No Evidence for Bacteriophage WO orf7 Correlation with *Wolbachia*-Induced Cytoplasmic Incompatibility in the *Culex pipiens* Complex (Culicidae: Diptera)

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ABSTRACT Gene flow between populations of *Culex pipiens* L. is relevant to observed differences in disease transmission, insecticide resistance, behavior, and physiology. Intracellular *Wolbachia* bacteria have been hypothesized to affect gene flow in insects. Specifically, *Wolbachia* cause a form of embryonic mortality known as cytoplasmic incompatibility (CI) in crosses between individuals with different *Wolbachia* types. Incompatibility in *Culex* is exceptional in that it represents the most complex CI pattern known, and yet *Culex* populations are not infected with divergent *Wolbachia* strains. This has led to the hypothesis that extrachromosomal factors such as phages or mobile genetic elements may be involved in determining CI phenotype. Recent molecular characterization of *Culex* laboratory strains has identified variation in the orf7 locus of the *Wolbachia*-associated bacteriophage WO. Here, crosses between eight *Culex* strains differing in their orf7 type were conducted to examine for the hypothesized involvement of bacteriophage WO in determining CI in *Culex*. Although crossing results show examples of compatibility, partial compatibility, and incompatibility, the results fail to show a correlation between the CI phenotypes and orf7 type. Specific examples include high egg hatch resulting in crosses between *Culex* strains that differ significantly in their orf7 type and low egg hatch resulting in crosses between *Culex* strains with similar orf7 types. Thus, the phage orf7 locus alone cannot predict CI type in the *Culex* strains examined in this study. However, rejection of the hypothesized role of WO phage in *Culex* CI will require the characterization of additional phage loci.

KEY WORDS cytoplasmic incompatibility, *Culex pipiens*, WO bacteriophage, mobile genetic elements

Wolbachia are obligate intracellular alpha-proteobacteria belonging to the order Rickettsiales and form a monophyletic lineage within the clade containing the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia* (Dumler et al. 2001). *Wolbachia* have drawn interest due to their frequent occurrence in invertebrates, the various reproductive alterations that they cause in their hosts (Stouthamer et al. 1999), hypothesized involvement in insect speciation (Laven 1967, Giordano et al. 1997, Bordenstein et al. 2001), and their potential applied use in the control of insects and insect-vectored diseases (Curtis and Sinkins 1998, Dobson et al. 2002, Taylor 2002, Dobson 2003).

Mosquitoes of the *Culex pipiens* complex are important vectors of emerging and reemerging infectious diseases worldwide (Hubalek and Halouzka 1999, Farid et al. 2001). The complex is formed of sibling species that differ significantly in their biology but vary little morphologically (Mattingly 1951, Byrne and Nichols 1999). Traditionally, the DV/D ratio of the male genitalia has been used to distinguish the different forms (Sundararaman 1949). Molecular tools also have been developed to segregate between the representatives of the complex (Severini et al. 1996, Bourguet et al. 1998, Cornel et al. 2003, Smith and Fonseca 2004). However, the taxonomic status of members of the group remains unclear.

Wolbachia induce cytoplasmic incompatibility (CI) in crosses between both sympatric and allopatric *Culex* populations (Laven 1951, Magnin et al. 1987, Barr 1980, Guillemaud et al. 1997). In *Culex* and other insects, CI occurs when a *Wolbachia* infected male mates with a female that is either uninfected or infected with a different *Wolbachia* strain (O'Neill and Karr 1990, Yen and Barr 1973). CI results in the loss of the paternal pronucleus during early mitotic division, resulting in embryonic death (Laven 1951, Tram and Sullivan 2002). Reduced hybridization success caused by CI has been hypothesized to restrict gene flow in the *Cx. pipiens* complex and other insect species (Laven 1967, Bordenstein et al. 2001). In addition to the relevance to basic science, gene flow between *Culex* populations is also relevant to the dissemination of insecticide resistance genes (Bourguet et al. 1998) and observed natural variation in vector competency (Fonseca et al. 2004).

Although the CI mechanism remains unknown, a mod/resc model has been hypothesized (Charlat et al.

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2001). According to the model, *Wolbachia* infections in the male "modify" (mod) the sperm, such that karyogamy failure results after fertilization. *Wolbachia* is not transmitted through the sperm. The *Wolbachia* infection in the female and embryo is able to "rescue" (resc) the modified paternal pronucleus, resulting in normal development. The maternal *Wolbachia* infection often does not rescue the mod mechanism of a divergent *Wolbachia* strain (Clancy and Hoffmann 1996, Poinsot et al. 2003).

Although the mod/resc model serves to explain observed CI phenomena in many insects, it cannot readily be extended to *Culex.* Most populations of *Cx. pipiens* are infected with *Wolbachia* (Ricci et al. 2002, Cornel et al. 2003, Ruang Areerate et al. 2003, Rasgon and Scott 2004). Previous phylogenetic characterizations with commonly used *Wolbachia* loci (16S rRNA, ftsZ, and wsp gene sequences) have failed to show variation in *Culex* infections (Guillemaud et al. 1997, Ricci et al. 2002, Ruang Areerate et al. 2003, Rasgon and Scott 2004, Sanogo and Dobson 2004). In contrast with the uniformity of infection types, over seventeen CI types have been reported in *Culex* (Laven 1951). Thus, CI in *Culex* is not due to distantly related *Wolbachia* infections (Guillemaud et al. 1997). This has led to hypotheses of additional extrachromosomal genetic elements (plasmids, phages, and transposons) involvement in determining CI pattern in insects (Stouthamer et al. 1999).

Evidence is accruing suggesting that phages and other mobile genetic elements have shaped the evolution of *Wolbachia* and may be involved in the phenotypic manifestations of *Wolbachia* in their hosts (Bordenstein and Wernegreen 2004, Wu et al. 2004). Electron microscopy has described phage-like particles associated with *Wolbachia* infection (Wright et al. 1978). Studies in *Nasonia vitripennis* suggest that CI determination involves two components: *Wolbachia* and a smaller agent (<0.2 μm) (Williams et al. 1993). The entire genome of a *Wolbachia*-associated phage (WO) has been sequenced in *Ephestia kuehniella* (Masui et al. 2000) and in *Cadra cautella* (Fujii et al. 2004). Three prophage elements have been reported in the genome of a *Wolbachia* infection (*w*Mel) in *Drosophila melanogaster* (Meigen) (Wu et al. 2004). Recently, phage orf7 sequences have been reported in *Wolbachia*-infected *Culex* (Sanogo and Dobson 2004). Comparison of different *Culex* laboratory populations demonstrated variation in both orf7 sequences and copy number.

In the current study, we examine for the hypothesized involvement of WO phage in determining CI type in *Culex.* Reciprocal crosses were conducted between *Culex* laboratory populations known to differ in their orf7 type. Support for the hypothesized phage involvement in CI would be provided by the observation of increased compatibility levels between *Culex* strains with similar orf7 types. In contrast, the results fail to show a correlation between the orf7 loci and observed CI pattern. The results are discussed in relation to the hypothesized role of bacteriophage WO in determining cytoplasmic incompatibility in the *Cx. pipiens* complex and *Wolbachia* evolution.

Materials and Methods

Mosquito Strains. Eight *Wolbachia*-infected *Cx. pipiens* strains were used in this study: Edit (ED), Crisse (CR), Espro (ES), Tunis (TU), Kunu (KU), S-LAB (SL), and *Culex quinquefasciatus* Say (CQ). All strains are as described previously (Guillemaud et al. 1997, Sanogo and Dobson 2004). An uninfected strain (LNT) generated by tetracycline treatment also was used (Rasgon and Scott 2003). The orf7 pattern for each strain has been described in a previous study (Sanogo and Dobson 2004). In brief, three orf7 variants have been identified: orf7a occurs in all strains examined in this study; orf7b occurs in KU, TU, ES, and CQ; a truncated orf7b occurs in CR; orf7c occurs in SL, CR, and CQ.

The mosquito colonies were maintained in 0.3-m^3 cages at 28 $\pm$ 2°C, 75 $\pm$ 10% RH, and a photoperiod of 18:6 (L:D) h. Larvae were fed with a suspension of fish food (VitaPro Plus; M. Reed's Enterprises, Sutter Creek, CA). Adults had continuous access to a 10% sucrose solution and were blood fed with chicks (*Gallus domesticus*) at 3 d posteclosion.

Crosses and Phylogenetic Analysis. Virgin females were obtained for crossing experiments by isolation as pupae. Approximately 20 virgin females (2–3 d posteclosion) were crossed with males of an equivalent number and age. The mosquitoes were allowed 3 d to mate and were then blood fed. Engorged females were isolated for oviposition. CI was measured by counting the number of hatched and unhatched eggs for each egg raft. Eggs also were observed for embryo development. Normally developing eggs typically show an eye spot and faint segmentation $\approx$30–32 h postfertilization (Laven 1951). This also is observed in eggs that fail to hatch due to cytoplasmic incompatibility. Thus, rafts with no sign of embryonic development were suspected to result from unfertilized females. Females producing the latter egg rafts were dissected, and their spermathecae were examined to confirm insemination. Rafts from uninseminated females were excluded from the data set. Statistical comparisons were conducted using StatView version 5.0.1 (SAS Institute, Cary, NC).

Sequences of the orf7 variants were aligned using the program CLUSTAL W (Thompson et al. 1994). Phylogenetic trees were constructed by the maximum parsimony method using PAUP 4.0 (Swofford 2002). A heuristic search was carried out using tree bisection-reconnection branch swapping to search for the maximum parsimony trees. In total, 1000 bootstrap replicates were used to estimate the tree branch nodes reliability. Consistency index was calculated using PAUP 4.0 in its default setting. In separate analyses, gaps were treated either as missing data or as new character states. When considering two or more of orf7a, orf7b, and orf7c sequences in strain comparisons, artificial fusions were generated by placing the

Table 1. Results of reciprocal crosses between strains of *Cx. pipiens*

Female type	Male type							
	TU	KU	ES	CQ	CR	ED	SL	LNT
TU	83.2 ± 5.3; 5	43.2 ± 16.2; 4	96.2; 1	50.9 ± 11.6; 6	55.6 ± 12.3; 8	60.6 ± 15.5; 7	27.6 ± 5.2; 3	77.7; 1
KU	74.9 ± 9.5; 6	80.5 ± 6.0; 2	84.8 ± 7.8; 3	43.7 ± 16.3; 5	42.1 ± 36.0; 2	57.6 ± 8.0; 14	46.0 ± 14.4; 8	62.2 ± 10.4; 9
ES	58.7 ± 9.4; 5	71.7 ± 7.3; 7	84.9 ± 3.5; 5	71.9 ± 23.3; 4	57.6 ± 5.8; 33	18.9 ± 6.0; 20	62.4 ± 20.6; 4	65.5 ± 13.1; 7
CQ	70.8 ± 7.9; 11	74.2 ± 6.8; 12	94.6 ± 1.3; 7	81.0 ± 6.5; 5	60.4 ± 10.4; 14	78.9 ± 4.2; 15	73.2 ± 22.1; 4	80.7 ± 9.8; 10
CR	46.8 ± 5.5; 9	72.4 ± 9.2; 9	81.6 ± 3.0; 25	77.6 ± 9.6; 10	75.5 ± 9.6; 9	0.0 ± 0; 12	66.5 ± 9.5; 11	88.0 ± 4.7; 9
ED	79.9 ± 6.6; 4	12.3 ± 11.9; 7	72.8; 1	67.7 ± 9.4; 3	47.5 ± 6.6; 10	72.7 ± 6.2; 6	32.9 ± 16.4; 4	69.1 ± 10.5; 5
SL	78.1 ± 5.3; 11	61.2 ± 6.9; 16	86.2 ± 2.1; 13	58.8 ± 7.1; 21	62.4 ± 9.4; 14	7.2 ± 5.9; 11	86.9 ± 3.0; 11	91.4 ± 2.3; 4
LNT	0.0 ± 0; 9	0.0 ± 0; 7	0.0 ± 0; 2	0.0 ± 0; 4	0.0 ± 0; 3	0.0 ± 0; 3	0.0 ± 0; 4	67.5 ± 13.8; 4

Percentage of egg hatch resulting from crosses is shown as the average ± SE followed by the number of egg rafts.

sequences adjacently. Gaps were used for strains that lacked orf7a, orf7b, or orf7c.

Results and Discussion

Cross Results. Crosses between *Culex* strains resulted in a diverse range of egg hatch rates (Table 1). Crosses between aposymbiotic LNT females with males from all *Wolbachia*-infected strains resulted in no egg hatch. The reciprocal crosses, LNT males mated with infected females of all strains, resulted in high egg hatch rates (average of 62–91%). A significant difference in egg hatch was not observed in comparisons of the latter crosses ($P > 0.095$, df = 6, Kruskal–Wallis). Matings between females and males of the same strain also resulted in high egg hatch (average 68–92%). No difference in egg hatch was observed in comparisons of the latter crosses ($P > 0.22$, df = 7, Kruskal–Wallis).

Crosses between different *Wolbachia*-infected strains resulted in a broad range of egg hatch rates (0–96%; Table 1). To simplify interpretation, crosses were grouped into three categories: compatible (>60% hatch), partially compatible (30–60% hatch), and incompatible (<30% hatch). As shown in Table 1, a majority of crosses between different *Wolbachia* infected strains were compatible (24 of 42 cross types). Five cross types were incompatible (0–28% hatch). The remaining 13 cross types were categorized as partially compatible (33–58% hatch). No difference in egg hatch was observed among the cross types within the compatible ($P > 0.25$, df = 23, Kruskal-Wallis), partially compatible ($P > 0.9$, df = 12, Kruskal–Wallis), or incompatible ($P > 0.071$, df = 4, Kruskal–Wallis) categories. Egg hatch differed significantly between the three categories ($P < 0.0001$, pairwise Mann–Whitney *U* test). The crossing results are diagrammed (Fig. 1) by using a method similar to that of Laven (1951).

Egg hatch results also were categorized based upon reciprocal cross pairs. Seven cross pairs were identified as compatible in both directions: CQxES, CQxED, CQxCR, CRxSL, ESxKU, ESxSL, and EDxTU. As described above, crosses between mates of the same strain also fall into this category. Eight cross pairs are compatible in one direction and partially compatible in the reciprocal cross: CQxKU, CQxSL, CQxTU, CRxKU, CRxES, KUxSL, KUxTU, and ESxTU. One cross pair (CRxTU) is partially compatible in both directions. Two pairs are compatible in one direction and incompatible in the reciprocal cross (unidirectional incompatibility): SLxTU, and EDxES. As described above, crosses of *Wolbachia*-infected strains with the aposymbiotic LNT strain fall into the latter category. Three crosses are partially compatible in one direction and incompatible in the other direction: EDxKU, EDxSL, and EDxCR. No cross pairs were observed to be incompatible in both directions (bidirectional incompatibility).

The cross results confirm prior reports demonstrating complex CI patterns occurring in *Culex* strains infected with similar *Wolbachia* infection types. The pattern of unidirectional hybridization between *Wolbachia* infected and uninfected strains is consistent with expectations for CI in *Culex* (Yen and Barr 1973). Although examples of incompatibility were observed, a majority of crosses between the *Wolbachia* infected strains were compatible. Examples of bidirectional incompatibility were not observed. In agreement with previous reports (Laven 1951), mating was observed

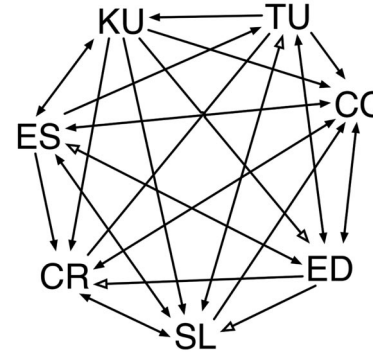

Fig. 1. Diagram representing cross results between *Wolbachia*-infected *Culex* strains. Arrows indicate the cross direction, pointing from male type toward female type. Filled arrows indicate compatibility. Hollow arrows indicate incompatibility. The absence of an arrow head on lines indicates partial compatibility. Thus, the line between KU and TU indicates that TU males mated with KU females results in compatible crosses. The reciprocal cross between KU males and TU females are partially compatible. KU and ES are bidirectionally compatible. Reciprocal crosses of CR and TU are partially compatible. SL females are incompatible with ED males.

between the examined *Culex* strains. Females of the CQ strain were compatible with all of the male types used in this study. Males of the ES strain were compatible with all of the female types used in this study. However, ES males were observed to be incompatible with other *Culex* strains (Barriol and Mart) in a previous study (Guillemaud et al. 1997).

Phylogenetic Tree Construction. The primary hypothesis tested in this study was the correlation of bacteriophage orf7 loci with cytoplasmic incompatibility type. Because the role of the three previously identified orf7 loci is not known, multiple phylogenetic trees were generated using one orf7 locus or combinations of orf7 loci. Because all orf7 variants were not present in all of the *Culex* strains, phylogenetic trees were examined that assume that either the absent sequence data are "missing data" or that absent data are a "new character state."

Although the orf7a locus was present in all of the *Culex* strains examined in this study, little sequence variation occurs. The strains either have identical orf7a sequences or differ by a single base pair (Sanogo and Dobson 2004). Therefore, orf7a sequence variation permits no resolution between the strains used in this study (branch support <50% bootstrap values).

The orf7b locus has not been detected in the SL or ED strains. The orf7c locus is absent from the ES, KU, and TU strains (Sanogo and Dobson 2004). Similar to orf7a, the paucity of sequence variation within the orf7b and orf7c loci allows no resolution between strains when the loci are examined individually. However, if absence of loci is considered as a new character state, the *Culex* strains segregate into clusters of those with and those without loci.

Phylogenetic relationships also were examined using combinations of the three previously reported orf7 loci (Sanogo and Dobson 2004). Similar to trees based upon single loci, no resolution was obtained if absent loci were considered missing data (branch support <60% bootstrap values). However, if absent loci are considered as a new character state, distinct groups are identified (Fig. 2). One group consists of ED and SL and is distinguished primarily by the absence of orf7b, which is present in all of the other strains examined here. Similarly, a second group consists of TU and KU and is distinguished primarily by the absence of orf7c. The ES strain also lacks orf7c, but differs from the TU and KU strains in the orf7b sequence. The orf7b3 sequence occurs in ES and the orf7b1 sequence occurs in TU and KU (Sanogo and Dobson 2004). The CR strain is primarily distinguished in that it is the only examined strain with the orf7c2 sequence. CQ differs from the other examined strains in that it is the only strain with orf7b2 and the only example of orf7c1 (Sanogo and Dobson 2004).

Additional trees were examined that were based upon all combinations of two of the orf7 loci. Ignoring orf7a had no effect on the topology and little effect on bootstrap values shown in Fig. 2. The orf7a+orf7b and orf7a+orf7c combinations were similar to trees based solely on orf7b or orf7c, respectively (described above).

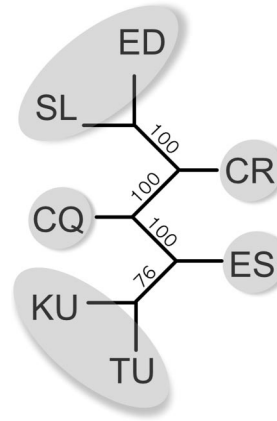

Fig. 2. Phylogenetic tree derived from the three combined orf7 loci of phage WO-infecting *Wolbachia* in *Cx. pipiens* strains. Bootstrap values above 50% are displayed. To generate the tree, gaps and missing data in the sequence data are treated as new character state.

No Correlation Observed between CI Cross Pattern and orf7 Phylogenetic Trees. A primary goal of this study was to examine for the hypothesized role of bacteriophage WO in *Culex* cytoplasmic incompatibility. Thus, phylogenetic trees inferred from orf7 loci were compared with the observed crossing patterns. The results fail to support the hypothesis. Inconsistent with hypothesis predictions, crosses between strains differing in their orf7 type were not observed to result in higher incompatibility levels relative to strains with similar orf7 types. As specific examples, the cross pairs ESxCQ, ESxSL, SLxCR, EDxTU, and EDxCQ each represent crosses between strains that differ in their orf7 (Fig. 2). The latter crosses are bidirectionally compatible, resulting in >60% hatch (Table 1). Crossing results with the ED and SL strains also contradict hypothesis predictions that strains with similar orf7 sequences should show greater levels of compatibility. ED and SL are indistinguishable in their orf7 sequences, but crosses result in hatch rates of <33%.

Although orf7 loci were not observed to predict CI type in *Culex*, the results presented here do not exclude the involvement of phage in determining CI in *Culex*. The orf7 locus is predicted to encode a phage capsid protein and is unlikely to be directly linked to the hypothesized phage locus that affects CI. Therefore, the use of orf7 loci to test the hypothesized involvement of phage in determining *Culex* CI type assumes a linkage between orf7 and the hypothetical CI-affecting phage locus. Furthermore, the orf7 locus provides relatively little sequence variation. For these reasons, characterization of additional phage loci is needed to better address the role of bacteriophage WO in *Culex* cytoplasmic incompatibility.

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