# Characterization of *Wolbachia* Infections and Interspecific Crosses of *Aedes* (*Stegomyia*) *polynesiensis* and *Ae.* (*Stegomyia*) *riversi* (Diptera: Culicidae)

JEFFRY L. DEAN AND STEPHEN L. DOBSON

Department of Entomology; University of Kentucky; Lexington, KY 40546

J. Med. Entomol. 41(5): 894–900 (2004)

**ABSTRACT** Prior studies have identified a complicated pattern of interspecific hybridization between members of the *Aedes* (*Stegomyia*) *scutellaris* (Walker) mosquito group, which includes medically important vectors of bancroftian filariasis and dengue. Here, we report that two members of the group, *Aedes polynesiensis* Marks and *Aedes riversi* Bohart & Ingram, are both infected with intracellular *Wolbachia* bacteria. Sequencing of the *Wolbachia wsp* gene demonstrates that the infections differ from each other and from *Wolbachia* infections previously reported in mosquitoes. *Aedes polynesiensis* is the first mosquito identified with a *w*Mel *Wolbachia* type. Intraspecific crosses of infected and aposymbiotic lines generated via antibiotic treatment show that the *Wolbachia* infections in both species cause high levels of cytoplasmic incompatibility. Interspecific crosses show that the two species are reproductively isolated. However, repeating the interspecific crosses with aposymbiotic mosquito strains demonstrates that the *Wolbachia* infections play a role in preventing hybrid offspring. We discuss *Wolbachia* infections in relation to better defining the evolutionary relationships and causes of speciation within the group, understanding the basis for the observed east-to-west gradient in filarial refractoriness, and developing novel genetic control measures.

**KEY WORDS** symbiosis, mutualism, speciation, population replacement, *Wolbachia*

THE *Aedes* (*Stegomyia*) *scutellaris* (Walker) complex of mosquitoes is comprised of ≈30 morphologically similar species spread throughout the Malaysian and Australasian zones, eastward through Polynesia, and northward to Japan (Macdonald 1976). Members are largely allopatric and include important vectors of bancroftian filariasis and dengue. Morphological similarities suggest that the group has a common ancestor and that they have been isolated relatively recently, perhaps with the development of the Fiji Basin and the destruction of the Outer Melanesian Arc 5–10 million years ago (Karig 1972, Trpis et al. 1981, McLain et al. 1985).

Geographic separation onto single islands or island groups separated by large stretches of water combined with limited dispersal abilities (Belkin 1962) is the most striking mechanism of reproductive isolation between species of this group. However, subtle pre- and postmating isolation mechanisms also have been documented within the group, including ethological isolation (Ali and Rozeboom 1973, McLain et al. 1985). Members of the group can be colonized relatively easily, allowing the artificial production of hybrids. Nonreciprocal fertility was first reported in the *Ae. scutellaris* group in crosses between *Ae. scutellaris scutellaris* Walker and *Aedes scutellaris katherinensis* Woodhill (Woodhill 1949). Subsequently, numerous studies have examined interspecific hybridization between members of the *Ae. scutellaris* group with interest in better defining the evolutionary relationships and the causes of speciation within the group, in understanding the basis for the observed east-to-west gradient in filarial refractoriness, and in the hope of developing novel genetic control measures (summarized in Macdonald 1976).

Rickettsia-like infections of *Wolbachia* bacteria are known to affect hybridization success between members of the *Ae. scutellaris* complex. Maternally inherited *Wolbachia* endosymbionts have been observed infecting the cytoplasm of several members of the *Ae. scutellaris* group (Beckett et al. 1978; Wright and Barr 1980, 1981; Wright and Wang 1980; Meek 1984). Crossing studies have shown that *Wolbachia* infections within the *Ae. scutellaris* complex can cause cytoplasmic incompatibility (CI), which results in reduced egg hatch from crosses between individuals that differ in their *Wolbachia* infection type (Dobson 2003a). Among mosquitoes, *Wolbachia*-induced CI is best known in *Culex pipiens* L. The original description of *Wolbachia pipientis* was in *Cx. pipiens* (Hertig 1936). The CI phenotype also was originally described in *Cx. pipiens* (Laven 1951), which later became the basis for field trials by using CI to reduce a medically important *Culex* population (Laven 1967a). Furthermore, it was experiments with *Cx. pipiens* in which *Wolbachia* was demonstrated to be the etiological agent of CI (Yen

0022-2585/04/0894–0900$04.00/0 

and Barr 1971). *Wolbachia*-induced CI in the *Ae. scutellaris* group has attracted significant research effort to define the role of *Wolbachia* in the reproductive isolation between species of the group (Trpis et al. 1981, Sherron and Rai 1983, Meek and Macdonald 1984). Studies have demonstrated that the removal of *Wolbachia* infections (generating aposymbiotic strains) can increase egg hatch in crosses of *Aedes polynesiensis* Marks with members of the *Ae. scutellaris* group (Trpis et al. 1981, Sherron and Rai 1983, Meek 1984).

Here, we examine the role of *Wolbachia* in preventing hybridization between *Ae. polynesiensis* and *Aedes riversi* Bohart & Ingram. *Ae. polynesiensis* has a wide distribution, ranging from Fiji to the Tuamotu archipelago, and is an important vector of bancroftian filariasis (Rosen et al. 1976). Due to its broad distribution (naturally overlapping with other species within the *Ae. scutellaris* group) and medical importance, much of the crossing experiments within the *Ae. scutellaris* group have included *Ae. polynesiensis* (Macdonald 1976). In contrast, there has been relatively little research examining the specific relationships of *Ae. riversi* (Toma and Miyagi 1989). This likely reflects its relatively limited distribution and medical importance. *Ae. riversi* is known from a relatively small set of islands of southern Japan (Ryukyu archipelago and islands north and west of Kyushu) (Mogi 1976). Although a biting pest, *Ae. riversi* is not reported to be an important disease vector.

Our results demonstrate that both *Ae. polynesiensis* and *Ae. riversi* are infected with *Wolbachia.* Sequencing of the *wsp* gene show that the *Wolbachia* infections differ from each other and previously reported *Wolbachia* types. Crossing studies demonstrate that the reproductive isolation between the two species is complete. However, upon removing the *Wolbachia* infections, crosses of the aposymbiotic strains result in hybrid offspring.

## Materials and Methods

**Mosquito Maintenance and Crosses.** Three strains of *Ae. polynesiensis* obtained from Unité d'Entomologie Médicale, Institut Louis Malardé were used in this study: Rangiora (APA), Raiatea (API), and Maupiti (APM). An *Ae. riversi* strain (AR) was obtained from Motoyoshi Mogi, Saga Medical School. All mosquito colonies had been maintained in laboratory conditions for >50 generations. For rearing and all experiments, standard conditions of 28 ± 2°C, 75 ± 10% RH, and a photoperiod of 18:6 (L:D) h were maintained. Larvae were fed ad libitum with a liver powder solution until pupation. Crosses consisted of 10 virgin females and 10 virgin males (2–3 d old). A constant supply of a 10% sucrose solution was made available for adult mosquitoes. Mosquitoes were provided weekly with a mouse for blood feeding. After blood feeding, oviposition cups lined with paper were placed into cages. Eggs were collected for three consecutive days. After maturation, eggs were submerged in deoxygenated water and given 4 d to hatch. Egg hatch rates were determined through visual inspection of eggs by using a dissecting microscope. Statistical comparisons of fecundity and egg hatch rate were accomplished via Kruskal–Wallace and Mann–Whitney by using StatView 5.0.1 (SAS Institute, Cary, NC).

**Tetracycline Treatment.** Initial attempts to remove *Wolbachia* infection from *Ae. polynesiensis* (APM and APA) and *Ae. riversi* (AR) by using a technique developed for *Ae. albopictus* (Otsuka and Takaoka 1997) were unsuccessful due to larval mortality. Therefore, a second technique previously developed for tetracycline treating *Ae. albopictus* adults was used (Dobson and Rattanadechakul 2001). The tetracycline concentration previously used for *Ae. albopictus* (1 mg/ml) was found to result in high mortality. Therefore, lower concentrations of tetracycline were tested: 0.2, 0.5, or 0.75 mg/ml tetracycline dissolved in a 10% sucrose solution and buffered to pH 7 by using unbuffered Tris (pH 11). Females treated with 0.2 mg/ml tetracycline were the only individuals to produce viable offspring. The resulting adults (G2) were again treated with the 0.2 mg/ml tetracycline sucrose solution. The resulting strains (APMT, APAT, and ART, respectively) were demonstrated to be aposymbiotic by using the polymerase chain reaction (PCR) technique (described below) and were maintained as described above.

**PCR Amplification and Sequencing.** Initial detection of *Wolbachia* infections used primers 81F and 691R (Braig et al. 1998). The PCR conditions were as described previously (Dobson et al. 2002b). Ovaries and testes were dissected from individual mosquitoes and homogenized in 100 μl of STE (0.1 M NaCl, 10 mM Tris HCl, and 1 mM EDTA, pH 8.0). Proteinase K was added to a concentration of 0.4 mg/ml. The mixture was incubated at 56°C for 1 h followed by 95°C for 15 min. One microliter of sample was then amplified in a solution of 50 mM KCl, 20 mM Tris HCl, pH 8.4, 0.25 mM dNTPs, 1.5 mM MgCl, 0.5 μM primers, and 1 U of *Taq*DNA polymerase (Invitrogen, Carlsbad, CA). Using a PTC-200 thermal cycler (MJ Research, Watertown, MA), samples were denatured for 3 min at 94°C, cycled 35 times at 94, 55, and 72°C for 1 min each, followed by a 10-min extension period at 72°C. Ten microliters of sample was loaded into 1% agarose gels and electrophoresed. Samples were then stained with ethidium bromide and visualized under UV illumination. Subsequent *Wolbachia* clade type was determined using diagnostic primers *w*AlbA (primers 328F and 691R) and *w*AlbB (primers 183F and 691R) (Zhou et al. 1998). For samples failing to amplify using *Wolbachia*-specific primers (e.g., the aposymbiotic strains), 12S primers were used to amplify mitochondria DNA as a positive control for template DNA quality (O'Neill et al. 1992). For direct sequencing of PCR products, amplification products were purified using a Qia-Quick kit (QIAGEN, Valencia, CA). Samples were then sequenced using a CEQ autosequencer (Beckman Coulter Inc., Los Angeles, CA). The sequences obtained in this study have been deposited in GenBank under the accession numbers *Ae. polynesiensis,* AY535013, and *Ae. riversi,* AY535014.

Phylogenetic comparisons were accomplished following Zhou et al. (1998). Specifically, a 3′ region

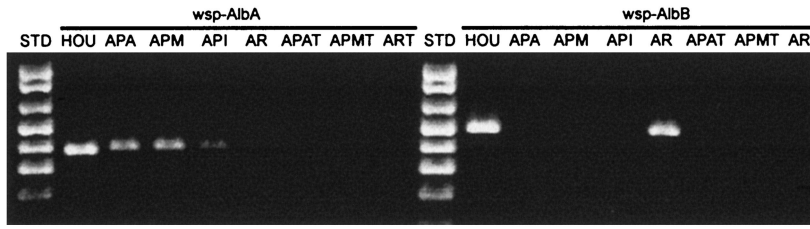


**Fig. 1.** PCR results using *Wolbachia* group-specific *wsp* primers (AlbA and AlbB). The mosquito strain abbreviations are as described in the text. As a positive control, an *Ae. albopictus* mosquito (HOU; Houston strain) is included. The *Ae. albopictus* Houston strain is superinfected with *Wolbachia* types from both the A and B clades (Sinkins et al. 1995). STD indicates a molecular weight marker (123 ladder; Invitrogen).

corresponding to the third hypervariable region of the *wsp* gene was deleted. An ≈40-bp region also was deleted from the 5′ end, because this region was not obtained from all taxa. Sequences were aligned using the ClustalW program (Thompson et al. 1994) and checked manually. Phylogenetic trees were constructed based upon *wsp* DNA sequences by using maximum-parsimony analysis in PAUP 4.0 (Swofford 2002). Bootstrap values were generated using a heuristic search with 1000 replicates. Due to the absence of an outgroup and to allow comparison with published phylogenies, the phylogenetic tree was midpoint rooted (Zhou et al. 1998).

## Results

***Wolbachia* Infection Type.** PCR amplification demonstrated all three *Ae. polynesiensis* strains (APM, APA, and API) and the *Ae. riversi* strain (AR) to be *Wolbachia* infected (Fig. 1). By using primers diagnostic for different *Wolbachia* clades (Zhou et al. 1998), the *Ae. polynesiensis* and *Ae. riversi* strains were shown to harbor *Wolbachia* types from the A clade and B clade, respectively. Sequencing of the *wsp* gene from the *Ae. polynesiensis* strains revealed that all three mosquito strains harbored an identical *Wolbachia* type (Fig. 2). Based upon previously defined criteria (Zhou et al. 1998), the *Ae. polynesiensis* infection falls within the *w*Mel group of the A clade (≤2.5% divergence). The *Ae. riversi* infection is similar to members of the *w*Con group within the *Wolbachia* B clade.

PCR amplification of tetracycline-treated lines (APMT, APAT, and ART) demonstrated that the *Wolbachia* infections had been successfully removed (Fig. 1). The DNA quality of the mosquito preparations were verified using 12S mitochondrial primers. The DNA extractions from the APMT, APAT, and ART strains resulted in a 12S amplicon but no *wsp* amplicon. Thus, cytoplasmic DNA was present in the preparations but did not include *Wolbachia* DNA (O'Neill et al. 1992).

**Intraspecific Crosses.** Crosses were conducted between the *Ae. polynesiensis* strains to determine compatibility levels. As shown in Table 1, 56 to 77% average egg hatch was observed in the nine cross types. No difference was observed in egg hatch rates ($P > 0.59$, df = 8; Kruskal–Wallis) or total egg number ($P > 0.12$, df = 8; Kruskal–Wallis). Intrastrain crosses between infected males and aposymbiotic females (generated by tetracycline treatment) demonstrated that the *Wolbachia* infection in the APM and APA strains induced almost perfect CI (≤0.1% egg hatch; Table 1). To confirm that the reduced egg hatch was due to CI and not reduced fertility of aposymbiotic males, aposymbiotic males were also crossed with aposymbiotic females (Table 1). High egg hatch resulted in the latter crosses (72% hatch), similar to that observed in crosses of the corresponding infected strains. Thus, the *Wolbachia* infection in *Ae. polynesiensis* induces strong unidirectional CI, resulting in a significantly lower egg hatch relative to compatible crosses ($P < 0.0001$; Mann–Whitney).

A 45% egg hatch rate was observed with the *Ae. riversi* strain (AR; Table 1). Egg hatch ($P > 0.21$, df = 9; Kruskal–Wallis) and fecundity ($P > 0.13$, df = 9; Kruskal–Wallis) were not different from that observed in the *Ae. polynesiensis* strains. Crosses between aposymbiotic ART females and infected AR males were conducted to examine CI levels induced by the AR *Wolbachia* infection. As shown in Table 1, the resulting egg hatch (1%) was significantly lower ($P < 0.012$; Mann–Whitney) than compatible crosses in which both the female and male were uninfected (59% hatch).

**Interspecific Crosses.** Crosses between *Wolbachia*-infected *Ae. polynesiensis* and *Ae. riversi* did not result in any egg hatch (Table 1), confirming that the two species were reproductively isolated. To determine the role of *Wolbachia* in preventing hybridization, aposymbiotic strains of *Ae. polynesiensis* (APAT and APMT) and *Ae. riversi* (ART) were crossed (Table 1). Interspecific crosses of the aposymbiotic strains did result in hybrid offspring, demonstrating that *Wolbachia* infections are at least partially responsible for the reproductive isolation. As shown in Table 1, matings of uninfected *Ae. polynesiensis* females and *Ae. riversi* males resulted in >2% egg hatch. The reciprocal crosses resulted in similar fecundity levels, but a significantly higher egg hatch rate ($P < 0.0011$; Mann–Whitney). Female and male hybrids survived to adult and were maintained for four generations.

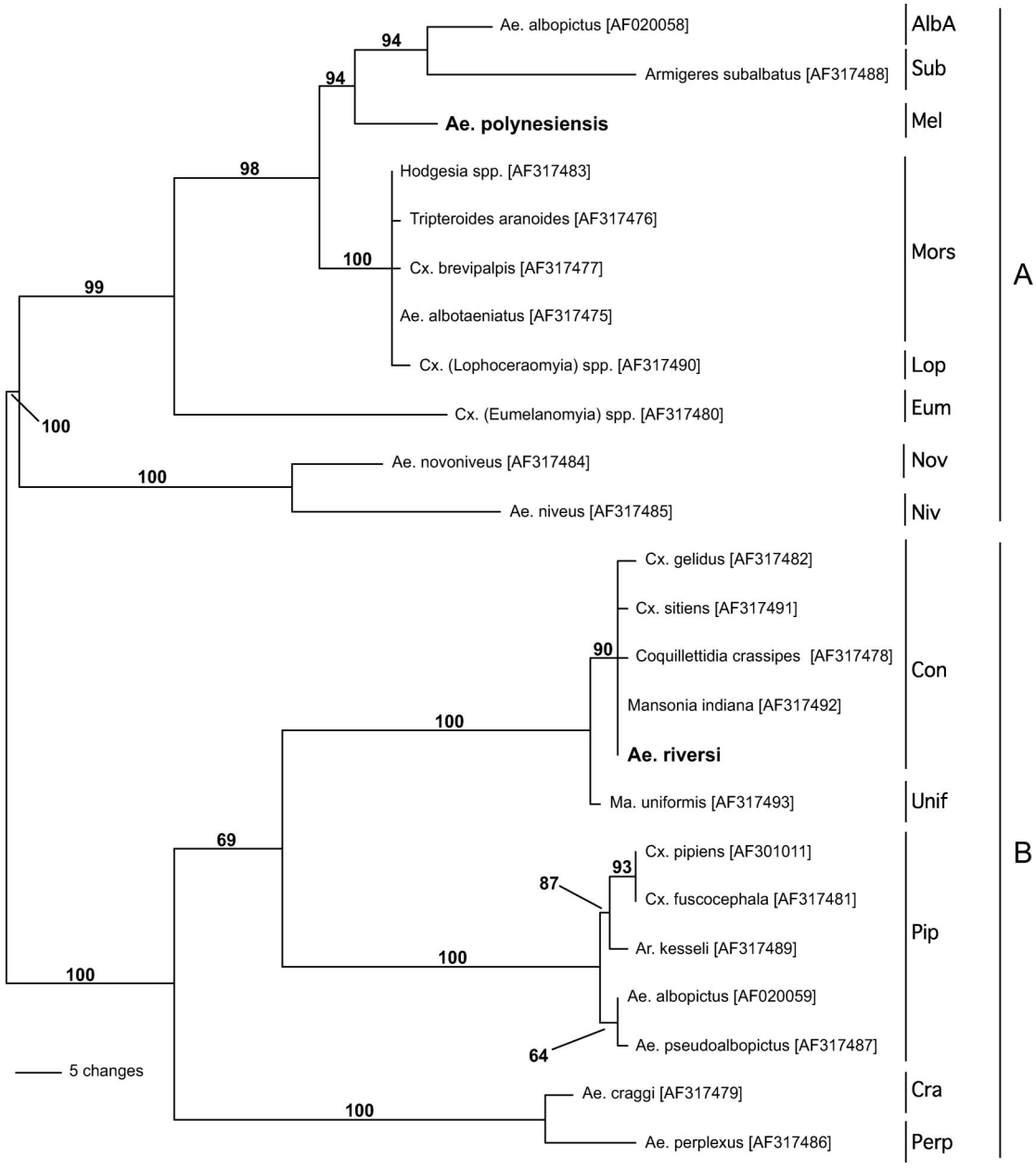


**Fig. 2.** Phylogenetic tree of *Wolbachia* infections in mosquitoes. For comparison, the *Ae. polynesiensis* and *Ae. riversi* infections are shown with other *Wolbachia* infections previously reported in Southeast Asian mosquitoes (brackets indicate the GenBank accession number) (Ruang Areerate et al. 2003). The *Wolbachia* groups and clades are indicated on the right. The tree is midpoint rooted. Branch lengths are scaled according to the number of substitutions (scale shown in lower left). Bootstrap values >50 are labeled on the branches. Tree length, 359; consistency index, 0.7521; retention index, 0.9102. See the text for additional description.

## Discussion

Here, we have identified and characterized the *Wolbachia* types present in *Ae. polynesiensis* and *Ae. riversi.* In addition to demonstrating that the *Wolbachia* infections can cause unidirectional cytoplasmic incompatibility within each of the mosquito species, we have observed that the *Wolbachia* infections can prevent hybridization between the two species. By removing the *Wolbachia* infection, egg hatch was increased from 0% egg hatch in interspecific crosses of naturally infected strains to >47% egg hatch in interspecific crosses of aposymbiotic strains. The latter results are similar to prior studies in which tetracycline clearing of *Wolbachia* from *Ae. polynesiensis* was

**Table 1. Crossing results**

| Intra- and Interspecific crosses of *Wolbachia*-infected strains | | | | |
|---|---|---|---|---|
| | Male type | | | |
| % Egg hatch[a] | APA | APM | API | AR |
| Female type | | | | |
| APA | 70.0 ± 5.4; $n = 8$ | 56.6 ± 17.6; $n = 4$ | 71.1 ± 8.7; $n = 6$ | 0.0 ± 0.0; $n = 3$ |
| APM | 62.8 ± 6.8; $n = 7$ | 62.2 ± 8.4; $n = 6$ | 66.9 ± 5.6; $n = 7$ | 0.0 ± 0.0%; $n = 4$ |
| API | 63.7 ± 6.5; $n = 5$ | 77.3 ± 4.3; $n = 5$ | 76.8 ± 6.0; $n = 7$ | 0.0 ± 0.0; $n = 4$ |
| AR | 0.0 ± 0.0; $n = 3$ | 0.0 ± 0.0; $n = 3$ | 0.0 ± 0.0; $n = 5$ | 45.3 ± 1.2; $n = 4$ |
| Fecundity[b] | | | | |
| Female type | | | | |
| APA | 328.6 ± 52.1; $n = 8$ | 67.0 ± 20.7; $n = 4$ | 279.7 ± 127.9; $n = 6$ | 182.3 ± 100.7; $n = 3$ |
| APM | 264.9 ± 62.3; $n = 7$ | 461.0 ± 95.8; $n = 6$ | 316.3 ± 71.1; $n = 7$ | 199.8 ± 125.7; $n = 4$ |
| API | 210.6 ± 61.0; $n = 5$ | 250.4 ± 69.8; $n = 5$ | 417.9 ± 117.3; $n = 7$ | 251.5 ± 144.8; $n = 4$ |
| AR | 167.0 ± 83.9; $n = 3$ | 83.7 ± 10.5; $n = 3$ | 147.0 ± 17.5; $n = 5$ | 237.8 ± 31.2; $n = 4$ |

| Aposymbiotic crosses | % Egg hatch[a] | Fecundity[b] |
|---|---|---|
| APAT × APAT* | 72.1 ± 11.3; $n = 4$ | 261.5 ± 83.3; $n = 4$ |
| APAT × APA | 0.0 ± 0.0; $n = 4$ | 350.5 ± 40.7; $n = 4$ |
| APMT × APMT | 72.5 ± 3.0; $n = 4$ | 184.8 ± 32.9; $n = 4$ |
| APMT × APM | 0.1 ± 0.1; $n = 5$ | 298.2 ± 94.9; $n = 5$ |
| ART × ART | 59.8 ± 6.5; $n = 6$ | 227.5 ± 28.3; $n = 6$ |
| ART × AR | 1.0 ± 0.6; $n = 3$ | 117.7 ± 17.4; $n = 3$ |
| Interspecific crosses | | |
| APAT × ART* | 0.4 ± 0.4; $n = 3$ | 164.3 ± 54.9; $n = 3$ |
| APMT × ART | 2.4 ± 2.0; $n = 3$ | 130.0 ± 74.7; $n = 3$ |
| ART × APMT | 28.1 ± 6.4; $n = 3$ | 220.7 ± 73.2; $n = 3$ |
| ART × APAT | 47.2 ± 6.8; $n = 7$ | 157.1 ± 19.0; $n = 7$ |

* female × male.
[a] % hatch ± SE; number of crosses.
[b] Egg number ± SE; number of crosses.

shown to increase interspecific hybridization between aposymbiotic *Ae. polynesiensis* with *Aedes kesseli* Huang and Hitchcock, *Aedes cooki* Belkin, *Aedes scutellaris malayensis*, or *Aedes scutellaris katherinensis* Woodhill (Trpis et al. 1981). Research with *Ae. riversi* is more limited, but one study has demonstrated the ability of *Ae. riversi* to hybridize with *Aedes alcasidi* Huang and *Ae. scutellaris* (Toma and Miyagi 1989). Although *Wolbachia* infection was not examined in the prior study, their observed hybridization pattern is consistent with a CI-inducing *Wolbachia* infection in *Ae. riversi.*

Based upon sequencing the *wsp* gene, the *Wolbachia* infection in *Ae. riversi* is indistinguishable from that of *Mansonia indiana* Edwards (GenBank #AF317492), and falls within the *w*Con group of the *Wolbachia* B clade (Zhou et al. 1998). The infection in *Ae. polynesiensis* falls within the *w*Mel group (Wu et al. 2004) of the *Wolbachia* A clade, which was originally described in *Drosophila melanogaster* (Meigen). This is the first report of a *w*Mel group infection in mosquitoes.

Comparison of our results with prior intraspecific crossing results with *Ae. polynesiensis* shows a similar egg hatch resulting from crosses of *Ae. polynesiensis* strains (Sherron and Rai 1983, Meek and Macdonald 1984). However, the CI level previously reported in crosses of aposymbiotic *Ae. polynesiensis* females and *Wolbachia*-infected males is higher (8%) than that observed in this study (≤0.1%). The difference in observed CI levels may be due to an incomplete elimination of *Wolbachia* in the prior study. The larval tetracycline treatment technique used in the prior studies has been shown to result in an incomplete or transient removal of *Wolbachia* from *Ae. albopictus* (Dobson and Rattanadechakul 2001). As stated in the prior studies, the reliance upon direct microscopic observation as an indication of *Wolbachia* infection may fail to detect a weak *Wolbachia* infection (Sherron and Rai 1983, Meek 1984). The PCR technique used here provides a more sensitive *Wolbachia* detection assay. We emphasize that the fecundity and hatch rates reported here do not necessarily reflect the rates occurring in the field. For example, we have not examined insemination of all females used in crosses. Insemination failure would result in an underestimation of egg hatch rate.

It is unclear what role *Wolbachia* has played in the evolution and speciation of *Ae. polynesiensis* and *Ae. riversi* and the role that *Wolbachia* currently plays in restricting natural gene flow between the groups. There is currently much debate about the role of *Wolbachia*-induced cytoplasmic incompatibility in speciation events (Turelli 1994, Hurst and Schilthuizen 1998). Potential examples of *Wolbachia* involvement in speciation events include *Culex* (Rozeboom and Kitzmiller 1958, Laven 1967b), *Nasonia* (Breeuwer and Werren 1995, Werren 1998, Bordenstein et al. 2001, Wade 2001), and *Drosophila* (Shoemaker et al. 1999, Rokas 2000). The current geographic isolation makes it unlikely that *Wolbachia* plays an important role in restricting natural gene flow between the *Ae. polynesiensis* and *Ae. riversi.* Although *Ae. polynesiensis* is relatively widespread throughout Polynesia, the

closest its range approaches that of *Ae. riversi* (southern Japan) is Fiji and the Ellice Islands. Thus, naturally occurring opportunities for hybridization are likely to be rare. To better address the role of *Wolbachia* in the speciation of the *Ae. scutellaris* group, future studies should include examining for additional types of pre- and postmating isolation between *Ae. polynesiensis* and *Ae. riversi*, including the genetic compatibility of the hybrid offspring. Although the hybrids were determined to survive to adult and were fertile, backcrossing experiments are needed to examine for F2 hybrid breakdown. Future experiments should also examine for additional premating or postmating isolation mechanism(s) responsible for the reduced egg hatch that is observed in interspecific crosses of *Wolbachia*-uninfected strains relative to intraspecific crosses.

The results presented here are useful for applied studies that propose manipulating *Wolbachia* infections with the goal of suppressing or modifying medically important vector populations. Specifically, proposed *Wolbachia* strategies targeting both population replacement and population suppression rely on releases of individuals with *Wolbachia* infections that differ from that which naturally occurs in the targeted field population (Sinkins and O'Neill 2000; Dobson et al. 2002a, Dobson 2003b). At present, much effort is focused on developing *Wolbachia* transfection techniques, primarily using embryonic microinjection to generate novel *Wolbachia*–host associations (Boyle et al. 1993, Braig et al. 1994, Rousset et al. 1999, Sasaki and Ishikawa 2000, Poinsot and Mercot 2001, Hartmann et al. 2003). The results presented here suggest a possible alternative to microinjection. Crosses of infected *Ae. riversi* with aposymbiotic *Ae. polynesiensis* males could potentially be used to introgress the *Ae. polynesiensis* genotype with the *Ae. riversi Wolbachia* infection type. This approach would be similar to prior vector control strategies used with *Cx. pipiens* (Laven 1967a).

## Acknowledgments

We thank Osee Sanogo and John Webb for assistance with mosquito rearing. Sequencing was performed at the Advanced Genetics Technologies Center (University of Kentucky) with the support of USDA grant 2002-34457–11844. This research was supported by a grant from the National Institutes of Health (AI51533). This is publication 04-08-018 of the University of Kentucky Agricultural Experiment Station.

## References Cited

**Ali, S. R., and L. E. Rozeboom. 1973.** Comparative laboratory observations on selective mating of *Aedes (Stegomyia) albopictus* Skuse and *A. (S.) polynesiensis* Marks. Mosq. News 33: 23–28.

**Beckett, E. B., B. Boothroyd, and W. W. Macdonald. 1978.** A light and electron microscope study of rickettsia like organisms in the ovaries of mosquitoes of the *A. scutellaris* group. Ann. Trop. Med. Parasitol. 72: 277–283.

**Belkin, N. J. 1962.** Mosquitoes of the South Pacific. University of California Press, Berkeley, CA.

**Bordenstein, S. R., F. P. O'Hara, and J. H. Werren. 2001.** *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia.* Nature (Lond.) 409: 707–710.

**Boyle, L., S. L. O'Neill, H. M. Robertson, and T. L. Karr. 1993.** Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila.* Science (Wash. DC) 260: 1796–1799.

**Braig, H. R., H. Guzman, R. B. Tesh, and S. L. O'Neill. 1994.** Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. Nature (Lond.) 367: 453–455.

**Braig, H. R., W. Zhou, S. L. Dobson, and S. L. O'Neill. 1998.** Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis.* J. Bacteriol. 180: 2373–2378.

**Breeuwer, J.A.J., and J. H. Werren. 1995.** Hybrid breakdown between two haplodiploid species - the role of nuclear and cytoplasmic genes. Evolution 49: 705–717.

**Dobson, S. L. 2003a.** *Wolbachia pipientis:* Impotent by association. *In* K. Bourtzis and T. A. Miller [eds.], Insect symbiosis. CRC LLC, Boca Raton, FL.

**Dobson, S. L. 2003b.** Reversing *Wolbachia*-based population replacement. Trends Parasitol. 19: 128–133.

**Dobson, S. L., and W. Rattanadechakul. 2001.** A novel technique for removing *Wolbachia* infections from *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 38: 844–849.

**Dobson, S. L., C. W. Fox, and F. M. Jiggins. 2002a.** The effect of *Wolbachia*-induced cytoplasmic incompatibility on host population size in natural and manipulated systems. Proc. R. Soc. Lond. Biol. 269: 437–445.

**Dobson, S. L., E. J. Marsland, and W. Rattanadechakul. 2002b.** Mutualistic *Wolbachia* infection in *Aedes albopictus:* accelerating cytoplasmic drive. Genetics 160: 1087–1094.

**Hartmann, N., H. Stuckas, R. Lucius, W. Bleiss, F. Theuring, and B. H. Kalinna. 2003.** Trans-species transfer of *Wolbachia:* microinjection of *Wolbachia* from *Litomosoides sigmodontis* into *Acanthocheilonema viteae.* Parasitology 126: 503–11.

**Hertig, M. 1936.** The Rickettsia. *Wolbachia pipientis* (Gen. et Sp. N.) and associated inclusions in the mosquito, *Culex pipiens.* Parasitology 28: 453–486.

**Hurst, G.D.D., and M. Schilthuizen. 1998.** Selfish genetic elements and speciation. Heredity 80: 2–8.

**Karig, D. E. 1972.** Remnant arcs. Geological Soc. Am. Bull. 83: 1057–1068.

**Laven, H. 1951.** Crossing experiments with *Culex* strains. Evolution 5: 370–375.

**Laven, H. 1967a.** Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature (Lond.) 216: 383–384.

**Laven, H. 1967b.** Speciation and evolution in *Culex pipiens*, pp. 251–275. *In* J. Wright and R. Pal [eds.], Genetics of insect vectors of disease. Elsevier, Amsterdam, The Netherlands.

**Macdonald, W. W. 1976.** Mosquito genetics in relation to filarial infections. *In* A.E.R. Taylor and R. Muller [eds.], Genetic aspects of host-parasite relationships. Blackwell, Oxford, United Kingdom.

**McLain, D. K., K. S. Rai, and P. N. Rao. 1985.** Ethological divergence in allopatry and asymmetrical isolation in the South Pacific *Aedes scutellaris* subgroup. Evolution 39: 998–1008.

**Meek, S. R. 1984.** Occurrence of Rickettsia-like symbionts among species of the *Aedes scutellaris* group Diptera Culicidae. Ann. Trop. Med. Parasitol. 78: 377–382.

**Meek, S. R., and W. W. Macdonald. 1984.** Crossing relationships among seven members of the group of *Aedes scutellaris* (Walker) (Diptera: Culicidae). Bull. Entomol. Res. 74: 65–78.

**Mogi, M. 1976.** Notes on the northern records of *Aedes (Stegomyia) riversi* Bohart and Ingram. Mosq. Syst. 8: 347–352.

**O'Neill, S. L., R. Giordano, A. M. Colbert, T. L. Karr, and H. M. Robertson. 1992.** 16S *rRNA* phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. U.S.A. 89: 2699–2702.

**Otsuka, Y., and H. Takaoka. 1997.** Elimination of *Wolbachia pipientis* from *Aedes albopictus.* Med. Entomol. Zool. 48: 257–260.

**Poinsot, D., and H. Mercot. 2001.** *Wolbachia* injection from usual to naive host in *Drosophila simulans* (Diptera: Drosophilidae). Eur. J. Entomol. 98: 25–30.

**Rokas, A. 2000.** *Wolbachia* as a speciation agent. Trends Ecol. Evol. 15: 44–45.

**Rosen, L., L. E. Rozeboom, W. C. Reeves, J. Saugrain, and D. J. Gubler. 1976.** A field trial of competitive displacement of Aedes polynesiensis by *Aedes albopictus* on a Pacific atoll. Am. J. Trop. Med. Hyg. 25: 906–13.

**Rousset, F., H. R. Braig, and S. L. O'Neill. 1999.** A stable triple *Wolbachia* infection in *Drosophila* with nearly additive incompatibility effects. Heredity 82: 620–627.

**Rozeboom, L. E., and J. B. Kitzmiller. 1958.** Hybridization and speciation in mosquitoes. Annu. Rev. Entomol. 3: 231–248.

**Ruang Areerate, T., P. Kittayapong, V. Baimai, and S. L. O'Neill. 2003.** Molecular phylogeny of *Wolbachia* endosymbionts in Southeast Asian mosquitoes (Diptera: Culicidae) based on *wsp* gene sequences. J. Med. Entomol. 40: 1–5.

**Sasaki, T., and H. Ishikawa. 2000.** Transinfection of *Wolbachia in the Mediterranean flour moth, Ephestia kuehniella,* by embryonic microinjection. Heredity 85: 130–135.

**Sherron, D. A., and K. S. Rai. 1983.** Genetics of Speciation in the *Aedes* (Stegomyia) *scutellaris* group (Diptera: Culicidae). J. Med. Entomol. 20: 520.

**Shoemaker, D. D., V. Katju, and J. Jaenike. 1999.** *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria.* Evolution 53: 1157–1164.

**Sinkins, S. P., and S. L. O'Neill. 2000.** *Wolbachia* as a vehicle to modify insect populations, pp. 271–287. *In* A. M. Handler and A. A. James [eds.], Insect transgenesis: methods and applications. CRC, Boca Raton, FL.

**Sinkins, S. P., H. R. Braig, and S. L. O'Neill. 1995.** *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. Proc. R. Soc. Lond. (Biol.) 261: 325–330.

**Swofford, D. L. 2002.** PAUP: Phylogenetic analysis using parsimony (and other methods) computer program, version 4.0 Sunderland, MA.

**Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–80.

**Toma, T., and I. Miyagi. 1989.** Reproductive isolation among *Ae. riversi,* Ae. alcasidi and *Ae. scutellaris* of the *Aedes* (*Stegomyia*) *scutellaris* subgroup (Diptera: Culicidae). J. Sanit. Zool. 40: 323–332.

**Trpis, M., J. B. Perrone, M. Reisseg, and K. L. Parker. 1981.** Control of cytoplasmic incompatibility in the *Aedes scutellaris* complex. J. Hered. 72: 313–317.

**Turelli, M. 1994.** Evolution of incompatibility-inducing microbes and their hosts. Evolution 48: 1500–1513.

**Wade, M. J. 2001.** Infectious speciation. Nature (Lond.) 409: 675–677.

**Werren, J. H. 1998.** *Wolbachia* and speciation, pp. 245–260. *In* D. Howard and S. Berlocher [eds.], Endless Forms. Oxford University Press, Oxford, United Kingdom.

**Woodhill, A. R. 1949.** A note on experimental crossing of *Aedes (Stegomyia) scutellaris* Walker and *Aedes (Stegomyia) scutellaris katherinensis* Woodhill (Diptera, Culicidae). Proc. Linn. Soc. N.S.W. 74: 224–226.

**Wright, J. D., and A. R. Barr. 1980.** The ultrastructure and symbiotic relationships of *Wolbachia* of mosquitoes of the *Aedes scutellaris* group. J. Ultrastruct. Res. 72: 52–64.

**Wright, J. D., and A. R. Barr. 1981.** *Wolbachia* and the normal and incompatible eggs of *Aedes polynesiensis* (Diptera: Culicidae). J. Invertebr. Pathol. 38: 409–418.

**Wright, J. D., and B. T. Wang. 1980.** Observations on *Wolbachiae* in mosquitoes. J. Invertebr. Pathol. 35: 200–208.

**Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, et al. 2004.** Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2: 0327–0341.

**Yen, J. H., and A. R. Barr. 1971.** New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature (Lond.) 232: 657–658.

**Zhou, W., F. Rousset, and S. L. O'Neill. 1998.** Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. Biol. 265: 509–515.

*Received 18 February 2004; accepted 13 June 2004.*