary decision as to which sequences represented separate bacterial strains, only one randomly selected sequence of each *Wolbachia* gene was included in the analysis. The total data set consisted of 171 *wsp* sequences and 124 *ftsZ* sequences. The 5′ and 3′ ends of both genes were deleted as they were not present in all the sequences. Additionally, the highly variable 3′ end of the *wsp* gene was removed due to the difficulty of aligning homologous sites.

The pairwise genetic distance between sequences was estimated using the HKY85 model of sequence evolution (Hasegawa *et al*. 1985). For each arthropod host genus where multiple sequences were available, the mean was calculated of all possible pairwise comparisons within that genus. Next, we tested whether the mean pairwise distance within a host genus is less than the mean of the entire data set. Because the pairwise comparisons are not independent, we used a randomization procedure. For example, if there were 10 *Acraea* sequences, then 1000 replicate data sets of 10 randomly selected sequences were generated, and the test statistic (mean pairwise genetic distance) was recalculated for each data set. This was then taken as the null distribution.

Phylogenetic reconstruction

The alignments of the *wsp* and *ftsZ* genes described above were reduced in size so that they only included the cases where multiple *Wolbachia* sequences had been isolated from the same insect genus. The *groE* sequences were aligned by eye to all other published sequences. The phylogeny was reconstructed using the program paup* (Swofford 1998). The neighbour-joining tree of the *ftsZ* and *wsp* genes was calculated and the robustness of the inferred topology was assessed by 10 000 nonparametric bootstrap replicates. The choice of the neighbour-joining method was unavoidable as other approaches were too computationally intensive to bootstrap such a large data set. For the smaller *groE* alignment, maximum likelihood was used as described in detail elsewhere (Jiggins *et al*. 2001c).

Results

Two species of *Acraea* showed evidence for infection with more than one strain of *Wolbachia.* In both *A. pentapolis* and *A. acerata* the *wsp* PCR product produced double peaks when sequenced and restriction fragment length polymorphisms when digested with restriction enzymes. The *wsp* PCR product was then cloned and two sequences (corresponding to the double peaks and differing at the enzyme cutting site) were isolated. Similarly, direct sequencing of *groE* produced double peaks, and two sequences were obtained following digestion with *Xmn*1. Two *ftsZ* sequences were obtained from the *A. acerata* clones, but only one *ftsZ* sequence was found in the five clones sequenced from *A. pentapolis*. Furthermore, no double peaks were visible when the PCR product was sequenced directly.

The numbers of the sequences obtained from each species are listed in Table 1. These have been deposited in GenBank under the following accession numbers: 1, AJ130716; 2, AJ271196; 3, AJ312256; 4, AJ271198; 5, AJ312257; 6, AJ271194; 7, AJ271197; 8, AJ271195; 9, AJ130892; 10,

Table 1 Species of *Acraea* whose *Wolbachia* infections shared identical gene sequences

Host	Phenotype	*wsp* sequence	*ftsZ* sequence	*groE* sequence
A. encedon U	SR	1	9	17
A. encedana	SR	1	9	17
A. alcinoe	(CI)	2	10	18
A. pharsalus	(CI)	2	10	19
A. macarista	(SR)	2	11	18
A. peneleos	(SR)	2	12	19
A. acerata	(CI)	2 & 3	11 & 13	19 & 20
A. encedon T	SR	4	13	21
A. pentapolis	(CI)	4 & 5	13	19 & 20
A. eponina	SR	6	14	22
A. althoffi	(CI)	7	15	19
A. equatoria	(CI)	8	16	23

The phenotype of the bacterium is shown as either sex ratio-distorting (SR) or cytoplasmic incompatibility (CI). Sequences with the same numbers are identical. Where the phenotype has been shown in brackets, it has been inferred from the prevalence of infection in the two sexes (Jiggins *et al*. 2001a), otherwise it is known from breeding experiments.

Table 2 The mean pairwise distances between *wsp Wolbachia* sequences from the different host genera

		Pairwise distance	
Host genus	n	Mean	Median
Acraea	14	0.08***	0.02
Aedes	7	0.21	0.23
Culex	10	0.15*	0.16
Bactocera	8	0.16	0.22
Armadillidium	3	0.00***	0.00
Drosophila	4	0.14	0.13
Trichogramma	14	0.15*	0.19
All sequences	171	0.18	0.21

Whether the mean is significantly less than that of the entire data set is given as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

AJ271202; 11, AJ271204; 12, AJ318488; 13, AJ271199; 14, AJ271200; 15, AJ271203; 16, AJ271201; 17, AJ318482; 18, AJ318483; 19, AJ318481; 20, AJ318484; 21, AJ318485; 22, AJ318486; 23, AJ318487.

The sequences obtained all belong to the B group of *Wolbachia*, and within this group they tended to be closely related to each other, as illustrated by three separate analyses. First, many bacterial sequences isolated from different *Acraea* species were identical (Table 1). Second, the mean pairwise distance of both the *wsp* and *ftsZ Acraea* sequences is significantly lower than that of randomly selected sequences ($P < 0.001$ in both cases; Tables 2 and 3). The mean pairwise distance for both *Acraea Wolbachia* genes is lower than for any other host genus for which more than three sequences were available (Tables 2 and 3). Finally, on the phylogenies of all three genes the *Acraea Wolbachia* tend to cluster together (Figs 1, 2, 3). However, the *Acraea Wolbachia* do not form a monophyletic group.

Table 3 The mean pairwise distances between *ftsZ Wolbachia* sequences isolated from different host genera

		Pairwise distance	
Host genus	n	Mean	Median
Acraea	13	0.013***	0.016
Drosophila	4	0.005***	0.005
Phyllonorycter	5	0.014**	0.014
Leptopilina	2	0.000*	0.000
Nasonia	5	0.100	0.153
Armadillidium	3	0.000**	0.000
Diplolepsis	10	0.080	0.012
Diabrotica	3	0.015	0.021
Trichogramma	13	0.034**	0.011
Gryllus	5	0.016*	0.000
All sequences	124	0.087	0.034

Whether the mean is significantly less than that of the entire data set is given as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

The phylogenetic position of the *Acraea Wolbachia* was not consistent across the three loci sequenced, and the groups of strains sharing identical sequences differ depending on the gene (Table 1, Figs 1, 2, 3). Where this inconsistency receives strong bootstrap support, as in the differing position of *A. encedon* U on Figs 2 and 3, then it may be caused by recombination between strains. The evidence for recombination is discussed in detail elsewhere (Jiggins *et al*. 2001c). However, in most cases the inconsistencies (Table 1) are not reflected in any significant difference in the tree topologies (Figs 1, 2, 3).

SR and CI *Acraea Wolbachia* were found to be closely related at all three gene loci (Table 1, Figs 1, 2, 3). For example, an SR strain in *A. macarista* and a CI strain in *A. acerata* were identical in *ftsZ* and *wsp* sequence, and differed only by a single substitution in *groE* sequence.

The phylogeny and mean pairwise distance of *ftsZ* and *wsp* sequences was also estimated for all the other host genera in which three or more sequences have been published. Considering only the cases where five or more sequences have been published, in no case do the *Wolbachia* from a single host genus form a monophyletic group (Figs 2 and 3). However, in the majority of cases there is a tendency for at least some of the *Wolbachia* strains to cluster together (Figs 2 and 3). Furthermore, many of these have significantly reduced mean pairwise distances (Tables 2 and 3). It

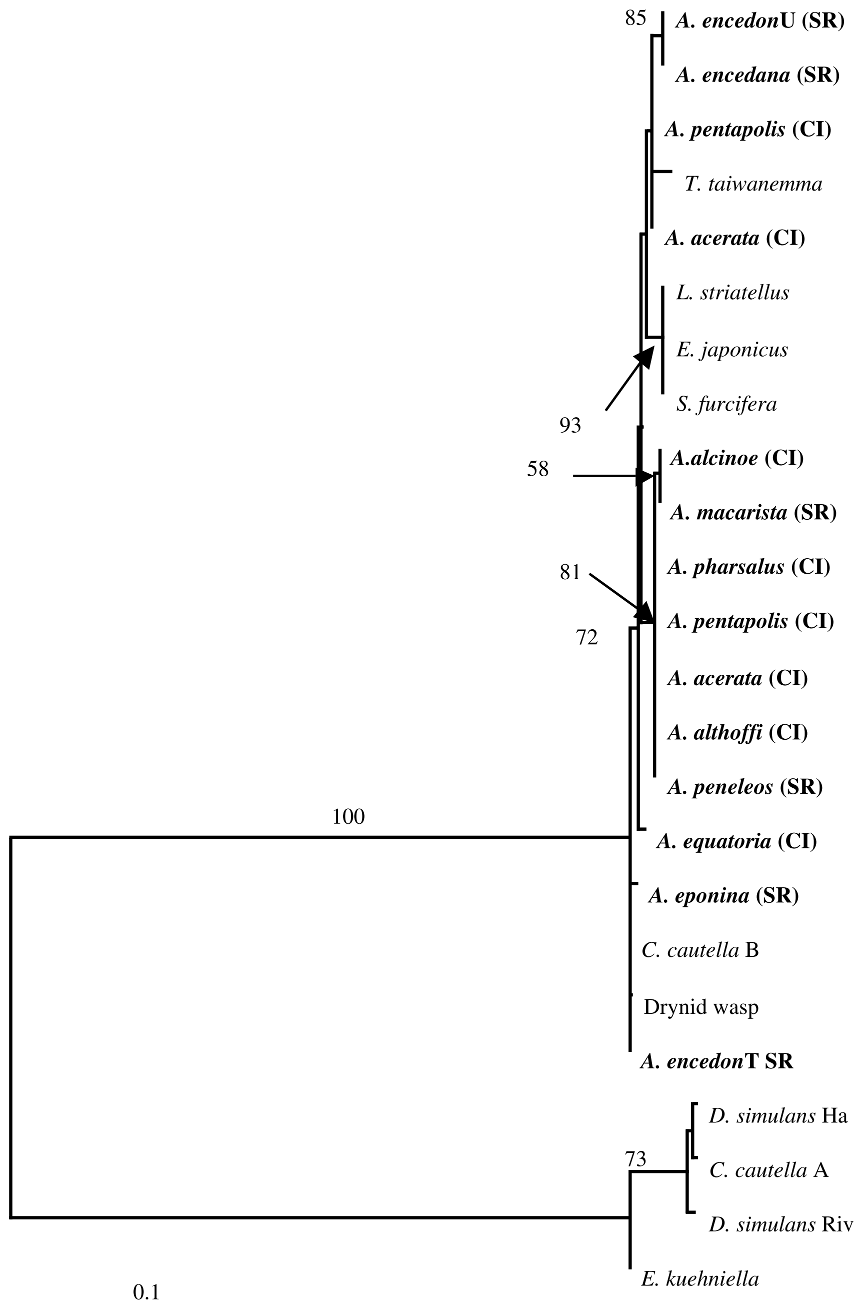

Fig. 1 Maximum likelihood tree of the *Wolbachia groE* gene labelled by host species. The *Acraea Wolbachia* are labelled with their phenotype (CI, cytoplasmic incompatibility; SR, sex ratio distortion). Parentheses indicate that the phenotype has been inferred from the distribution of *Wolbachia* in males and females rather than from breeding experiments (see text). The percentage of 1000 bootstrap replicates supporting each clade is shown when it exceeds 50%. The tree is rooted between the A and B groups.

is not clear from our analysis whether there are significant differences in the extent to which *Wolbachia* from different host genera cluster together.

The mean pairwise distance does not always identify taxa that cluster on the phylogeny. For example, *Wolbachia* strains from the *Diplolepsis* group together on the phylogeny (Fig. 3), but have a mean pairwise distance similar to that of the entire sample (Table 3). This occurs because the closely related clusters of *Diplolepsis* sequences are separated by large genetic distances.

Discussion

Changes in phenotype

A striking feature of our data is the observation that *Wolbachia* strains exhibiting different phenotypes shared indistinguishable or closely related gene sequences in a pattern that was replicated across gene loci. For example, the SR strain in *Acraea macarista* and the CI strain in *A. acerata* differed by only a single substitution across all three

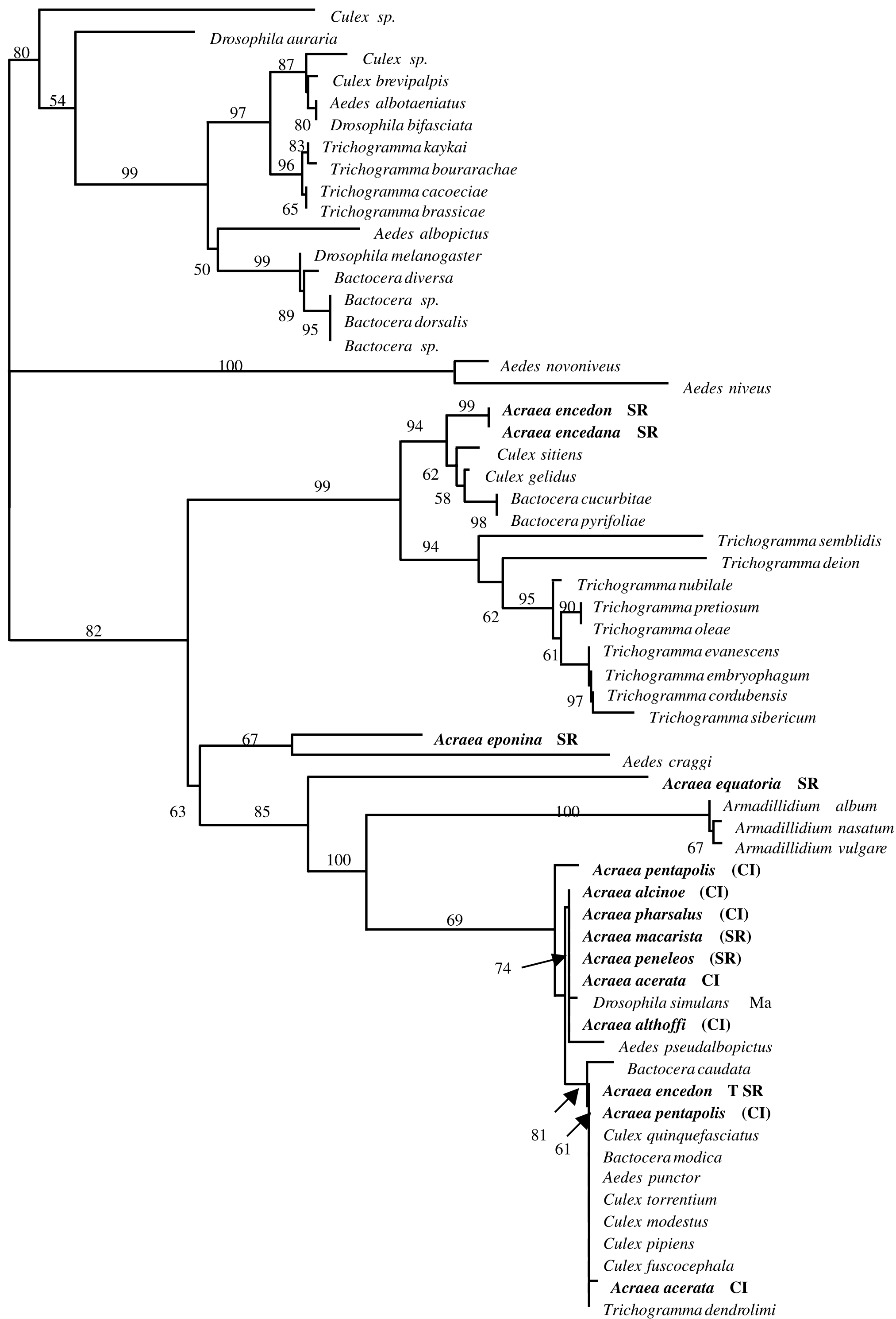

Fig. 2 Neighbour-joining tree of the *Wolbachia wsp* gene labelled by host species. The *Acraea Wolbachia* are labelled with their phenotype (CI, cytoplasmic incompatibility, SR, sex ratio distortion). Parentheses indicate that the phenotype has been inferred from the distribution of *Wolbachia* in males and females rather than breeding experiments (see text). The percentage of 10 000 bootstrap replicates supporting each clade is shown when it exceeds 50%. The rooting of the tree is uncertain.

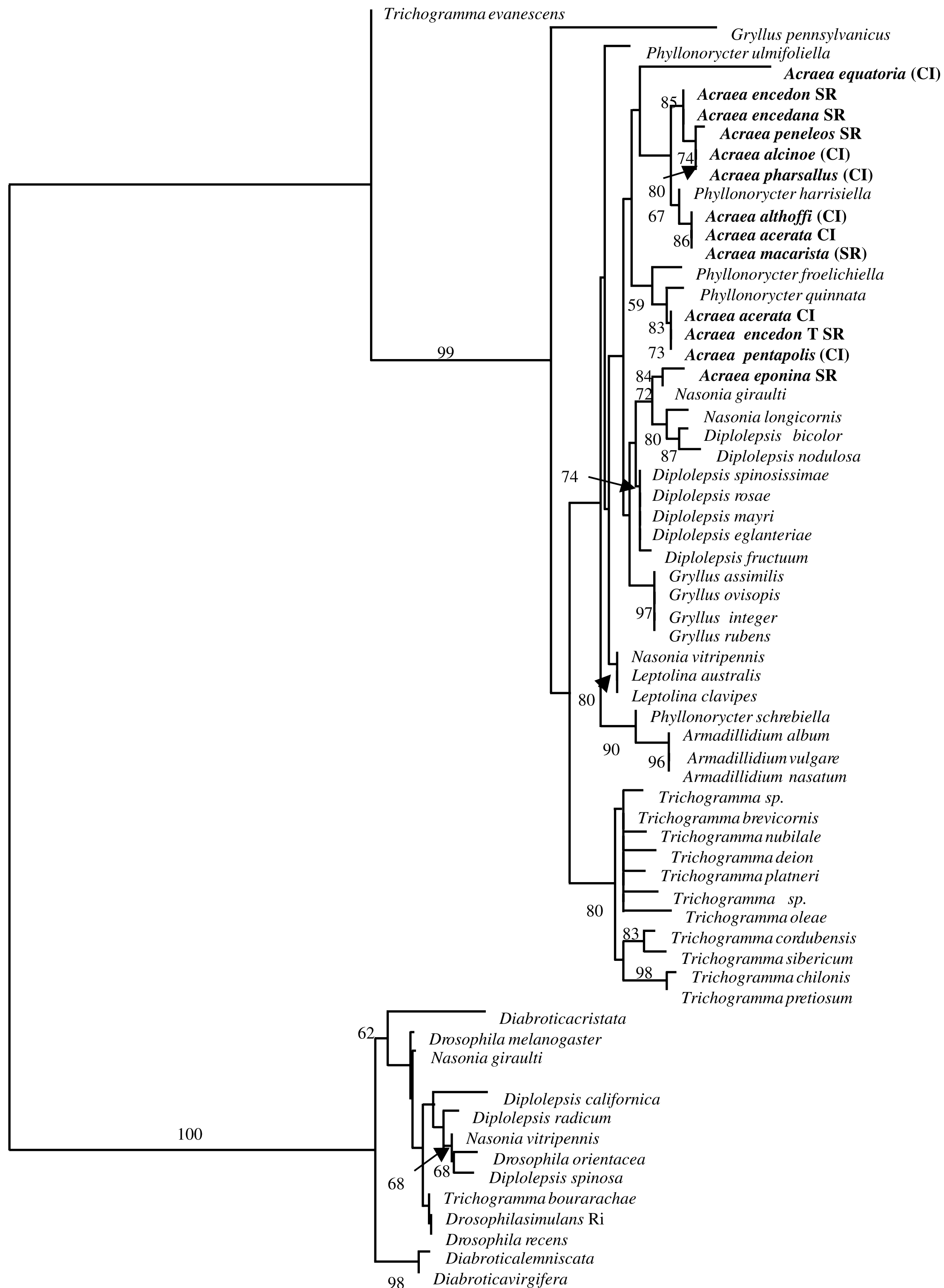

Fig. 3 Neighbour-joining tree of the *Wolbachia ftsZ* gene labelled by host species. The *Acraea Wolbachia* are labelled with their phenotype (CI, cytoplasmic incompatibility; SR, sex ratio distortion). Parentheses indicate that the phenotype has been inferred from the distribution of *Wolbachia* in males and females rather than from breeding experiments (see text). The percentage of 10 000 bootstrap replicates supporting each clade is shown when it exceeds 50%. The tree is rooted between the A and B groups.

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loci. Similarly, identical sequences across two loci occur in both the *A. encedon* T sex ratio distorter and the *A. pentapolis* CI strain, and again in *A. peneleos* (SR)/*A. pharsalus* (CI). This suggests that these bacterial strains are similar across a significant proportion of their genomes, and that this pattern cannot be explained by the isolated exchange, between unrelated *Wolbachia*, of the gene used for phylogenetic reconstruction. A similar situation is found in a far smaller data set in which a male-killing *Wolbachia* from the flour beetle *Tribolium madens* shared four gene sequences with a CI strain from *Tribolium confusum* (Fialho & Stevens 2000).

We can conclude that the *Acraea Wolbachia* have changed in phenotype recently and probably frequently. This may have been due to either mutation, exchange of genes involved in the phenotype recombination, or changes in host genotype. This final hypotheses could be tested by transferring these infections between host species by microinjection. If host genotype is important, the phenotype will change in transinfected strains. If *Wolbachia* genotype is important, then the phenotype will remain the same as in the natural host. Distinguishing between mutation and recombination would require the genes involved in the expression of the trait to be sequenced. One interesting possibility is that the genes involved in the expression of the phenotype are carried on the bacteriophage WO which was recently discovered in *Wolbachia* (Masui *et al*. 2000).

Theory predicts that CI *Wolbachia* are easily invaded and replaced by SR mutants (Hurst *et al*. 2002). Once the CI infection has been lost from the population, the sex ratio distorter then either remains at equilibrium, or is itself lost, leaving behind an uninfected host population. Our data raise the possibility that, on an evolutionary timescale, such changes in phenotype may occur frequently. This supports the hypothesis that the invasion of sex ratio distorters may represent a 'turnover' process that limits the longevity of CI infections in a given host population (Hurst *et al*. 2002). Should this be true, the hypothesis predicts that the proportion of species infected with CI *Wolbachia* will be lowest in those groups most susceptible to the invasion of male-killers.

Host specialization

The second notable feature of our phylogenies is that the *Wolbachia* infections of *Acraea* butterflies cluster together, and identical *Wolbachia* sequences are often isolated from different *Acraea* species. *Wolbachia* from five host species all shared a single *wsp* sequence, a second sequence being isolated from three host species, and a third from two hosts. Similar results were obtained from the other genes studied.

In contrast to the pattern in *Acraea*, early analyses of *Wolbachia* found that the bacterial phylogenies tend to be unrelated to those of their hosts (O'Neill *et al*. 1992; Stouthamer *et al*. 1993; Werren *et al*. 1995b). For instance, the infections found in *Drosophila simulans* and *D. melanogaster* are widely distributed across both the A and B *Wolbachia* groups. However, subsequent studies detected host specialization without co-cladogenesis both in the parthenogenesis inducing *Wolbachia* of *Trichogramma* wasps (Schilthuizen & Stouthamer 1997), and the feminizing *Wolbachia* of crustaceans (Bouchon *et al*. 1998; Cordaux *et al*. 2001). Therefore, the data suggested that sex ratio distortion may limit the taxonomic range of the bacterium. However, we can reject this hypothesis in the case of the *Acraea Wolbachia*, as related bacteria both cause CI and distort the sex ratio.

In the case of the *A. encedon* and *A. encedana* male-killers, where both the bacteria and butterflies are closely related, the bacterium may have been inherited from a common ancestor to these butterflies (Jiggins *et al*. 2000). This hypothesis is, however, untenable for the other species, as there is no congruence between our phylogeny of the bacteria and a morphology-based phylogeny of the butterflies (Pierre 1987), and identical *Wolbachia* sequences occur in both of the *Acraea* subgenera. The more extreme hypothesis that the ancestor to the *Acraea* was infected by all these bacterial strains that have subsequently been lost from most lineages, seems extremely implausible.

Our analysis of published *Wolbachia* sequences from other insect genera found that there was some degree of clustering on the bacterial phylogeny in the majority of the insect genera studied. In no case for which there were substantial data sets do the bacteria from a single host genus form a monophyletic group. The level of host specialization ranges from the majority of bacteria belonging to the same clade in *Trichogramma*, to the apparently random distribution in *Phyllonorcyter* leaf miners (West *et al*. 1998; vanMeer *et al*. 1999). However, our analysis did not test whether differences between host genera are significant. Therefore, the pattern observed in *Acraea* appears to be common across arthropod hosts.

We can therefore conclude that *Wolbachia* bacteria are more likely to move horizontally within a host genus than between distantly related hosts. This may be because the bacteria are adapted to certain host physiologies and/or related hosts share vectors for the transmission of the bacteria. If the latter is true, then parasitoids appear to be obvious candidates in the case of *Acraea* butterflies, as the different species share neither host plants nor habitats. Studies of different insect groups have provided evidence both for (Werren *et al*. 1995b) and against (West *et al*. 1998) the role of parasitoids in transmitting the bacterium.

Acknowledgements

Francis Jiggins was funded by a NERC small grant and an Emmanuel College Research Fellowship. Greg Hurst was funded

by a BBSRC D. Phillips Research Fellowship. This work was in collaboration with the Institute of Environment and Natural Resources, Makerere University, Uganda.

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This work is part of ongoing work by F. Jiggins on the evolution of *Wolbachia* bacteria. It is part of a larger research program in the laboratories of M. Majerus and G. Hurst into the diversity, mechanisms and evolutionary consequences of bacterial sex ratio distorters. J. Bentley is a graduate student in the UCL lab.