Molecular evidence for single *Wolbachia* infections among geographic strains of the flour beetle *Tribolium confusum*

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SUMMARY

Infections with the rickettsial microorganism *Wolbachia* are cytoplasmically inherited and occur in a wide range of insect species and several other arthropods. *Wolbachia* infection often results in unidirectional cytoplasmic incompatibility (CI): crosses between infected males and uninfected females are incompatible and show a reduction of progeny or complete inviability. Unidirectional CI can also occur when males harbouring two incompatible *Wolbachia* strains are crossed with females infected with only one of the two strains. In the flour beetle *Tribolium confusum*, *Wolbachia* infections are of particular interest because of the severity of incompatibility. Typically, no progeny results from the incompatible cross, whereas only partial incompatibility is observed in most other hosts. Werren *et al.* (1995*a*) reported that *Wolbachia* infections in *T. confusum* consist of two bacterial strains belonging to distinct phylogenic groups, based on PCR amplification and sequence analysis of the bacterial cell division gene *fts*Z. However, Fialho & Stevens (1996) showed that eight strains of *T. confusum* were infected with a single and common incompatibility type. Here we report analysis of the *fts*Z gene by specific PCR amplification. Diagnostic restriction enzyme assays revealed no evidence of double infections in 11 geographic strains of *T. confusum*, including the strain examined by Werren *et al.* (1995*a*). Further, sequence analysis of the *Wolbachia fts*Z gene and an internal transcribed spacer (ITS) region in two of these strains displayed no nucleotide variation or evidence of polymorphisms. Results suggest that *T. confusum* is infected with B-group *Wolbachia* only.

1. INTRODUCTION

Wolbachia are endosymbiotic bacteria found in a variety of insects and other arthropods (Stevens 1993). *Wolbachia* infections are maternally inherited and often associated with cytoplasmic incompatibility (CI), but other reproductive abnormalities have also been described (Rousset *et al.* 1992).

Recently, Sinkins *et al.* (1995*a*) considered incompatibility patterns in infections with two distinct and bidirectionally incompatible *Wolbachia* strains. They predicted that such double infections should spread in polymorphic populations and, therefore, are expected to occur naturally. In fact, natural double infections have been reported in several insect hosts (Rousset & Solignac 1995; Merçot *et al.* 1995; Sinkins *et al.* 1995*b*; Breeuwer *et al.* 1992; Werren *et al.* 1995*a*). A survey of neotropical arthropods detected double *Wolbachia* infections in 5.8% of 154 insect species tested (Werren *et al.* 1995*b*). Furthermore, Werren *et al.* (1995*a*) found double infections in 23% of insects examined in a phylogenetic analysis of their *Wolbachia* symbionts.

The flour beetle, *Tribolium confusum*, is among the several insect species reported by Werren *et al.* (1995*a*) to harbour two strains of *Wolbachia*. These strains were found to belong to two distinct phylogenetic groups, designated Atc and B, with a proposed divergence time of 58–67 Myr BP (Werren *et al.* 1995*a*). *Wolbachia* infections in flour beetles are of particular interest because of the severity of incompatibility observed. Complete incompatibility appears to be the rule in these insects (Wade & Stevens 1985). There are no data yet available on the effects of double *Wolbachia* infections on host fitness or on the degree of CI as compared to single infection effects.

It should be noted that in the above reports, double *Wolbachia* infections were determined solely by PCR-based methods. The high sensitivity of PCR and the large number of insects examined in some of these studies allow for the possibility of contamination, resulting in false positives. The recent work of Perrot-Minnot *et al.* (1996) is the first to address this issue by using Southern analysis of genomic DNA to confirm double infections in *Nasonia vitripennis*.

Recently, Fialho & Stevens (1996) examined eight infected strains of *T. confusum* for differences in compatibility types. Results suggested that all strains were infected with a single and common incompatibility type. In corroboration with these results, here we present molecular data suggesting that only one infection exists naturally in this species.

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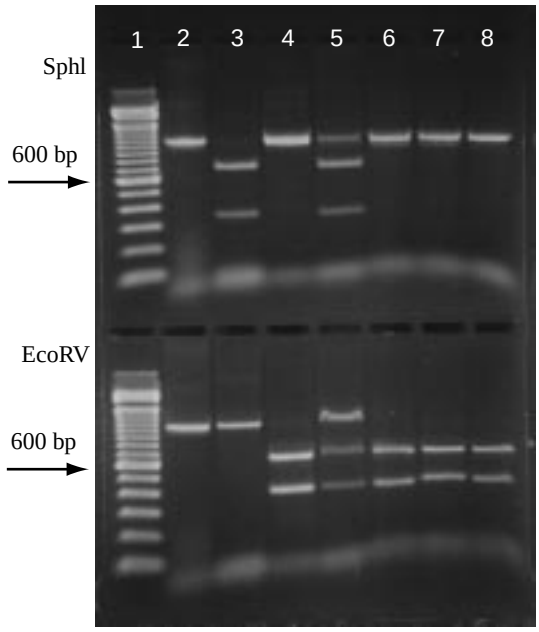

Figure 1. Diagnostic restriction patterns of *Sph*I (*fts*Z A-cutter, top) and *EcoR*V (*fts*Z B-cutter, bottom). Lane 2: uncut *fts*Z; lane 3 is *Melittobia digitata fts*Z; lane 4 is *Gryllus pennsylvanicus*; lane 5 is *fts*Z amplified from a mixture of 50:50% *M. digitata* and *G. pennsylvanicus* DNA extractions, and shows the expected pattern for double infection with Atc and B; lanes 6–8 are the following strains of *T. confusum*: +, Mississippi and bYI. All other strains yielded the same restriction pattern. Lane 1 is Gibco BRL 100 bp ladder.

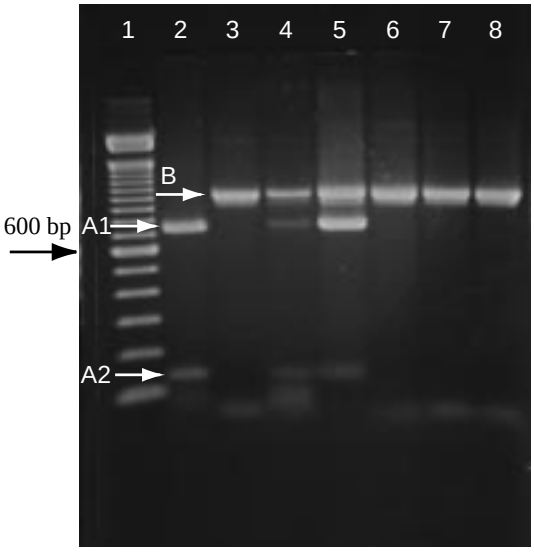

Figure 2. *fts*Z A- and B-specific PCR amplifications. The products of three PCR reactions (two are A-specific yielding bands of 737 [A1] and 129 bp [A2], and one B-specific PCR yielding bands of *ca.* 1000 bp [B]) were run on the same lanes. Lane 2 is *Melittobia digitata* (both A bands are seen); lane 3 is *Gryllus pennsylvanicus* (B band only); lanes 4 and 5 simulate an insect that is double-infected with Atc and B *Wolbachia*: the template in PCR reactions in lane 4 is a mixture of *M. digitata* and *G. pennsylvanicus* DNA extractions at 10:90% total DNA concentration; template in lane 5 is a mixture of *fts*Z products obtained with general primers of Werren *et al.* (1995) in a 1:99% *M. digitata*:*G. pennsylvanicus* concentration ratio. Lanes 6–8 are the following strains of *T. confusum*: +, Mississippi and bYI. Lane 1 is Gibco BRL 100 bp ladder.

2. METHODS AND RESULTS

PCR and diagnostic restriction enzyme (RE) assays were used to probe the two putative *Wolbachia* strains in 11 geographical strains of *T. confusum* (+, Georgia, McGill, Pakistan, Thailand, Uganda, China, Kansas and Mississippi, and two strains from Croatia: bYI and bYK). One of these strains (Mississippi) is the same strain examined by Werren *et al.* (1995*a*) (M. Wade, personal communication) and was obtained from M. Wade, the original source. All insects in this study were found to be infected by PCR amplification using *Wolbachia*-specific primers (O'Neill *et al.* 1992).

The field cricket *Gryllus pennsylvanicus* and the parasitic wasp *Melittobia digitata* were used as positive controls in PCR reactions and RE assays. According to the phylogeny proposed by Werren *et al.* (1995*a*), the bacteria in these two insects are the sister taxa of the two bacterial strains claimed to infect *T. confusum*. Specifically, *G. pennsylvanicus* is infected with *Wolbachia* belonging to the B subdivision and shows 98% *fts*Z sequence similarity with *Wolbachia* found in *T. confusum*. Similarly, *M. digitata* harbours *Wolbachia* belonging to the Atc group (*sensu* Werren *et al.* 1995*a*) which shares a nearly identical *fts*Z sequence (99.89% similarity or one difference out of 957 bp) with the *Wolbachia* that Werren *et al.* (1995*a*) reportedly found in the flour beetle. The almond moth (*Ephestia cautella*), found to harbour double *Wolbachia* infections with Adm and B bacteria (Werren *et al.* 1995*a*), was used as a natural double infection positive control. DNA was extracted as in Werren *et al.* (1995*a*).

The general *fts*Z primers of Werren *et al.* (1995*a*) were used to generate PCR product for diagnostic RE assays. Analysis of GenBank sequences (U28192 and U28194) revealed two diagnostic sites for the *fts*Z gene: *Sph*I (A-cutter) and *EcoR*V (B-cutter). Results showed that the general *fts*Z primers (Werren *et al.* 1995*a*) are capable of amplifying both Atc (*M. digitata*) and B (*G. pennsylvanicus*) when a 1:1 DNA extraction mixture (corrected for total DNA concentration) was used as a template. These diagnostic assays also consistently showed complete digestion of *fts*Z products obtained from all strains of *T. confusum* by *EcoR*V (B-cutter) and no digestion by *Sph*I (A-cutter) (figure 1). Digestion of general *fts*Z product of *E. cautella* with both *Sph*I and *EcoR*V yielded incomplete digestions and restriction patterns indicative of double infection with A and B type *Wolbachia*.

*fts*Z primers specific for the A and B groups of *Wolbachia* were also used to probe infections. Two A-specific forward primers (*fts*ZAF221: 5′-CAGTTAAGGATAGAGCGC-3′; *fts*ZAF829: 5′-GGTCGCAATAATAAATCA-3′) and one reverse primer (*fts*ZAR: 5′-TTCAGCTGGTTTTGTTTC-3′) were designed based on GenBank sequences (U28191 and U28192). PCR conditions and B-specific primers are those described in Werren *et al.* (1995*a*) except for annealing temperature of A primers (52 °C). The A primers proved to be specific by amplifying fragments of 737 base pairs (bp) (*fts*ZAF221 and *fts*ZAR) and 129 bp (*fts*ZAF829 and *fts*ZAR) from *M. digitata* and *E. cautella* only. They were also capable of amplifying in the presence of both Atc and B templates, as mixtures of DNA extractions of *M. digitata* and *G. pennsylvanicus* (10:90% total genomic DNA, respectively) and as a mixture of *fts*Z products obtained

with general primers (1:99% *M. digitata*:*G. pennsylvanicus* concentration ratio). In these diagnostic amplifications, *M. digitata* samples consistently yielded A bands only. *G. pennsylvanicus*, and all strains of *T. confusum* showed no A amplification and yielded *fts*Z B bands only (figure 2). *E. cautella* samples showed both A and B amplifications.

Approximately 1050 base pairs of the *fts*Z gene and an internal transcribed spacer (ITS) region (between the 23S and 5S rDNA genes) of *Wolbachia* in *T. confusum* (+ and Mississippi strains) were sequenced directly from PCR products. *fts*Z product was obtained using the general primers of Werren *et al.* (1995*a*) and Holden *et al.* (1993). The ITS region was amplified and sequenced using the following primers: 23S-2839F: 5′-AAGACYACCACGTTGATAGG-3′ and 5S100R:5′-AGAGTARGTCGCCGCCARGY-3′ (courtesy of H. Robertson). Sequences obtained showed no nucleotide variation between the two *T. confusum* strains. They are available in GenBank under accession numbers U97351–U97354.

3. DISCUSSION

We were unable to replicate the results of Werren *et al.* (1995*a*), who reported double *Wolbachia* infections in the flour beetle *Tribolium confusum*. Analysis of the bacterial cell division gene *fts*Z of *Wolbachia* by specific PCR amplifications and diagnostic RE assays failed to probe the Atc infection in all 11 geographic strains examined. The results presented here indicate that *T. confusum* is infected with *Wolbachia* belonging only to the B subdivision (*sensu* Werren *et al.* 1995*a*). Although PCR is sensitive enough to amplify DNA at very low copy numbers, it is also competitive in nature. Therefore, probing multiple infections can be problematic, especially if one of the target templates is found at significantly lower concentrations. We addressed this issue by using general and specific PCR primers for the *fts*Z gene and also by showing that these primers can amplify the Atc strain of *Wolbachia* when both Atc and B templates are present in the reaction. These primers efficiently amplified Atc *Wolbachia* (found in the positive control insect, *M. digitata*) from template mixtures, even when template ratio of Atc:B was as disparate as 10:90% (genomic DNA mixture) and 1:99% (PCR product mixture). Finally, the PCR and diagnostic restriction enzyme assays used here also proved capable of detecting the two strains of *Wolbachia* (Adm and B) in *E. cautella*, an insect found to harbour a natural double infection (Werren *et al.* 1995*a*).

Although our methods could not have detected infections with multiple B-type bacteria, *fts*Z and ITS sequence data revealed no evidence of polymorphisms. In addition, O'Neill *et al.* (1992) and Rousset *et al.* (1992) sequenced the 16S rDNA of *Wolbachia* in *T. confusum* and did not report any polymorphisms in this gene. The results presented here are in accordance with compatibility data on eight strains of *T. confusum* reported earlier (Fialho & Stevens 1996), which suggested that, despite being genetically distinct, these strains are infected with a single and common compatibility type.

It has been predicted that double *Wolbachia* infections should be common in nature (Sinkins *et al.* 1995*a*). However, loss of one infection due to stochasticity in cytoplasmic transmission or environment-mediated cures make it less likely that such double infections would be prevalent among multiple populations of one species. Sinkins *et al.* (1995*a*) reported 16S rDNA and *fts*Z polymorphisms suggestive of double *Wolbachia* infections in ten mainland stocks of *Aedes albopictus*, but only one of such putative bacterial strains in two island populations. Recently, Perrot-Minnot *et al.* (1996) showed that prolonged larval diapause of double-infected *Nasonia vitripennis* can result in stochastic loss of one or both *Wolbachia* strains. The single infection scenario for multiple strains of *T. confusum*, proposed by Fialho & Stevens (1996) and supported here by molecular data, appears more plausible and parsimonious.

We agree that infections with multiple *Wolbachia* strains may indeed be common, as predicted. As the *Wolbachia* genome becomes better characterized, it is likely that more hosts will be reported as harbouring multiple strains. Multiple *Wolbachia* infections can be confirmed by Southern analysis of genomic DNA whenever PCR-based methods detect their presence. This is especially warranted when large numbers of host species are examined due to greater likelihood of contamination.

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