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Wolbachia in the Culex pipiens Group Mosquitoes: Introgression and Superinfection

THOMAS WALKER, SHEWU SONG, AND STEVEN P. SINKINS

Abstract

Wolbachia bacteria in mosquitoes induce cytoplasmic incompatibility (CI), where sperm from Wolbachia-infected males can produce inviable progeny. The #Pip strain in the Culex pipiens group of mosquitoes produces a complexity of CI crossing types. Several factors are thought to be capable of influencing the expression of CI including Wolbachia strain type and host genotype. In this study, the unidirectional CI that occurs between 2 C. pipiens complex laboratory strains, Col and Mol, was further investigated by nuclear genotype introgression. The unidirectional CI between Col and Mol was not found to be influenced by host genetic background, in contrast to a previous introgression study carried out using bidirectionally incompatible C. pipiens group strains. A line containing both #Pip strain variants superinfection was also generated by embryonic cytoplasmic transfer. The same crossing type as the parental Col strain was observed in the superinfected line. Quantitative polymerase chain reaction demonstrated a low density of the injected #PipMol variant in the superinfected line after 18 generations, which was considered likely to be responsible for the crossing patterns observed. The Wolbachia density was also shown to be lower in the parental Mol strain males compared with Col strain males, and no inverse relationship between WO phage and Wolbachia density could be detected.

Key words: cytoplasmic incompatibility, Wolbachia
(Laven 1967), although there are questions concerning the sustainability of this control approach. Genetic replacement strategies targeting vector competence could also depend on CI and achieving a better understanding of how complex patterns of incompatibility between infected populations are generated would be important in this respect.

The mechanisms of Wolbachia-induced CI are not well characterized. CI is now generally accepted to involve sperm modification that interferes with the process of karyogamy and a rescue component provided by the Wolbachia present in the egg, which restores normal male and female pronuclear karyogamy in compatible crosses (Werren 1997). There are several factors that are thought to influence the expression of CI in insects including Wolbachia density (Bordenstein et al. 2006), Wolbachia strain type (Sasaki and Ishikawa 2000; Sakamoto et al. 2005), and host genotype (McGraw et al. 2001; Sasaki et al. 2005). The bidirectional CI between the Pel and Bei strains of C. quinquefasciatus was previously shown to be influenced by modifying effects by introgressing the Pel nuclear genome into a Bei cytoplasmic background (Sinkins et al. 2005). In this comparative study, the unidirectional CI that occurs between 2 mosquito strains as donor and recipient, to investigate the effects on CI/crossing type.

Materials and Methods

Mosquito Colonies and Crossing Experiments

Culex pipiens complex laboratory strains Mol (C. molestus, China) and Col (C. quinquefasciatus, Colombia) were reared using standard mosquito rearing procedures at low larval densities in insectary conditions (26 °C, 70% relative humidity) with a 12:12 h light:dark circadian cycle. Mass crossing experiments were carried out using 50 virgin individuals of each sex. Virgin male and female mosquitoes were obtained through isolation and sexing of pupae. The F1 generation progeny from crosses were analyzed by calculating the hatch rate of hatched embryos from a minimum of 8 egg rafts, each containing between 50 and 110 eggs per raft, as a measure of the CI phenotype. Female spermathecae were examined for the presence of sperm if the hatch rate was zero to confirm insemination. Unidirectional incompatibility between Mol and Col strains allowed nuclear replacement by crossing Col males to females containing the Mol cytoplasmic background. In the backcrossing experiment, F1 females from the cross between Mol females and Col males were backcrossed with Col males, and offspring females backcrossed with Col males for a further 4 generations. From the third backcross generation, males were crossed with Col females to examine whether crossing type was maintained.

Discrimination of wPip Strain Variants

The Mol and Col strains are infected with the wPip strain of Wolbachia. No sequence polymorphism has been found for ftsZ (Guillemaud et al. 1997) or the highly variable Wolbachia surface protein (wsp) genes for the Wolbachia present in the Mol and Col strains (Sinkins et al. 2005). Further sequence analysis of wPip ankyrin repeat domain (ANK) genes revealed variation in both nucleotide sequence and predicted amino acid sequence for only 2 prophage-associated ANK genes. One of these genes, pk1, shows nucleotide sequence variability between the Col and Mol colonies, and discrimination of the wPipMol variant in the Mol colony from the wPipCol variant in the Col colony was carried out using pk1 primers previously described (Sinkins et al. 2005).

Microinjection of C. pipiens Embryos

Microinjection of Culex embryos was carried out using a method developed for injection of Anopheles embryos (Bossin and Benedict 2005). Donor Mol and recipient Col preblastoderm embryos were aligned against a thin hydrophilic blotting membrane in contact with moist filter paper. Microinjection was carried out under ×100 magnification using a Femtojet microinjector system (eppendorf) with type II femtotip microinjection needles (eppendorf). A Narishige micromanipulator attached to a Nikon compound microscope was used to manipulate the microinjection needles. After breakage of the needle tip against the membrane, cytoplasm was withdrawn from the donor wPipMol-infected embryos and subsequently injected into the posterior poles of the recipient wPipCol-infected embryos. G0 females were mated to colony Col males and blood fed to establish isofemale lines.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) analysis was carried out on 5 individual DNA extracts of C. pipiens colony adult mosquitoes. DNA was extracted using a modified version of the Livak buffer method with ethanol precipitation (Collins et al. 1987). Estimation of Wolbachia density was undertaken by comparing in vivo gene copy numbers: ftsZ gene copy counts were used to estimate the total Wolbachia abundance in the DNA extracts. The single copy C. pipiens gene was used to normalize the data, controlling for variation in the amount of DNA extracted or mosquito size. In order to estimate the relative density of the wPipMol variant in the superinfected line compared with the parental Mol strain, relative copy numbers of the wPipMol pk1 gene variant were measured. The pk1 gene is present in 3 of the integrated prophage copies in the wPip (Pel strain) genome sequence (Walker et al. 2007), and copy number was corrected for in the analysis. Bacteriophage WO density was estimated by comparing in vivo copy numbers of the phage capsid orf7 gene (Masui et al. 2000) to ftsZ copy numbers.

Long oligonucleotide standards were designed along with corresponding primer sets, and standards were diluted
from $10^6$ copies to $10^7$ copies for each gene for generation of standard curves. Primer sequences were as follows: qS7F CCGAGCTGATCAGC8, qS7R ATCGCTCGGAAATGTAAGATCTA (standard = 120 bp); qFtsZF TGGTTGTTAAGGTTGACCGAG, qFtsZR CAGTTACACCAACCCATAAC (FtsZ standard = 102 bp); qpk1MolF ATTTGGCAGCTGATTGGGA, qpk1MolR CATCGGTGCTGTAATTTCGTG (standard = 101 bp); qopt7F AAGTAGCAGGACATATAAGATTTG, qopt7R GCCAAAATATAGACCTGCTC (standard = 98 bp).

The relative quantity of template DNA was estimated using an Option 2 Continuous Fluorescence Detection System (GRI) together with Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). Template DNA was PCR amplified using primers at 10 μM, and 1.0 μl of DNA was added in a total volume of 20 μl per reaction. qPCR cycling conditions consisted of an initial denaturation step of 95 °C for 15 min followed by 40 cycles of denaturing at 95 °C (15 s) and primer annealing at 50 °C (30 s). A melting curve was analyzed to check for any nonspecific amplification or primer dimers. Included in the assays were DNA extracts from the Wolbachia-uninfected Pel U colony and sterilized water controls to confirm the absence of contaminating DNA.

Results and Discussion

Crossing and Introgression

Crossing experiments between uPip-infected C. pipiens complex laboratory strains Mol and Col previously revealed unidirectional incompatibility (Walker et al. 2007). Col females mated to Mol males resulted in 0.44 ± 0.19% embryo hatch in contrast to 93.49 ± 1.17% hatch when Mol females mated with Col males. To investigate whether host modifying effects could influence this crossing pattern, as was previously observed in a cross between 2 bidirectionally incompatible Culex strains (Sinkins et al. 2005), 5 generations of backcrossing, introgressing the Col nuclear genotype into a uPipMol-infected cytoplasm expected to result in around 97% Col genotype, was undertaken as shown in Table 1.

<table>
<thead>
<tr>
<th>Cross/backcross, female × male</th>
<th>Progeny name</th>
<th>Test cross, female × male</th>
<th>% hatch (no. rafts/embryos)</th>
</tr>
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<tbody>
<tr>
<td>F1 females from the cross between Mol females and Col males were backcrossed with Col males, and offspring females backcrossed with Col males for a further 4 generations. From the third backcross generation (BCIII), males were crossed with Col females and embryo hatch rates counted.</td>
<td></td>
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</table>

The resulting line was fully anautogenous (requiring a blood meal for egg laying, unlike the autogenous C. molestus).

As shown in Table 1, close to complete incompatibility was observed when males from the introgressed line were crossed with Col females, showing no evidence for a host modifying effect in this particular example of CI. Host modifying effects on CI are thus not universal in this complex but dependent on the particular host strain variant combination being introgressed, and this pattern may contribute to the complexity of crossing type variation in the complex.

Introgression and replacement of the Col nuclear genotype into uPipMol-infected cytoplasm also confirm that the interstrain incompatibilities between Mol and Col are not controlled by host nuclear divergence but by Wolbachia. This introgression experiment has in effect created a novel crossing type in C. quinquefasciatus because the C. molestus nuclear genome has been largely replaced. The uPipMol variant would be expected to be able to spread through populations of C. quinquefasciatus of the Col crossing type.

uPip Strain Variant Superinfection

In order to create a line containing both the uPipMol and uPipCol variants, Col embryos were injected with uPipMol-infected preblastoderm embryo cytoplasm; of 350 injected embryos, there was a 15.1% hatch rate and 8.9% survival of injected embryos to eclosion (31/350). To detect the presence of the uPipMol variant in the Col G0 females and resulting isofemale lines, PCR analysis was carried out using primers that were designed to discriminate between variants of the WO prophage-associated pk1 gene present in the 2 uPip strains (Sinkins et al. 2005). An isofemale line, termed uPipCol(uPipMol), was successfully established and maintained.

Crossing experiments were undertaken with generation 11 (G11) of the superinfected uPipCol(uPipMol) line to analyze the effect on CI crossing type. PCR analysis for the pk1Mol variant confirmed the presence of the uPipMol variant in all 5 G11 males assayed, suggesting no heterogeneity in the line. As shown in Figure 1, the very low embryo hatch resulting from crossing uPipCol(uPipMol) G11 females to uPipMol males was similar to the low hatch when uPipCol females were crossed to uPipMol males. The presence of the uPipMol variant in uPipCol(uPipMol) females would be expected to result in compatibility with Mol males, based on the model that the females contain the correct “rescue” factor for both singly and superinfected males (Sinkins et al. 1995). However, crossing analysis revealed that the uPipMol variant in females of the superinfected line was unable to rescue the modification factor in sperm produced by uPipMol males. Likewise, crossing the uPipCol(uPipMol)-infected G11 males with uPipCol-infected females produced a high hatch rate, very similar to the hatch seen when uPipMol females were crossed to uPipCol males. Thus, the presence of the uPipMol variant did not produce CI in this cross as had been expected.
and 50 virgin females (F), and the hatch rate of the F1 progeny
were calculated from a minimum of 10 egg rafts, each containing
50–120 eggs per raft, as a measure of CI.

Crossing analysis to determine the effect of the 
\textit{w}PipMol variant in the superinfected \textit{w}PipCol(\textit{w}PipMol) line.

Crossing experiments were performed with 50 virgin males (M)
and 50 virgin females (F), and the hatch rate of the F1 progeny
was calculated from a minimum of 10 egg rafts, each containing
50–120 eggs per raft, as a measure of CI.

Density of \textit{w}Pip Strain Variants

A possible explanation for the crossing data described is that the
\textit{w}PipMol variant did not reach a sufficiently high density
in the superinfected line to be able to induce CI when super-
infected males were crossed to Col females or to rescue CI
when superinfected females were crossed to Mol males.
qPCR density assays were carried out on the G18 generation
allowing a stable equilibrium density to be reached. Although
all individual G18 males assayed were positive for the
\textit{w}PipMol based on PCR followed by gel electrophoresis,
a very low comparative density of the \textit{w}PipMol \textit{pk}1 variant
was detected by qPCR as shown by the \textit{pk}1\textit{Mol:ftsZ} gene
ratios, equivalent to 1:250 (Table 2). Although naturally
occurring \textit{Wolbachia} strain superinfections such as that seen
in \textit{Aedes albopictus} can be stable despite significant density
differences between strains (Dutton and Sinkins 2004), the
density differences between them were lower than those
seen here.

The quantitative PCR assay is expected to amplify both
integrated \textit{WO} prophage copies of the \textit{pk}1 gene in the
\textit{Wolbachia} genome and any lytic phage particles containing
phage DNA that may be produced from those copies
containing \textit{pk}1. Thus, an alternative explanation is that the

\textit{Wolbachia} and Bacteriophage \textit{WO} Density in Parental Col
and Mol Strains

As shown in Table 2, the \textit{Wolbachia} density in Mol males was
significantly lower than for Col males ($P = 0.03$, Student’s t-test).
The \textit{w}PipMol variant may have a slower growth rate
compared with \textit{w}PipCol, and this could be responsible for it
being unable to establish itself to a sufficiently high density
when indirect intrahost competition with \textit{w}PipCol. Mol males
carrying the lower density \textit{w}PipMol variant are incompat-
ible with females carrying \textit{w}PipCol (Walker et al. 2007).
This pattern is the opposite of what would be expected if
density differences were causally responsible for the dif-
ferent patterns of CI observed between these strains. The
density of \textit{Wolbachia} in \textit{C. pipiens} testes was also found to be
strain dependent and did not appear to influence CI in other
\textit{C. pipiens} strains (Duron et al. 2007).

A threshold level of \textit{Wolbachia} density (and/or even
distribution within the testes) may be required for induction
of complete CI. However, if \textit{Wolbachia} density differences
influence the penetrance of CI only when the density falls
below a threshold level, the varying \textit{w}Pip densities between the
Mol and Col strains may all be above this threshold and
therefore irrelevant with respect to CI induction/rescue.
However, when the \textit{Wolbachia} variants are superinfecting the
same host, then density of the \textit{w}PipMol variant may have
fallen below this necessary threshold due to effects such as
direct competition between strains.

The overall density of bacteriophage \textit{WO} was also
assessed in Col and Mol, measured as a ratio of the phage
capsid \textit{orf}7 gene copy number to \textit{Wolbachia} \textit{ftsZ} copy number
(because not all prophage copies contain \textit{pk}1), and was not
significantly different between Mol males (8.04 ± 2.42) and
Col males (7.07 ± 2.18) ($P = 0.43$, Student’s t-test). The
densities of bacteriophage \textit{WO} in males were not inversely
correlated with \textit{Wolbachia} density (Pearson $r = -0.35$) with
no evidence for increased bacteriophage \textit{WO} lytic activity in
Mol males. These data suggest that the inverse relationship
between phage density and \textit{Wolbachia} density, and tripartite
association as an explanation for unidirectional CI presented

\begin{figure}
\includegraphics{f1.png}
\caption{Crossing analysis to determine the effect of the 
\textit{w}PipMol variant in the superinfected \textit{w}PipCol(\textit{w}PipMol) line.
}
\end{figure}

\begin{table}
\centering
\begin{tabular}{lll}
\hline
Strain/line & Overall \textit{w}Pip density (\textit{ftsZ}: \textit{S7}) & \textit{w}PipMol relative density (\textit{pk}1\textit{Mol:ftsZ}) \\
\hline
Mol & 0.12 ± 0.04 & 1.278 ± 0.189 \\
Col & 0.39 ± 0.11 & 0 ± 0 \\
\textit{w}PipCol(\textit{w}PipMol) G18 & 0.42 ± 0.12 & 0.004 ± 0.001 \\
\hline
\end{tabular}
\caption{qPCR analysis to estimate the \textit{w}PipMol density in the
superinfected \textit{w}PipCol(\textit{w}PipMol) line.}
\end{table}

qPCR was carried out on 4 individual DNA extracts of adult Col and Mol
male mosquitoes and on 8 adult male mosquitoes of the G18 generation of
the \textit{w}PipCol(\textit{w}PipMol) line. The overall \textit{Wolbachia} density was estimated from the \textit{ftsZ}:\textit{S7} gene copy ratio, and the relative density of the \textit{w}PipMol
variant was estimated using the gene copy ratio of \textit{pk}1\textit{Mol:ftsZ}.
for Nasonia by Bordenstein et al. (2006), do not apply in this case. The observed phage copy numbers per Wolbachia are broadly consistent with the 5 prophage regions containing the opr7 gene identified in the wPip (Pel strain) genome, implying that bacteriophage WO is primarily temperate rather than lytic in wPip.

The development of intraspecific transfer of wPip strain variants by microinjection, coupled with the development of specific PCR assays to separate these variants and estimate their density (Sinkins et al. 2005; Walker et al. 2007) provide useful tools to investigate the control of CI in the C. pipiens complex. Future experiments can now be contemplated within the Culex genus involving other wPip variants with more similar growth rates/relative densities; the effects of introducing novel Wolbachia strains from other species can also be investigated. Meanwhile, the replacement of host genotype by introgression has provided a novel crossing type in C. quinquefasciatus, furthering understanding of CI in the complex and potentially providing a platform for CI-based mosquito control strategies.

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References


