

CRAVEN, AUSTIN N., M.S. The Impact of Endoparasitic *Wolbachia* on the Evolution of Reproductive Barriers During Speciation in *Drosophila ananassae* from Southeast Asia and the South Pacific (2013)

Directed by Dr. Malcolm D. Schug. 43pp.

There is still much work to be done in Biology to study and understand the mechanisms that drive the generation of new species. The model organism *Drosophila ananassae* represents an ideal model to untangle these issues. Previous genetic and mate discrimination studies of *D. ananassae* showed evidence that populations in Southeast Asia, and the South Pacific may be at a nascent stage of speciation (Schug *et al.* 2007, 2008). Subsequent preliminary studies demonstrated a potential postmating isolation barrier may exist between Bogor, Indonesia isofemale line 13 (BOG13) and females from Trinity Beach, Australia isofemale line 12 (TB12), which when hybridized and backcrossed to BOG13 females showed a decrease in offspring production. This may reflect a genetic isolation barrier, or alternatively, the effects of infection of one population or the other with endoparasite *Wolbachia* which is known in other organisms to cause postmating reproductive barriers. My study tested the hypothesis that the postmating barrier present between these two populations is driven by *Wolbachia* infection.

I found that TB12 was infected with *Wolbachia* and BOG13 was not. A full reciprocal backcross performed between these isofemale lines, and replicate using a TB12 isofemale line cured of the *Wolbachia* infection, revealed results consistent with *Wolbachia*-induced cytoplasmic incompatibilities (CI), that were removed when cured of the *Wolbachia* infection. A screen of additional strains previously shown to have high

levels of mate discrimination showed the possibility that *Wolbachia* infection may have influenced the evolution of postmating reproductive barriers in additional populations of *D. ananassae* from throughout Southeast Asia and South Pacific. However, my mate discrimination experiments using infected versus cured isofemale lines from Bogor, Indonesia and Trinity Beach, Australia indicated that it is unlikely that *Wolbachia* infections directly influence mate discrimination behaviors, but are likely having an influence on postmating reproduction. Taken together this suggests that *Wolbachia* infections in populations of *D. ananassae* throughout its range in Southeast Asia and the South Pacific may have a significant influence on population divergence and speciation.

THE IMPACT OF ENDOPARASITIC *WOLBACHIA* ON THE EVOLUTION OF
REPRODUCTIVE BARRIERS DURING SPECIATION IN *DROSOPHILA*
ANANASSAE FROM SOUTHEAST ASIA AND THE SOUTH PACIFIC

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

One major goal of evolutionary biologists is to determine the mechanisms driving the generation of new species. The most widely accepted explanation of the genetic mechanisms underlying speciation is the Dobzhansky and Muller model, which proposes that when populations become sexually isolated accumulation of genetic mutations at multiple loci causes incompatibilities that prevent hybrid fertility when the populations are reintroduced to each other (Dobzhansky, 1936; Coyne and Orr 2004). A key component of this model is a period of sexual isolation when genetic incompatibilities accumulate. Current research in evolutionary biology has therefore focused on aspects of an organism's environment, physiology, and behavior that cause the necessary sexual isolation that leads to speciation (Coyne 1994, Coyne and Orr 2004).

The process of species formation

The most widely accepted model describing the process of speciation (reviewed in Coyne and Orr 2004) involves a period of time during which populations are geographically isolated while pre-mating reproductive barriers evolve that prevent mating events between populations when they experience secondary contact. Dobzhansky/Muller incompatibilities may also evolve during this time that cause genetic incompatibilities in hybrid offspring between populations. If these incompatibilities hamper the formation of the zygote, they may be considered post-mating prezygotic isolation mechanisms, and if

they occur within the hybrid zygote, either lowering fitness or fecundity, they are classified as postmating postzygotic isolation. It has also been shown that in some cases, premating isolation barriers may evolve, and during secondary contact, the reduced viability of hybrid offspring between populations drives or reinforces the divergence and may cause speciation (Noor 1999). Furthermore, theory suggests that the interaction between pre- and postmating isolation barriers may strengthen each others' effects (Servedio and Saetre 2003). Interestingly, mechanisms other than genetic incompatibilities may also be involved in hybrid incompatibilities between populations that are separated for some time and then experience secondary contact. The most commonly observed mechanism in many arthropod species is cytoplasmic incompatibility effects caused by the arthropod endoparasite *Wolbachia*.

D. ananassae as a model for speciation research

D. ananassae has traditionally been used as an alternative to *D. melanogaster* for testing predictions about the influence of natural selection on genetic variation in structured populations (Tobari 1993). This organism inhabits an extensive tropical and subtropical geographic range with a species center found on Java Indonesia (Vogl *et al.* 2003, Schug *et al.* 2007). Previous genetic analysis of Southeast Asian populations has shown structured populations along geographic clines, which is suggestive of local adaptation in these populations (Chen *et al.* 2000, Schug *et al.* 2007, 2008). There is also evidence of a strong level of population sub-structuring in the South Pacific island populations (Johnson *et al.* 1966, Schug *et al.* 2007, 2008).

Previous studies of *D. ananassae* showed evidence of possible speciation events between populations in Southeast Asia and the South Pacific. In some cases, genetic differentiation is higher between some *D. ananassae* populations than the genetic differentiation between *D. ananassae* populations and its sister species, *D. pallidosa* (Schug *et al.* 2007). This shows that there is limited gene flow between these populations, and possible reproductive isolation barriers other than geographic distance. Schug *et al.* 2008 demonstrated high levels of mate discrimination between populations of *D. ananassae*, particularly between Indonesia, Australia, and South Pacific Islands and argued that the levels of mate discrimination are consistent with these populations being nascent species. A definitive test of the species status among these populations requires a test for the presence of postmating isolation barriers to determine if hybridization is possible, and if so, if hybrids are viable.

Preliminary studies in which females from these populations were mated in no-choice situations demonstrated that populations that exhibit pre-mating reproductive barriers also show reduced hybrid fitness (Schug pers. comm.). Since it appears that the populations have evolved in isolation for significant time, the evolution of postzygotic barriers may represent Dobzhansky-Mueller genetic incompatibilities or non-genetic effects such as differential infection among populations by *Wolbachia*. There is reason to believe that *Wolbachia* infection may be integrally involved in the evolution of *D. ananassae* populations because Hotopp *et al.* (2007) found that the *D. ananassae* genome has incorporated the entire genome of the *Wolbachia* from a lateral gene transfer event. Hotopp *et al.* (2007) argued that this event appears to be an ancestral state for this species

implying *D. ananassae* has been historically infected with *Wolbachia* from an ancestral state (Hotopp *et al.* 2007).

Potential affects of Wolbachia on species formation

The potential effects of *Wolbachia* infection on population divergence and speciation in *D. ananassae* populations is an open and important question. *Wolbachia pipientis* *Wolbachia* is well known to have a wider range of effects on host species that may modify is reproductive fitness both within and between populations and closely related species (Werren 2008). It is an obligate endoparasite estimated to infect > 65% of arthropod species (Hilgenboecker *et al.* 2008). The infection is typically spread by vertical transfer, and is inherited from the mother, but there is evidence of rare horizontal transmissions (Werren *et al.* 2008). Due to this form of transmission, *Wolbachia* has evolved several methods of modifying host reproduction. Observed effects include; cytoplasmic incompatibilities (CI) when infected males mate with non-infected females, feminization of genetically male individuals, the killing of male offspring when infected females mate with uninfected males, and parthenogenesis in females (Werren *et al.* 2008). This has led to the investigation of *Wolbachia* as a possible factor in speciation.

Wolbachia infections have been implicated in speciation events in the *Nasonia* species complex. This group consists of three sister species; *N. giraulti*, *N. vitripennis*, and *N. longicornis*. *Nasonia* are a parasitic wasp with a haplo-diploid sex determination, and are infected with CI-inducing *Wolbachia*. Work performed by Breeuwar *et al.* (1990) showed that there were post-mating isolation barriers between *N. giraulti*, and *N.*

vitripennis, as mating events between these two species resulted in non-hybrid male offspring, that were the result of unfertilized eggs. When *Wolbachia* infection was cured hybrid females could be made (Breeuwer 1990). Additional work performed by Bordenstein *et al.* showed post mating isolation barriers between *N. giraulti*, and *N. longricornis* including genetic effects, and *Wolbachia*-induced CI. The results from this study suggest that *Wolbachia*-induced CI effects were evolutionarily older than genetic effects (Bordenstein *et al.* 2001). This information suggests that *Wolbachia* was necessary for the divergence of these species.

Wolbachia infection is known to historically appear in *D. ananssae* populations and could be a candidate for causing the observed postzygotic barriers. Evidence of *Wolbachia*-induced postmating reproductive barriers is common in many arthropods. For example, Vala *et al.* observed increased mortality in offspring of spider mite populations differentially infected with *Wolbachia* (Vala *et al.* 2000). This increase in mortality was alleviated when the crosses were repeated using tetracycline cured populations. This indicates that a partial reproductive barrier caused by *Wolbachia* exists between these differentially infected populations. The postmating barrier created by the CI effects have also been documented to lead to premating barriers through reinforcement, where postmating barriers lead to premating barriers through the natural selection of mating behaviors that avoid unfit hybrids (Werren *et al.* 2008, Zouros *et al.* 1980).

Theoretical support of Wolbachia-induced speciation

One theoretical study by Telschow *et al.* (2007) demonstrated that *Wolbachia* may drive the evolution of premating isolation barriers when certain criteria are met. Their model assumes a mainland island model of migration, with an initially uninfected island, and an infected mainland, and a locally adapted gene that acts as a cue for female mate preference that is divergent between the populations. A second gene locus for female mate preference was included and allowed to diverge after the beginning of the simulation. This model demonstrated that with a sufficiently low migration rate from mainland to island, and either incomplete *Wolbachia* transmission or a reduction in female fecundity due to infection, the infection polymorphism between populations is maintained. Furthermore, presence of *Wolbachia*-induced CI effects were shown to select for premating isolation in the island population. The chances of premating isolation evolving in the island population were increased if CI effects were intermediate, and if female fecundity is decreased by infection, as these help maintain a low level of infection in the island population. Since we know that limited migration is a feature among *D. ananassae* populations, and the population distribution and history is consistent with a mainland-island model, it is possible that the theoretical predictions in the Telschow *et al.* (2007) model may apply to the populations of *D. ananassae* studied by Schug *et al.* (2008) for which both genetic divergence and mate discrimination were prominent features. Furthermore, there is additional empirical evidence that *Wolbachia*-induced CI will cause reinforcement for uninfected populations found in sympatry with infected

populations. In addition to causing premating barriers for the uninfected population towards the infected population, the uninfected populations also developed premating barriers towards uninfected allopatric populations which indicates that natural selection has acted upon the willingness to mate with outside populations (Jaenike *et al.* 2006).

I propose to test the hypothesis that *Wolbachia* is involved in the evolution of postzygotic isolation between populations of *Drosophila ananassae* from Asia and the South Pacific. Furthermore, I will test the potential effect of *Wolbachia* infections on mate discrimination predicted by the Telschow *et al.* (2007) model. If premating isolation barriers are observed, they may be caused by the detection of *Wolbachia* infection cues by the uninfected population, as this has been observed in other *Drosophila* species (Gazla 2011). I will thus test the prediction that mate discrimination (pre mating barrier) observed by Schug *et al.* is correlated with the infection status of populations from Bogor, Indonesia, Trinity Beach, Australia, Thursday Island, Australia, and Apia, Samoa.

Goal of this study

A survey of potential reductions in hybrid viability between population crosses between *D. ananassae* populations from Indonesia, Australia, and the South Pacific demonstrated that Bogor, Indonesia (BOG) and Trinity Beach, Australia (TB) show reduced viability consistent with traditional Dobzhansky-Muller effects (Schug, personal communication.). Due to the inferred historic relationship between *Wolbachia* and *D. ananssae* (Hotopp *et al.* 2007) it is likely that at least some of the populations assayed for mate discrimination by Schug *et al.* (2008) are infected with *Wolbachia*. Therefore I will

focus on the BOG and TB populations and assay for *Wolbachia* infection to determine if *Wolbachia*-induced CI effects are the causative agent in any observed reduction in hybrid viability. As there is theoretical evidence that *Wolbachia* is capable of inducing premating isolation in the form of mate discrimination, I will determine the infection status of populations of *D. ananassae* whose mate discrimination has already been assayed. I will use isofemale lines from previous studies performed by Killon-Atwood (2007), and Schug *et al.* (2008) to determine if distribution of *Wolbachia* in these populations is consistent with the observed patterns of mate discrimination. Finally I will perform mate-discrimination assays between the BOG and TB populations to determine if it is a function of *Wolbachia* infection status.

Specific Aims

1) Determine if differences in Wolbachia infection between populations cause a decrease in offspring number in crosses between populations that show partial or full hybrid inviability.

I predict that if *Wolbachia* infection is causing the decrease in offspring count from backcrosses between the TB12 and BOG13 isofemale lines then *Wolbachia* will be present in only one of these populations.

If one of the cultures is infected, as predicted, I will create a replicate culture and treat it with tetracycline to eliminate the *Wolbachia* infection. If cured I expect to see a recovery of hybrid fitness consistent with intra-population crosses. To prevent any

confounding factors from the removal of natural flora or toxic effects from the treatment cured isofemale lines will be propagated for 3 generations prior to use in any cross. To control for beneficial antibiotic treatment effects I will also produce a reinfected isofemale line by breeding a cured TB12 male to a native (infected) female, as there is preliminary PCR evidence of infection in TB12. This will incorporate *Wolbachia* into this line while retaining the history of a cure treatment. If *Wolbachia* is causing the hybrid inviability then I expect to see a recovery of intrapopulation offspring count when using a cured isofemale line to generate the hybrids. I will also expect to see the same effects when using the reinfected population as with the native TB12 isofemale line. If these results are not obtained then the observed hybrid inviability between TB12 and BOG13 cannot be attributed to *Wolbachia*. Therefore genetic approaches would be required to determine the cause of the hybrid inviability.

2) *Determine if previous mate discrimination in D. ananassae is associated with Wolbachia-induced CI effects.*

I predict that if *Wolbachia* is causing premating reproductive barriers in *Drosophila ananassae* then an association will be observed between crosses involving infected males and un-infected females, and high levels of asymmetric mate discrimination.

I will perform PCR on samples from the mate discrimination assay performed by Schug *et al.* (2008) to determine *Wolbachia* infection status of these populations. The

infection status will be compared to the mate discrimination data collected on these populations to determine if there is an association between the occurrence of mate discrimination, asymmetric mate discrimination, and mating across infection status. If an association between mate discrimination and *Wolbachia* infection status is observed, or if asymmetrical mate discrimination and *Wolbachia* infection status is associated then this would indicate that *Wolbachia* may be responsible for mate discrimination in populations of *D. ananassae*. If there is no correlation then either *Wolbachia* does not influence mate discrimination, or there was an insufficient sample size to detect effects.

3) *Determine if Wolbachia infection leads to premating reproductive isolation with noninfected populations.*

I predict that if *Wolbachia* is causing premating isolation barriers in *Drosophila ananassae* then number of copulation events will be decreased in comparison to intrapopulation mating events. I also expect to see an increase in male courtship time as noninfected females should be more reluctant to mate with infected males.

No-choice mating assays will be performed with BOG,13 TB12, TB12 cured, and TB12 reinfected isofemale lines to determine if *Wolbachia* can cause mate discrimination in *D. ananassae*. The use of the cured and reinfected isofemale lines in this cross will show if the flies are capable of detecting *Wolbachia* infection status of potential mates and are able to modify their behavior accordingly. If the flies are capable of detecting *Wolbachia* infection, and there is premating isolation due to *Wolbachia* then I expect to

observe an increase in mate discrimination for the native and reinfected TB12 isofemale lines, and a recovery of intrapopulation discrimination for the cured isofemale line. If there is no increase in discrimination between BOG13 and the native TB12 isofemale line then there is no evidence of *Wolbachia*-induced mate discrimination. If the cured TB12 isofemale line experiments do not show a recovery of intrapopulation discrimination then it is unlikely I will be able to determine the cause of the discrimination but it will be clear that the females are not detecting *Wolbachia* and modifying their behavior.

CHAPTER II

MATERIALS AND METHODS

Stock samples

Isofemale lines from native populations have been formed by capturing wild females and rearing them in lab on molasses/yeast/agar media with tegero-sept for fungal control as described by Schug *et al.* (2007, 2008). Lines were stored in a 25°C incubator with constant moisture and a 12:12 hour dark/light cycle. Preserved F1 progeny from the initial females were suspended in ethanol and stored at -80°C. Samples that were cured of *Wolbachia* infection were treated with tetracycline (TET) in their food for three generations. The concentration of TET were as follows; 0.25 g/L for the first generation, 0.8 g/L for the second, and 1g/L for the third. The isofemale lines were allowed to recover from TET effects for three additional generations prior to use in experiments. *Wolbachia* absence was confirmed by PCR using controls. Re-infected populations were created by breeding a native infected female with a cured male and the presence of *Wolbachia* infection in progeny was confirmed by PCR. This generated an isofemale line of flies with TB12 genomes that have a history of antibiotic treatment and *Wolbachia* infection.

PCR assay for Wolbachia Infection

DNA was extracted from whole flies by grinding living or preserved flies in 100ul of cell lysis solution (Qiagen Puregene core kit A), and incubating at 65°C for 15 min. proteinase-k was then added to degrade proteins, and the samples incubated at 45°C for 1 hr for fresh samples or overnight for preserved samples. The liquid solution was then transferred to a new 1.5 ml microfuge tube to minimize large particles in the final solution. An initial DNA precipitation step was performed by adding 300 uL of isopropyl alcohol and centrifuged for 10 min at 14k rpm. The supernatant was then removed and 100uL of 70% ethanol were added. The samples were then vortexed and centrifuged again at 14k rpm for 10 min. The ethanol supernatant was then removed and the samples were allowed to air dry for 30 min prior to being re-suspended in 1x TE buffer. *Wolbachia* specific PCR primers were designed according to Simões *et al.* (2011) and amplified using an initial denaturation step at 72°C for 5 min, followed by 40 cycles of 95°C for 1 min, 59°C for 35 s, 72°C for 2.5 min, and a final step of 72°C for 5 min. PCR product separated on a 1.5% agarose gel and stained with ethidium bromide. PCR primers targeted a 436bp region of the *Wolbachia* 16s ribosomal DNA. Primer sequences can be found in Table 1. *A. D. recens* isofemale line infected with *Wolbachia* was used as a positive control for *Wolbachia* (provided by Kelly Dyer, University of Georgia).

Wolbachia Cure Full Reciprocal Backcross

Previous studies in the Schug lab (Schug pers. comm.) showed a decrease in offspring count when hybrids formed by TB females crossed to BOG males were backcrossed to BOG females. To determine if *Wolbachia* is the causative agent of this effect, I performed 10 replicates of a full reciprocal back-cross using the native TB isofemale lines, cured TB isofemale lines and iso-breeding crosses for each population where iso-breeding lines represent intrapopulation crosses as controls. A full reciprocal backcross entails taking females from one population and males from another to generate a hybrid class. The reciprocal of that cross is then performed to generate another hybrid class. Both of these hybrid classes are then backcrossed to all gender combinations in the initial parent population. Virgin females were collected every 24 hours and maintained for 3 days to assure virginity. Flies were then allowed to mate for 7 days with 1 female and 1 male per vial. After 7 days, parents were removed and stored in 70% ethanol at -80°C. Offspring counts were recorded 3 times during a 10 day period.

Re-infected and Cured Backcross

Backcrosses using hybrids generated with TB 12 females and Bog13 males backcrossed to BOG13 females were performed using TB native, TB cured, or TB re-infected females. This cross was selected because it is the initial cross that fecundity loss was observed with. These crosses were replicated 10 times. Virgin female and data collection were performed as described previously.

Wolbachia F1 Crosses

Since *Wolbachia*-induced CI effects are typically documented to reduce the offspring count in the F1 generation of crosses it was important to assay potential F1 generation effects to determine if *Wolbachia* is causing reproductive barriers (Gazla 2011, Jaenike *et al.* 2006, Rousset and Solignac 1995). I thus crossed TB males and BOG females and additionally crossed cured and re-infected TB isofemale lines. I also crossed individuals within each population/treatment as controls for comparison to hybrid crosses. Virgin female and data collection were performed as described previously.

Association with mate choice experiments

Population samples from throughout the species geographic range in Southeast Asia and the South Pacific that were assayed for mate-discrimination by Schug *et al.* (2008) were assayed for infection status. PCR analysis was performed on F1 progeny samples from nature preserved in EtOH at -80° C for each collection site. Infection status was compared to mate discrimination data collected by Schug *et al.* (2008) to determine if there is an association between mate discrimination and *Wolbachia* infection status.

No-choice Mate Experiments

To assay premating reproductive barriers between TB and BOG a no-choice mate experiment was performed as described by Castrezana *et al.* (2008). Female *D. ananassae* were introduced to a single male in vial with molasses/cornmeal fly media without using anesthesia on the female. Preliminary trials showed the optimal conditions

for copulation involved starting experiments within 30 min after first light in the morning, performing the crosses at approximately 70% humidity, maintaining temperature at 28°C, isolating individual males prior to the experiment, using 3 day-old males, and using 5 day-old females. Time was recorded from initial introduction of the female to the beginning of male courting (male song, male contact with female reproductive organs, and attempts at copulation). This male lag time will act as an index of male mate discrimination. Time between male courting and female acceptance of copulation was recorded to assay female discrimination, and will be referred to as female lag time. Twenty replicate no-choice trials were performed for BOG X BOG, TB X TB, BOG females X TB males, and BOG females X cured TB males.

Statistical Analysis

All statistical analysis was performed in R version 2.14.1. Additional packages used for analysis included STATS, and LME4.

CHAPTER III

RESULTS

Tests for viability effects of Wolbachia

My assay for *Wolbachia* infection using PCR to detect *Wolbachia* 16s ribosomal DNA showed that the TB12 isofemale line is infected with *Wolbachia* and the BOG13 isofemale line is not. Multiple individuals were screened but a sample gel with only sample from each population is shown in Figure 1.

I performed a full reciprocal backcross of TB12 and BOG13 hybrids to test for reductions in viability, which could be caused by either Dobzhansky/Muller genetic interactions, or *Wolbachia*-induced CI. Crosses within the BOG13 and TB12 isofemale line populations were performed under identical conditions, and on the same dates as the backcross to act as intrapopulation controls.

I found a reduction in viability (number of offspring produced) for male TB12/BOG13 hybrid offspring backcrossed to BOG13 females. For the full reciprocal backcross of hybrids to both males and females of each parental isofemale line, there was an overall significant variation in viability using the aov function in R (Table 2, $F = 3.314$, $P < 0.001$). Using the TukeyHSD function in R, a posthoc Tukey-Kramer test revealed a significant decrease in the number of offspring produced for the backcross to BOG13 females using male hybrids from TB12 females crossed to BOG13 males (mean

= 13.5, se = 5.62) relative to the TB12 intrapopulation control crosses (mean = 46, se = 2.61, mean difference = 32.5, 95% CI 6.48 – 58.52, TK test, $P < 0.05$), and for the backcross to TB12 males using the female hybrid from TB12 males crossed to BOG13 females (mean = 18, se = 4.04) relative to TB12 intrapopulation control crosses (mean = 46, se = 2.61, mean difference = 28.0, 95% CI 1.98 – 54.02, TK test, $P < 0.05$).

To confirm that the reduced viability observed is a function of *Wolbachia* infection rather than a genetic effect that may reflect incipient speciation, I performed the same full reciprocal backcross using a TB12 isofemale line for which *Wolbachia* had been eliminated by treatment with tetracycline (TB cured). Using the aov function I found no significant variation between crosses when using TB12 cured isofemale lines (Table 3, $F = 1.49$, $P = 0.154$). Figure 2 shows the plot of means for the offspring count of the backcross using native (infected) and cured TB flies.

I performed an ANOVA using the lm function in R to compare the two crosses with predicted CI effects against other crosses. Predicted CI effect crosses include, [BOG male x TB female] male x BOG female, and [TB male x BOG female] female x TB male. There was a significant decrease in hybrid fecundity for crosses predicted to experience CI (mean = 13.45) compared to other crosses (mean = 35.5) when these crosses were performed with infected TB isofemale lines (Table 4, $F = 20.41$, $P < 0.001$). This decrease in fecundity was not observed when the crosses were performed with the cured TB12 isofemale line (Table 5 $F = 0.13$, $P = 0.719$). Using the lm function in R, I found that crosses that were predicted to show CI (native infected TB12) also showed a significant decrease in offspring production relative to offspring production using cured

(uninfected) TB12 in the same cross (Table 6, decreased from 42.8 to 15.75, $F = 18.13$, $P < 0.001$).

Re-infected and Cured Backcross experiments

To confirm that the effect of reduced viability observed in backcrosses of hybrids to BOG13 females was due to *Wolbachia* curing and not the tetracycline treatment, I re-infected the TB12 isofemale line that had been cured of *Wolbachia* with the tetracycline treatment, and performed a backcross to BOG13 females using hybrids formed by the mating of these TB12 re-infected females to BOG13 males, the same cross in which the reductions in fecundity were first observed in preliminary data. As a control, I performed the same experiment with infected TB12 females, tetracycline cured TB12 females, and intrapopulation control crosses under identical conditions, and on the same dates as the backcross. Using the aov function in R, I found a significant variation in offspring number across all crosses (Table 7, $F = 13.96$, $P < 0.001$). This effect was due to a reduced viability of the TB12 native isofemale lines and TB re-infected isofemale lines relative to the controls (Figure 3, Tukey-Kramer $P < 0.01$). This result was found with the TukeyHSD function from R.

These results support my hypothesis that *Wolbachia*-induced CI is causing this decrease in offspring for the specific cross. They indicate that the difference between the offspring counts for the cured and native isofemale lines is not due to antibiotic treatment of the cured TB population.

Wolbachia F1 Cross

To confirm the presence of *Wolbachia*-induced CI effects in the F1 generation of offspring between BOG and TB mating events, I performed a cross between BOG and TB populations and recorded the number of offspring. This also removes any genetic effects that would decrease hybrid fecundity from the experiment, and decrease the likelihood of type one error. These crosses were repeated using the cured and re-infected TB populations. Intrapopulation control crosses were performed at the same time in the same conditions to provide a point of comparison.

I performed an ANOVA using the `aov` function in R and found a significant variation in offspring counts of groups (Table 8, $F = 7.108$, $P < 0.001$). A Tukey-Kramer test was then performed using the `TukeyHSD` function in R to determine which crosses show significant difference from intrapopulation control crosses. I found a significant decrease for crosses using TB native isofemale lines, and TB re-infected isofemale lines when compared to re-infected and cured controls (Figure 4, $P < 0.01$). TB cured isofemale lines did not show a significant difference from controls. Figure 4 shows the plot of means for the offspring count of this cross.

Additional analysis using the `lm` function revealed a significant decrease in fecundity for crosses where CI effects were predicted in comparison to other crosses (Table 9, decreased from 60 to 23.6, $F = 29.96$, $P < 0.001$). These include intercrosses performed with TB native, and TB reinfected.

Geographic distribution of Wolbachia infection in populations that demonstrate mate discrimination

It is clear that *Wolbachia*-induced CI effects are present between populations of *D. ananassae*. No previous assays for viability of hybrids between populations in Southeast Asian, and the South Pacific have shown evidence for reduced viability (Schug, unpublished data). However, it is possible that *Wolbachia* infections are affecting mate discrimination patterns previously studied in these populations (Killon-Atwood 2007, Schug *et al.* 2008). Therefore I assayed individuals from these previously described populations to determine if *Wolbachia* infection is present in these populations, and to determine if its presence is consistent with high levels of mate discrimination.

I used PCR to amplify *Wolbachia* 16s RNA using F1 samples from isofemale lines collected from Apia (Samoa, N=2), Thursday Island (Australia, N=9), Trinity Beach (Australia, N=7), Bogor (Indonesia, N=28). Apia and Thursday Island samples were negative for *Wolbachia*, while samples from Trinity Beach were all positive. The Bogor populations were polymorphic for the infection (N=28, 28.6% infected). These data are summarized in Table 10, with a list of lines assayed in Table 11.

In laboratories *Wolbachia* infection status can easily be identified in infected host species with *Wolbachia* specific PCR primers (Simões *et al.* 2011), but due the incorporation of the *Wolbachia* genome into the *D. ananassae* genome there were concerns about false positive detection of *Wolbachia* in *D. ananassae*. However, preliminary work with primers designed by Simões *et al.* did not show false positives

(Simões *et al.* 2011). It is likely that due to the large number of transposable elements which have been found in the inserted genetic material that the chance of unspecific priming is low (Hotopp *et al.* 2007).

Mate discrimination data (Killon-Atwood 2007, Schug *et al.* 2008) showed that crosses between these populations all demonstrated significant mate discrimination, some of which was asymmetric where females from one population discriminated against males from the other, but not *visa versa* (Table 12). The specific crosses with asymmetric discrimination included TB ♀ x Thursday Island ♂, and Apia ♀ x Bog ♂. The Bog ♀ x TB ♂ cross was found to be statistically suggestive of asymmetric discrimination.

Mating events which follow the pattern of typical CI events include any mating event using a TB male, or a Bog male, as these are the only crosses involving an infected male. Potential CI crosses with asymmetric discrimination in the appropriate direction include Apia ♀ x Bog ♂ and Bog ♀ x TB ♂.

No-choice Mate Experiments

To determine the presence and strength of mate discrimination between the BOG and TB populations no-choice mate discrimination experiments were performed. To determine if these effects are removed with the removal of *Wolbachia* infection these crosses were repeated with the cured TB isofemale line. Intrapopulation control crosses were performed on the same dates and in the same conditions to act as a point of comparison. Both female lag time and male lag time were recorded to analyze mate discrimination from either gender.

Both lag times were analyzed separately. For each dependent variable, and population an ANOVA model was fit using the `lm` function in R, and found no significant differences ($P = \text{N.S.}$). A Tukey Kramer using the pooled variance from each ANOVA model, and the `TukeyHSD` function, was also performed and found no significant differences. Figure 5 shows the plot of means for the male and female lag times for these crosses. This does not support my prediction that *Wolbachia* is causing premating isolation barriers in this population.

CHAPTER IV

DISCUSSION

As predicted by my first Specific Aim, decreases in BOG13/TB12 hybrid offspring count were alleviated when the *Wolbachia* infection was cured from the TB12 population. Native TB12 cross offspring counts were recoverable if the cured TB12 populations were re-infected with *Wolbachia* (Figure 2, Table 2, 3, 4, 5, and 6). These results support my hypothesis that *Wolbachia* is a causative agent in post mating isolation barriers between these two populations. Similar, but weaker, effects were observed in crosses between infected male TB12 flies and non-infected BOG13 females (Figure 4, Table 8 and 9). This shows that *Wolbachia*-induced CI is acting as a reproductive barrier for these populations during the first and second generation of contact. As all of these events were observed in crosses between infected males and non-infected females, it suggests that this post mating isolation barrier is caused by the CI effect of *Wolbachia*.

The *Wolbachia*-induced CI effects between the BOG13 and TB12 populations were less significantly different from intrapopulation control crosses when observed in cross between parental lines, then in crosses using hybrids (Figure 2, 4). This effect could be explained by disadvantageous cryptic genetic interactions in the hybrids. If the TB12 and BOG13 populations have already differentiated enough to allow for some traditional Dobzhansky/Muller interactions to accumulate these could add an additional reduction in offspring count in backcross design experiments.

Telschow *et al* (2007) has described possible effects of different *Wolbachia* infection status between two populations when coming into secondary contact based on a mainland-island model, and *Wolbachia*-induced CI effects between the populations. This work showed given an infected mainland and an initially uninfected island that gene flow to the island is reduced in the presence of CI effects. In addition it showed that given stable infection polymorphism equilibrium in the island population CI effects can select for premating isolation in the island population. These effects were observed to be stronger when the CI effects were intermediate. The levels of CI were observed to be intermediate between BOG13 and TB12 as a significant decrease in fecundity was observed but these crosses were still able to produce offspring (Figures 2, 3, 4). This suggests that it is possible that the Bogor, Indonesia and Trinity Beach, Australia populations may be driven towards speciation by *Wolbachia*-induced CI effects.

The analysis of infection status for the F1 progeny of environmentally collected samples, from the populations used in the 2008 Schug *et al.* study revealed only the Trinity Beach and Bogor populations possess any *Wolbachia* infection. Trinity Beach samples showed the infection at fixation in this population; however the Bogor population was found to be polymorphic for the infection with a frequency of 0.28 (Table 10). The Bogor population represents an oddity because an infection frequency at this level is higher than theoretical equilibrium frequencies given migration from an infected source (Telschow *et al.* 2007). A *Wolbachia* infection should move towards fixation in a population unless it is being maintained by other factors. The two most likely explanations are, either a *Wolbachia* invasion/extinction in the Bogor population, or

subpopulation structure.

There is suggestive evidence for subpopulation structure in Bogor population presented by Schug *et al.* (2007) in which microsatellite analysis of population structure using the program Bayesian Analysis of Population Structure (BAPS) clustered individual genotypes in the Bogor population did not show evidence of admixture. This may potentially represent subpopulations within the Bogor population, which could explain the high *Wolbachia* infection frequency observed in Bogor. If the Bogor population is indeed separated into assortative mating groups the high-level infection frequency may be explained by a non-infected and an infected population living in sympatry in Bogor. Additional research should determine if there is a subpopulation structure in Bogor, and what effects *Wolbachia* has had in maintaining it. Theoretical studies by Flor *et al.* (2006) indicate that such population sub-structuring can be maintained with a mosaic of infected populations. Such populations can closely co-exist if gene flow rates stay below a critical level. This critical level is increased when CI levels are intermediate, as we have observed in *D. ananassae*.

My prediction that previous mate discrimination work performed by Schug *et al.* (2008) would correlate with mating events across *Wolbachia* infection status was not confirmed. The results from Schug *et al.* (2008) showed that significant mate discrimination was present in all populations discussed here (Table 12). If *Wolbachia* were the primary cause of mate discrimination in these populations we would expect to see the highest levels of mate discrimination in crosses using infected males and uninfected females. This was not the case, as highest levels of non-infected female mate

discrimination where against a non-infected population (Thursday Island.) In addition no trend of increased discrimination from uninfected females against infected males is evident.

There is some suggestive evidence that indicates *Wolbachia* may be influencing the mate discrimination in these populations. Theoretical studies have shown that *Wolbachia*-induced mate discrimination (from CI effects) should lead to asymmetric mate discrimination, such that the uninfected population should express a higher degree of discrimination than the infected population. There were two crosses from the Schug *et al.* (2008) mate discrimination experiments that showed asymmetric mate discrimination. If we consider the Bogor population infected then one of these asymmetric crosses (Bog x Apia) is across *Wolbachia* infection. If we consider the Bogor population non-infected then there is suggestive evidence that the Bogor and Trinity Beach populations also show asymmetric mate discrimination. These results, however, must be interpreted considering the possible population substructure or invasion/extinction of *Wolbachia* in the Bogor population. If subpopulation structure is present in the Bogor population then this could explain the low degree of discrimination that this population experienced in all crosses, as there may have been some discrimination between these sub populations (Bogor strains were pooled for the Schug *et al.* (2008) mate discrimination assays.) Additional studies of subpopulation structure in Bogor should be performed to investigate these possibilities.

My prediction that mate discrimination should be observed in no-choice mate assays between BOG13 and TB12 was not supported, nor was there a significant change between crosses using the native and cured TB12 isofemale lines (Figure 5). This result

fails to agree with previous work published by Schug *et al.* (2008). This discrepancy could be due to lack of expertise in no-choice mate experiments with *D. ananassae* as there are no previously published examples of this experimental design, or it may represent a difference reflected in the pooled population choice experiments performed by Schug *et al.* (2008) and the single isofemale line no-choice assay I performed. There is also the possibility with these behavioral assays that discrimination behavior or cue expression is environmentally dependent, and that the environment the study was performed in did not offer the same components. As the strains used in this experiment have been maintained in lab for several years there is also the possibility that over this time these populations have lost the discrimination trait. These effects could possibly be mitigated in future experiments using populations more recently collected from the environment, with a larger sample size.

The evidence provided here indicates that *Wolbachia* infection may be playing an important part in driving speciation in *D. ananassae*. I have presented strong evidence that *Wolbachia* is causing postmating isolation barriers between two populations. My results suggest that theoretical models may need to be refined to include incidences of polymorphic or extinction/reinfection dynamics in populations to determine if premating isolation barriers may evolve. They further suggest that population substructure within specific geographic regions may play an important role in the dynamics of pre- and postmating reproductive isolation. *D. ananassae* is an excellent model organism for these studies because of the clear variability in pre-and post-mating isolation, and differential infection with *Wolbachia* throughout its geographic range.

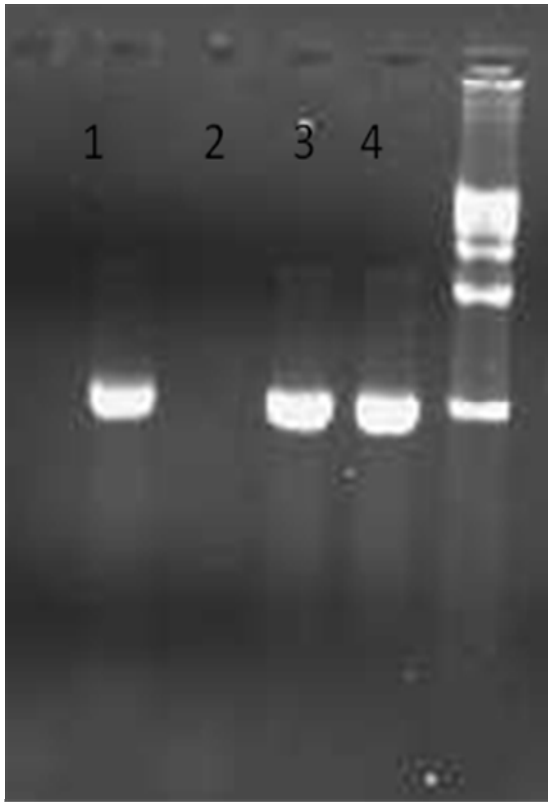


Figure 1. Gel image of PCR results for the *Wolbachia* Detection Assay. Lane 1 shows a positive result for the TB12 isofemale line. Lane 2 shows a negative result for the BOG13 isofemale line. Lanes 3 and 4 show known positives.

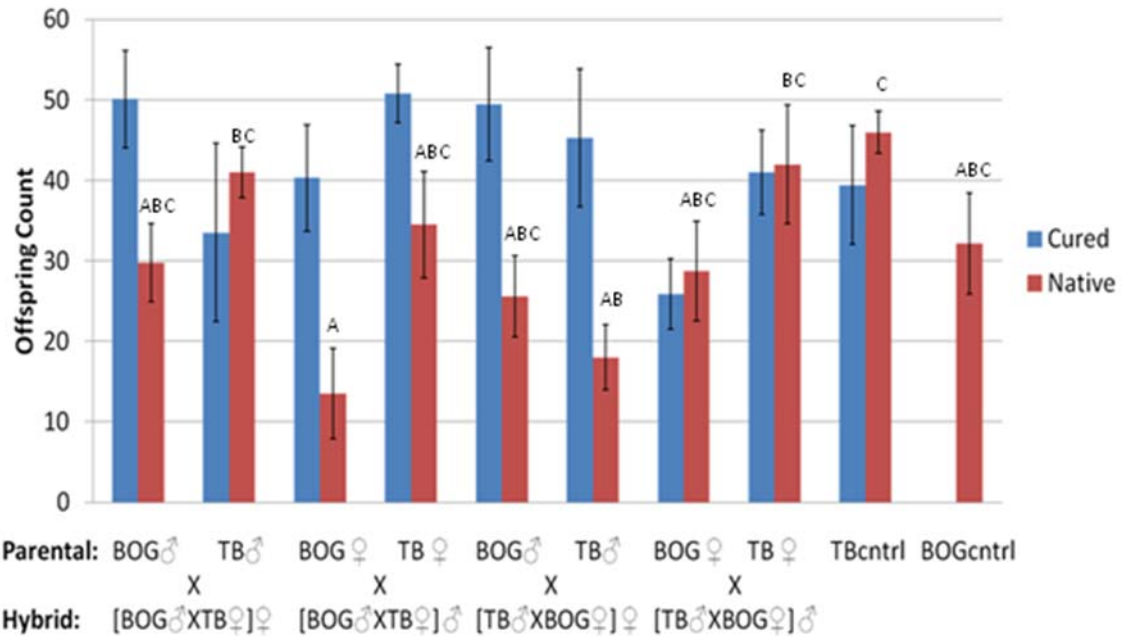


Figure 2. Full Reciprocal Backcross between the BOG13 and TB12 Isofemale Lines. Significance notation shows significant groups according to the Tukey-Kramers comparison of the crosses performed with native TB12 isofemale lines ($P < 0.05$). Error bars represent standard error of the mean. Cured crosses used the cured TB12 isofemale line and native crosses used the TB12 isofemale line. Statistical approaches summarized in Tables 2, 3, 4, 5, and 6.

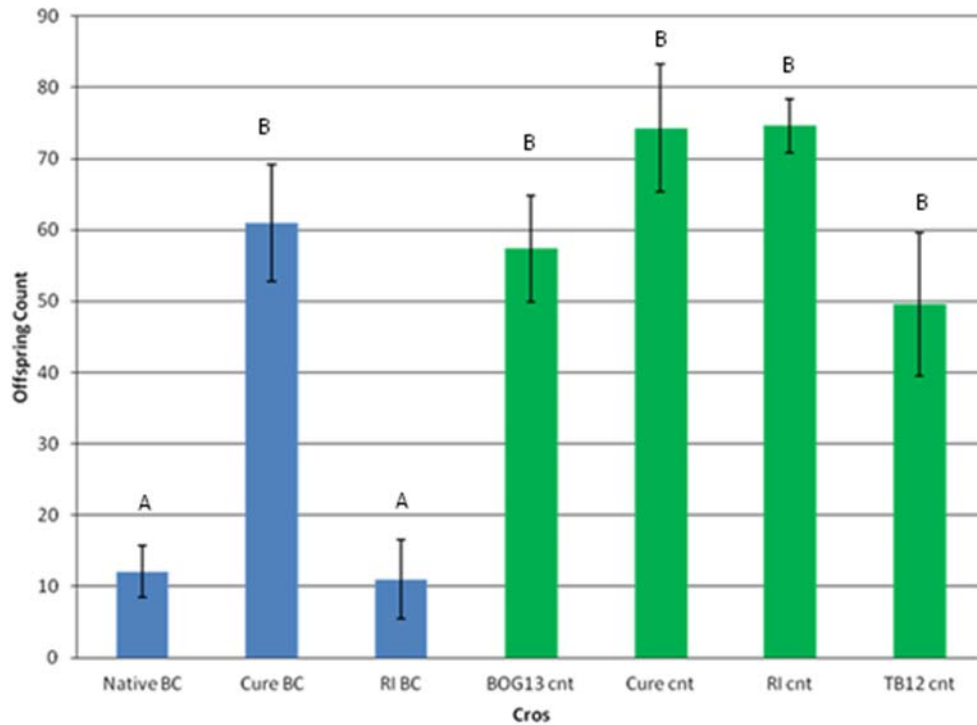


Figure 3. Offspring Count for [TB ♀ /BOG♂] ♂ X BOG♀ with Cured and Re-infected TB Isofemale lines. These hybrids were then backcrossed to BOG13 females. TB12 isofemale lines used include native (infected), cured, and re-infected. Significance notation shows significant groups according to the Tukey-Kramers comparison of the crosses ($P < 0.05$). Statistical approaches summarized in Table 7.

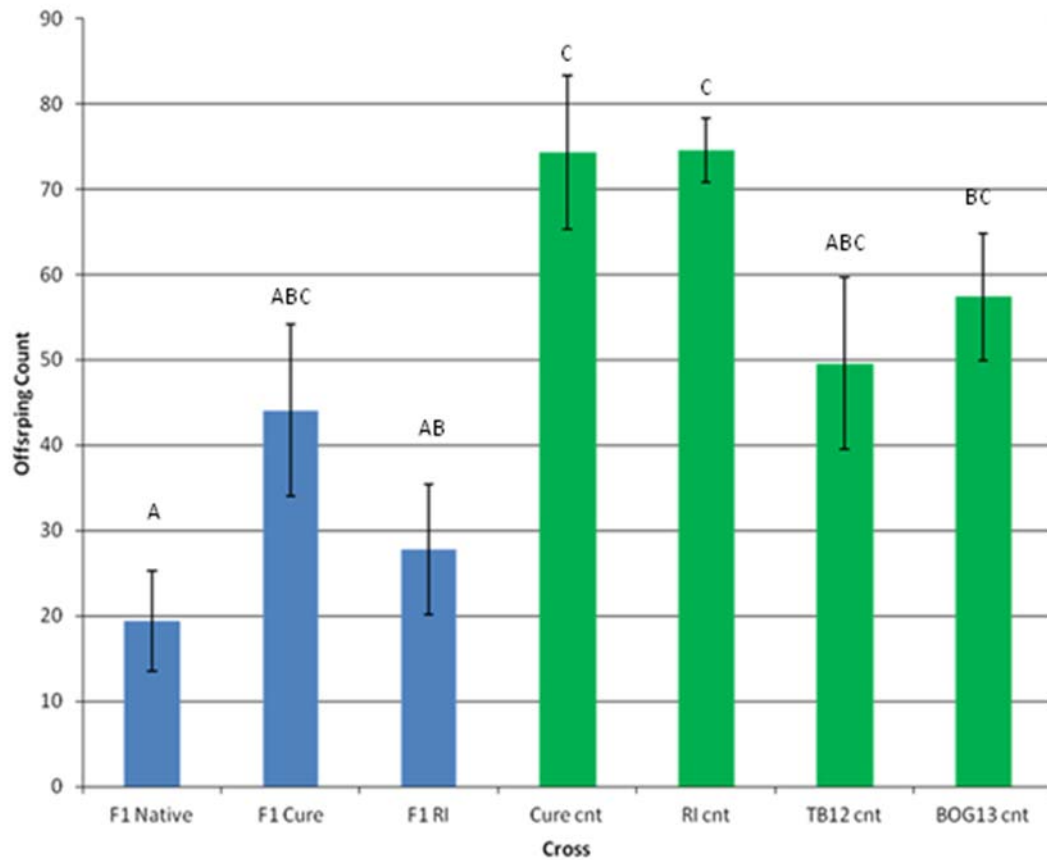


Figure 4. Plot of means of Offspring Count for the F1 crosses using BOG13 females, and TB12 males. TB12 isofemale lines used include TB12 Native, Cured, and Re-infected. Significance notation shows significant groups according to the Tukey-Kramers comparison of the crosses ($P < 0.05$). Statistical approaches summarized in Table 9.

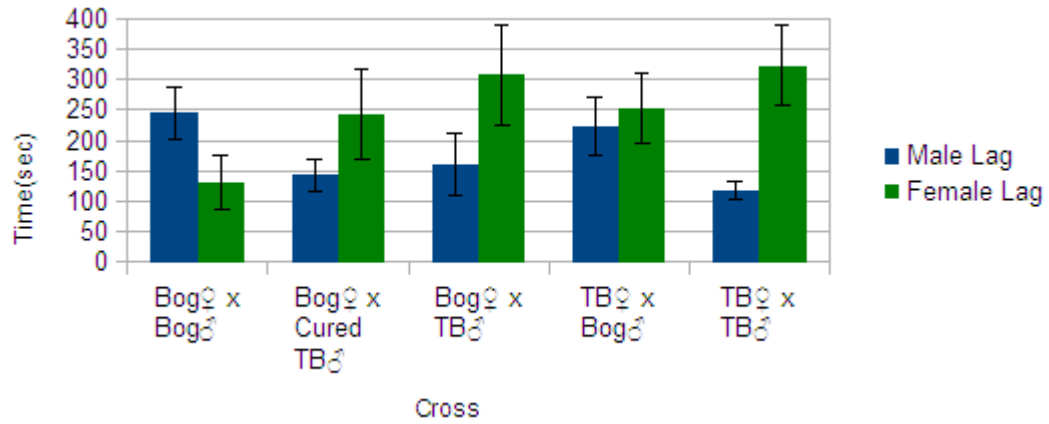


Figure 5. Male and Female Lag times During No-Choice Mate Discrimination Assay. Where lag time for males is measured between introduction and first mate display, and lag times for females is measured between first mate display and acceptance of copulation. No significant differences were detected.

Table 1. DNA Sequence of *Wolbachia* Specific Primers. Primers are displayed from the 5' to the 3' direction. These primers flank a portion of the 16s ribosomal RNA gene of *Wolbachia*.

Direction	Sequence
Forward	CATACCTATTCTGAAGGGATAG
Reverse	AGCTTCGAGTGAAACCAATTC

Table 2. ANOVA Results for Offspring Counts of full Reciprocal Backcross between BOG13 and TB12 native (infected) Isfemale Lines. Significant variation observed between crosses.

	Df	Sum of Squares	Mean Square	F value	P value
Cross	10	10328	1032.8	3.314	0.000958
Residuals	99	30853	311.6		

Table 3. ANOVA Results for Offspring Counts of full Reciprocal Backcross between BOG13 and TB12 cured Isfemale Lines. No significant variation observed between crosses.

	Df	Sum of Squares	Mean Square	F value	P value
Cross	10	6585	658.5	1.49	0.154
Residuals	99	43761	442		

Table 4. ANOVA Results for Offspring Counts of Full Reciprocal Backcross with Native TB12 populations Comparing Predicted CI Crosses to all Others. Predicted CI crosses include [BOG male x TB female]male x BOG female, and [TB male x BOG female]female x TB male. This ANOVA tests if predicted CI crosses show significant variation among non-predicted CI crosses when the TB isofemale line is infected

	DF	Sum of Squares	Mean Square	F value	P value
PRED	1	6361	6361	20.412	1.73e-05
PRED:Cross	9	3967	441	1.414	0.192
Residuals	99	30853	312		

Table 5. ANOVA Results for Offspring Counts of Full Reciprocal Backcross with Cured TB12 populations Comparing Predicted CI Crosses to all others. Predicted CI crosses include [BOG male x TB female]male x BOG female, and [TB male x BOG female]female x TB male. This ANOVA tests if predicted CI crosses show significant variation among non-predicted CI crosses when the TB isofemale line is not infected.

	DF	Sum of Squares	Mean Square	F value	P value
PRED	1	57	57	0.130	0.7193
PRED:Cross	8	6403	800	1.824	0.0812
Residuals	100	43886	439		

Table 6. ANOVA Results for Offspring Counts of Full Reciprocal Backcross Comparing Effect of Infection Status in Crosses with Predicted CI. Predicted CI crosses include [BOG male x TB female]male x BOG female, and [TB male x BOG female]female x TB male. This ANOVA tests if there is a significant variation among cured, and infected for the predicted CI crosses.

	DF	Sum of Squares	Mean Square	F value	P value
CURE	1	7317	7317	18.13	0.00014
Cure:Cross	1	125	125	0.31	0.51217
Residuals					

Table 7. ANOVA Results for Offspring Counts of [TB ♀ /BOG♂] ♂ X BOG♀ with Reinfected Controls. These crosses were performed with TB12 native, TB12cured, and TB12 reinfected isofemale lines. This ANOVA tests if there is any significant variation among type of TB12 isofemale line used.

	Df	Sum of Squares	Mean Square	F value	P value
Cross	6	43147	7191	13.96	5.03e-10
Residuals	63	32460	515		

Table 8. ANOVA Results for Offspring Counts of crosses between BOG13 females and TB12 Males. This cross was performed with native cured and reinfected TB12 populations. This ANOVA tests if there was a significant variation among crosses.

	Df	Sum of Squares	Mean Square	F value	P value
Cross	6	27165	4522	7.108	8.23E-006
Residuals	63	40089	636		

Table 9. ANOVA Results for Offspring Counts of F1 Cross Comparing Predicted CI crosses to all others. Crosses predicted to exhibit CI include those performed with native and reinfected TB12 isofemale lines.

	Df	Sum of Squares	Mean Square	F value	P value
Cross	10	6585	658.5	1.49	0.154
Residuals	99	43761	442		

Table 10. *Wolbachia* Infection Status in Selected Populations of *D. ananassae* in the South Pacific. Numbers in parenthesis are infection frequencies.

Population	Number of Lines S creened	Number of Lines Infected
Apia	2	0
Bogor	28	8 (0.286)
Trinity Beach	7	7(1.0)
Thursday Island	9	0

Table 11. Summary of Isofemale Lines Screened for *Wolbachia* Infection from Populations in the South Pacific.

Population	Lines Infected	Lines Uninfected
Apia		69, 770
Bogor	6, 7, 11, 22, 24, 57, 60, 65	2, 4, 8, 12, 17, 18, 21, 23, 26, 51 52, 53, 58, 59, 61, 62, 63, 64, 68, 160
Trinity Beach	12,18, 24, 29, 39, 42, 43	
Thursday Island		7, 8, 10, 12, 17, 33, 71, 74, 76

Table 12. Summary Results from Schug *et al.* 2008 Mate Discrimination Study. Values represent frequencies of intrapopulation mating events during a multiple mate choice experiment. All crosses showed significant mate discrimination based upon *Ipsi*. Asterisk denote crosses which showed significant asymmetry in mate discrimination. NS, denotes non significant asymmetry after multiple comparison Bonferroni corrections.

Population A	Population B	Population A is Female	Population B is Female
Apia	Bogor	0.67*	0.54
Apia	Trinity Beach	0.71	0.75
Apia	Thursday Island	0.93	0.93
Bogor	Trinity Beach	0.6*(NS)	0.59
Bogor	Thursday Island	0.72	0.72
Trinity Beach	Thursday Island	0.92*	0.85

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