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 wPip (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
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

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 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

orf7 amplification products consistent with the mother and distinct from the paternal pattern of orf7 amplicons (Fig. 4).
of active phage particles reported in a Wolbachia-infected cricket Teliogriillus taiwanemima (Masui et al., 2001). The lack of congruence between the phylogey 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 wMel 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., 1997). The orf7 locus was amplified using primers phgWOI and phgWOr (Masui et al., 2000). Specific orf7 primers were: orf7a (orf7-A1 GCTAATGCAAGAATCAGAAC, orf7-A2 ATTTCTCTAC- GACATCTCC); orf7b (orf7-B1 CCCACATGAGCCTGACG- TCTG, orf7-B2 CTAGGCTATCATGCTTTCAGGT(CT)CAG); and orf7c (orf7-C1 CCCACATGAGCCTGAGCTTCTG, orf7-C2 TTACGCT- CTCAACTTACACTT). Single-strand conformation polymorphism (SSCP) assay of PCR products generated by the phgWOI 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 ClustalW 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; orf7a2 – CR (AY505098), ED (AY505099); orf7a3 – LX (AY505100); orf7b1 – CR (AY505083), KU (AY505085), TU (AY505094); orf7b2 – CQ (AY505090); orf7b3 – ES (AY505093); orf7c1 – CQ (AY505095), LX (AY505102); orf7c2 – CR (AY505101); orf7c3 – SL (AY505105), ED (AY505089).

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References


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Wolbachia and bacteriophage WO infections in Culex

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