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Evaluation of Fitness and Genetic Variation in *Aphytis melinus* DeBach, an Important Biological Control Agent of *Aonidiella aurantii* (Maskell) in California

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by

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ABSTRACT OF THE DISSERTATION

Evaluation of Fitness and Genetic Variation in *Aphytis melinus* DeBach, an Important Biological Control Agent of *Aonidiella aurantii* (Maskell) in California

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Doctor of Philosophy, Graduate Program in Entomology
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Aphytis melinus DeBach is a parasitoid wasp that is used for biological control of California red scale, *Aonidiella aurantii* (Maskell), in California and other areas of the world. The quality of *A. melinus* reared in five California insectaries was determined by measuring the sex ratio, size, and percentage of individuals still alive on certain sampling days. All five of the insectaries displayed reduced *A. melinus* quality and fitness parameters during certain times of the year. *Wolbachia*, an endosymbiotic bacterium, was also found to cause cytoplasmic incompatibility in *A. melinus* and associated fitness costs were seen in infected individuals based on longevity and fecundity measurements.

Classical biological control has been utilized for decades to help manage pests, but little research has been done on how these introductions alter genetic variation in natural enemies. We developed 10 microsatellites for assessing the genetic variation of and between different populations of *A. melinus*. The original material used to propagate *A. melinus* for mass release came from four relatively small collections in Pakistan and India and we sought to determine how much genetic diversity remains in this potentially

inbred original colony, in comparison with that found in other captive colonies, California field populations, and in populations from the native range of *A. melinus*. Not surprisingly, the field samples from Pakistan had the highest average number of alleles per locus and captive colonies the lowest. A number of unique alleles were also found in both Pakistan and California.

Lastly, a well-known issue in the field of biological control is the lack of accurate identification of pests and natural enemies. We collected *Aphytis lingnanensis* Compere in the University of California, Riverside's campus biological control grove, a species that was previously thought to have been competitively excluded from the inland areas of southern California by *Aphytis melinus*. We also used 28S-D2 and COI sequences to examine the genetic variation and relatedness of *Aphytis* individuals in native, field-released and captive populations as well as in crossing experiments between the captive populations of *A. melinus*.

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Introduction

The parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) is a biological control agent of California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae). California red scale has been considered the number one arthropod pest of citrus in California since its appearance in the state in the 1870's (Comstock 1880, Ebeling 1959, Flint et al. 1991, Morse et al. 2007). From 1889 to the 1950's, at least 35 attempts were made at introducing biological control agents for California red scale control (reviewed in Clausen et al. 1978) but the prospects seemed so bleak that Harold Compere (1961) concluded that biological control would probably never control California red scale in California. However, the introduction of *A. melinus* in 1957 apparently displaced *Aphytis lingnanensis* Compere in inland areas (DeBach and Sundby 1963, Luck and Podoler 1985; but see Vasquez et al. 2010) and by 1962, *A. melinus* had become established as an effective agent of biological control, adding to the biological control exerted by other parasitoids and predators (Luck 2006) to a degree such that chemical control was rarely needed in southern California (Flint et al. 1991, Morse et al. 2007). The original material used to propagate *A. melinus* for mass release came from four relatively small collections in Pakistan and India that were started as four separate cultures inside a quarantine facility (DeBach 1959). All four cultures were determined to interbreed and were combined to form a single quarantine culture, which was mass released in the field during the fall of 1957.

Until the mid 1980s, quality control of natural enemies had rarely been considered or discussed in the scientific literature. Today there are quality control guidelines for some natural enemies, but testing is rare and there are no criteria specifically for *A. melinus* (van Lenteren 2008). The methods commercial insectaries employ to produce *A. melinus* were developed in the late 1950's for *A. lignanensis* (DeBach and White 1960), have changed little since then, and there has been almost no evaluation of quality of the produced insects. In this study, we estimate the quality of *A. melinus* reared in five California insectaries by measuring the sex ratio, size, and percentage of individuals still alive on certain sampling days.

Aphytis spp. are also known to harbor *Wolbachia* infections. *Wolbachia* is a gram-negative obligate intracellular bacterium that is estimated to infect from 16% up to 76% of arthropod species (Werren et al. 1995, Jeyaprakash and Hoy 2000, Hilgenboecker et al. 2008) and is likely to be found in a large percentage of biological control agents (Floate et al. 2006). *Wolbachia* can alter its host's reproduction and sex ratios and is therefore of great interest to pest control practitioners. Although there are conflicting hypotheses on the effect that *Wolbachia* has on its host, with some stating that it is likely to impart a physiological cost in its host (Stouthamer et al. 1999) and others expecting a mutualistic relationship to evolve over time (Werren et al. 2008), evidence of how *Wolbachia* affects fitness has been inconclusive, ranging from harmful to neutral to beneficial.

The most common *Wolbachia*-induced phenotype is cytoplasmic incompatibility (CI). Unlike the other phenotypes, CI can cause reproductive isolation between some

populations. Sperm from an infected male is unable to properly fertilize an egg of an uninfected female or a female that is infected with a different *Wolbachia* strain (Werren et al. 2008). In haplo-diploid species this results in a sex ratio shift towards the haploid (unfertilized) sex, which is usually the male (Bourtzis 2008). In biological control, where the female is the more effective sex in controlling pests, CI can be potentially detrimental to control efforts. Here, we verified that *A. melinus* is infected with a CI-*Wolbachia*, determined whether the CI is partial or complete, and investigated the mechanism that *Wolbachia* uses to skew the sex ratio (affecting male development vs. female mortality). Subsequently, we determined whether this infection confers fitness costs or benefits in *A. melinus* by measuring differences in longevity under ideal nutrition conditions versus nutritional stress, as well as investigating differences in female fecundity in an infected versus a *Wolbachia*-cured strain. Additionally, we completed a survey of five commercial insectaries and 40 field sites in central and southern California to determine *Wolbachia* prevalence and infection frequency in the field. Experiments were performed to see if high temperatures, similar to those found in the Central Valley of California during summer, can cure *A. melinus* of their *Wolbachia* infection, a phenomenon that has been observed in other species (Clancy and Hoffmann 1998, Mouton et al. 2006).

We also used microsatellite markers to determine how much genetic diversity remains in the potentially inbred original *A. melinus* colony (instigated with a total of only 157 females and 43 males), in comparison with that found in other captive colonies, the introduced California field population, and in populations from the native range of *A. melinus* in Pakistan. The processes of collecting subsamples from the native range,

testing and rearing in quarantine, and exponentially increasing population sizes for subsequent release into the field are all steps in which genetic diversity may be lost. If population size is small due to bottlenecks or founder events, genetic drift may cause the loss or fixation of rare alleles (Nei et al. 1975). Reduced genetic diversity may result in inbreeding depression and associated fitness impacts (for example Hufbauer 2002, Spielman and Frankham 1992, Woodworth et al. 2002), and therefore, may alter the effectiveness of biological control agents (Hopper et al. 1993, Roush 1990).

Lastly, upon collection of *A. melinus* for the genetic variation study above, other species of *Aphytis* were found. A well-known issue in the field of biological control is the lack of accurate identification of pests and natural enemies (reviewed in Gordh and Beardsley, 1999). Parasitoid wasps in the genus *Aphytis* Howard are a good example of natural enemies that are important for biological control, but are often misidentified. There are more than 100 species described in the genus *Aphytis* (Kim 2003, Noyes 2003), all of which are tiny (< 1 mm) ectoparasitoids of armored scale insects (Diaspididae) (Rosen and DeBach 1979). Here we used DNA sequences to investigate the taxonomy of the *Aphytis* spp. in the *lingnanensis* group.

Aphytis lingnanensis, previously thought to have been competitively excluded from the inland areas of southern California by *Aphytis melinus*, was collected on the University of California, Riverside's campus biological control grove in 2003. The displacement of *A. lingnanensis* by *A. melinus*, has been well documented in the literature (DeBach and Sundby 1963, DeBach 1966, Luck and Podoler 1985) and is considered one the most famous cases of competitive displacement (Murdoch et al. 1996). Originally, it

was believed that *A. melinus* had better searching abilities and was able to outcompete *A. lingnanesis* in harsher climates (DeBach and Sundby 1963, DeBach 1965, DeBach 1966). A second hypothesis emerged stating that *A. melinus* is able to utilize smaller red scales, especially for female progeny, and thus was able to exclude *A. lingnanensis* by exploiting the host before it reached the ideal size for *A. lingnanensis* parasitism (Luck and Podoler 1985). No accounts of this species have been reported in this area since 1965.

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Chapter 1

Fitness Components of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) Reared in Five California Insectaries

Abstract

Shipments of approximately 50,000 commercially reared *Aphytis melinus* DeBach were obtained from each of the five insectaries that sell this parasitoid to citrus growers in California for control of California red scale, *Aonidiella aurantii* (Maskell). Shipments were received from each insectary every two months over a period of a year in order to assess variability in quality through time and between insectaries. As indices of quality, we assessed the percentage of live parasitoids (both sexes) 1, 3, 7, 14, and 28 days after receipt of the shipment, shipment sex ratio, and the size of female wasps. We found a fair amount of variation in the percentage of *A. melinus* that were alive on different sampling days. Despite the fact that all insectaries rear *A. melinus* in temperature controlled rooms and all of our studies were done at 22ºC, wasp mortality occurred more quickly in the colder months of November, January/February, and March. Similar trends were observed with sex ratios; many of the insectaries had male-biased sex ratios in the colder months, especially January/February. Wasp size varied significantly for each of the insectaries throughout the year, with the summer months of July/August yielding significantly smaller females than other months. Collectively our results have important implications for biological control on citrus in California.

Introduction

California red scale, *Aonidiella aurantii* (Maskell), first appeared in California in the 1870's (Comstock 1880) and has historically been considered the number one arthropod pest of California citrus (Ebeling 1959, Clausen 1978a, Flint et al. 1991, Morse and Luck 2003, Morse et al. 2007). At least 35 attempts were made to import predators and parasitoids for red scale biological control between 1889 and the 1950's (reviewed in Clausen et al. 1978b). In 1961, Harold Compere (1961) concluded that biological control probably would never be able to control this pest in citrus. However, with the introduction of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) into California in 1956-1957 from Pakistan and India, by the early 1960's, good biological control of red scale was achieved in southern California. Although *Aphytis lingnanensis* Compere was already established and controlled red scale in California coastal areas, *A. melinus* proved to be a much more effective biological control agent in the inland coastal valleys (Luck and Podoler 1985). Insectaries began rearing and releasing *A. melinus*, adapting methods previously developed for *A. lingnanensis* (DeBach and White 1960), and by 1962, *A. melinus* had become established as an effective agent of biological control, adding to the biological control exerted by other parasitoids and predators (Luck 2006) to a degree such that chemical control was rarely needed in southern California (Flint et al. 1991, Morse et al. 2007).

Nearly 70% of California's citrus acreage is now located in the San Joaquin Valley (SJV) of California and it was previously believed that biological control of red scale was ineffective in this region due to extreme temperatures in the summer and winter

(Riehl et al. 1980, Morse et al. 2007). This perspective changed following a biologically-based IPM research/demonstration project in Tulare Co. of the SJV run over the period 1987 – 1991 (Haney et al. 1992, Forster and Luck 1997, Luck et al. 1997). The key new component of this IPM program was augmentative release of 247,100 insectary-reared *Aphytis melinus* per ha per year to control red scale (16 releases, one every two weeks mid-Feb. to mid-Sept.), coupled with the management of other arthropod pests using economic treatment thresholds and use of selective pesticides that allowed *A. melinus* to persist.

With the success of the aforementioned demonstration project, the biologically-based IPM program for red scale became quite popular in the SJV, especially as the insect began to exhibit resistance to available organophosphate and carbamate insecticides (Grafton-Cardwell 1994, Grafton-Cardwell et al. 1997, 1998, 2001). However, in 1998, an emergency use exemption was granted allowing use of pyriproxyfen for red scale control. Just prior to the introduction of pyriproxyfen, *A. melinus* use for red scale control peaked with augmentative releases on ca. 30% of SJV groves (Rill et al. 2008). Because of the high efficacy of pyriproxyfen, less than 10% of SJV groves still use augmentative release of wasp parasitoids (Rill et al. 2008).

Despite the recent success of pyriproxyfen, relying solely on chemical control has long-term ramifications. Pesticide resistance is inevitable in a purely chemically-based control program where a single class of chemistry is used repeatedly, requiring growers to apply increased numbers of applications and consequently increasing the cost of chemical control. Biological control, on the other hand, is more sustainable and less

costly in the long run in addition to being safer for consumers and the environment (van Lenteren 2008).

Until the mid 1980s, quality control of natural enemies had rarely been considered or discussed in scientific literature. Today there are quality control guidelines for some natural enemies, but testing is rare and there are no criteria specifically for *A. melinus* (van Lenteren 2008). The methods commercial insectaries employ to produce *A. melinus* were developed in the late 1950's for *A. lignanensis* (DeBach and White 1960), have changed little since then, and there has been almost no evaluation of quality of the produced insects. It is well known that providing large host scales is critical to producing a female-biased sex ratio as well as large and fit *Aphytis* (Luck et al. 1982, Luck and Podoler 1985, Opp and Luck 1986, Walde et al. 1989, Murdock et al. 1992). Parasitoid size and longevity can by improved by manipulating host size and diet regimes (Collier 1995, Heimpel and Rosenheim 1995, Heimpel et al. 1997, Luck and Forster 2003) but insectaries normally attempt to maximize wasp production and seldom, if ever, examine *Aphytis* size, sex ratio, or fitness.

In this study, we estimate the quality of *A. melinus* reared in five California insectaries by measuring the sex ratio, size, and percentage of individuals still alive on certain sampling days. Commercial insectary shipments can vary greatly in their sex ratios and because females are the effective sex in biological control, sex ratio differences can greatly influence the efficacy of biological control efforts (Heimpel and Lundgren 2000). Size was also used as a fitness proxy because it is often positively correlated with the field performance of adult female parasitoids (Kazmer and Luck 1995, Bennett and

Hoffmann 1998, Roitberg et al. 2001, Sagarra et al. 2001). We hypothesize that fitness parameters for *A. melinus* will differ by insectary as well as the time of year the wasps are produced.

Materials and Methods

Sampling. Adult *Aphytis melinus* were ordered from the five California insectaries that reared this insect at the time (Table 1.1). Data were randomly coded (A-E) to maintain anonymity. Each insectary overnight-shipped approximately 50,000 individuals every two months over the period of a year (six total shipments per insectary). *Aphytis* were sent in a paper cup with a plastic lid that had pin-holes for aeration and honey applied to the inner surface for a food source. The entire cup was wrapped in wet newspaper and shipped chilled on ice. Shipment methods were exactly the same as those used to ship to growers. Upon arrival of each shipment at UC Riverside, a hole was punched in the cup lid and *A. melinus* were anesthetized with CO_2 for 30 seconds (see the following section on CO_2 anesthesia). While anesthetized, approximately 150 *A. melinus* were spooned into each of fifteen 50 mm plastic Petri dishes (Fisherbrand, Pittsburgh, PA). Petri dishes were then gently placed into individual wide mouth, pint size Kerr® Mason jars (Jarden Corporation, Rye, NY). Jars were covered with a fine mesh cloth that was streaked with honey. Upon transfer, live wasps were then allowed to move out of the Petri dish and into the jar for 20 minutes. Individuals that did not move from the dish to the jar were presumed dead and were discarded. This procedure ensured that we started our study with only individuals that were alive at the time the shipment arrived at UC

Riverside. For a food source, organic honey was added to each jar every three days by applying thin streaks of honey through the mesh cloth cover of the jar. All studies were carried out in a laboratory at a temperature of approximately 22ºC.

Three jars from each shipment were randomly selected to be destructively sampled 1, 3, 7, 14, and 28 days after the arrival of each shipment. For destructive sampling, *A. melinus* were anesthetized with CO_2 and then killed by saturation with 70% ethanol. These animals were then collected on 70 mm filter paper (Whatman, Piscataway, NJ) using a 186 mL Buchner funnel (Fischerbrand, Pittsburgh, PA) and were immediately counted and sexed. When processing jars on sampling days, individuals that were desiccated were counted as having died sometime between the setup and destruction of the jar and those that were not desiccated were counted as alive. The eyes and abdomen of *A. melinus* begin to desiccate within 4 hours of being killed with ethanol, allowing for accurate determination of whether an individual was living or dead at the time of destructive sampling (C.J.V., personal observation). This method was used to assess the percent alive and dead (those dying between when the shipment arrived and when the destructive sampling was done) as well as sex ratio measurements. Five live females and five dead females (when available) were then randomly selected from each of these samples to measure adult size. In total, 150 size measurements (75 live, 75 dead) per insectary were performed every two months. Size was calculated in 0.01 mm increments with an ocular micrometer by measuring the hind tibia length (HTL). HTL is used here as a proxy for overall adult body size because it can be linearly measured and is resistant to desiccation (Nicol and Mackauer 1999).

CO_2 Anesthesia. There is conflicting evidence on the effects of CO_2 anesthesia on insects (Nicolas and Sillans 1989). Some studies report adverse effects ranging from a decrease in male fertility (Champion de Crespigny and Wedell 2008), reduced longevity (Perron et al. 1972) and fecundity (Dawson 1995), while others report no effect at all (Parkman and Pienkowski 1991, Perrotti and Maroli 1993). We therefore performed our own experiment to test the effect of CO_2 exposure on *A. melinus* longevity prior to collecting data for this study. We exposed adults to a constant pressure of 100% CO_2 for 0 seconds (control), 1.5, 3, 5, 10, and 30 minutes. We found no significant difference in the percentage of *A. melinus* alive three days after exposure (C.J.V., unpublished data) and therefore assumed an anesthesia period of 30 seconds would not adversely affect these animals.

Statistical Analyses. All statistical analyses were performed using SAS® 9.2 software for Windows (SAS Institute 2008). In order to determine the effect of the three key factors (1) shipment date, (2) insectary, and (3) sample day (day of destructive sampling post receipt of the shipment) on size data, we performed a three-way analysis of variance. A logistic regression model (Hosmer and Lemeshow 2000) was used to determine the effects of the explanatory variables (shipment date, insectary, sample day) and their interaction on the percentage measurements that required arc-sine square root transformation (sex ratio and percentage alive). Data were converted to a binary response (i.e. females were coded as 1 and males as 0, live individuals were coded as 1 and dead as 0) and tested with a Wald Chi-square test.

Results

Percentage Alive. Neither the shipment date (time of year) nor the source insectary had a significant effect on the percentage of females (Table 1.2) or males (data not shown) that remained alive on destructive sampling days. Sample day (period of time after receipt of shipment) as a main factor did have a significant effect on the percentage of live females and males. All of the interactions (shipment*insectary, shipment*sample day, insectary*sample day, shipment*insectary*sample day) had a significant effect on the percentage of living females. Table 1.3 shows the average number of females that remained alive for each of the sampling days. Insectaries B and D consistently had the highest female survival rate whereas insectary E had the lowest. Results are represented graphically in Figure 1.1.

Sex Ratio. Sampling day was not included as a main effect in the logistic regression model for sex ratio because the jar replicates were randomly assigned to a sampling day and therefore should not differ in their sex ratio. Both of the main effects, shipment date (Wald χ^2=3191.90, df=5, $p<0.0001$) and insectary (Wald χ^2=1246.46, df=4, $p<0.0001$), and their interaction (Wald χ^2=4643.36, df=20, $p<0.0001$) had significant impacts on sex ratio. Table 1.4 shows the mean proportion females and the differences in sex ratio between shipments and insectaries can be seen in Figure 1.2. All of the insectaries had male-biased sex ratios in shipment 4 (January and February). Although insectary B had a higher total percentage of females (59.1% female), this difference was not significant when averaged over all shipments.

Size. All of the main effects (shipment date, insectary, sample day) and the interaction between shipment date and insectary significantly affected the size of both live (Table 1.5) and dead females (data not shown). Table 1.6 shows the mean live female sizes after combining data from the first two sampling days. These data were chosen because they are the most biologically relevant to growers and the pool of live *A. melinus* individuals was large enough from which to choose truly random samples. When averaged over all shipments, insectary B had the largest females (0.257 mm) while insectary C had the smallest (0.232 mm).

Discussion

Our results suggest that all five of the insectaries display reduced *A. melinus* quality and fitness parameters during certain times of the year. Decreased longevity and male-biased sex ratios were evident in the cooler months of November – March. Smaller females were produced in the warmer months of July – August. Data from insectary B, when averaged over the six shipments, showed the highest percentage females, the largest size of live females, and tied for the highest female survival rate. Variation in longevity, sex ratio, and size persisted despite a relatively constant production environment of approximately 60% relative humidity and 25ºC at all of the insectaries.

One caveat of our study is that we did not evaluate the percentage of *A. melinus* that were alive upon arrival of the shipments, nor the number of wasps that were actually sent. Because we began our experiments with only live wasps, it is unknown what percentage of the remaining wasps were alive or dead. Knowing this would be valuable

to buyers (i.e. commercial citrus growers) because if the majority of the wasps they receive are dead, then it will not matter greatly how fit the few remaining wasps. Another limitation to this study is the lack of field performance measures, which may or may not correlate with laboratory results. Flight tests would be particularly relevant because dispersal ability can deteriorate in mass rearing conditions as well as the preparation and shipment of these fragile wasps. Identifying an easily measured parameter in the laboratory that predicts field success would be ideal for producers and growers (van Lenteren 2008).

Although they are ultimately released into citrus groves for the control of California red scale, all insectaries produce *A. melinus* on oleander scale, *Aspidiotus nerii* Bouché, growing on squash plants. Insectaries use oleander scale on squash as a surrogate host instead of red scale for several reasons: the scales are larger, resulting in more wasps produced as well as a more female-biased sex ratio, the scales are uniparental and therefore easier to rear, and squash plants are cheaper and easier to work with than citrus. Hare et al. (1997) and Hare and Morgan (1997) hypothesized that the lack of *A. melinus* exposure to the target host or target host plant cues may reduce parasitism rates in the field and consequently lower the efficacy of biological control efforts. These authors identified a kairomone, *O*-caffeoyltyrosine, that is used by *A. melinus* for red scale host selection. This kairomone is not present in oleander scale and, in California red scale, the compound theoretically may be used to "prime" insectary-reared wasps to attack scales on citrus. Although a small scale field experiment showed so-called "primed" wasps to have a higher than expected population growth rate (Hare et al. 1997),

it has not been demonstrated that these primed wasps would provide better biological control in commercial citrus. It is therefore possible that the lack of exposure to the target host or host plant in addition to the innate differences in offspring reared on oleander scale could potentially have affected the results of this study. Such impacts would also likely be seen when these parasitoids are released for control of California red scale in commercial citrus groves.

Although all five evaluated insectaries adapted rearing methods from the same protocol (DeBach and White 1960), they differed in some ways that appeared to affect production and fitness of *A. melinus*. For example, some of the facilities maintain *A. melinus* adults in a cup with honey for up to three days before shipping them out to growers. This practice could certainly affect wasp longevity. Also, some insectaries will sell females that have previously been used to parasitize oleander scale to produce the next generation of *A. melinus*. This practice undoubtedly affects the fecundity and quite possibly the life expectancy of these synovigenic females that growers purchase.

It is often suggested that wild genetic stock be infused into cultures of biological control agents in order to avoid loss of genetic variation under the benign conditions of domestication (Hopper et al. 1993, Hufbauer and Roderick 2005). Each of the insectaries evaluated varies in its implementation of this practice with some insectaries not attempting to bring in new genetic material at all, some integrating field collections to the ongoing insectary population, and some completely replacing their populations each fall with new collections. Each of these methods brings with it potential benefits and detriments. Completely replacing the insectary population on an annual basis could

reduce "lab" adaptation problems associated with inbreeding; such effects have been documented to negatively affect the fitness of *Drosophila melanogaster* L. (Spielman and Frankham 1992, Woodworth et al. 2002). On the other hand, it is important to know what one is replacing the lab population with and whether it is actually more "fit" (i.e. are field-collected animals actually increasing genetic variation or have they been collected from a population with little variation). Adaptation to captivity actually increases with higher initial genetic diversity and can occur quite rapidly. Similarly, mixing new genetic material with an established colony could increase genetic diversity, potentially leading to hybrid vigor (a.k.a. outbreeding enhancement or heterosis) and greater efficacy of biological control (Hopper et al. 1993, Margan et al. 1998, Nunney 2002, 2003, 2006). Conversely, if the populations are genetically distinct, the combination could result in outbreeding depression in which the offspring actually have lower fitness.

Introducing new genetic stock into an established colony is generally recommended because of the ramifications of inbreeding and lab adaptation; however, not doing so obviates the potential problems above that are associated with using field populations. Several studies have shown that biological control agents suffer from bottlenecks in population size based on neutral genetic variation (Baker et al. 2003, Hufbauer et al. 2004, Lloyd et al. 2005). Unfortunately, we can only speculate on these impacts at this time, as it is not known how genetic diversity correlates with the field success of insect biological control agents. We hypothesize that the wide variation in insectary methods for introducing new genetic stock into established captive populations

could have fitness consequences for the *A. melinus* that growers receive and release into the field.

Lastly, recent discoveries suggest additional factors that may influence the fitness of insectary reared *A. melinus*. A previously introduced and established *Aphytis* species, *A. lingnanensis*, was thought to have been displaced by *A. melinus* (Luck and Podoler 1985, Murdoch et al. 1996) decades ago. However, our recent survey of UC Riverside's on-campus Biological Control Grove (Field 21) has turned up multiple samples of *A. lingnanensis* based on molecular identification (C.J.V., unpublished data). This finding could have obvious implications for those insectaries that are collecting field material to add to their colonies. Typically, insectaries do not identify their specimens upon field collection and assume that all collected individuals are *A. melinus*. In addition, we have detected *Wolbachia* in some California insectary and field populations of *A. melinus* that causes cytoplasmic incompatibility (CI) (C.J.V., unpublished data). Because *Wolbachia* can impart varying degrees of fitness costs and benefits in natural enemies (for examples see Stouthamer and Luck 1993, Tagami et al. 2001, Huigens et al. 2004, Miura & Tagami 2004), we suggest further investigation into *A. melinus Wolbachia* infections and the effects it has on the success of red scale biological control. Unfortunately the *Wolbachia* infection status of the individuals used in this study was not known and thus could have influenced our results.

Even though our study clearly shows that certain times of the year are of concern to insectaries producing *A. melinus*, much research needs to be done to tease apart the reasons for these production problems. One possibility would be to examine squash scale

size in the winter and summer months. It is known that increasing temperature reduces the size of second and third instar red scale (Yu and Luck 1988) and that *A. melinus* will lay more males than females in small scales. In addition, females emerging from small scales are themselves smaller (Luck et al. 1982). Another consideration is the quality of the squash used as a host plant. Determining which squash qualities (age, color, volatiles, etc.) produce the most and largest oleander scales could result in more and larger *A. melinus* females. Alternatively, looking into other host plants or artificial diets might not only improve the quality of *A. melinus* but could also lower production costs and save insectaries time and space.

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Table 1.1. List of California insectaries supplying *Aphytis melinus* for our studies.

Insectary	Phone	Address
Associates Insectary	(805) 933-1301	1400 E. Santa Paula St. Santa Paula, CA 93060
Foothill Agricultural Research	(951) 371-0120	550 Foothill Parkway Corona, CA 92882
Good Bugs Insectary (a.k.a. The Plant People)	(559) 592-1681	30761 Road 216 Exeter, CA 93221
Mulholland Citrus	(559) 626-4441	5505 South Hills Valley Road Orange Cove, CA 92646
Sespe Creek Insectary	(559) 562-6464	P.O. Box 176 Lindsay, CA 93247

Table 1.2. Percent Live Females: probabilities produced from a logistic regression model testing the effect of independent predictors (Shipment, Insectary, Sample Day) and their interactions on the percentage of live female *A. melinus.*

Variable	*df*	Wald χ^2	*p*
Shipment	5	0.0262	1.0000
Insectary	4	0.0137	1.0000
Sample Day	4	912.27	<.0001
Shipment*Insectary	20	231.46	<.0001
Shipment*Sample Day	20	578.75	<.0001
Insectary*Sample Day	16	216.25	<.0001
Shipment*Insectary*Sample Day	80	913.87	<.0001

Table 1.3. Proportion Live Females. Mean number of females alive, averaged over shipment, for each insectary (A-E) within each of the five sampling days. Means sharing a letter within a shipment are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

Sampling Day	A	B	C	D	E
1	0.9786 *ab*	0.9889 *a*	0.9797 *ab*	0.9798 *ab*	0.9139 *b*
3	0.7584 *b*	0.9701 *a*	0.9225 *a*	0.9629 *a*	0.6082 *c*
7	0.5730 *a*	0.6601 *a*	0.5853 *a*	0.6655 *a*	0.4750 *a*
14	0.3297 *a*	0.3694 *a*	0.3449 *a*	0.5173 *a*	0.0865 *b*
28	0.0380 *ab*	0.0198 *ab*	0.0167 *ab*	0.0668 *a*	0.0035 *b*

Table 1.4. Proportion Female. Means are shown for each insectary (A-E) within each of the six shipments. Means sharing a letter within a shipment are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

Shipment	A	B	C	D	E	Shipment Means
1 – July/Aug. 2006	0.5866 *b*	0.6293 *b*	0.6416 *b*	0.6066 *b*	0.7547 *a*	0.6437 *a*
2 – Sept./Oct. 2006	0.6729 *a*	0.6567 *a*	0.3555 *d*	0.4967 *c*	0.5737 *b*	0.5536 *ab*
3 – Nov. 2006	0.4902 *b*	0.5385 *b*	0.7055 *a*	0.3972 *c*	0.4792 *b*	0.5207 *ab*
4 – Jan./Feb. 2007	0.3639 *b*	0.4221 *ab*	0.4760 *a*	0.2816 *c*	0.4084 *ab*	0.3927 *b*
5 – March 2007	0.6338 *a*	0.6177 *a*	0.2732 *c*	0.4709 *b*	0.3132 *c*	0.4606 *ab*
6 – May 2007	0.3901 *d*	0.6812 *a*	0.4467 *c*	0.3521 *d*	0.5700 *b*	0.4913 *ab*
Insectary Means	0.5252 *a*	0.5912 *a*	0.4836 *a*	0.4357 *a*	0.5164 *a*	

Table 1.5. Live Female Size: probabilities produced from an ANOVA model testing the effect of independent predictors (Shipment, Insectary, Sample Day) and their interactions on the size of live females.

Variable	*df*	Wald χ^2	*p*
Shipment	5	12.54	<.0001
Insectary	4	36.32	<.0001
Sample Day	4	7.59	<.0001
Shipment*Insectary	20	3.99	<.0001
Shipment*Sample Day	19	1.31	0.1791
Insectary*Sample Day	16	1.09	0.3693
Shipment*Insectary*Sample Day	65	0.78	0.8800

Table 1.6. Live Female Size (mm). Sizes of live females on sampling days 1 and 3 combined. Means are shown for each insectary (A-E) within each of the 6 shipments. Means sharing a letter within a shipment are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

Shipment	A	B	C	D	E	Shipment Means
1 – July/Aug. 2006	0.2363 *ab*	0.2458 *a*	0.2230 *c*	0.2270 *bc*	0.2437 *a*	0.2348 *b*
2 – Sept./Oct. 2006	0.2417 *bc*	0.2665 *a*	0.2355 *cd*	0.2271 *d*	0.2544 *ab*	0.2449 *a*
3 – Nov. 2006	0.2411 *b*	0.2581 *a*	0.2355 *b*	0.2488 *ab*	0.2495 *ab*	0.2466 *a*
4 – Jan./Feb. 2007	0.2415 *bc*	0.2540 *ab*	0.2358 *c*	0.2520 *ab*	0.2567 *a*	0.2482 *a*
5 – March 2007	0.2535 *ab*	0.2573 *a*	0.2338 *c*	0.2440 *abc*	0.2400 *bc*	0.2450 *a*
6 – May 2007	0.2333 *b*	0.2586 *a*	0.2305 *b*	0.2360 *b*	0.2566 *a*	0.2429 *a*
Insectary Means	0.2413 *c*	0.2569 *a*	0.2322 *d*	0.2392 *c*	0.2499 *b*	

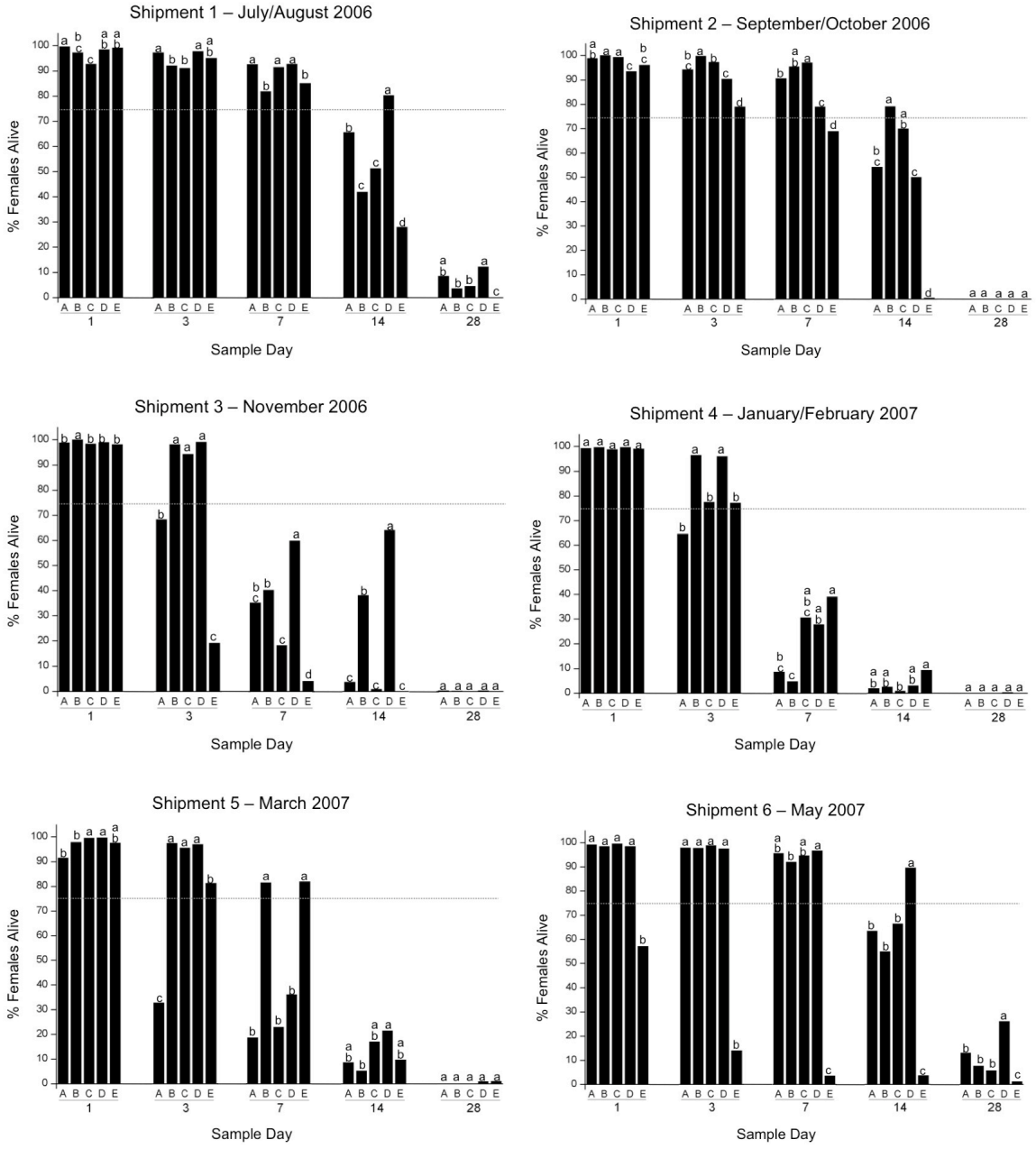

Figure 1.1. Percentage live females on all five sampling days (1, 3, 7, 14, and 28 days post receipt of a shipment) are given for each of the five insectaries (A-E) and each of the six shipments. Dotted horizontal lines are drawn at 75% for reference between graphs. Data were transformed for statistical analyses using an arc-sine square root transformation and untransformed means are shown. Means for bars sharing a letter within each sample day are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

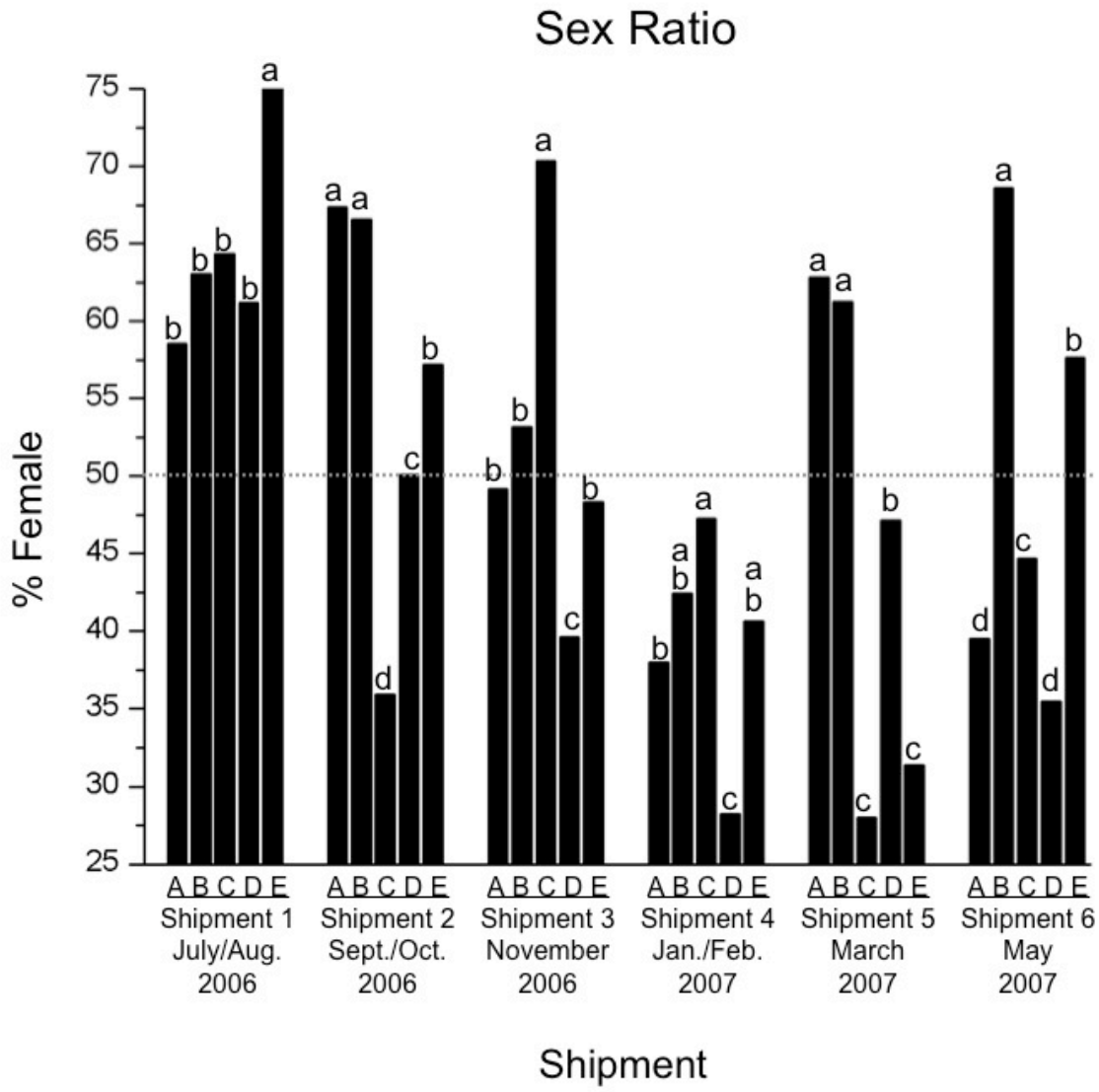

Figure 1.2. Sex ratio (% females) averaged over sampling day for each of the five insectaries (A-E) and each of the six shipments. Bars above the dotted horizontal line at 50% are female-biased and those below are male-biased. Data were transformed for statistical analyses using an arc-sine square root transformation and untransformed means are shown. Means for bars sharing a letter within each shipment are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

Chapter 2

Discovery of a CI-inducing *Wolbachia* and its Associated Fitness Costs in the Biological Control Agent *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae)

Abstract

Wolbachia is an endosymbiotic bacterium that infects a large percentage of arthropods and can affect the fitness if its host. Here we verified for the first time that the biological control agent *Aphytis melinus* DeBach is infected with a *Wolbachia* that causes complete cytoplasmic incompatibility and conducted an insectary and field survey to determine the infection frequency. *Aphytis melinus* appears to suffer fitness costs associated with infection based on measurements of longevity and fecundity. We also quantified the *Wolbachia* titers of *A. melinus* reared at different temperatures and found that, although not completely cured, increased temperature resulted in a significant reduction in the number of *Wolbachia* copies found in an individual wasp. Implications of our results for biological control are discussed.

Introduction

Wolbachia is a gram-negative obligate intracellular bacterium that is estimated to infect from 16% up to 76% of arthropod species (Werren et al., 1995; Jeyaprakash and Hoy, 2000; Hilgenboecker et al., 2008) and is likely to be found in a large percentage of biological control agents (Floate et al., 2006). It is a reproductive parasite in the α subdivision of the Proteobacteria that is vertically transmitted from mother to offspring,

although horizontal transmission has been observed (Haine et al., 2005) and can alter its host's reproduction via cytoplasmic incompatibility (CI), parthenogenesis induction (PI), feminization of genetic males, and male-killing (MK) (Stouthamer et al., 1999; Hurst et al., 1999; Hunter et al., 2003; Merçot and Poinsot, 2009).

Because it can severely modify host reproduction and sex ratios, *Wolbachia* is of great interest to pest control practitioners. It has been proposed as an environmentally-friendly method that might be used to manage pest species, transform disease vectors, and/or improve beneficial insects (Bourtzis, 2008). Although there are conflicting hypotheses on the effect that *Wolbachia* has on its host, with some stating that it is likely to impart a physiological cost in its host (Stouthamer et al., 1999) and others expecting a mutualistic relationship to evolve over time (Werren et al., 2008), evidence of how *Wolbachia* affects fitness has been inconclusive ranging from harmful to neutral to beneficial.

The most severe fitness cost is probably the discovery by Min and Benzer (1997) of a virulent *Wolbachia* strain, named *popcorn*, which drastically reduces adult lifespan in *Drosophila melanogaster* Meigen. The virulent strain has been horizontally transferred between species, it has been proposed as a potential means of reducing vector-borne disease transmission by driving desirable genes into populations (McGraw et al., 2001; Brownstein et al., 2003), and such a strategy was recently implemented via transinfection in the dengue fever mosquito, *Aedes aegypti* (L.) (Turley et al., 2009). *Drosophila simulans* Sturtevant males infected with *Wolbachia* experienced a significant decrease in sperm production and sperm competitiveness, even though sperm do not actually transmit

the bacterium (Snook et al., 2000; de Crespigny and Wedell, 2006). The parasitoids *Trichogramma deion* Pinto & Oatman and *T. pretiosum* Riley suffered a decrease in fecundity (Stouthamer and Luck, 1993) and an increase in embryonic mortality (Tagami et al., 2001); *T. kaykai* Pinto & Stouthamer suffered a decreased competitive ability (Huigens et al., 2004), a lower net reproductive rate, and an improved intrinsic rate of natural increase (Miura and Tagami, 2004) when infected with *Wolbachia*.

There are even some examples of advantageous mutualistic relationships between *Wolbachia* and its host. In *Drosophila melanogaster*, *Wolbachia* infection has been correlated with increased survival in the laboratory for both unmated males and females (Fry and Rand, 2002). Alexandrov et al. (2007) found that by removing *Wolbachia* from female *D. melanogaster* using antibiotics, the lifespan and competitiveness of the cured (but genetically similar) females decreased. A control study showed that the observed effects were not the result of antibiotic treatment. The psocid *Liposcelis tricolor* Badonnel also experienced decreased fitness when cured of its *Wolbachia* infection (Dong et al., 2007). Recently it has been shown that some hosts cannot survive without *Wolbachia*. For example, the moth *Ostrinia furnacalis* (Guenée) is infected with a male killing *Wolbachia*. After curing females of this infection, all female progeny died instead of restoring a 1:1 sex ratio (Sakamoto et al., 2007). This type of relationship would be considered more obligate than mutualistic in nature.

Yet there are many studies that have shown variable or no fitness effects of *Wolbachia* infection. Another study with *D. melanogaster* showed no evidence of fitness effects caused by *Wolbachia* when looking at larval competiveness and adult fecundity

(Montenegro et al., 2006). Poinsot and Merçot (1997) found only a temporary decrease in female fitness after curing *Drosophila simulans* of their *Wolbachia* infection. *Drosophila melanogaster* fitness has been shown to differ depending on the nuclear background and also depending on whether the studies were performed in the laboratory or the field (Olsen et al., 2001; Fry et al., 2004). In the flour beetle *Tribolium confusum* Jacquelin du Val, *Wolbachia* caused a decrease in female fecundity but an increase in male fertility (Wade and Chang, 1995). Four strains of cured *Trichogramma pretiosum* displayed a reduction in total fecundity whereas cured *T. evanescens* Westwood showed no difference in fecundity (Grenier et al., 2002) or diapause (Pintureau et al., 2003). *Leptopilina heterotoma* (Thomson), a parasitoid of *Drosophila*, was negatively affected by *Wolbachia* in some fitness traits but not in others (Fleury et al., 2000). The mosquito *Aedes albopictus* (Skuse) showed different *Wolbachia*-induced fitness effects for males and females and between adult and immature life stages (Dobson et al., 2004; Islam and Dobson, 2006; Calvitti et al., 2009).

Biological control generally relies on the fitness and reproduction of a biological control agent, usually the females, and any means of enhancing its efficacy are of interest to practitioners. *Wolbachia* has the ability to increase the number of female offspring produced (via parthenogenesis induction, male killing, and feminization) resulting in a higher intrinsic rate of increase, fewer resources wasted on male production, and greater biological control (Stouthamer, 1993). Because of the varied fitness costs and benefits that *Wolbachia* imparts, it is recommended that biological control agents be routinely screened for its presence (Werren, 1997; Stouthamer et al., 1999; Floate et al., 2006). For

example, Silva et al. (2000) discovered a *Wolbachia* infection conferring parthenogenesis induction in two parasitoids, *Trichogramma cordubensis* Vargas & Cabello and *T. deion*. Despite a decrease in fecundity due to the infection, the benefit of increased female production would outweigh the fecundity costs.

The most common *Wolbachia*-induced phenotype is cytoplasmic incompatibility (CI). Unlike the other phenotypes, CI can cause reproductive isolation between some populations. Sperm from an infected male is unable to properly fertilize an egg of an uninfected female or a female that is infected with a different *Wolbachia* strain (Werren et al., 2008). In diploid species, this causes a significant decrease in fertility, but in haplo-diploid species it results in a sex ratio shift towards the haploid (unfertilized) sex which is usually the male (Bourtzis, 2008). The reciprocal cross and any crosses between individuals with the same infection status are compatible. In biological control, where the female is the more effective sex in controlling pests, CI can be potentially detrimental to control efforts. Infection status can be particularly important in any biocontrol program that will be combining different populations before or after release. Releasing infected insects into an area that has an uninfected native population is similar to what occurs with the sterile insect technique and could jeopardize the establishment or success of biological control agents. Some populations of *Cotesia sesamiae* (Cameron), a biological control agent of stem borers, have *Wolbachia* infections that cause CI and could significantly reduce the population growth rate if care is not taken to match infection statuses in the laboratory and the field (Mochiah et al., 2002). Severe CI has also been detected in the predator *Macrolophus pygmaeus* (Rambur), a biological control agent of

glasshouse pests (Machtelinckx et al., 2009), and *Spalangia cameroni* Perkins, a parasitoid of filth flies (Kyei-Poku et al., 2006).

We have recently detected a CI-inducing *Wolbachia* infection in the biological control agent *Aphytis melinus* DeBach (R. Stouthamer, unpublished data). *Aphytis melinus* was imported into California in the mid-1950s from Pakistan and India and has been important for many years in the biological control of California red scale, *Aonidiella aurantii* (Maskell), in southern California. Other species of *Aphytis* have been found to possess the PI *Wolbachia* strain (Zchori-Fein et al., 1995, 1998; Gottlieb et al., 1998) and several previous studies failed to detect *Wolbachia* in *A. melinus* (Zchori-Fein et al., 1995, 1998). Here, we verify that *A. melinus* is infected with a CI-*Wolbachia*, determine whether the CI is partial or complete, and the mechanism that *Wolbachia* uses to skew the sex ratio (affecting male development vs. female mortality). Subsequently, we investigate whether this infection confers fitness costs or benefits in *A. melinus* by measuring differences in longevity under ideal nutrition conditions versus nutritional stress as well as differences in female fecundity in an infected and *Wolbachia*-cured strain. Additionally, a survey was completed by the labs of Dr. Richard Stouthamer and Dr. Robert F. Luck (UC Riverside, Entomology) of five commercial insectaries and 40 field sites in central and southern California to determine *Wolbachia* prevalence and infection frequency in the field. Experiments were performed in the laboratory of Dr. Richard Stouthamer to see if high temperatures, similar to those found in the Central Valley of California during summer, can cure *A. melinus* of their *Wolbachia* infection, a phenomenon that has been considered in other species (Clancy and Hoffmann, 1998;

Mouton et al., 2006). This could have important implications for biological control depending on the infection status of the biological control agent being released.

Materials and Methods

Source of specimens and creation of infected and uninfected isofemale lines. Wasps were collected from five commercial insectaries (listed in Table 1.1) and 40 orchards in 2004-2005 (Table 2.1). Insectaries shipped *A. melinus* to our lab overnight and 50 live adult females were collected via aspiration from each insectary shipment and placed in 95% EtOH. Wasps from the field were collected by bringing citrus fruit naturally infested with California red scale into the lab and aspirating at least 20 emerging females (when possible, see results), which were also placed into 95% EtOH. All samples were stored at -20ºC for subsequent DNA extraction.

In order to be able to attribute differences in fitness to *Wolbachia*, we created genetically identical, infected and uninfected isofemale lines. At the outset, a single *Wolbachia*-infected virgin female from the Sespe Creek Insectary (Table 1.1) was allowed to mate with one of her own sons, ensuring the production of both male and female offspring and minimizing the genetic differences between individuals in subsequent generations.

Adult F1 females were aspirated and placed in individual pint size Kerr® Mason jars (Jarden Corporation, Rye, NY) with a lemon infested with late second to early third instar oleander scale, *Aspidiotus nerii* Bouchè. Because *Aphytis* normally mate immediately after emerging (Rao and DeBach, 1969), we assumed these adult females

had already mated. Organic honey was provided as a food source and the jars were covered with a fine mesh cloth and stored at 25°C. Females were allowed to oviposit for 7 days and were then collected and tested for *Wolbachia* (see "*Wolbachia* screening" section below). Females (and their progeny) that tested negative were discarded. *Wolbachia*-infected F1 females that produced only male offspring were also discarded, because this indicated they had probably not mated. Female offspring produced by the remaining *Wolbachia*-infected mothers (i.e. those that produced mixed sex broods) were placed in jars and allowed to oviposit on oleander scale-infested lemons. Prior to the emergence of the next generation, individual pupae were isolated by flipping over the oleander scale cover and gently transferring *A. melinus* pupae to individual vials. Upon emergence, the females were combined with a brother for 24 hours and allowed to mate to ensure fertilization and the production of female offspring. After mating, each female was placed in a separate cage and given oleander scale-infested lemons and honey each week.

In order to attribute *Wolbachia* infection to any losses in fitness, the infected isofemale colony was split into two colonies, one of which was cured of its *Wolbachia* infection by isolating females and feeding them a 0.2% mixture of rifampicin (Sigma, St. Louis, MO) in organic honey for 24 hours before providing hosts to ensure ingestion of the antibiotic. Oleander scale-infested lemons were provided to each female every 7 days for oviposition. Honey with the antibiotic was provided continuously by streaking the mesh cloth covering of the Mason jar with thin lines of honey every three days. The first batch of infested lemons used for oviposition after curing were discarded before the

progeny emerged because the first eggs are usually still infected (R. Stouthamer, unpublished data). As we allowed the *Aphytis* numbers to increase again before they were tested for *Wolbachia* or used in any experiments, antibiotic honey was continuously applied to each new lemon as a food source and to increase the probability that all wasps were ”cured”. Only after approximately six generations, did we begin providing non-antibiotic honey as the adult food source.

Extraction of DNA. DNA was extracted from individual wasps via two techniques. Initially, extractions for the infection frequency survey and temperature experiments were done using a "salting out" technique adapted from Sunnucks and Hales (1996). Individual wasps were removed from 95% EtOH, allowed to dry on filter paper for several minutes, and then individually transferred into 0.6 ml centrifuge tubes containing 1μl of TNES buffer (50 mM Tris, 0.4 M NaCl, 100 mM EDTA, 0.5% SDS). Wasps were ground up using a glass pestle and an additional 99μl of TNES buffer and 2μl of proteinase K (>600 mAU/ml; Qiagen, Inc., Valencia, CA) were added before the tubes were incubated overnight at 37°C. Following incubation, proteins were precipitated by the addition of 28 μl of 5 M NaCl to each tube. Tubes were subsequently centrifuged at 13,200 rpm for 5 minutes and the supernatant was transferred to a clean centrifuge tube. DNA was precipitated by the addition of 128 μl of ice-cold 100% EtOH. DNA was pelleted by centrifuging again at 13,200 rpm for 5 minutes. The supernatant was removed and the DNA pellet was washed with 200 μl of ice-cold 70% EtOH and again centrifuged at 13,200 rpm for 5 minutes. The supernatant was discarded and the DNA pellet was

allowed to air dry (approximately 30 minutes) before being eluted in 20 μl of sterile double distilled water and stored at -20ºC.

In later extractions, DNA was obtained from wasps taken from isofemale colonies using a commercial extraction kit. Live adult *A. melinus* were collected via aspiration and euthanized in 95% EtOH. DNA was extracted using the EDNA HiSpEx Tissue Kit (Saturn Biotech, Perth, Australia) and following the manufacturer's protocol for extraction from 1 mm^3 of tissue but halving the volumes of each proprietary solution to compensate for the small size of *Aphytis*. Extracted DNA was stored at -20ºC for subsequent PCR.

***Wolbachia* screening.** Two methods were used to screen for evidence of *Wolbachia* infection. To detect *Wolbachia* in the infection frequency survey, a hemi-nested polymerase chain reaction (PCR) technique was adopted following Weeks et al. (2003). PCR products were visualized on 1% agarose gels stained with ethidium bromide. In this survey, samples that did not test positive for *Wolbachia* using this hemi-nested method were tested with a set of universal insect 28S ribosomal DNA PCR primers to ensure the presence of genomic DNA, i.e. that the DNA extraction was successful, thus avoiding recording false negatives (Choudhury and Werren, 2006). Samples for which 28S did not amplify were considered failed extractions and excluded from further analyses. Those that initially tested negative for *Wolbachia* but for which 28S amplified, were tested a second time using a different set of 16s rDNA *Wolbachia* specific primers (Choudhury and Werren, 2006). Samples that amplified with either set of 16s ribosomal *Wolbachia*-specific primers were scored as infected.

The infection status of our isofemale colonies was also assessed using the method of Choudhury and Werren (2006) alone. Fifteen individuals from each colony were tested before and after our study to ensure that the infection status did not vary over the duration of the experiment.

Effect of heat on the prevalence of *Wolbachia* in *Aphytis melinus*. A *Wolbachia*-infected colony of *A. melinus* was reared at 27.5°C for 62 days (approximately 3 generations) and eggs of the third generation laid on oleander scale-infested lemons were divided into five treatment groups and were reared at different temperatures: 32.5, 30, 27.5 (control), 25, and 24°C. Upon emergence, live adult wasps were preserved in 95% EtOH. Each of 10 female wasps was measured, dorsal side up, from the top of the head to the tip of the abdomen using an ocular micrometer. Individuals that were chosen for analysis were between 0.90 and 0.96 mm long. *Wolbachia* titer in individual wasps from the different temperature treatments was estimated using quantitative PCR (qPCR) as described by Jeong and Stouthamer (2009).

Crossing experiments. To verify *Wolbachia* infection and the presence of CI, we also performed all possible crosses between infected and uninfected females and males ($♀^{W-} \times ♂^{W-}$, $♀^{W-} \times ♂^{W+}$, $♀^{W+} \times ♂^{W+}$, $♀^{W+} \times ♂^{W-}$). Individual pupae were isolated in 0.25-dram glass shell vials (Fisher Scientific, Pittsburgh, PA) topped with cotton. After eclosion, all adults were given honey to feed on for 24 hours. Next, a single virgin adult male was added to each virgin female vial for 24 hours to ensure mating, after which the pair of wasps was transferred to a Mason jar with an oleander scale-infested lemon (see "source of specimens and creation of infected and uninfected isofemale lines" above for

methods). The female and male were removed after 14 days, offspring were allowed to emerge, and were then counted and sexed (see "effect of *Wolbachia* infection on fitness" section below). Each cross was replicated 20 times for a total of 80 crosses. Crosses that did not produce any offspring were excluded from the data analyses.

Effect of *Wolbachia* infection on fitness. Two measure of fitness were used to investigate the impact infection with *Wolbachia* may have on *Aphytis*: longevity, measured as days from eclosion; and fecundity, measured as the number of offspring emerging. To test the effects of *Wolbachia* on longevity, we isolated individual pupae following the same procedures as detailed above ("Source of specimens and creation of infected and uninfected isofemale lines" section). Equal numbers of females and males from both the infected and cured colonies were tested, and half were provided honey throughout their lifespan while the other half received no honey. A food treatment was included to determine whether *Wolbachia* confers starvation resistance. Individuals were checked daily and their day of death recorded. We used 20 individuals for each treatment and, with three replications over time, a total of 480 individuals were evaluated (2 strains [infected, cured] x 2 food treatments [honey, no honey] x 2 sexes x 3 replications over time x 20 individuals = 480).

To test fecundity, we isolated 15 *Wolbachia*-infected female pupae and 15 uninfected female pupae in vials, each originating from a host oleander scale in which they were the only developing individual, to ensure there was no competition for larval resources. Each of the virgin females was placed in a Mason jar covered with a fine mesh cloth that was streaked with honey and was given a lemon infested with oleander scale.

Each female was given a new oleander-infested lemon every 7 days until she died. Females that did not survive for at least two weeks were discarded and not included in this study. The emerged offspring from each lemon were killed using 70% ethanol, collected on filter paper using a Buchner funnel, and immediately counted. All offspring were male because the female was unmated. This experiment was repeated 3 times for a total of 45 infected and 45 uninfected females. All studies were carried out in a laboratory kept at a temperature of approximately 22ºC.

Size measurements. In order to be able to attribute any fitness differences to *Wolbachia*, as opposed to being the result of differences in size, we measured the size of females used in the longevity experiment. Size was calculated in 0.01 mm increments with an ocular micrometer by measuring the hind tibia length (HTL). HTL is used here as a proxy for overall adult body size because it can be linearly measured and is resistant to desiccation (Nicol and Mackauer, 1999).

Statistical analyses. All statistical analyses were performed using Statistical Analysis Software (SAS) 9.2 for Windows (SAS Institute Inc., Cary, NC). Data were analyzed using a general linear model procedure and means were separated using the REGWQ multiple range test.

Results

Infection frequency. Nearly all of the 40 field sites sampled showed high frequencies of *Wolbachia*-infected *A. melinus* (Table 2.1). All five of the California insectaries had high levels of *Wolbachia*-infected *A. melinus*, which varied from 85% to

100% infection (Table 2.2). Overall, the insectaries did not have a significantly different infection frequency compared to the field sites ($F_{1,58} = 0.67$; $P = 0.4156$).

Effect of heat on the prevalence of *Wolbachia* in *Aphytis melinus*. The number of *Wolbachia* copies significantly decreased as temperature increased ($F_{4,45} = 16.26$; $P < 0.0001$), although the highest temperature we used (32.5°C) still did not completely cure *A. melinus* of the infection (Table 2.3). There was no significant difference in the number of *Wolbachia* copies at the two highest temperatures tested, i.e. 30°C and 32.5°C.

Crossing experiments. None of the crosses between an uninfected female and an infected male produced female offspring, indicating complete cytoplasmic incompatibility. The mean (± S.E.M.) number of males produced from the incompatible cross ($♀^{W-}$ x $♂^{W+}$ = 5.26 ± 0.83) was not significantly different ($F_{3,71} = 0.76$; $P = 0.5183$) from the number of males produced from compatible crosses ($♀^{W-}$ x $♂^{W-}$ = 5.84 ± 1.02, $♀^{W+}$ x $♂^{W+}$= 4.26 ± 0.55, $♀^{W+}$ x $♂^{W-}$= 4.67 ± 0.70).

Effect of *Wolbachia* infection on longevity. There was a significant difference ($F_{1,112} = 7.70$; $P = 0.0065$) between the lifespan of *Wolbachia*-infected and uninfected females given honey, with uninfected females living longer on average. Infected females lived 32.03 ± 1.41 (mean ± S.E.M.) days whereas uninfected females lived 37.85 ± 1.56 days on average. There was a similar trend ($F_{1,122} = 10.00$; $P = 0.002$) with honey-fed males, with uninfected males living 41.08 ± 0.70 days and infected males 35.66 ± 1.62 days.

There was no significant difference ($F_{1,108} = 0.21$; $P = 0.6494$) between the lifespan of *Wolbachia*-infected and uninfected females given no honey. Infected females

lived an average of 2.08 ± 0.10 days whereas uninfected females lived 2.15 ± 0.10 days on average. Similar results were obtained with males not provided access to honey. Infected males lived an average of 2.42 ± 0.10 days whereas uninfected males lived 2.26 ± 0.08 days ($F_{1,130} = 1.69$; $P = 0.1956$).

Size. *Wolbachia* infection did not appear to affect female size. The HTL of females used in the longevity experiment was not significantly different between infected and uninfected wasps (($F_{1,191} = 1.67$; $P = 0.1979$).

Effect of *Wolbachia* infection on Fecundity. There was a significant difference ($F_{1,85} = 4.45$; $P = 0.0378$) between the number of male offspring produced from *Wolbachia*-infected and uninfected females, with the uninfected females having higher fecundity on average. Infected females produced an average of 13.89 ± 1.37 male offspring whereas uninfected females produced an average of 18.07 ± 1.44 male offspring.

Discussion

This study verifies for the first time that the biological control agent, *Aphytis melinus*, is infected with a *Wolbachia* that induces complete cytoplasmic incompatibility (CI). DNA analysis (PCR results) and crossing data both support this conclusion. Additionally, we found there was no significant difference between the number of males produced from the incompatible and compatible crosses, implying that *Wolbachia* functions by killing the female eggs in *A. melinus*, and not by allowing the incompatible eggs to develop into males.

The results of this study suggest that *Wolbachia* does impart a fitness cost in *Aphytis melinus*, as is evident from the decreased fecundity of virgin females and the decreased longevity of both female and male wasps that were fed honey. We tested longevity without a food source to emulate stressful nutritional conditions and looked for signs of adult starvation resistance because it has been suggested that bacteria may provide hosts nutritional assistance with novel metabolic pathways as a route to endosymbiosis (Douglas, 1994). Harcombe and Hoffmann (2004) found no nutritional fitness benefits of a *Wolbachia* infection in *Drosophila melanogaster*. We also found no fitness benefits in starved *Wolbachia*-infected *A. melinus*. However, this could be because the wasps live for only a few days and were only checked on a daily basis instead of hourly, resulting in us overlooking differences in longevity. We found no evidence of size difference between infected and uninfected females used in this study, a variable that is often correlated with differences in fitness, and we therefore conclude that *Wolbachia* did not cause difference in *A. melinus* female sizes.

The infection survey shows a high frequency of *Wolbachia* infection in both field and insectary *A. melinus*. Some of the frequencies were lower than 100%, which may somewhat hamper reproductive potential of the wasps, but does not indicate a need to rear and release "cured" *A. melinus* into the field. A *Wolbachia* infection can reach equilibrium in a population but will never go to complete fixation due to the imperfection of vertical transmission, which usually produces several uninfected offspring each generation (Turelli, 1994).

Although rearing *A. melinus* at high temperatures (up to 32.5°C) did not cure the wasps of their *Wolbachia* infection, it did decrease *Wolbachia* titer. A decrease in *Wolbachia* titer may potentially restore partial mating compatibility, which could be examined by performing crosses with individuals held at different temperatures. Additionally, testing the *Wolbachia* titer at even higher temperatures, similar to those found in the summers of inland California, and for multiple generations at elevated temperatures, could provide insight into the level of curing that actually occurs in the field.

CI-*Wolbachia* infection is usually considered beneficial to females because it allows them to fertilize their eggs and protects them from any mortality induced by CI. When CI-*Wolbachia* infection is infrequent in a population, infected males confer a reproductive fitness cost because they are unable to successfully pass on their genes when they mate with an uninfected female. On the other hand, if CI-*Wolbachia* infection is prevalent in a population then infection would be beneficial, allowing males to fertilize both infected and uninfected females and result in viable progeny (Merçot and Poinsot, 2009). It is commonly assumed that symbionts will eventually evolve towards a mutualistic relationship, conferring fitness benefits in order to facilitate their spread throughout the host population. However, here we see a decrease in fitness when *A. melinus* is infected. Hoffmann et al. (1986) saw similar fitness costs in *Drosophila simulans* infected with *Wolbachia* in southern California in the 1980s. A follow up study done 20 years later, however, showed that the fitness costs of infection had disappeared and that infected *D. simulans* at that time had higher fecundity than uninfected

individuals due to *Wolbachia* evolution (Weeks et al., 2007). Whether this rapid evolution will occur in infected *A. melinus* remains to be seen. Until more evidence emerges on the fitness effects of *Wolbachia*, we recommend that all biological control agents be screened for *Wolbachia* and tested for fitness costs associated with infection that could be potentially detrimental to biological control efforts.

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Table 2.1. Field sites in California where *A. melinus* were collected in 2004-2005 and tested for *Wolbachia.*

Site	County	Collection date	*Aphytis* releases in 2003/2004?	# tested	# infected	% infected
1	Fresno	10/27/04	yes	4	4	100
2	Fresno	8/22/05	yes	2	1	50
3	Imperial	5/31/04	?	8	7	87.5
4	Imperial	6/11/04	yes	12	12	100
5	Kern	10/27/04	yes	1	1	100
6	Kern	10/27/04	yes	20	20	100
7	Kern	10/27/04	yes (2003)	9	9	100
8	Kern	10/27/04	yes	20	20	100
9	Kern	10/27/04	yes	20	20	100
10	Kern	10/27/04	yes	20	20	100
11	Kern	10/29/04	yes	16	16	100
12	Kern	10/29/04	no	20	20	100
13	Kern	11/1/04	yes	20	20	100
14	Riverside	7/12/04	?	20	16	80
15	Riverside	9/28/04	yes	20	15	75
16	Riverside	9/28/04	yes	20	20	100
17	Riverside	9/29/04	?	11	11	100
18	Riverside	9/29/04	no	13	0	0
19	Riverside	10/7/04	yes	2	2	100
20	Riverside	8/26/05	yes	19	18	94.7
21	Riverside	8/26/05	yes	10	8	80
22	Riverside	8/27/05	yes	1	0	0
23	Riverside	8/29/05	yes	1	1	100
24	Riverside	8/29/05	yes	10	9	90
25	Riverside	8/30/05	yes	2	2	100
26	Riverside	8/30/05	yes	3	3	100
27	Riverside	8/30/05	yes	3	3	100
28	Riverside	9/6/05	?	8	6	75
29	San Bernardino	11/11/04	?	19	17	89.5
30	San Diego	5/31/05	no	3	3	100
31	San Diego	8/12/05	?	14	14	100
32	San Diego	8/22/05	?	7	7	100
33	San Diego	8/22/05	?	10	4	40
34	Tulare	9/30/04	yes (2004)	20	19	95
35	Tulare	10/26/04	no	19	19	100
36	Tulare	10/27/04	no	20	19	95
37	Tulare	11/1/04	no	20	20	100
38	Tulare	11/1/04	no	20	18	90
39	Tulare	11/1/04	no	20	20	100
40	Tulare	11/1/04	yes (2003)	15	15	100

Table 2.2. Commercial insectaries in California from which *A. melinus* were collected in 2004-2005 and tested for *Wolbachia*. Insectaries are randomly coded to maintain anonymity.

Insectary	Collection date	# tested	# infected	% infected
1	2/16/05	80	73	91.3
1	6/13/05	40	40	100
1	8/11/05	39	38	97.4
2	5/25/04	80	80	100
2	3/2/05	60	57	95
2	4/21/05	40	40	100
2	6/14/05	40	38	95
2	8/16/05	40	40	100
3	5/25/04	38	34	89.5
3	2/15/05	60	54	90
3	5/3/05	40	37	92.5
3	6/15/05	40	39	97.5
3	8/11/05	40	39	97.5
4	5/13/04	38	36	94.7
4	4/18/05	40	40	100
4	6/23/05	40	40	100
5	2/10/05	80	68	85
5	4/15/05	60	59	98.3
5	6/16/05	40	40	100
5	8/11/05	40	40	100

Table 2.3. Mean *Wolbachia* copy numbers (copy/µl) in 10 females of similar size reared at different temperatures for three generations. Means sharing a letter are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

Temperature (°C)	Mean (copy/µl)
24	397.6[a]
25	240.32[b]
27.5	146.76[bc]
30	85.36[c]
32.5	96.07[c]

Chapter 3

Reappearance of *Aphytis lingnanensis* Compere (Hymenoptera: Aphelinidae) in Inland Southern California

Abstract

Aphytis lingnanensis, previously thought to have been competitively excluded from the inland areas of southern California by *Aphytis melinus*, was collected on the University of California, Riverside's campus biological control grove in 2003. No accounts of this species have been reported in this area since 1965. This finding is significant because the disappearance of *A. lingnanensis* has been used as a classic example of competitive displacement in the ecological and biological control literature. The implications of this finding for biological control practitioners are unknown and need to be further investigated.

Introduction

Aphytis lingnanensis Compere was first introduced into California in 1948 from southern China for the control of California red scale, *Aonidiella aurantii* (Maskell), in an attempt to find a more effective biological control agent than *Aphytis chrysomphali* (Mercet) (DeBach and White, 1960). *Aphytis chrysomphali* was presumably accidentally imported around 1900 from the Mediterranean area and was semi-successful as a biological control agent in the mild coastal areas of California (DeBach and Sisojevic, 1960). Ten years after its introduction, *A. lingnanensis* had displaced most populations of *A. chrysomphali* (DeBach and Sundby, 1963). However, *A. lingnanensis* was not as

successful in the more extreme inland climatic regions, such as Riverside, apparently due to the high temperatures and low humidity (DeBach et al., 1955; DeBach and Sisojevic, 1960; DeBach, 1965).

During 1956-1957 another ectoparasitoid species, *Aphytis melinus* DeBach, was imported from Pakistan and India in the hopes of controlling red scale in the more extreme interior conditions of the inland valley (DeBach, 1957). *Aphytis melinus* quickly became a well established and effective biological control agent, particularly in the interior citrus areas of southern California (DeBach and Landi, 1961a, 1961b). Only a few years after its introduction, *A. melinus* had replaced *A. lingnanensis* in all citrus areas except for the mildest climates of San Diego County (DeBach and Sundby, 1963).

The replacement of *A. chrysomphali* by *A. lingnanensis*, which was subsequently displaced by *A. melinus*, has been well documented in the literature (DeBach and Sundby, 1963; DeBach, 1966; Luck and Podoler, 1985) and is considered one the most famous cases of competitive displacement (Murdoch et al., 1996). Originally, it was believed that *A. melinus* had better searching abilities and was able to outcompete *A. lingnanesis* in harsher climates (DeBach and Sundby, 1963; DeBach, 1965; DeBach, 1966). A second hypothesis emerged stating that *A. melinus* is able to utilize smaller red scales, especially for female progeny, and thus was able to exclude *A. lingnanensis* by exploiting the host before it reached the ideal size for *A. lingnanensis* parasitism (Luck and Podoler, 1985).

By 1965, the only *Aphytis* species found in the inland areas of southern California was *A. melinus* (Rosen and DeBach, 1979). The geographical distribution of the three *Aphytis* species up until 1972 shows the changes in composition and apparent exclusion

of *A. lingnanensis* and *A. chrysomphali* (Rosen and DeBach, 1979; Luck and Podoler, 1985). No distribution data exists after 1972 and since then it has been assumed that either *A. melinus* continued to expand its geographical range, further excluding and possibly extirpating *A. lingnanensis* from southern California, or that the two species have reached a stable distribution. Here, we report findings of *A. lingnanensis* in Riverside County, a discrepancy with current understanding that *A. melinus* had excluded *A. lingnanensis* from inland regions. Because of their small size, the unresolved taxonomic relationships of Aphelininae, and the lack of distinguishable morphological criteria, identification was based solely on molecular methods (Narasimham and Chacko, 1987; Kim, 2003).

Materials and methods

Source of specimens. Citrus fruit infested with California red scale were collected on 16 June 2003 from the University of California, Riverside's on-campus Biological Control Grove (Field 21) and brought back to the laboratory to rear out *Aphytis* for a *Wolbachia* infection frequency survey (Vasquez et al., 2010). Upon emergence, live adults were collected via aspiration and placed in 95% EtOH and stored at -20ºC for subsequent DNA extraction. A total of 15 adults (8 males, 7 females) were used for DNA analysis from this location.

DNA extraction, amplification and sequencing. DNA was extracted from whole individual adult wasps using the EDNA HiSpEx Tissue Kit (Saturn Biotech, Perth, Australia) and following the manufacturer's protocol for extraction from 1 mm^3 of tissue

but halving the volumes of each proprietary solution to compensate for the small size of *Aphytis*. Extracted DNA was stored at -20ºC. Specimen carcasses were subsequently slide-mounted and deposited at the Entomology Research Museum, University of California, Riverside.

The polymerase chain reaction (PCR) was used to amplify the D2 region of ribosomal DNA using the universal insect primers and protocol from Choudhury and Werren (2006). PCR was performed in 25 μl volumes consisting of: 1X ThermoPol PCR buffer (New England Biolabs, Ipswich, MA), 200 μM dNTPs (Fermentas, Glen Burnie, MD), 1 U *Taq* polymerase (New England Biolabs), 0.2 μM of each primer, and 2 μl of template DNA (concentration not determined). Amplification was performed in a Mastercycler 5331 (Eppendorf North America, Inc., New York, NY) following the thermocycling profile: 94°C for 2 minutes; followed by 38 cycles of 94°C (30 sec), 58°C (50 sec), and 72°C (1.5 min); and a final extension at 72°C for 10 minutes. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

Amplified PCR products were cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and sequenced in both directions at the University of California, Riverside's Institute for Integrative Genome Biology Genomics Core Facility using the Applied Biosystems 3730xl DNA Sequencer (Applied Biosystems, Foster City, CA).

Analysis of sequences. Sequences were aligned manually using BioEdit version 7.0.9 (Hall, 1999) and compared to *Aphytis melinus* and *Aphytis lingnanensis* sequences retrieved from GenBank (*A. melinus* accession numbers: AY635342, AY635343,

AY635344, AY635345; *A. lingnanensis* accession numbers: AY635333, AY635334, AY635335). GenBank sequences were based on work by Kim and Heraty on the intertribal relationships of Aphelinidae (Kim, 2003).Trimming and alignment of our sequences to match those from GenBank, resulted in a data matrix consisting of 22 sequences (15 of our own plus the 7 from GenBank) each of which was 456 base pairs long. A sequence identity matrix was created to determine the percent similarity between each of the aligned sequences.

Results

Of the 15 *Aphytis* specimens sequenced from this field, 3 haplotypes were detected. The DNA sequence of the most common haplotype, shared by 12 individuals (7 males, 5 females), was identical to the *A. lingnanensis* sequences retrieved from GenBank. The second haplotype was shared by two specimens (one male, one female) and was identical to the *A. melinus* accession number AY635344. The last haplotype was found in a single female and was identical to the *A. melinus* accession number AY635343.

The two *A. melinus* sequences were 99.7% similar, differing by only one base pair. *Aphytis lingnanensis* was most dissimilar to *A. melinus* AY635344 (91.9% similarity) with 37 base pair differences and a difference of 36 base pair with AY635343 (92.1% similarity).

Discussion

The discovery of *A. lingnanensis* in Riverside County does not support the traditional explanation involving competitive displacement of *A. lingnanensis* by *A. melinus* from the inland valleys of southern California. DeBach and Sundby (1963) were cited 117 times (according to Web of Science, as of July 6, 2010) and were the first to suggest that this displacement was due to differences in searching ability and tolerance to climatic conditions. Luck and Podoler (1985), proposing that competitive exclusion of *A. lingnanensis* was due to *A. melinus*' ability to produce female offspring on smaller hosts, have been cited 85 times (according to Web of Science, as of July 6, 2010). This example has been used to model competitive displacement (see Murdoch et al., 1996) and is cited widely in ecology and biology textbooks (for example: van Driesche and Bellows, 1996; Price, 1997; Bellows and Fisher, 1999; Ricklefs and Miller, 2000; Purves et al., 2001).

The collection area for our samples in Riverside is approximately 83 kilometers from the closest of the last locations in San Diego County known to contain *A. lingnanensis* in 1972 (Luck and Podoler, 1985) and approximately 64 kilometers from the nearest coastal region. Riverside has an average high temperature of 34.4°C in the summer and an average low temperature of 5°C in the winter (Intellicast, 2010). At this stage, it is unclear if the presence of these specimens are remnants of the displaced population, or if they represent a "self introduced" population with life history characteristics different from the displaced population. Finally, it may also be possible that the specimens indeed represent the original introduction that is now once again capable of invading the inland areas due to environmental changes over time such as

modifications to chemical control measures since their original exclusion. Additional studies are needed in to determine whether this was an isolated event and what the implications may be for biological control practitioners.

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Chapter 4

Comparing Levels of Genetic Variation in Captive, Field-released, and Native Populations of a Classical Biological Control Agent, *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae)

Abstract

Classical biological control has been utilized for decades to help manage pests, but little research has been done on how these introductions alter genetic variation in natural enemies. In this study we developed ten microsatellites for use in the parasitoid *Aphytis melinus* (Hymenoptera: Aphelinidae), a biological control agent of the California red scale, *Aonidiella aurantii* (Hemiptera: Diaspididae). The original material used to propagate *A. melinus* for mass release came from four relatively small collections in Pakistan and India and we sought to determine how much genetic diversity remains in this potentially inbred original colony, in comparison with that found in other captive colonies, California field populations, and in populations from the native range of *A. melinus*. Not surprisingly, the field samples from Pakistan had the highest average number of alleles per locus and captive colonies the lowest. A number of unique alleles were found in both Pakistan and California. Possible explanations of our findings and their implications for biological control are discussed.

Introduction

Classical biological control, i.e. introduction of natural enemies from their home range to help control pests introduced into new environments, has been utilized for decades to help regulate pests, but little research has been done on how these introductions alter genetic variation in natural enemies. The processes of collecting subsamples from the native range, testing and rearing in quarantine, and exponentially increasing population sizes for subsequent release into the field are all steps in which genetic diversity may be lost. If population size is small due to bottlenecks or founder events, genetic drift may cause the loss or fixation of rare alleles (Nei et al. 1975). Reduced genetic diversity may result in inbreeding depression and associated fitness impacts (for example Hufbauer 2002, Spielman and Frankham 1992, Woodworth et al. 2002), and therefore, may alter the effectiveness of biological control agents (Hopper et al. 1993, Roush 1990). To alleviate these consequences, it has been suggested that practitioners collect from as many different locations as possible to ensure maximum genetic diversity in the new range. The yet unknown role that genetic diversity plays in adaptation will drive future decisions on collecting and preserving biological control agents (Hopper et al. 1993, Roderick and Navajas 2003).

Microsatellites are highly polymorphic, neutral markers that are frequently used to estimate genetic diversity (Sunnucks 2000). Several recent studies utilizing microsatellites have provided evidence that biological control agents do experience bottlenecks in population size (Baker et al. 2003, Hufbauer et al. 2004, Lloyd et al. 2005). Although the link between neutral genetic variation and variation in ecologically

important traits is poorly understood, several meta-analyses have found significant correlation between genetic diversity and quantitative fitness traits (Merilä and Crnokrak 2001, Reed and Frankham 2003).

In this study, we developed ten microsatellites for use in the parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), a biological control agent of California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae). California red scale has been considered the number one arthropod pest of citrus in California since its appearance in the 1870's (Comstock 1880, Ebeling 1959, Flint et al. 1991, Morse et al. 2007). From 1889 to the 1950's, at least 35 attempts were made at introducing biological control agents for California red scale control (reviewed in Clausen et al. 1978) but the prospects seemed so bleak that Harold Compere (1961) concluded that biological control would probably never control California red scale in California. However, the introduction of *A. melinus* in 1957 apparently displaced *Aphytis lingnanensis* Compere in inland areas (DeBach and Sundby 1963, Luck and Podoler 1985; but see Vasquez et al. 2010) and by 1962, the former had become such an effective biological control agent in southern California that chemical control was rarely needed (Flint et al. 1991, Morse et al. 2007).

The original material used to propagate *A. melinus* for mass release came from four relatively small collections in Pakistan and India that were started as four separate cultures inside a quarantine facility (DeBach 1959). The first, and largest sample came from New Delhi, India on 5 September 1956 (S&R [Sending & Receiving] No. 1643; these are numbers filed by UC Riverside Quarantine staff to track shipments) and

consisted of 108 females and 27 males. On 15 September 1956, the second sample from Lahore, Pakistan (S&R No. 1646) contained 14 females and 2 males and the third sample from Gurgaon, India on 26 October 1956 (S&R No. 1651) contained 24 females and 12 males. All three of these collections were from California red scale on roses. The fourth and final sample was collected on 3 April 1957 from Rawalpindi, Pakistan (S&R No. 1737) from yellow scale on orange and pummelo and contained only 11 females and 2 males. All four cultures were determined to interbreed and were combined to form a single quarantine culture, which was mass released in the field during the fall of 1957. If we assume that the cultures were reared at 25°C then the lifecycle would take approximately 17 days, thus allowing for up to 11 generations in the lab if they were combined immediately after the last shipment was received and released in late September. This original colony, from which all known California releases have been made, has been maintained at the University of California, Riverside's Insectary and Quarantine Facility for more than 50 years. The purpose of our study was to determine how much genetic diversity remains in this potentially inbred original colony (instigated with a total of only 157 females and 43 males), in comparison with that found in other captive colonies, the introduced California field population, and in populations from the native range of *A. melinus* in Pakistan.

Materials and Methods

Collection. Adult *Aphytis melinus* were collected from four laboratory-maintained colonies (UCR), five California insectaries, California field sites, and

Pakistan field sites (Table 4.1). Live adults were collected via aspiration and these were immediately placed into 95% EtOH for preservation and stored at -20ºC for subsequent DNA extraction. Paul DeBach's original colony, propagated from a total of 157 females and 43 males and released into California fields during the fall of 1957 (DeBach 1959), has been maintained at the University of California, Riverside's Insectary and Qurantine facility since then. The Taxila, Pakistan colony (S&R No. 88-05) was collected by R. F. Luck in 1988 and was started from 6 females and 2 males. The Ming Ho, China colony (S&R No. 90-05), also collected by R. F. Luck, was started in 1990 from an unknown number of males and females. The Queensland, Australia colony (S&R No. 92-62) was started in 1992 from 150 live adults shipped from a commercial insectary. These three colonies have also been maintained at the University of California, Riverside's Insectary and Qurantine facility. In addition, five commercial insectaries (listed in Table 1.1 and coded A-E for anonymity) shipped *A. melinus* to our lab overnight. Two California field sites were sampled by bringing citrus fruit infested with California red scale into the lab to rear out *Aphytis*. Both field sites had not received insectary-reared *Aphytis* releases for at least two years prior to sampling. Similar field samples from Pakistan were collected by rearing out adult *A. melinus* from infested citrus fruit collected in 2007 and 2008.

DNA extraction, amplification and sequencing. DNA was extracted from whole individual adult wasps using the EDNA HiSpEx Tissue Kit (Saturn Biotech, Perth, Australia) and following the manufacturer's protocol for extraction from 1 mm^3 of tissue but halving the volumes of each proprietary solution to compensate for the small size of *Aphytis*. Extracted DNA was stored at -20ºC.

To verify the species identity of specimens, the polymerase chain reaction (PCR) was used to amplify the D2 domain of 28S ribosomal DNA using universal insect primers and a protocol from Choudhury and Werren (2006). PCR was performed in 25 μl volumes consisting of: 1X ThermoPol PCR buffer (New England Biolabs, Ipswich, MA), 200 μM dNTPs (Fermentas, Glen Burnie, MD), 1 U *Taq* polymerase (NEB), 0.2 μM of each primer, and 2 μl of template DNA (concentration not determined). Amplification was performed in a Mastercycler 5331 (Eppendorf North America, Inc., New York, NY) following the thermocycling profile: 94°C for 2 minutes; followed by 38 cycles of 94°C (30 sec), 58°C (50 sec), and 72°C (1.5 min); and a final extension at 72°C for 10 minutes. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

Amplified PCR products were cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and sequenced in both directions at the University of California, Riverside's Institute for Integrative Genome Biology Genomics Core Facility using an Applied Biosystems 3730xl DNA Sequencer (Applied Biosystems, Foster City, CA).

Sequences were aligned manually using BioEdit version 7.0.9 (Hall, 1999) and compared to *Aphytis melinus* sequences retrieved from GenBank (*A. melinus* accession numbers: AY635342, AY635343, AY635344, AY635345). GenBank sequences were based on work by J. Kim and J. M. Heraty on the intertribal relationships of Aphelinidae (Kim 2003). Only those specimens whose sequences matched one of the *A. melinus* GenBank sequences were used for microsatellite analyses.

Microsatellite development. Four enriched microsatellite libraries (repeat motifs:

CA-, GA-, ATG-, and CATC-) were prepared by Genetic Identification Services (GIS; http://www.genetic-id-services.com, Chatsworth, CA) using DNA from adult *A. melinus* obtained from one of the commercial insectaries in California. Primers were designed using DesignerPCR version 1.03 (Research Genetics, Inc., Huntsville, AL). Ten polymorphic microsatellite primers were chosen and are listed in Table 4.2. Forward primers were labeled at the 5' end using the fluorescent dyes 6-FAM™, VIC™, NED™, and PET™ (Applied Biosystems).

Microsatellite protocols. All ten microsatellite markers were amplified in 151 female *A. melinus*. Because *A. melinus* are haplo-diploid, only the diploid females were used in this study. Microsatellite loci were amplified via PCR in 20 µl volumes consisting of: 1X ThermoPol PCR buffer (NEB), 1.6 µl BSA (NEB), 1 mM $MgCl_2$, 200 µM dNTPs (Fermentas), 0.5 U *Taq* polymerase (NEB), 0.3 µM of each primer, and 2 µl of template DNA (concentration not determined). Amplification was performed in a Mastercycler 5331 (Eppendorf) following a thermocycling profile of: 94°C for 3 minutes; followed by 40 cycles of 94°C (30 sec), 55°C (40 sec), and 72°C (30 sec); and a final extension at 72°C for 4 minutes. PCR products were visualized on 1% agarose gels stained with ethidium bromide. PCR products were combined with an internal size standard, GeneScan®-500 LIZ™ (Applied Biosystems), and separated on an ABI 3100® genetic analyzer (Applied Biosytems) at the University of California, Riverside's Institute for Integrative Genome Biology Genomics Core Facility. Each plate contained a positive (known genotype) and negative (sterile water) control. Alleles were sized manually using GENEMAPPER® software v. 3.7 (Applied Biosystems).

Microsatellite analyses. Estimates of observed and expected heterozygosity , F_{IS} values, allele frequencies, tests of Hardy-Weinberg equilibrium and linkage disequilibrium, were all calculated using Genepop 4.0.10 as implemented at "Genepop on the Web" (Raymond and Rousset 1995, Rouset 2008; http://genepop.curtin.edu.au/). Hardy-Weinberg equilibrium and linkage disequilibrium were assessed using exact probability tests and default Markov chain parameters (1000 dememorization steps, 100 batches, 1000 iterations per batch). Inter-population differences in the average number of alleles per locus were examined with a generalized linear model using SAS® 9.2 software for Windows (SAS Institute 2008).

Results and Discussion

The number of alleles for each of the ten microsatellite loci ranged from five to 20. After controlling for multiple comparisons, significant deviations from Hardy-Weinberg equilibrium were observed in 9 out of the 70 possible locus/population combinations (Table 4.3). All significant deviations were due to deficits in heterozygosity. Seven of these deviations (four in the combined insectaries and three in Pakistan field samples) likely represent a Wahlund effect in which heterozygosity appears reduced due to population structuring. The combined insectary population actually represents a pool of samples from five different insectaries and the Pakistan field population is from four different collection sites that are geographically distinct. The other two significant deviations occurred in colonies and likely resulted from null alleles and/or small sample sizes and not from non-random mating. Only three out of the 315

locus pairs for all seven populations exhibited significant linkage disequilibrium after Bonferroni correction (corrected $\alpha = 0.00016$) and we therefore assume the loci to be independent.

A total of 39 *A. melinus* females were collected in Pakistan, 22 near Peshawar, 14 near Bhalwal, 2 near Islamasbad, and 1 near Kot Momin. Microsatellite data for aggregate data from these four Pakistani collections sites were denoted as Pak-Fld whereas data in aggregate for samples from the five insectaries were denoted as Ins-All (n=47) (Table 4.4). The relative abundance of different alleles at each locus varied across populations and, as expected, the combined Pakistan field samples had the highest average number of alleles per locus (9.9 alleles). This was significantly higher than the number of alleles for all other populations except for the combined insectary "population" (7.9 alleles; Table 4.4). Considering the insectaries separately, the average number of alleles ranged from 3.9 to 5.5. This difference from the 7.9 alleles found in the combined insectary data would suggest that introductions from multiple insectaries would be best for conserving genetic diversity. Next in allelic abundance were the California field samples (6.4 alleles). The four colonies had considerably fewer alleles, all of which differed significantly from the combined Pakistan field population, the combined insectary population the California field population: the DeBach colony and Pakistan colony both had 2.9 alleles followed by the Australia (2.2 alleles), and then the China colony (1.4 alleles). All four of the colonies were also the only populations that showed fixed alleles at any of the ten loci. All colonies were fixed at locus B120, the DeBach colony at one additional locus, and the China colony at five additional loci. It is

surprising to see the allelic paucity in the DeBach colony samples from which all of the California populations were established. Although this could represent the diversity originally introduced into California, a more likely explanation is that alleles have been lost over the years due to inbreeding and density dependent fluctuations that cause many colonies to exhibit boom and bust cycles.

Out of a total of 126 alleles, 23 were unique to the Pakistan field samples. These unique alleles generally occurred at low frequencies (less than 10% abundance) and indicates that there is variation in Pakistan that was not captured in the initial sampling in 1956-1957 and/or that rarer alleles have been lost from the introduced population simply by chance. On the other hand, there are 27 alleles in the other populations that were not found in the Pakistan field samples we collected. There are several potential explanations for this. One possibility is that the alleles have changed due to mutation over the past 50 years. Generally, microsatellite mutation rates are significantly higher than point mutations in coding DNA, ranging from 10^{-6} to 10^{-2} mutations per locus for each generation (Bhargava and Fuentes, 2010). Given the generation time at 25ºC, we estimate that the DeBach colony has passed through well over 1,000 generations in captivity which, based on the highest mutation rate (Bhargava and Fuentes, 2010), would lead to a maximum of approximately 10 mutations at a single locus. A second explanation is that our recent sampling in Pakistan was not large enough to have picked up most of the alleles that were originally collected. Lastly, it is important to emphasize that the original colony was initiated from a mixture of samples taken from Pakistan and India. Indeed,

the majority of the original DeBach colony was collected in India and it is quite possible that Indian populations harbor at least some of the unique alleles we see in California.

Locus B125 has a unique set of alleles (287-295bp) found only in the insectary samples. This could be due to unsampled alleles from Pakistan and India or it could be the result of a mutation. Microsatellites have high mutation rates due to replication slippage and these rates can vary drastically depending on the loci, repeat number, repeat type, and overall length of the microsatellite (Ellegren 2000). Long loci with a large number of dinucleotide repeats are expected to have the highest mutation rate (Bhargava and Fuentes, 2010). Locus B125 has 25 repeats of a dinucleotide and is one of the longest loci in our study, perhaps explaining the possible mutations we see in the insectary samples. Large expansions in the repeat region, as seen in this locus, could also be caused by processes such as unequal crossing over or gene conversion (Bhargava and Fuentes, 2010).

In summary, although we see the highest number of alleles in the Pakistan field samples, the California insectary and California field samples showed a surprising amount of genetic diversity in spite of the small number of *A. melinus* that were initially imported. This could be the result of successfully collecting much of the genetic variation to begin with, or the restoration of alleles, via slippage mutation, that were initially lost through sampling or a population bottleneck. A survey of the *A. melinus* in India would provide additional information that could help us refine some of the explanations of our results. Lastly, it is unknown whether genetic variation plays a role in the efficacy of biological control agents in the field. If the low number of alleles found in the original

DeBach colony are indicative of what was actually brought over and introduced into California, then perhaps allelic diversity is not as important as once thought, because *A. melinus* has been a successful biological control agent in southern California. Indeed, literature on invasion biology suggests that large amounts of genetic variation are not necessary for the successful introduction of an organism (Roderick and Navajas 2003, Hufbauer and Roderick 2005). Alternatively, perhaps *A. Melinus* could achieve even better biological control with added genetic diversity, particularly in the desert areas of the Coachella Valley and in California's largest citrus production area, the San Joaquin Valley. Many authors have hypothesized about the importance of genetic diversity (Hopper et al. 1993, Margan et al. 1998, Nunney 2002, 2003, 2006), but field trials are desperately needed.

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Table 4.1. Collection records of *A. melinus* used in microsatellite analyses.

Collection location	Abbreviation	*N*	Collector	Year
California insectaries - combined	Ins-All	47		
Insectary A	Ins-A	15	C. Vasquez	2005, 2007
Insectary B	Ins-B	10	C. Vasquez	2005, 2007
Insectary C	Ins-C	10	C. Vasquez	2005, 2007
Insectary D	Ins-D	5	C. Vasquez	2005
Insectary E	Ins-E	7	C. Vasquez	2007
California field sites - combined	Cal-Fld	16		
Redlands, San Bernardino County		14	C. Boisseranc	2005
Field 21 – UCR's biological control grove, Riverside County		2	?	2003
DeBach's original colony from 1956-7	DeB-Col	20	C. Vasquez	2008
Queensland, Australia colony from 1992	Aus-Col	7	C. Vasquez	2008
Taxila, Pakistan colony from 1988	Pak-Col	12	C. Vasquez	2008
Ming Ho, China colony from 1990	Chi-Col	10	C. Vasquez	2008
Pakistan field sites - combined	Pak-Fld	39		
Peshawar, Pakistan	Pak-Pesh	22	I. Khan	2007
Bhalwal, Pakistan	Pak-Bha	14	I. Khan	2008
Islamasbad, Pakistan		2	I. Khan	2008
Kot Momin, Pakistan		1	I. Khan	2008

Table 4.2. Characteristics of ten microsatellite loci in *Aphytis melinus* used in this study: locus name, GenBank Accession no., primer sequences (* indicates the primer that was dye-labeled for visualization), PCR annealing temperatures (T_a), fluorescent dye, repeat structure, number of alleles, size range of amplified alleles, and average observed (H_O) and expected (H_E) heterozygosities.

Locus (Accession #)	**Primer sequences (5'→ 3')**	**Repeat Motif**	**# of Alleles**	**Size Range (bp)**	H_O	H_E
A106 (HQ007894)	F:*CGCTACCTGGGAATATAACGT R: AGGAAGGTGATTAGCACAGGAG	$(CA)_{10}GC(CA)_5$	12	150-189	0.5035	0.7589
B120 (HQ007891)	F:*GTCGTGATCGGACGTTGTAG R: GCTCGCTCAGTTGACAAATC	$(AC)_7GCATACGCGAGCGCACGT(CA)_7$	5	214-229	0.2897	0.3167
D4a (HQ007893)	F:*TGTGCCCTTGCTATACCTAC R: CTCTCTCTCTCTCGGCTAGAG	$(CA)_{25}$	16	219-269	0.6250	0.8039
B125 (HQ007899)	F:*CCGTAGAGCTACTCCACTCAC R: TAAATCAAATAGCGTCCACATC	$(CT)_{24}$	16	245-295	0.4851	0.8460
B103 (HQ007897)	F:*ACCAACATAGACGCCGTTAC R: ACTTTCGTTTTCCGCTAAATC	$(AG)_{23}$	20	160-204	0.6689	0.8592
A107 (HQ007895)	F:*TAACGCAAGCCAATTATCG R: TGCCCGACAGCTACTACAG	$(AC)_{12}$	12	190-223	0.5510	0.8450
B127 (HQ007900)	F:*ACTGGAACAACGCACATTG R: TCGCTTATTGCTCCTTCG	$(CT)_{16}$	15	251-279	0.4722	0.8622
A128 (HQ007896)	F:*CTCGCTCTCTCTCTTTCTGAC R: TTACCAGTAAATCGAGCAACTC	$(AC)_{13}$	11	170-198	0.4558	0.5027
B6 (HQ007892)	F:*GAGTAAAGCGAGAGAGTCAGTC R: GGATGTTTCTCAGCTCTTTG	$(TC)_{15}$	12	133-170	0.3129	0.7619
B123 (HQ007898)	F:*AGGAGGAGCACACGTAGAG R: ATCCCGAGGAGAGAAAGAG	$(GA)_{12}$	7	273-287	0.6069	0.7572

Table 4.3. Average inbreeding coefficients (F_{IS}) for each population at each locus. Positive inbreeding coefficient (F_{IS}) values indicate less heterozygosity than expected whereas negative F_{IS} values indicate more than expected. Asterisks denote significant deviation from Hardy-Weinberg equilibrium, based on a Bonferroni adjusted $\alpha = 0.0007$. N/A signifies loci that were monomorphic.

locus	INS-All (n = 47)	Cal-Fld (n = 16)	DeB-Col (n = 20)	Aus-Col (n = 7)	Pak-Col (n = 12)	Chi-Col (n = 10)	Pak-Fld (n = 39)
A106	-0.0270	0.1318	N/A	-0.3333	0.4915	-0.0588	0.1617
B120	0.0394	0.0722	N/A	N/A	N/A	N/A	-0.0876
D4a	0.2215	-0.1563	-0.1043	0.3939	-0.0526	N/A	-0.0009
B125	0.2050*	0.2097	0.6739*	0.5000	1.000*	N/A	0.1034
B103	-0.0100	-0.0474	-0.1613	-0.3333	0.000	N/A	0.2846*
A107	0.4427*	0.1538	-0.1314	0.3684	-0.0539	N/A	0.0588
B127	0.3921*	0.2458	-0.1029	1.0000	0.2183	-0.2000	0.3639*
A128	-0.1830	-0.1250	-0.2147	-0.0435	N/A	N/A	0.0577
B6	0.7221*	0.1892	0.7216	N/A	0.4737	N/A	0.3876*
B123	0.1370	0.0652	0.0769	-0.2000	-0.0526	0.2500	0.1662

Table 4.4. Allele frequencies for each of the *A. melinus* populations at each of the loci. Populations Ins-All (all insectary data combined) and Pak-Fld (all Pakistan field data combined) are also shown as subsamples. The most frequent alleles for each locus in each population are shaded. Sample sizes and the average number of alleles per locus are given for each population. Mean number of alleles sharing a letter (a-f) are not significantly different (REGWQ within SAS Institute 2008, p = 0.05).

Locus	size	DeB-Col n=20	Pak-Col n=12	Chi-Col n=10	Aus-Col n=7	*Ins-A* n=15	*Ins-B* n=10	*Ins-C* n=10	*Ins-D* n=5	*Ins-E* n=7	Ins-All n=47	Cal-Fld n=16	*Pak-Pesh* n=22	*Pak-Bha* n=14	Pak-Fld n=39
A106	150	-	-	-	-	-	-	0.050	-	-	0.011	-	-	-	-
	152	-	-	0.100	-	-	-		-	-	-	-	-	-	-
	156	-	-	-	-	-	-	0.050	0.100	-	0.021	-	-	-	-
	164	-	-	-	-	0.200	0.150	0.100	0.100	0.429	0.192	-	0.079	0.036	0.111
	166	-	-	-	-	-	-		-	-	-	-	0.026	-	0.014
	168	-	-	-	-	-	0.100	0.050	-	-	0.032	-	0.079	0.036	0.069
	170	-	-	-	-	0.533	0.350	0.350	0.600	0.071	0.394	0.833	0.500	0.321	0.389
	172	-	-	-	0.300	-	0.050	0.050	-	-	0.021	0.067	-	0.214	0.097
	174	-	0.500	-	0.700	0.067	-	0.150	-	-	0.053	-	0.184	0.179	0.167
	176	1.000	0.500	0.900	-	0.200	0.350	0.100	0.200	0.500	0.255	0.067	-	0.179	0.069
	178	-	-	-	-	-	-	0.050	-	-	0.011	0.033	0.132	0.036	0.083
	189	-	-	-	-	-	-	0.050	-	-	0.011	-	-	-	-
B120	214	-	-	-	-	-	-	-	-	-	-	0.031	-	-	-
	216	-	-	-	-	-	-	-	-	-	-	0.031	-	-	-
	225	-	-	-	-	-	0.050	-	-	-	0.011	0.188	0.150	0.214	0.162
	227	1.000	1.000	1.000	1.000	0.933	0.750	0.833	0.800	0.714	0.826	0.594	0.775	0.571	0.676
	229	-	-	-	-	0.067	0.200	0.167	0.200	0.286	0.163	0.156	0.075	0.214	0.162
D4a	219	-	-	0.050	-	-	-	-	-	-	-	-	-	-	-
	227	0.132	-	-	0.667	0.033	0.050	0.056	-	0.143	0.054	0.233	0.050	-	0.027
	229	0.026	-	-	0.250	0.133	-	0.111	-	-	0.065	0.233	0.100	0.036	0.095
	231	-	-	-	-	-	-	0.056	0.100	-	0.022	-	-	0.036	0.014
	235	-	-	-	-	-	-	0.056	-	-	0.011	-	0.050	-	0.027
	239	-	-	-	-	-	-	-	-	-	-	-	-	0.036	0.014
	241	-	-	-	-	-	-	0.056	-	-	0.011	-	-	-	-
	249	-	-	-	-	-	-	-	-	-	-	-	0.025	-	0.014
	251	-	-	-	-	0.067	-	-	-	-	0.022	-	0.100	0.143	0.122
	253	-	-	-	-	0.033	-	-	-	0.143	0.033	0.067	0.175	0.143	0.149
	255	0.105	0.455	-	-	0.067	-	-	-	0.357	0.076	0.033	0.125	0.179	0.162
	257	-	-	-	0.083	0.067	0.100	0.056	0.200	0.071	0.087	0.300	0.250	0.286	0.257
	259	-	-	-	-	-	-	0.056	-	0.071	0.022	-	0.125	-	0.068
	261	0.711	0.546	0.950	-	0.533	0.850	0.444	0.500	0.214	0.533	0.100	-	-	-
	263	-	-	-	-	0.067	-	0.111	0.200	-	0.065	0.033	-	0.143	0.054
	269	0.026	-	-	-	-	-	-	-	-	-	-	-	-	-
B125	245	-	-	-	0.083	-	-	-	-	-	-	-	-	-	-
	249	-	0.556	-	0.083	0.133	-	-	-	-	0.044	0.033	0.025	0.107	0.056
	251	-	-	-	-	-	-	-	-	-	-	-	0.025	-	0.014

	255	-	-	-	-	-	-	-	-	-	-	0.033	0.025	-	0.014
	257	-	-	-	-	0.033	0.100	0.056	0.200	0.214	0.098	0.033	0.050	-	0.028
	259	-	-	-	-	-	-	-	-	-	-	-	0.075	0.143	0.097
	261	0.063	-	-	-	0.500	0.050	0.333	0.400	-	0.283	0.133	0.375	0.321	0.347
	263	0.313	0.111	-	-	0.100	0.150	0.111	0.100	0.071	0.109	0.633	0.225	0.286	0.236
	265	0.594	0.222	1.000	0.833	-	0.050	-	-	-	0.011	-	0.100	-	0.083
	267	-	-	-	-	-	-	-	-	-	-	-	0.100	0.107	0.097
	269	0.031	0.111	-	-	-	-	-	-	-	-	-	-	0.036	0.014
	271	-	-	-	-	-	-	-	-	-	-	-	-	-	0.014
	287	-	-	-	-	-	-	-	-	0.071	0.011	-	-	-	-
	289	-	-	-	-	0.033	-	0.111	-	0.214	0.065	0.100	-	-	-
	291	-	-	-	-	0.200	0.650	0.333	0.300	0.429	0.370	0.033	-	-	-
	295	-	-	-	-	-	-	0.056	-	-	0.011	-	-	-	-
B103	160	-	-	-	-	-	0.050	0.111	-	-	0.033	-	-	-	0.026
	162	-	-	-	-	0.067	0.050	-	-	0.143	0.056	0.063	0.091	0.071	0.077
	164	-	-	-	-	-	-	-	-	-	-	-	-	0.036	0.013
	166	0.105	-	-	0.714	0.133	0.150	0.167	-	0.143	0.133	0.219	0.023	0.143	0.064
	168	-	-	-	-	0.200	0.150	-	0.250	0.071	0.133	0.094	0.068	0.071	0.090
	170	-	-	-	-	-	-	-	-	0.143	0.022	-	0.159	0.107	0.128
	172	0.105	0.375	-	0.286	0.267	0.250	0.278	0.375	-	0.233	0.313	0.068	0.036	0.064
	174	0.790	0.542	1.000	-	0.033	0.100	-	0.125	0.286	0.089	0.031	0.136	0.179	0.141
	176	-	-	-	-	-	-	-	-	-	-	-	0.068	0.071	0.064
	178	-	-	-	-	-	-	0.111	-	0.071	0.033	-	0.114	0.036	0.077
	180	-	-	-	-	0.067	0.050	-	-	-	0.033	0.031	0.046	0.179	0.090
	182	-	0.083	-	-	-	-	-	-	-	-	-	0.046	-	0.026
	184	-	-	-	-	-	-	-	-	-	-	0.063	-	-	-
	188	-	-	-	-	0.067	0.050	-	-	0.071	0.044	0.156	-	0.036	0.013
	190	-	-	-	-	0.033	-	0.111	-	0.071	0.044	-	0.023	-	0.013
	192	-	-	-	-	-	-	-	-	-	-	-	0.091	0.036	0.064
	194	-	-	-	-	-	-	-	-	-	-	-	0.023	-	0.013
	196	-	-	-	-	-	-	-	-	-	-	0.031	-	-	-
	198	-	-	-	-	0.133	0.150	0.222	0.250	-	0.144	-	0.023	-	0.026
	204	-	-	-	-	-	-	-	-	-	-	-	0.023	-	0.013
A107	190	-	-	-	-	0.167	0.250	0.222	0.250	0.143	0.200	-	-	-	-
	198	0.105	0.083	-	0.286	0.367	0.300	0.500	0.750	0.500	0.433	0.033	-	-	-
	204	-	0.042	-	-	-	-	-	-	-	-	0.100	0.114	0.107	0.128
	206	-	-	-	-	-	-	-	-	-	-	0.067	0.182	0.286	0.231
	208	-	-	-	-	-	-	-	-	-	-	0.167	0.091	0.036	0.064
	210	-	-	-	0.714	0.067	0.050	-	-	-	0.033	0.100	0.023	0.071	0.039
	212	-	-	-	-	-	-	-	-	-	-	0.233	0.091	0.036	0.064
	215	0.658	0.417	-	-	0.400	0.350	0.278	-	0.357	0.322	0.233	0.114	0.214	0.167
	217	-	-	-	-	-	-	-	-	-	-	-	0.250	0.036	0.154
	219	0.237	0.458	1.000	-	-	0.050	-	-	-	0.011	0.067	0.068	0.107	0.077
	221	-	-	-	-	-	-	-	-	-	-	-	0.046	0.071	0.051
	223	-	-	-	-	-	-	-	-	-	-	-	0.023	0.036	0.026
B127	251	-	-	-	-	-	-	-	-	-	-	-	0.025	-	0.014

	253	-	0.125	-	-	0.133	0.050	0.100	0.200	0.071	0.106	0.033	0.075	0.214	0.122
	255	-	-	-	-	-	0.050	-	-	0.071	0.021	-	0.150	0.143	0.135
	257	0.094	0.125	-	0.571	0.367	0.550	0.250	0.100	0.643	0.394	0.533	0.125	0.036	0.122
	259	0.063	-	-	0.429	-	-	0.100	0.100	0.071	0.043	0.133	0.325	0.179	0.243
	261	0.844	0.417	0.200	-	0.067	-	0.050	0.100	0.071	0.053	0.100	0.050	0.071	0.054
	263	-	0.042	-	-	-	-	-	-	-	-	-	0.100	-	0.054
	265	-	-	-	-	-	-	-	-	-	-	0.033	0.075	-	0.041
	267	-	-	-	-	-	-	-	-	-	-	-	0.025	0.143	0.068
	269	-	-	-	-	0.033	0.100	-	0.200	-	0.053	-	-	0.071	0.027
	271	-	-	-	-	0.167	0.050	0.200	-	-	0.106	-	-	-	0.014
	273	-	-	-	-	0.167	-	-	-	0.071	0.064	0.033	-	0.143	0.054
	275	-	0.292	0.800	-	-	-	-	-	-	-	0.033	0.025	-	0.027
	277	-	-	-	-	-	-	0.100	0.100	-	0.032	0.067	-	-	0.014
	279	-	-	-	-	0.067	0.200	0.200	0.200	-	0.128	0.033	0.025	-	0.014
A128	170	-	-	-	-	-	-	-	-	-	-	-	0.023	-	0.013
	172	-	-	-	-	-	-	0.056	-	-	0.011	-	-	-	-
	174	-	-	-	-	-	-	-	-	0.071	0.011	-	-	-	-
	176	0.026	-	-	0.071	-	-	-	-	-	-	0.033	-	-	-
	178	-	-	-	-	-	-	-	-	-	-	-	0.023	-	0.013
	180	0.316	0.042	-	-	0.033	-	-	-	0.071	0.022	0.400	0.182	0.429	0.308
	182	-	-	-	-	-	-	-	-	-	-	-	0.318	0.071	0.218
	184	-	-	-	-	-	-	-	-	-	-	-	-	0.107	0.039
	190	0.658	0.958	1.000	0.857	0.900	0.900	0.778	0.500	0.643	0.800	0.567	0.432	0.393	0.397
	192	-	-	-	-	-	-	-	-	-	-	-	0.023	-	0.013
	198	-	-	-	0.071	0.067	0.100	0.167	0.500	0.214	0.156	-	-	-	-
B6	133	-	-	-	-	-	-	-	-	-	-	0.063	-	-	-
	139	-	-	-	0.071	-	-	-	-	-	-	-	-	-	-
	143	0.579	0.455	-	0.929	0.533	0.600	0.500	0.200	0.857	0.553	0.188	0.225	0.143	0.176
	145	-	-	-	-	-	-	-	0.100	-	0.011	0.250	0.250	0.321	0.297
	147	-	0.273	-	-	0.033	-	-	0.100	-	0.021	0.219	0.075	0.143	0.122
	151	-	-	-	-	-	-	-	-	-	-	0.031	0.100	0.143	0.108
	154	-	-	-	-	-	-	-	0.100	-	0.011	-	0.075	0.036	0.054
	156	-	-	-	-	-	-	-	-	-	-	0.094	0.225	0.071	0.162
	158	-	-	-	-	0.100	0.050	0.050	0.300	0.071	0.096	-	0.025	0.036	0.027
	162	-	-	-	-	-	-	-	-	-	-	-	-	0.071	0.027
	168	0.342	0.273	1.000	-	0.333	0.350	0.350	0.200	0.071	0.287	0.156	0.025	0.036	0.027
	170	0.079	-	-	-	-	-	0.100	-	-	0.021	-	-	-	-
B123	273	-	-	-	-	0.133	-	-	-	-	0.044	0.125	0.025	0.036	0.027
	275	0.316	0.091	-	0.214	0.167	0.150	0.111	0.250	0.071	0.144	0.125	0.225	0.179	0.203
	277	0.105	0.455	0.250	-	0.333	0.200	0.111	-	0.357	0.233	0.250	0.375	0.393	0.405
	279	0.579	0.455	0.750	0.786	0.167	0.150	0.167	0.125	0.214	0.167	0.438	0.275	0.143	0.216
	281	-	-	-	-	0.167	0.450	0.333	0.375	0.357	0.311	-	0.075	0.179	0.108
	285	-	-	-	-	-	-	-	-	-	-	0.063	-	-	-
	287	-	-	-	-	0.033	0.050	0.278	0.250	-	0.100	-	0.025	0.071	0.041
avg alleles		2.9 *ef*	2.9 *ef*	1.4 *f*	2.2 *ef*	5.3 *bcde*	4.6 *cdef*	5.5 *bcde*	3.9 *def*	4.4 *def*	7.9 *abc*	6.4 *bcd*	8.2 *ab*	7.3 *abcd*	9.9 *a*

Chapter 5

Genetic Relatedness of Species of *Aphytis* in the *lingnanensis* Group with Notes on their Specific Status

Abstract

Aphytis melinus DeBach is recognized worldwide as one of the most effective natural enemies of California red scale, *Aonidiella aurantii* (Maskell), and has become a classic example of successful biological control. It was originally introduced into California in 1957 from its native range of Pakistan and India. Several closely related and morphologically similar *Aphytis* spp. have also been described from the native and introduced ranges of *A. melinus*, but the status of these species has not always been clear. We used 28S-D2 and COI sequences to examine the genetic variation and relatedness of *Aphytis* individuals in native, field-released and captive populations as well as in crossing experiments between captive populations of *A. melinus*. We found evidence for a monophyletic *A. melinus* clade that was supported by our crossing experiments. *Aphytis lingnanensis* forms a complex that splits into two groups. Based on COI data this complex would be considered one species, but morphology and 28S-D2 structuring suggest more than one species. We detected possible evidence for hybridization between *A. melinus* and the *A. lingnanensis* complex based on several California field samples. Surprisingly more variation was observed in the 28S-D2 sequences than the COI sequences. The possible reasons for this variation and the need for multiple sources of evidence for species determination are discussed.

Introduction

A well-known issue in the field of biological control is the lack of accurate identification of pests and natural enemies (reviewed in Gordh and Beardsley, 1999). Parasitoid wasps in the genus *Aphytis* Howard are a good example of natural enemies that are important for biological control, but are often misidentified. There are more than 100 species described in the genus *Aphytis* (Kim, 2003; Noyes, 2003), all of which are tiny (< 1 mm) ectoparasites of armored scale insects (Diaspididae) (Rosen and DeBach, 1979). DeBach et al. (1971) attributed decades of delay in the control of California red scale (*Aonidiella aurantii* [Maskell]) to a lack of taxonomic knowledge. In California, *A. melinus* DeBach and *A. lingnanensis* Compere have been the two most economically important and effective *Aphytis* species.

The California red scale first arrived in California between 1868-1875 (Comstock, 1880) and has been considered one of the most damaging arthropod pests of citrus in the state (Ebeling, 1959; Morse and Luck, 2003; Morse et al., 2007). Since its introduction, there have been at least 35 attempts at importing natural enemies for its control (reviewed in Clausen, 1978). *Aphytis lingnanensis* was first introduced into California from southern China in 1948 when it was recognized as a different species from the already-established *A. chrysomphali* (Mercet) (Compere, 1955). It established quickly in all of the counties of California that had red scale infestations, displacing most *A. chrysomphali* populations, and was considered a moderately effective biological control agent (DeBach and Sundby, 1963; Clausen, 1978).

In 1957, another parasitoid, *A. melinus*, was introduced into California in the hope that it would provide better control in the more extreme inland climatic regions. The original material used to propagate *A. melinus* for mass release came from four relatively small collections, originally maintained as separate cultures inside the Quarantine facility at UC Riverside, California, USA (DeBach, 1959): two Indian collections from New Delhi (108 females, 27 males) and Gurgaon (24 females, 12 males); and, two Pakistani collections from Lahore (14 females, 2 males) and Rawalpindi (11 females, 2 males). DeBach determined that all four cultures would interbreed and combined them to form a single quarantine culture, that was mass-reared and released in California citrus groves during the fall of 1957. Within several years of its introduction, *A. melinus* had established and replaced *A. lingnanensis* in all citrus growing areas except for the mild coastal areas of San Diego County (DeBach and Sundby, 1963). Although the competitive exclusion of *A. lingnanensis* by *A. melinus* has been well documented in the literature (DeBach, 1966; Rosen and DeBach, 1979; Luck and Podoler, 1985), *A. lingnanensis* has recently been found in coexistence with *A. melinus* in the inland areas of southern California (Vasquez et al., 2010a).

The small size, lack of distinguishing morphological characteristics, and subsequent unresolved taxonomy of *Aphytis*, make molecular methods an attractive alternative for their identification (for other examples, see Monti et al. 2005; Pavan and Monteiro, 2007). In the past, Rao and DeBach (1969a, b, c) performed a series of experimental crosses to delineate species boundaries and determine the taxonomic relationships among *Aphytis* species. The crosses were performed under unnatural

conditions and the production of often poorly viable hybrids lead the authors to conclude that introgressive hybridization was very unlikely to occur in nature (Rao and DeBach, 1969c). For example, the reciprocal crosses between what they determined to be *A. lingnanensis* and *A. melinus* produced no female offspring, indicating that two species were reproductively isolated (Rao and DeBach, 1969a).

Here we use DNA sequences of a nuclear ribosomal gene as well as a mitochondrial gene to investigate the taxonomy of *Aphytis* spp. in the *lingnanensis* group. The *Aphytis* used in this study were initially collected in an effort to compare the neutral genetic variation that exists in *A. melinus* from its native range versus its introduced range (Vasquez, 2010).

Materials and methods

Source of specimens. Adult *Aphytis melinus* were collected from four laboratory-maintained colonies (UCR), five California insectaries, California field sites, and Pakistan field sites (Table 5.1). Live adults were collected via aspiration and these were immediately placed into 95% EtOH for preservation and storage at -20ºC for subsequent DNA extraction. Paul DeBach's original colony, propagated from a total of 157 females and 43 males, and released into California fields during the fall of 1957 (DeBach, 1959), has been maintained since then at the University of California, Riverside's Insectary and Quarantine facility. The Taxila, Pakistan colony (S&R [Sending & Receiving] No. 88-05; S&R numbers are filed by UC Riverside Quarantine staff to track shipments) was collected by R. F. Luck (Entomology, UC Riverside) in 1988 and was started from 6

females and 2 males. The Ming Ho, China colony (S&R No. 90-05), also collected by R. F. Luck, was started in 1990 from an unknown number of males and females. The Queensland, Australia colony (S&R No. 92-62) was started in 1992 from 150 live adults shipped from a commercial insectary. These three colonies also have been maintained at the University of California, Riverside's Insectary and Quarantine facility. These four colonies were also sequenced by Kim (2003) and these sequences are included in our 28S-D2 analyses. In addition, five commercial insectaries (listed in Table 1.1 and coded A-E for anonymity) were sampled for *A. melinus*. California field sites were sampled by bringing citrus fruit infested with California red scale into the lab to rear out *Aphytis*. Similar field samples from Pakistan were collected by rearing out adult *A. melinus* from infested citrus fruit collected in 2007 and 2008.

Species in the *Aphytis lingnanensis* group are notoriously difficult to key to species using Rosen and DeBach (1979). We were confident in our identifications of *A. melinus* and *A. africanus*, but for species close to *A. lingnanensis*, we were less certain. Four species that are part of this complex, and possibly our samples, include *A. lingnanensis, A. coheni* DeBach*, A. sp.* near *coheni* and *A. yanonensis* DeBach & Rosen as identified and based on sequences from Kim (2003). We choose herein to treat this latter group as simply part of what we call the "*lingnanensis* complex", a group that we assessed both morphologically and using genetic analyses. Vouchers of all material have been placed in the Entomology Research Museum at the University of California, Riverside.

DNA extraction, amplification and sequencing. DNA was extracted from whole individual adult wasps using the EDNA HiSpEx Tissue Kit (Saturn Biotech, Perth, Australia) and following the manufacturer's protocol for extraction from 1 mm^3 of tissue but halving the volumes of each proprietary solution to compensate for the small size of *Aphytis*. Extracted DNA was stored at -20ºC. Specimen carcasses were subsequently slide-mounted and deposited at the Entomology Research Museum, University of California, Riverside.

The polymerase chain reaction (PCR) was used to amplify a 454 bp section of the D2 domain of 28S-D2 ribosomal DNA using universal insect primers and a protocol from Choudhury and Werren (2006). PCR was performed in 25 µl volumes consisting of: 1X ThermoPol PCR buffer (New England Biolabs, Ipswich, MA), 200 µM dNTPs (Fermentas, Glen Burnie, MD), 1 U *Taq* polymerase (NEB), 0.2 µM of each primer, and 2 µl of template DNA (concentration not determined). Amplification was performed in a Mastercycler 5331 (Eppendorf North America, Inc., New York, NY) following the thermocycling profile: 94°C for 2 minutes; followed by 38 cycles each of 94°C (30 sec), 58°C (50 sec), and 72°C (1.5 min); and a final extension at 72°C for 10 minutes.

A 504 bp section of the cytochrome oxidase *c* subunit I (COI) gene of mitochondrial DNA was amplified in separate 25 µl PCR reactions using the primers mtD-7.2F (5'- ATTAGGAGCHCCHGAYATAGCATT-3') (Brunner et al., 2002) and C1-N-2329 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Simon et al., 1994). Reactions consisted of: 1X ThermoPol PCR buffer (NEB), 1.25 µl BSA (NEB), 1 mM $MgCl_2$, 200 µM dNTPs (Fermentas), 1 U *Taq* polymerase (NEB), 0.2 µM of each primer,

and 2 μl of template DNA (concentration not determined). The thermocycling profile was: 94°C for 2 minutes; 6 cycles each of 94°C (30 sec), 45°C (1.5 min), and 72°C (1.1 min); 36 cycles each of 94°C (30 sec), 51°C (1.5 min), and 72°C (1.1 min); and a final extension at 72°C for 5 minutes.

All PCR products were visualized on 1% agarose gels stained with ethidium bromide. Amplified PCR products were cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and were sequenced in both directions at the University of California Riverside's Institute for Integrative Genome Biology Genomics Core Facility using an Applied Biosystems 3730xl DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences were trimmed and aligned manually using BioEdit version 7.0.9 (Hall, 1999). COI sequences were translated at http://www.ebi.ac.uk/emboss/transeq to confirm that absence of stop codons, insertions, and deletions that are indicative of pseudogenes.

Phylogenetic analyses. 28S-D2 sequences were compared to sequences for members of the *lingnanensis* group and outgroups retrieved from GenBank (accession numbers AY635319-635348; Kim, 2003). 28S-D2 data were analyzed using PAUP* 4.0 (Swofford, 2003) with TBR branch swapping and 100 random search replicates followed by condensing all trees to collapse branches with 0 support, and then filtering for the shortest trees. The same analyses were conducted on each gene region independently and on a combined 28S+COI dataset. Successive Approximations character Weighting (SAW) analysis was applied using the rescaled consistency index and a base weight of 1000 to analyze data stability (Babcock et al., 2001). Boostrap analyses were conducted

on unweighted data with 100 random search replicates. A haplotype network for COI data was created using TCS version 1.21 (Clement et al., 2000) with an arbitrary connection limit set at 100 steps.

Reciprocal crosses. Reciprocal crosses were performed using individuals from three colonies of *A. melinus* that showed variation in 28S-D2 sequences: i.e. the colonies originating from Pakistan, China, and Australia. Virgin wasps were obtained by isolating individual pupae dissected out of oleander scale hosts, *Aspidiotus nerii* Bouchè, into 0.25-dram glass shell vials (Fisher Scientific, Pittsburgh, PA) topped with cotton. After eclosion, all adults were given honey to feed on for 24 h. Individual virgin females were then each provided with a single virgin adult male and allowed 24 h in which to mate. Each pair of wasps was then transferred to a Kerr® Mason jar (Jarden Corporation, Rye, NY) with a lemon infested with late second to early third instar oleander scale. Organic honey was provided as a food source and the jars were covered with a fine mesh cloth and maintained at 22°C. The parents were removed after 14 days, and all emerging offspring were sexed and counted. Each cross was replicated 20 times for a total of 120 crosses. Crosses that did not produce any offspring were excluded from analysis. As a control, 20 crosses were also performed between males and females from the same colony following the same protocol. 20 virgin females from each colony were also given oleander scale-infested lemons to oviposit on and were subjected to the same conditions as the crosses. Statistical analyses were performed using Statistical Analysis Software (SAS) 9.2 for Windows (SAS Institute Inc., Cary, NC). Data were analyzed using general linear models and means were separated using the REGWQ multiple range test.

Results

Phylogenetic analyses. Overall, there was surprisingly more variation in the 28S-D2 sequences than in the COI sequences. There was even 28S-D2 variation within colonies that have been kept in isolation for more than 15 years. For example, within the Pakistan colony there were at least five different genotypes sampled, differing by 1-6 bp. Variation was also found within an individual, with double peaks observed in some chromatograms at nucleotide positions where genotypes differed.

The parsimony analysis of 28S-D2 produced 335 equally parsimonious trees with 315 steps (retention index 0.93). Figure 5.1 depicts one of the 335 trees; branches in bold are supported in all trees. Overall, there were 22 28S-D2 genotypes across the entire data set. All specimens from captive populations and a majority of those from the California field samples nested together with genetic sequences from specimens previously identified as *Aphytis melinus*, to form a monophyletic clade with eight genotypes (Fig. 5.1). The remaining specimens (mainly from the native range in Pakistan, but also including several CA field specimens) formed a clade (the *A. lingnanensis* complex in Fig. 5.1 tree) with 14 genotypes. These grouped with sequences from specimens previously identified as *A. lingnanensis*, *A. coheni*, *A.* nr. *coheni*, and *A. yanonensis*. The *A. lingnanensis* complex can be further split into two groups each containing seven genotypes. The A group is paraphyletic and corresponds with sequences of *A. lignanensis*, *A. coheni* and *A. yanonensis*, but collapses in a concensus. The B group

corresponds with the sequence of *A.* near *coheni,* and is not only monophyletic, but groups into 5 subgroups based on single additive base pair differences.

There were 11 different COI haplotypes (Figure 5.2.) that fell into two clusters, for the most part supporting the same division indicated by 28S-D2. Three haplotypes (2 common, 1 rare), found only in specimens of *A. melinus* (M1-M3) differed from each other by only 1-2 bp. Within the *A. lignanensis* A & B complex, 8 haplotypes were recognized (L1-8) that were minimally 29bp (5.8%) different from the three *A. melinus* haplotypes and formed a cluster differing from each other by up to 13 bp (2.6%). Two distinct haplotypes (L7 & L8) were found only in the California field populations. These haplotypes differed from the other *A. lingnanensis* haplotypes (L1-L6) by 8-13 bp and from each other by 4 bp. Seven of these haplotypes were only found in individuals with a *lingnanensis* nuclear genotype, and four of these (L1, L2, L5, L6) were freely distributed across the A and B groups of the *A. lingnanensis* complex (Fig. 5.1 tree). The L7 COI haplotype was found both in individuals with *A. melinus* and *A. lingnanensis* genotypes, differing by 36 bp in their 28S-D2 sequence and morphologically corresponding with members of each group (J. Heraty, personal communication). The haplotype analysis, and an independent phylogenetic analysis propose that the L7 and L8 haplotypes are derived from within the *A. lingnanensis* nework (Fig. 5.1 & 5.2). The appearance of the L7 haplotype within *A. melinus* is likely the result of a horizontal transfer through hybridization.

A combined analysis of 28S-D2 and COI resulted in numerous trees (>42,000) of length 454 and an r.i. of 0.93. The L7-*A. melinus* specimen was the largest cause of the

instability. It was placed in a majority rule tree (75%) as the sister of the *A. lingnanensis* complex, and in other trees as the sister group of *A. melinus*. *Aphytis melinus* also became unstable, and was monophyletic in only 72% of the MR trees. Other relationships not resolved were the result of conflicts between the 28S-D2 and COI haplotypes within the *A. lingnanensis* species complex and *A. melinus*. This is a result of the scattered distribution of COI haplotypes within each group as shown on Fig. 5.1. This analysis provides clear evidence of the conflict that can arise from an uneven mixing of nuclear and mitichondrial genomes, and horizontal gene transfer.

Reciprocal crosses. All crosses of *A. melinus* produced offspring and overall, the crosses did not have a significantly different sex ratio ($F_{8,144} = 1.05$; $P = 0.4005$). Sex ratios ranged from 46% to 60% female, but none were significantly different from one another based on REGWQ. All of the control virgins produced only male offspring (as would be expected).

Discussion

The importance of accurate identifications in biological control cannot be over emphasized (Heraty, 2003). Many species of parasitoids used as natural enemies are small and notoriously difficult to identify, possibly representing cryptic species. Wasps in the genus *Aphytis* have been used in biological control for decades, but have proven difficult to identify based on morphological traits. In an attempt to better understand the taxonomic relationships of specimens collected from four cultures at UC Riverside, five

California insectaries, four California field sites, and four field locations in Pakistan, we employed the use of nuclear 28S-D2 and mitochondrial COI markers.

Based on our phylogenetic analyses, we conclude that all of the populations that fall into the *Aphytis melinus* clade (Fig. 5.1) are indeed one species. The three COI haplotypes (M1-M3) are found dispersed throughout the clade and the 28S-D2 differences represent intraspecific genetic variation. The *A. lingnanensis* complex creates a less straightforward picture. The Pakistan field samples and samples from two locations in southern California grouped in the *A. lingnanensis* complex, along with populations from South Africa identified as *A. coheni* and *A. sp.* near *coheni*, *A. lingnanensis*, and a population of *A. yanonensis* originally sampled from Japan. The distribution of COI haplotypes throughout the complex (both the A and B groups; Fig. 5.1), would suggest that we consider this as likely to be one species. However, the structure of 28S-D2 variation and observed morphological differences suggest that more than one species may be involved. These taxa need to be part of future investigations.

The expectation that 28S-D2 would show less variation than COI was not upheld in our study. 28S-D2 is a highly conserved region of the ribosomal cistron. Although repeated many times in each cell, concerted evolution is thought to typically result in complete homogeny of different repeats (Hillis and Dixon, 1991) within an individual and within different individuals of the same species. As such, it is often used for species identification (Stouthamer, 2006). In the present study, 28S-D2 was variable, both within and between individuals. Mitochondrial DNA is considered to be much more variable within a species as a result of its higher mutation rate (Brown et al., 1979) and is

frequently used in DNA barcoding (Hebert et al., 2003), although the usefulness of this gene for this purpose has been highly debated (Rubinoff et al., 2006). The recommendation of DNA barcoding that a 2-3% divergence in COI denotes a different species (Hebert et al., 2003) does not help to clarify the *A. lingnanensis* situation (we see a 2.58% difference).

In our study, relative to 28S-D2, we found COI to be conserved, particularly in the *A. melinus* group (Fig. 2 network). Mitochondrial haplotypes were divided into 2 groups, which were largely congruent with the 28S-D2 division (*A. lingnanensis* and *A. melinus*), but these were dispersed across the different nuclear genotypes in the corresponding 28S-D2 group indicating substantial interbreeding among the members within each group. We also found evidence for hybridization between the two groups, with two individuals from the CA field samples that possessed the nuclear genotype of *A. melinus*, having a mtDNA haplotype predominantly associated with the *A. lingnanensis* nuclear genotype. Although it is only two specimens, having a COI sequence of *A. lingnanensis* and a 28S-D2 sequence of *A. melinus* within an individual is indicative of hybridization in the field. Had we only considered one gene region in our study, such as COI, which is suggested by advocates of mitochondrial DNA barcoding (Hebert et al., 2003), our results would not have shown an accurate picture of the variation within *Aphytis* species. This alludes to the need for multiple sources of evidence for species determination as outlined by DeSalle et al. (2005). As well, the mix of COI haplotypes both within and between groups had severe implications on our combined analysis, with almost no resolution in a strict consensus of the numerous resulting tree topologies. Data

concordance between genomes is an issue, especially when confused with haplotypes of both genes that may differ only slightly and the possibility of gene introgression of COI haplotypes (L7) through hybridization events that do not affect species integrity.

One possible explanation for the relative lack of mtDNA variation could be the presence of cytoplasmic symbionts, such as *Wolbachia*, driving the spread of certain haplotypes. *Wolbachia* is an endosymbiotic bacterium that can manipulate host reproduction and has been associated with a decrease in mtDNA diversity via directional selection as it spreads throughout a population (Turelli et al., 1992, Jiggins, 2003, Shoemaker et al., 2004). *Aphytis melinus* is known to harbor a cytoplasmic incompatibility-inducing *Wolbachia* infection (Vasquez et al., 2010b), which may explain the low number of mitochondrial haplotypes found in this species with respect to the variation in 28S-D2. However, to our knowledge, *A. lingnanensis* has not been tested for the presence of *Wolbachia*.

Our conclusion that *A. melinus* is a single species is supported by the results of our crossing study, as based on the biological species concept in which interbreeding organisms are defined as the same species. However, our crossing study has several caveats. First, the actual individuals that were crossed were not the same ones that were sequenced and were used for our phylogenetic analyses. Although they were taken from the same colonies, we cannot say with certainty that the individuals that were crossed had different 28S-D2 or COI haplotypes. Also, the F1 offspring of the crosses were not tested for fitness or functionality, an important step in crossing and hybridization studies.

One way to know for certain whether the genotypes within the *A. lingnanensis* complex can be attributed to different species would be to conduct further crossing experiments. Fernando and Walter (1997) performed mating tests between different populations that all appeared to be *A. lingnanensis* based on morphological characteristics and found that populations collected from California red scale did not readily mate with those from the white louse scale, *Unaspis citri* Comstock. However, if reproductive isolation between two species is weak then hybridization may occur, clouding the results. It has been estimated that 10% of animal species hybridize (Mallet, 2005).

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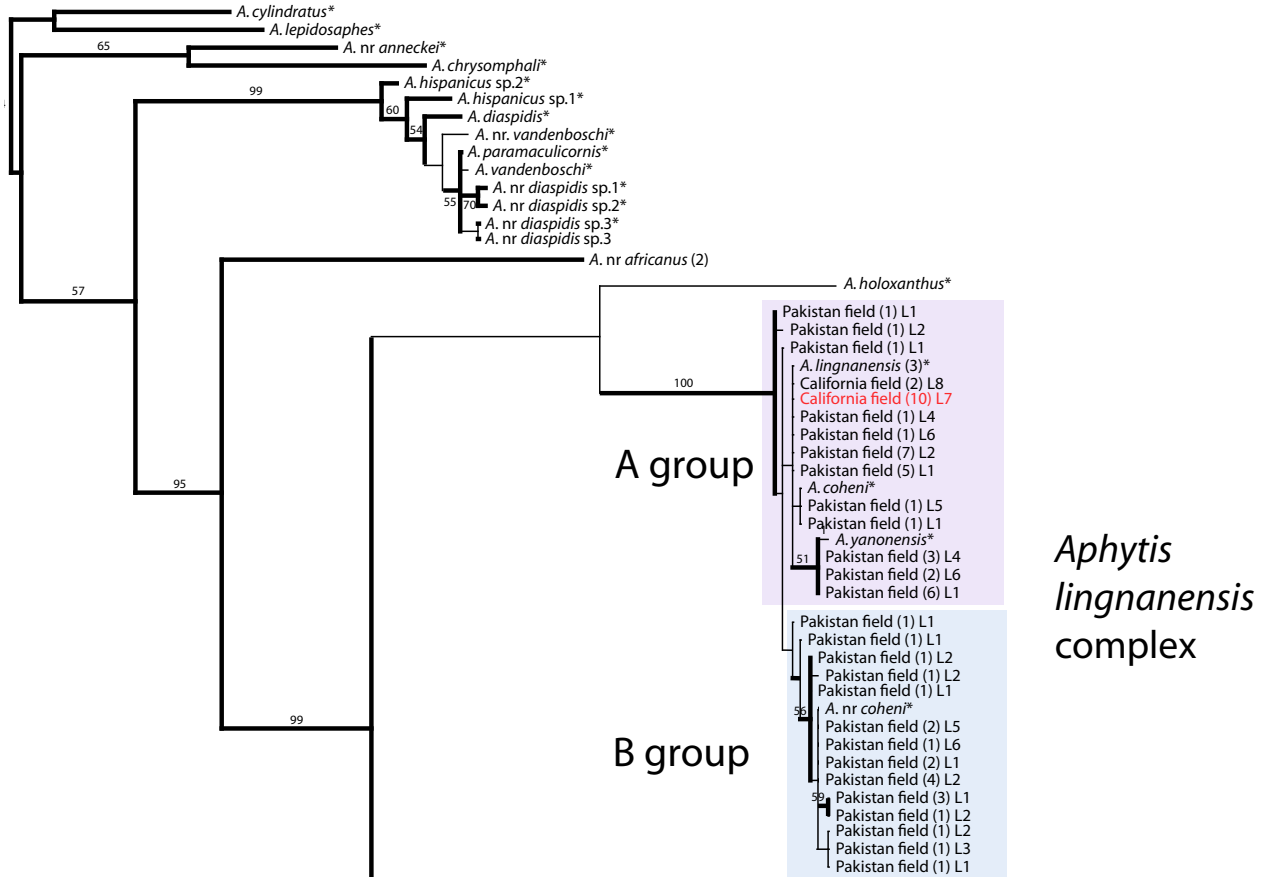
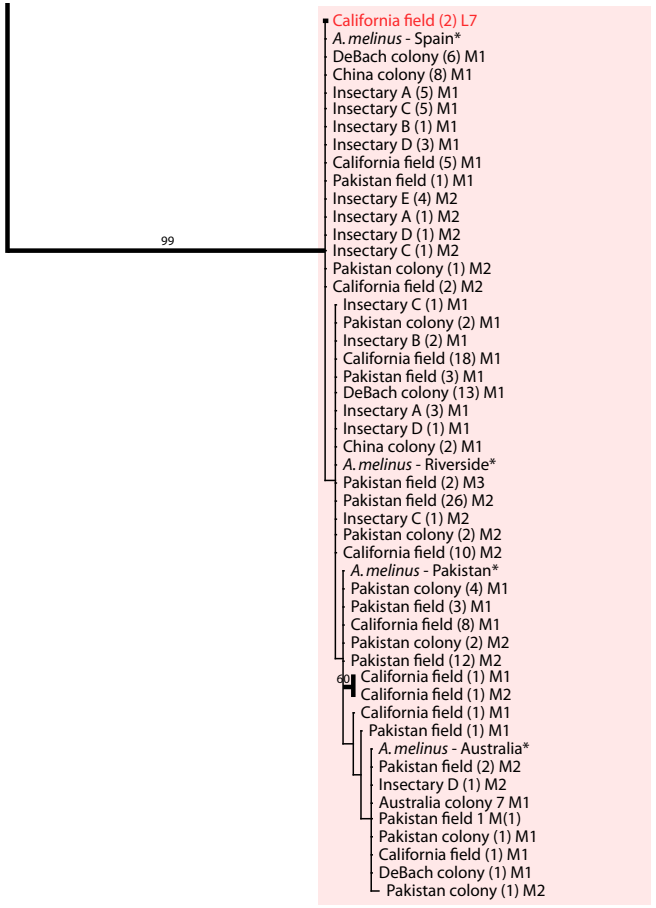
Table 5.1. Collection records of *A. melinus* used in phylogenetic analyses.

Collection location	*N*	Collector	Year
DeBach colony – original from 1957	20	C. Vasquez	2008
Pakistan colony – from Taxila – 1988	13	C. Vasquez	2008
China colony – from Ming Ho – 1990	10	C. Vasquez	2008
Australia colony –from Queensland – 1992	7	C. Vasquez	2008
California insectaries	28		
Insectary A	9	C. Vasquez	2005, 2007
Insectary B	3	C. Vasquez	2005, 2007
Insectary C	8	C. Vasquez	2005, 2007
Insectary D	6	C. Vasquez	2005
Insectary E	4	C. Vasquez	2007
California field sites	61		
Tulare County	26	B. Grafton-Cardwell	2004
Kern County	7	B. Grafton-Cardwell	2004
Riverside County	15	L. Forster	2003
San Bernardino County	13	C. Boisseranc	2005
Pakistan field sites	128		
Peshawar, Pakistan	99	I. Khan	2007, 2008
Bhalwal, Pakistan	21	I. Khan	2008
Islamasbad, Pakistan	5	I. Khan	2008
Kot Momin, Pakistan	3	I. Khan	2008

Figure 5.1. One of 335 equally parsomonious trees produced from analysis of 28S-D2 sequences analyzed in PAUP (315 steps; r.i. 0.93). Numbers on branches show bootstrap values based on 100 replicates. Thin branches collapse in a strict consensus tree. Numbers in parentheses next to the name are the number of individuals sequenced, with different *A. linganensis* or *A. melinus* COI haplotypes indicated last (Lx or Mx). Sequences obtained from Kim (2003), shown with an *, were identified via morphological and molecular data, but lack data from the same region of COI that was used in this study. Specimens in red are those that have the same mitochondrial haplotype but incongruent 28S-D2 sequences.



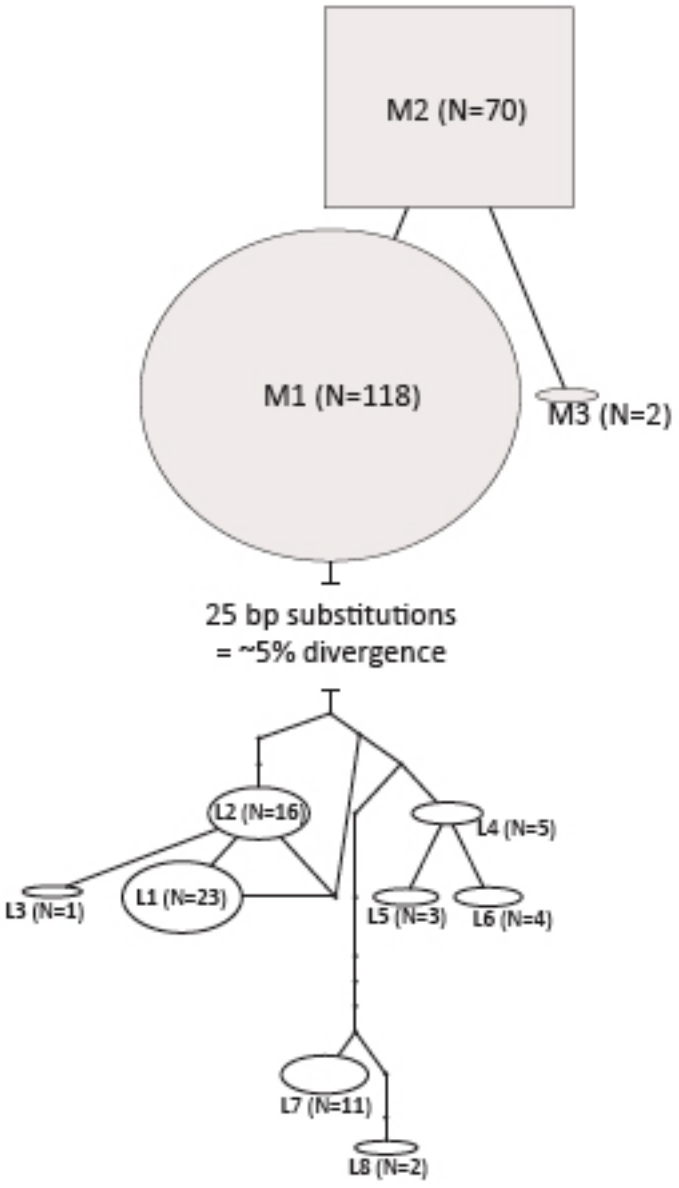


28S-D2
315 steps
r.i. 0.93
1 of 335 trees

- 1 change

Aphytis melinus

Figure 5.2. Mitochondrial network for the three *Aphytis melinus* haplotypes (M1-M3) and eight *Aphytis lingnanensis* haplotypes (L1-L8). The number of samples for each haplotype is listed in parentheses. Each line connecting haplotypes represents one nucleotide base change (with the exception of the the connection between the *A. melinus* group and *A. lingnanensis* group which, as indicated, is 25 bp) and small circles represent unobserved haplotypes.



Conclusion

Our results from the first study suggest that all five of the evaluated insectaries display reduced *A. melinus* quality and fitness parameters during certain times of the year. Decreased longevity and male-biased sex ratios were evident in the cooler months of November – March. Smaller females were produced in the warmer months of July – August. Data from insectary B, when averaged over the six shipments, showed the highest percentage females, the largest size of live females, and tied for the highest female survival rate. Variation in longevity, sex ratio, and size persisted despite a relatively constant production environment of approximately 60% relative humidity and 25ºC at all of the insectaries. Although all five evaluated insectaries adapted rearing methods from the same protocol (DeBach and White 1960), they differed in some ways that appeared to affect production and fitness of *A. melinus*.

The second study verifies for the first time that *A. melinus* is infected with a *Wolbachia* that induces complete cytoplasmic incompatibility (CI). DNA analysis (PCR results) and crossing data both support this conclusion. Additionally, we found there was no significant difference between the number of males produced from the incompatible and compatible crosses, implying that *Wolbachia* functions by killing the female eggs in *A. melinus*, and not by allowing the incompatible eggs to develop into males. The results of this study suggest that *Wolbachia* does impart a fitness cost in *Aphytis melinus*, as is evident from the decreased fecundity of virgin females and the decreased longevity of both female and male wasps that were fed honey. We found no evidence of size

difference between infected and uninfected females used in this study, a variable that is often correlated with differences in fitness, and we therefore conclude that *Wolbachia* did not cause differences in *A. melinus* female size.

The infection survey shows a high frequency of *Wolbachia* infection in both field and insectary populations of *A. melinus* in California. Some of the frequencies were lower than 100%, which may somewhat hamper reproductive potential of the wasps, but does not indicate a need to rear and release "cured" *A. melinus* into the field. Although rearing *A. melinus* at high temperatures (up to 32.5°C) did not cure the wasps of their *Wolbachia* infection, it did decrease *Wolbachia* titer.

CI-*Wolbachia* infection is usually considered beneficial to females because it allows them to fertilize their eggs and protects them from any mortality induced by CI. However, here we saw a decrease in fitness when *A. melinus* was infected. Until more evidence emerges on the fitness effects of *Wolbachia* from other species, we recommend that all biological control agents be screened for *Wolbachia* and tested for fitness costs associated with infection that could be potentially detrimental to biological control efforts.

The study of genetic variation in *A. melinus* showed the highest number of alleles in the Pakistani field samples, but the California insectary and California field samples also showed a surprising amount of genetic diversity in spite of the small number of *A. melinus* that were initially imported. Many authors have hypothesized regarding the importance of genetic diversity (Hopper et al. 1993, Margan et al. 1998, Nunney 2002, 2003, 2006), but field trials are desperately needed.

Lastly, during the collection of samples for the genetic variation study, we found specimens of other *Aphytis* spp. in some of our populations. Multiple samples of *Aphytis lingnanensis* were identified from the UC Riverside's biological control grove. This discovery does not support the traditional explanation involving competitive displacement of *A. lingnanensis* by *A. melinus* from the inland valleys of southern California.

Based on the phylogenetic analysis of our samples as well as the results from our crossing experiments, we conclude that all of the populations that fall into the *Aphytis melinus* clade are indeed one species. The *A. lingnanensis* complex creates a less straightforward picture. The distribution of COI haplotypes throughout the complex, would suggest that we consider this as likely to be one species. However, the structure of 28S-D2 variation and observed morphological differences suggest that more than one species may be involved. Additionally, the expectation that 28S-D2 would show less variation than COI was not upheld in our study. Relative to 28S-D2, we found COI to be conserved, particularly in the *A. melinus* group. We also found evidence for hybridization between the two groups, with two individuals from the CA field samples that possessed the nuclear genotype of *A. melinus*, having a mtDNA haplotype predominantly associated with the *A. lingnanensis* nuclear genotype.

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