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# Molecular discrimination of *Wolbachia* in the *Culex pipiens* complex: evidence for variable bacteriophage hyperparasitism

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**Abstract**

**The medically important members of the *Culex pipiens* species complex provide an enigma for systematists, evolutionary biologists, and vector biologists. The species complex is composed of forms that differ in their ecology, behaviour, physiology and vector competence. Cytoplasmic incompatibility (CI) caused by endosymbiotic *Wolbachia* bacteria is thought to play an important role in restricting gene flow and the evolution of the *Culex* complex. Here we describe the first molecular marker useful for discriminating between *Wolbachia* infections in *Culex*. A putative bacteriophage locus (*orf7*) varies between *Culex* forms in copy number and sequence. We provide evidence that the *orf7* loci are strictly associated with *Wolbachia* and are maternally inherited.**

**Keywords: *Culex pipiens*, *Wolbachia*, bacteriophage WO, molecular marker.**

## Introduction

Mosquitoes of the *Culex pipiens* complex represent one of the outstanding problems in current mosquito taxonomy, with opinions on *Culex pipiens* taxonomy ranging from distinct species to physiological forms with considerable genetic introgression (Cornel *et al*., 2003). The ability of differing *Culex* forms to hybridize has been implicated as playing a role in determining vectorial capacity in this medically important disease vector (Fonseca *et al*., 2004). The successful resolution of the *C. pipiens* complex will depend upon studies that further clarify the morphological, behavioural/physiological, and genetic issues that currently complicate taxonomic interpretation.

The role of intracellular *Wolbachia* bacteria in restricting hybridization and gene flow within the *Culex* complex is unclear. *Wolbachia* induced cytoplasmic incompatibility (CI) is widespread in insects and results in karyogamy failure and embryonic mortality in matings between individuals infected with different *Wolbachia* types (reviewed in Stouthamer *et al*., 1999). The most polymorphic example of CI occurs within the *C. pipiens* complex, with over seventeen distinct cytoplasmic incompatibility phenotypes ('cytotypes') that differ in their compatibility with other cytotypes (Laven, 1967; Guillemaud *et al*., 1997). In contrast with CI in other insects, the differing cytotypes in *Culex* are not correlated with divergent *Wolbachia* types. Indeed, previous molecular characterizations of *Culex* cytotypes have not observed any variation in the *Wolbachia* infections (Guillemaud *et al*., 1997; Ruang Areerate *et al*., 2003).

Here, we describe two loci in *Culex*: the *Wolbachia* outer surface protein (wsp, Zhou *et al*., 1998) and *orf7* (Masui *et al*., 2000). Although the former is invariable within the *C. pipiens* complex, the latter varies significantly. Multiple copies of *orf7* are observed in infected strains, and *orf7* is absent from an aposymbiotic strain. Intrastrain crosses demonstrate a pattern of maternal inheritance that is consistent with *Wolbachia* infection (Laven, 1967). We discuss the results as a new molecular marker useful for clarifying the role of *Wolbachia* in restricting gene flow within the *C. pipiens* complex.

## Results

Although amplification and sequencing of the *wsp* gene in the different *Wolbachia*-infected *Culex* strains yielded sequences identical to *w*Pip (GenBank AF301010; data not shown), electrophoresis of the *orf7* amplification products revealed multiple amplicons and a differing banding pattern between *Culex* strains (Fig. 1). Sequencing of cloned *orf7* amplicons revealed nine different sequences (Fig. 2) that cluster into three groups: *orf7a* (394 bp), *orf7b* (424 bp), and *orf7c* (409 bp). Although a comparison between the three groups reveals considerable sequence variation, comparison

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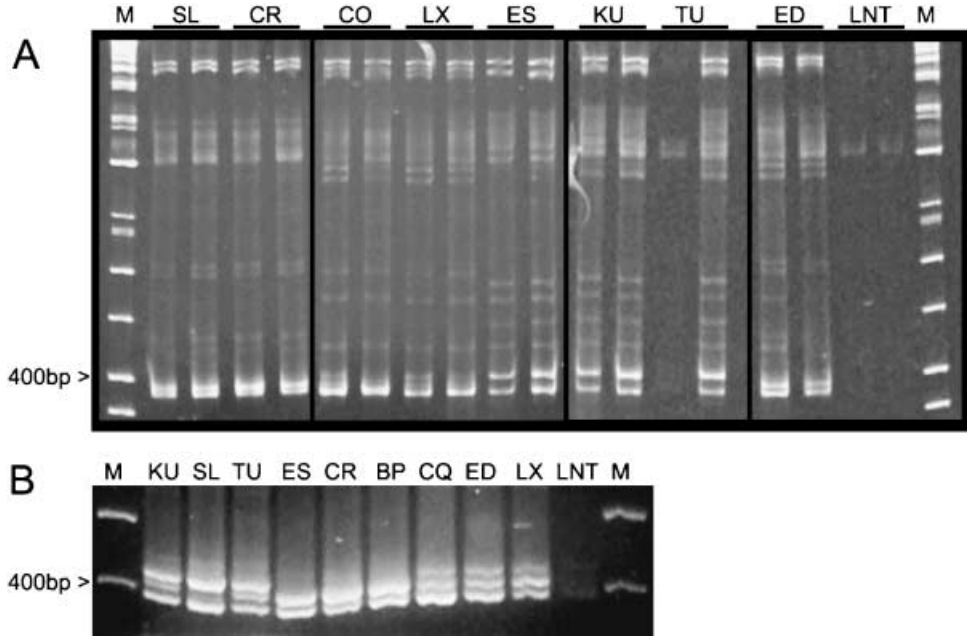


**Figure 1.** (A) Single-strand conformation polymorphism (SSCP) analysis of *orf7* amplification products from *Culex* strains. A male and female are shown for each strain (left and right, respectively). The TU male failed to amplify. (B) Agarose electrophoresis of *orf7* amplification products from *Culex* strains using phgWOf and phgWOr primers. The BP strain was lost and was not further characterized (not discussed in the text). *Culex* strain abbreviations are as shown in Table 1. M is the molecular weight marker (1kbPlus DNA ladder; Invitrogen).

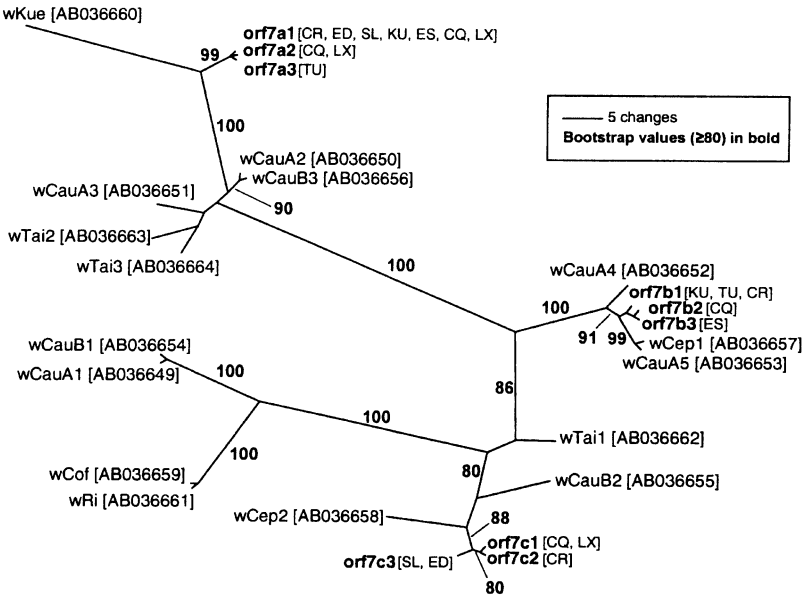


**Figure 2.** Unrooted phylogenetic tree based upon *orf7* DNA sequences using maximum parsimony. Bootstrap values were generated using a heuristic search with 1000 replicates (values above 80 are shown). The nine *Culex orf7* sequences are shown as bold text and are followed by brackets indicating the mosquito strains from which the sequences were isolated. Non-*Culex* sequences are shown followed by brackets containing the GenBank accession number.

of the predicted amino acid sequences within each of the three groups shows identical (*orf7a* group) or similar (*orf7b* and *orf7c*) sequences (Fig. 3).

Based upon the observed sequence variation, additional primer sets were designed and used to amplify the different *Culex* strains (Fig. 4). To confirm that the amplification products resulting from the specific primers were *orf7* amplicons, the TU product amplified using the orf7-B primers was sequenced and determined to be identical to the *orf7b1* sequence. The orf7-B primers failed to amplify a product from the CR strain (Fig. 4) from which an *orf7b* sequence was obtained. This may be due to a 5′-truncation of orf7b1 in the CR strain (AY505083).

The *orf7* group-specific primers were used to examine the pattern of *orf7* inheritance in *Culex*. Hybrid offspring from crosses between CQ and CR displayed a pattern of

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wKue    LKSEVNRLYGMLVELIARNRSLSIEAIKSTEAGLYFGEKAVEIGLADGVTTF-EFINNH 58
orf7a1  LQKEVDRLYEMFVQLIARNRNLSIEAIKSTEAGLYFGEKAVEIGLADGVTTF-EFINNH 58
wCauA5  LQKEVDRLYEMFVQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGVTAF-EFINK- 57
orf7b1  LQKEVDRLYEMFVQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGVTTF-EFINK- 57
orf7b2  LQKEVDRLYEMFLQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGVTTF-EFINK- 57
orf7b3  LQKEVDRLYEMFLQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGVTTF-EFINK- 57
wCauB2  LQKEVDRLYEMFLQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGITILS-SINKN 58
orf7c1  LQKEVGRLYEMFLQLIARNRGLSIEKIRSTEAGLYFGEKAAEIGLADGMTILS-SINKN 58
orf7c2  LQKEVGRLYEMFLQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGMTILS-SINKN 58
orf7c3  LQKEVDRLYEMFLQLIARNRGLSIEKIRSTEAGLYFGEKAVEIGLADGMTILS-SINKN 58
        *  ** *** *   ****** **** * ************ ******* *     **

wKue    ---SVSMTT-NEL-----TEEGYENCRREIVEIIRLCNVSKMPEKIGEFIEQGVSIEQAR 109
orf7a1  ---SVSMTT-NEL-----TE----NCRREILEIIRLCNLSKMPEKIGEFIEQGSSVEQAR 105
wCauA5  GENTMNKQITTDLEIDDLETDNLTKYRTEIVELIRLCNLSRMPEKIGEFIEQGVSIEQAR 117
orf7b1  GENTMNKQTTTDLEIDDLETDNLTKYRTEIVELIRLCNLSRMPEKIGEFIEQGVSIEQAR 117
orf7b2  GENTMNKQTTTGLEIDDLETDNLTKYRTEIVELIRLCNLSRMPEKIGEFIEQGVSIEQAR 117
orf7b3  GENTMNKQTTTDLEIDDLETDNLTKYRTEIVELIRLCNLSRMPEKIGEFIEQGVSIEQAR 117
wCauB2  RSITMNERTTTDL-----ETDNLTKYRNEVLELIRLCNLSRMPEKIGEFIEQSVSVEQAR 112
orf7c1  RSITMNEQTTNDL-----ETDNLTKYRTEVLELIRLCNLSKMPEKIGEFIEQSVSVEQAR 112
orf7c2  RSITMNEQTTNDL-----ETDNLTKYRTEVLELIRLCNLSKMPEKIGEFIEQSVSVEQAR 112
orf7c3  RSITMNEQTTNDL-----ETDNLTKYRTEVLELIRLCNLSKMPEKIGEFIEQSVSVEQAR 112
                  *             * *  * ***** * ***********  * ****
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**Figure 3.** Alignment of the deduced amino acid sequences of *Culex orf7* sequences. For comparison, the *orf7a*, *orf7b*, and *orf7c* sequences are grouped within boxes. Because the three *orf7a* DNA sequences are predicted to yield identical amino acid sequences, only *orf7a1* is shown. For each of the groups, a homologous sequence from *Ephestia kuehniella* (*w*Kue, AB036665) or *Cadra cautella* (*w*CauA5, BAA89608.1; *w*CauB2, AB036655.1) is included for comparison. Highlighted areas indicate sequence variation within each of the *orf7* groups. Stars indicate identical consensus across all groups. Gaps are indicated by dashes.

*orf7* amplification products consistent with the mother and distinct from the paternal pattern of *orf7* amplicons (Fig. 4).

## Discussion

Here we describe variation between *Culex* cytotypes in a *Wolbachia* bacteriophage locus. In contrast with other *Wolbachia* infected insects (Stouthamer *et al*., 1999), the variable cytotypes in the *C. pipiens* complex are not due to distantly related *Wolbachia* infection types. The *wsp* gene has been described as the most variable gene used in characterizing *Wolbachia* infection types (Zhou *et al*., 1998). Data presented here and in prior studies have identified no variation in *Wolbachia* from *C. pipiens* based upon DNA sequencing of the *wsp* (Rasgon & Scott, 2003; Ruang Areerate *et al*., 2003) or the *ftsZ* (Guillemaud *et al*., 1997) genes. However, our comparison of different *Culex* strains did reveal variation in both the copy number and sequence of *orf7* loci. This represents the first evidence of *Wolbachia*-related genetic variation within the *C. pipiens* complex. Thus, the *orf7* locus provides a useful marker for discriminating between *Wolbachia* infection types in *C. pipiens*. As shown in the phylogenetic analysis, greater homology of the *Culex orf7a*, *orf7b* and *orf7c* groups is observed with *orf7* sequences identified in other insects than to each other, suggesting horizontal movement of *orf7* within *C. pipiens*.

Directions for future experiments include examining whether the *orf7* locus represents an inactivated prophage in

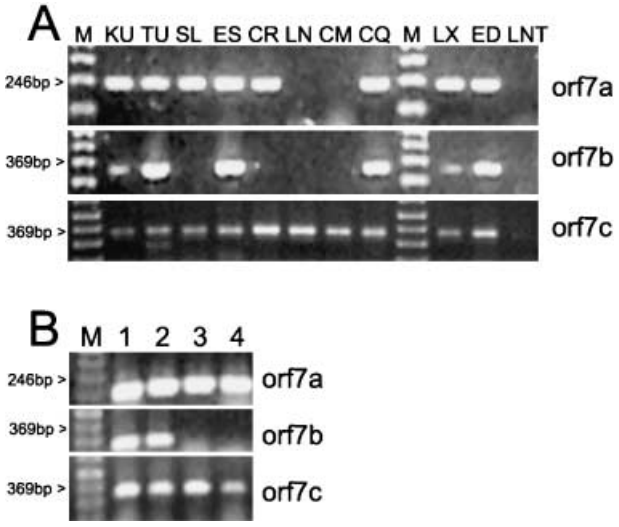


**Figure 4.** (A) Amplification of *Culex* strains using *orf7* group-specific primers. The CM strain is not discussed in the text. (B) Maternal inheritance pattern using *orf7* group-specific primers. Lanes are: (1) CQ female; (2) $F_1$ hybrid resulting from mating a CQ female and CR male; (3) CR female; (4) $F_1$ hybrid resulting from mating a CR female and CQ male. M is the molecular weight marker (123 ladder; Invitrogen).

*Culex* or an exogenic bacteriophage. The latter is suggested by prior reports of virus-like particles that have been described in electron microscopic sections of *Wolbachia* infected ovaries of *C. pipiens* (Wright *et al*., 1978) and the presence

**Table 1.** List of *Culex* strains used in the study

| Strain | Abbrev. | Source | |
|---|---|---|---|
| Lexington | LX | Authors | Fayette Co., Kentucky |
| Lincoln, CA | LN | Jason Rasgon | Lincoln, CA (Rasgon & Scott, 2003) |
| Lincoln; Aposymbiotic | LNT | Jason Rasgon | Tetracycline treated LN strain, (Rasgon & Scott, 2003) |
| ESPRO | ES | François Rousset | (Ben Cheikh & Pasteur, 1993) |
| SLAB | SL | François Rousset | (Georghiou *et al*., 1966) |
| TUNIS | TU | François Rousset | |
| EDIT | ED | François Rousset | |
| CRISSE | CR | François Rousset | |
| KUNU | KU | François Rousset | |
| *C. quinquefasciatus* | CQ | Dennis Wallette | Field collected; East Baton Rouge Mosq. Abatement & Rodent Control District. |

of active phage particles reported in a *Wolbachia*-infected cricket *Teliogrillus taiwanemma* (Masui *et al*., 2001). The lack of congruence between the phylogeny of *Wolbachia* and the incompatibility types has led some to propose that genes responsible for *Wolbachia* incompatibility are conveyed by extrachromosomal particles such as plasmids or phages (Guillemaud *et al*., 1997; Stouthamer *et al*., 1999). Thus, future studies must examine the potential role of phages in determining the high level of polymorphism in *Culex pipiens* cytoplasmic incompatibility types. The *orf7* variants described here are also expected to serve as markers useful in clarifying the role of *Wolbachia* in preventing hybridization between field populations of this important disease vector (Fonseca *et al*., 2004). Future research should also examine the role of bacteriophages in the evolution of the *Wolbachia* genome in *Culex*. Bacteriophages are known to facilitate lateral gene transfer between bacteria and may represent the predominant source of differences in closely related bacterial strains (Canchaya *et al*., 2003). Furthermore, a recent report demonstrates that bacteriophages constitute an important part of the *w*Mel *Wolbachia* genome in *Drosophila* (Wu *et al*., 2004).

## Experimental procedures

A list of the mosquito strains, their origin and their infection status is provided in Table 1. DNA was extracted from dissected ovaries and testes of individual mosquitoes using the STE preparation technique (O'Neill *et al*., 1992). The *Wolbachia* surface protein (*wsp*) gene was amplified using the primers wsp81F and wsp691R (Zhou *et al*., 1998). The *orf7* locus was amplified using primers phgWOf and phgWOr (Masui *et al*., 2000). Specific *orf7* primers were: *orf7a* (orf7-Af GCTAATAGCACGAAATCGAAAC, orf7-Ar ATTTCTCTACGACAGTTCTCC); *orf7b* (orf7-Bf CCCACATGAGCCAATGACGTCTG, orf7-Br CTAGGTCATCAATTTCTAGG(CT)CAG); and *orf7c* (orf7-Cf CCCACATGAGCCAATGACGTCTG, orf7-Cr TTGCTTGCTCAACACTTACACTT). Single-strand conformation polymorphism (SSCP) assay of PCR products generated by the phgWOf and phgWOr primers was as previously described (Black & DuTeau, 1997). For sequencing, *orf7* amplification products were gel purified, cloned into a PCR-II TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced using M13 primers.

Sequences were edited using SeqMan (Lasergene, DNASTAR Inc., Madison, WI) and visually checked for sequencing errors. The consensus sequence for each was aligned using the Clustal W program (Thompson *et al*., 1994). Phylogenetic trees were constructed using maximum-parsimony analysis based on DNA alignment using PAUP 4.0 (Swofford, 2002). GenBank accession numbers are: orf7 type – *Culex* strain (accession number). orf7a1 – ES (AY505084), KU (AY505086), SL (AY505087), CQ (AY505088, AY505097), CR (AY505098), ED (AY505101), LX (AY505104); orf7a2 – LX (AY505103), CQ (AY505096); orf7a3 – TU (AY505091); orf7b1 – CR (AY505083), KU (AY505085), TU (AY505094); orf7b2 – CQ (AY505090); orf7b3 – ES (AY505093); orf7c1 – CQ (AY505095), LX (AY505102); orf7c2 – CR (AY505100); orf7c3 – SL (AY505105), ED (AY505089).

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