DETECTION OF *XYLELLA FASTIDIOSA* IN INSECT VECTORS IN CALIFORNIA

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ABSTRACT

Recent spread of *Xylella fastidiosa (Xf)* to several agricultural commodities and ornamental plants in California has prompted great interest in understanding the comparative interactions between *Xf* and native and recently introduced insect vectors. The generally low titer of *Xf* in insect vectors limits the use of serological techniques, such as ELISA, for qualitative and quantitative analyses of *Xf* associated with different insect vectors. *Xf* detection by molecular techniques, such as PCR, can potentially overcome this limitation. The objective of this study was to compare standard PCR for detection of *Xf* in field-collected insects as well as in greenhouse-reared insect vectors using primers RST31/RST33 with newly developed primers HL5/HL6 in standard PCR and in Real Time PCR using the system HL5/HL6 and a probe labeled with FAM. Two native species the green sharpshooter (*Draeculacephala minerva*) and the red-headed sharpshooter (*Xyphon fulgida*), and the recently introduced glassy-winged sharpshooter (*Homalodisca coagulata)* were included in this study. Field-collected insects were obtained from *Xf*-infected grapevines and almonds in the San Joaquin Valley, California. Greenhouse-reared green and red-headed sharpshooters were also obtained from cultures maintained on a non-host of *Xf* in Parlier, California. Five-10 *Xf* cells per µL of insect head DNA sample were detected with the HL5/HL6 primer pair-FAM system. Also, using this system, the number of *Xf*-cells detected in field-collected and greenhouse reared insect was between 10^2-10^3/µL sample/reaction. This concentration of *Xf* cells was detected by visualization of the *Xf*-specific amplicon (221 bp) in gels following standard PCR with the HL5/HL6 primers. This level of pathogen in insect heads was below the limit of detection in standard PCR with primers RST31/RST33. Using Real-Time PCR quantification with the system HL5/HL6-FAM, the total amount of *Xf*-cells per insect head was estimated to be between 10^4-10^5. Implications of these results on the epidemiology of the disease are discussed.

EVALUATION OF A NOVEL, FIELD DEPLOYABLE, ELECTROCHEMICAL DETECTION SYSTEM FOR THE DETECTION OF *XYLELLA FASTIDIOSA* WITHIN GRAPEVINE PETIOLES

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ABSTRACT

We have tested a new electro-chemical detection (ECD) system designed by AnzenBio, Inc. for the quick detection of *Xylella fastidiosa* within grapevine petioles. Like standard ELISA this detection method relies on antibodies against the bacterium, but unlike ELISA it detects movement of electrons through the final product conversion, measuring current rather than color change. Using a hand-held meter and pre-coated chips the test can be done in a fraction of the time (1.5 vs. 5 hrs.). Comparison of 18 Cabernet Sauvignon petioles from a vineyard with Pierce's disease (PD) to 18 petioles guaranteed PD free showed the ECD readings per gram of tissue to be higher for PD petioles (31.3 vs. 6.2 microamps). This difference is statistically different using a t-test (p<0.0001). In another trial in South Texas, ECD was used to evaluate the petioles from three different varieties, Blanc du Bois, Black Spanish and Cynthiana, which have been shown to carry differing levels of *Xylella fastidiosa* within this area of high PD pressure. Petioles were also categorized into those from leaves with low, medium and high PD symptoms. Analysis of variance on ECD data from the 9 symptom variety categories with 6 replications showed that ECD could detect distinct significant differences between several of the categories (p<0.0001). Analysis of variance on ELISA data run on the same 54 samples found no significance between categories (p=.43). ECD appears to give more sensitive readings over a range of bacterial levels, potentially giving fewer false positives.

Section 5:
Control Strategies

ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES

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ABSTRACT

Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new hosts. *Alcaligenes xylosoxidans denitrificans* (*Axd*), an insect and plant symbiotic bacterium, occupies same niche as the plant pathogen *Xylella fastidiosa* (*Xf*) which causes Pierce's disease. We determined the fate of genetically altered *Axd* (*RAxd*) after introduction into grapevines to assess its feasibility as a symbiotic control organism to control *Xf*. *RAxd*, which expresses a fluorescent protein (DsRed), was applied to grapevines by needle inoculation, foliar spray application, or soil drench. The plants were covered with insect-resistant screening, to exclude arthropods from test plants. *RAxd* were detected in stems of several grapevines 2 weeks post-inoculation from each inoculation type. The amount detected at 4 weeks post-inoculation declined, and *RAxd* was absent 6 weeks post-inoculation. *RAxd* was not detected in grape berries or soil samples collected around *RAxd* positive grapevines. This work demonstrated that transgenic *Axd* became established in grapevines in the field but did not thrive there. A limited lifespan of transformed *Axd* in grapevines would keep its population increase in check in that host plant. Re-inoculation of grapevines at 6 wk intervals would be sufficient to keep anti-pathogen products present. *RAxd* thrives in GWSS and citrus. Therefore, there is a good chance that GWSS would pick up the *RAxd* as an antimicrobial symbiont from nearby sources to render GWSS vector-incompetent.

INTRODUCTION

Replacement therapy or symbiotic control employs symbiotic bacteria to deliver anti-disease compounds to target pathogens of plants to make vector insects unable to harbor the pathogen or to prevent a pathogen from being transmitted to healthy plants (1). *Alcaligenes xylosoxidans denitrificans* (*Axd*), was selected for further study and a fluorescent marker gene inserted. We followed the movement of genetically altered *Axd* (*RAxd*) in grapevines and in the vector insect, glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*.

Regulatory and industry acceptance of this approach requires knowing the fate of *Axd* in various locations and in plants at different times of the year. Our current detection methods employ PCR (polymerase chain reaction) and fluorescence microscopy (3, 4). QRT-PCR provides a quantitative measure of bacteria in the samples, which is missing from existing methods. This is important because it allows determining optimum doses and timing for application of the delivery organism and its expression of anti-*Xylella* products.

Fluorescent protein gene markers are now commonly used in genetics and are considered environmentally benign since they are based on natural products. The bacterial transformation cassette was inserted with so-called jumping genes (mobile or transposable elements) originally identified in *Drosophila mauritiana* and called mariners (7). The mariner elements have had their jump mechanism removed (so the inserted gene will not be remobilized) and all antibiotic genes used for selection have been removed (so no antibiotic factors can be moved inadvertently to other bacteria). The resulting transgenic strains are very stable and grow readily in culture. Little or no mutation or reversion has been observed.

Since the marker genes were placed next to an open reading site that is designed to contain the future anti-*Xf* compound, the bacteria we are using now are nearly complete. In other words it is close to the final product. Thus, we can study the biology of the genetically altered vehicle bacterium, *RAxd*, and its behavior in the vineyard ecosystem.

We prefer to do this in commercial vineyards because the laboratory experiments are never fully indicative of behavior in the field. We chose widely separated locations and in California and more than one variety of grapevine to test. A top priority was to determine if the transgenic endophyte lodged in the grape berries or otherwise contaminated the product of the vineyards.

OBJECTIVES
1. Track the movement of *Alcaligenes xylosoxidans denitrificans* (*Axd*) within plants with or without insect involvement and track movement in the environment.
2. Characterize transmission of *Axd* by glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*).
3. Develop an application method for transgenic *Axd* into the xylem of grape plants for delivery of an anti-*Xylella* strategy.

RESULTS
In July 2003, field sites were established at four locations in the state of California; Napa, Bakersfield, Temecula, and Riverside. At the Napa, Bakersfield, and Temecula sites, *RAxd* was applied to grapevines using 3 inoculation techniques; needle inoculation, foliar spray application, and soil drench. These plants were covered with insect-free screening, to exclude arthropods from test plants. Samples were taken throughout the growing season and processed. Grapevines at the Riverside field site were needle inoculated with *RAxd* and three concentrations of GWSS (0, 10, and 50) were placed on the plants to test the affect of GWSS feeding pressure on the translocation of *RAxd* in grapevines. We collected mature grapes and plant parts for analysis from grapevines at all four field sites.

Detection of RAxd in Grapevine Xylem: Napa Field Site
Grapevines were inoculated 41 days prior to harvest. Pre-harvest grapevines xylem samples were collected three times (2, 4, and 6 weeks post-inoculation). Only single samples from 2 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. These positives were from plants treated by needle inoculation and soil drench. Two weeks later, only a single sample from the soil drench-treated plant tested positive. There were no positive samples collected 6 weeks after inoculation. No control plants tested positive for the presence of *RAxd* on any date.

Bakersfield Field Site
Grapevines were inoculated 33 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Multiple samples from 8 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. Of these *RAxd* positives plants, 3/5 from foliar spray, 2/5 from needle inoculation and 3/5 from soil drench. Two weeks later, only two plants from the foliar spray-treated grapevines tested positive. No control plants tested positive for the presence of *RAxd* on any date.

Temecula Field Site
Grapevines were inoculated 43 days prior to harvest. Pre-harvest grapevines xylem samples were collected 3 times (2, 4, and 6 weeks post-inoculation). No samples on any collection date tested positive for the presence of *RAxd*.

Riverside Field Site
Grapevines were inoculated 26 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Only 10 samples collected 2 weeks after inoculation were positive for the presence of *RAxd*. Six of the positive samples were from grapevines with no GWSS included, while 4 of the positive samples were from grapevines with GWSS included. No significant differences in *RAxd* presence in grapevines could be attributed to the presence of GWSS (X^2=0.24 df=1, *p* value=0.624).

Detection of RAxd in Soil. RAxd
Detection of *RAxd* in soil. *RAxd* was not detected in soil samples collected from the base of any grapevines at any locations using the culture methods or RT-PCR.

Detection of RAxd in Grape Berries
In grape cluster samples collected on the date of harvest (Napa Aug. 27, Bakersfield Sept. 3, Temecula Sept. 2, and Riverside Aug. 18), *RAxd* was not detected by RT-PCR in whole grape samples from any location. Furthermore, *RAxd* was not detected in dissected grape berry samples of flesh, veins, seeds, peduncle, or stem from any location.

Detection at the Time of Field Plot Destruction
RAxd was not detected in grapevine, root, or soil samples at the time of removal.

Table 1. Detection of *RAxd* in grapevines from three field sites (2003).

Application Method	*RAxd* positive samples[1]					During grapevine removal[2]	
	Weeks post-inoculation				Berries[4]	Canes	Root[5]
	0	2	4	6[3]			
Bakersfield Foliar Spray	0	**3**	**2**	ND	0	0	0
Needle Inoculation	0	**2**	0	ND	0	0	0
Soil Drench	0	**3**	0	ND	0	0	0
Control	0	0	0	ND	0	0	ND
Napa Foliar Spray	0	0	0	0	0	0	0
Needle Inoculation	0	0	0	0	0	0	0
Soil Drench	0	**1**	**1**	0	0	0	0
Control	0	0	0	0	0	0	ND
Temecula Foliar Spray	0	0	0	0	0	0	0
Needle Inoculation	0	0	0	0	0	0	0
Soil Drench	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	ND

[1]Represents 6 samples from 5 grapevines per treatment per field site (n=30 per grapevine).
[2]Grapevines were removes >14 weeks after inoculations at all locations.
[3]ND = not determined.
[4]Berries were collected during final collection date.
[5]Root samples were taken only from *RAxd* treated vines.

2004 Field Project
Data are not complete and will not be reported here.

CONCLUSIONS
Grapevine inoculations were made after 50-80 days following grapevine flowering at all locations. After flowering, both the xylem and the phloem begin to fill the fruit with fluid (6). Between 60 and 70 days after flowering, the xylem stops filling the fruit, and phloem contributes all fluid for the development of the fruit. This flow continues to 120 days after flowering which is the average number of days to fruit harvest. At all field sites, *RAxd* inoculations were made 26 (Riverside) to 43 (Temecula) days prior to harvest. Considering grapevine physiology, inoculations were made after the xylem ceased to contribute fluid directly to the fruit in all cases. Therefore, it was not surprising that *RAxd* was not found in fruit at any location because it is a xylem-associated bacterium (3).

The most probable explanation for the inability of *RAxd* to survive in grapevines after 4 weeks was its lack of competitive fitness associated with the transgenic organism. Xylem contains diverse and sometimes extensive communities of microbes (2). In greenhouse studies, a strain of EGFP protein-expressing *Axd* was introduced into seedlings of several plant species (3). In that study, the genetically marked bacterium moved readily within the xylem vessels of the plants and was recovered 10 months later. However, presence of a well-established microbial community may have restricted the growth and colonization of transformed *Axd*, ultimately leading to its demise. Chromosomally transformed organisms are commonly less fit than native bacterial species due to the cost of the genetic insert (5). A comparison of the genetically modified *Axd* to the native *Axd* showed that the transformed strain was less fit in laboratory cultures (Lauzon, unpublished data). Although the experiments were not designed to test the relative fitness of transgenic *Axd,* the bacterium's inability to persist longer than 4 weeks provides additional support for the theory of reduced fitness.

Given the ubiquitous nature of *Axd* and its ability to colonize several plant hosts, including grapevines, in the greenhouse (3), we expected it to persist longer in field-grown grapevines. Even so, viability of 4 weeks may offer a large enough window for the delivery agent in a symbiont control strategy to dispense the necessary anti-pathogen factors to negatively affect *Xf*. Additionally, re-application of the symbiotic control agent may be necessary. Additionally, reduced fitness offers an internal controlled mechanism that will guard against transformed *Axd* population spread and persistence in the environment or consumer products.

REFERENCES
1. Beard, C.B., Cordon-Rosales, C., and Durvasula, R.V. 2002. Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. Annu. Rev. Entomol. 47:123-141.
2. Bell, C. R., Dickie, G. A., Harvey, W. L. G., and Chan, J. W. Y. F. 1995. Endophytic bacteria in grapevine. *Can. J. Microbiol.* 41:46-53.
3. Bextine, B., Lampe, D., Lauzon, C., Jackson, B.C., and Miller, T.A. 2004. Establishment of a genetically marked insect-derived symbiont in multiple host plants. Curr. Microbiol. In Press.
4. Bextine, B., Lauzon, C., Potter, S., Lampe, D., and Miller, T.A. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Curr. Microbiol. 48:327-331.
5. De Leij, F.A.A.M., Thomas, C.E., Bailey, M.J., Whipps, J.M., and Lynch, J.M. 1998. Effect of insertion site and metabolic load on the environmental fitness of a genetically modified *Pseudomonas fluorescens* isolate. Appl. Envir. Microbiol. 64:2634-2638.
6. Mullins, M.G., Bouquet, A., and Williams, L.E. 1998. The Biology of the Grapevine.
7. Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., and Mekalanos, J.J. 1999. *In vivo* transposition of mariner-based elements in enteric bacteria and mycobacteria. Proc Natl Acad Sci. 96:1645-1650.

PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: BIOLOGY OF ENDOPHYTIC BACTERIA IN GRAPE PLANTS AND BIOASSAY OF REAGENTS TO DISRUPT PIERCE'S DISEASE

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ABSTRACT

Xylella fastidiosa (*Xf*), which causes Pierce's disease (PD) in grapevines, is transmitted by the glassy-winged sharpshooter (GWSS). Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new host plants. *Alcaligenes xylosoxidans denitrificans* (*Axd*) was identified as a potential agent for paratransgenesis because it inhabits the foregut of GWSS and the xylem of plants, as does *Xf*. In this report, we describe the relationship between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control. Additionally, disruption of *Xf*-transmission by GWSS was demonstrated using two reagents, a single chained antibody fragment and an antibiotic peptide.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is the principal vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapevines. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission would control the disease.

Symbiotic control approaches have been developed to disrupt Triatomid transmission of *Trypanosoma cruzi* [3], to prevent colitis in mammals [4, 11], and to interfere with transmission of HIV [8]. Candidate microbes that live in close proximity to the pathogen in the vector insects and in host plant tissues would by ideal vehicles to control *Xf*.

Alcaligenes xylosoxidans denitrificans (Axd), originally isolated from the cibarium of GWSS, has been described as a non-pathogenic plant endophyte and a non-pathogenic soil-borne microbe [10, 12]. *Axd*, genetically marked with DsRed or EGFP protein, colonized the cibarium of GWSS for up to 35 days, the longest period tested [6]. *Axd* readily colonized the xylem vessels of several plants with citrus being the most hospitable to the bacterium. [5]

Two categories of anti-pathogen reagents, single-chained antibodies (scFV) and antibiotic peptides, were tested for activity against *Xf*. Screening of scFV uncovered an antibody fragment that was specific to *Xf* and may be specific to the PD-causing strain of *Xf*. Four toxic peptides were identified that inhibited the growth of *Xf*, but did not inhibit the growth of *Axd*.

OBJECTIVES

1. Identify relationships between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control.
2. Test the ability of anti-pathogens to disrupt *Xf* disease cycle.

RESULTS

Axd Movement and Colonization within Host Plants

In two trials, chrysanthemums (n=20) were needle inoculated with DsRed *Axd,* which contains a kanamycin-resistance gene. One week later phloem and xylem fluid samples were collected independent of one another using a Scholander pressure bomb [7]. The collection resulted in about 20-50 µl of phloem fluid and 100-150 µl of xylem fluid per stem. DNA was extracted from the remaining half of each phloem and xylem fluid sample from each plant using the Extract-N-Amp™ Plant kit (Sigma Aldrich, Steinheim, Germany). Presence of *Axd* was then determined using QRT PCR. The other half of each phloem and xylem fluid sample was inoculated into LB broth containing kanamycin and incubated for 48 h at 37ºC. After the incubation period, bacteria were screened for red fluorescence using a MZ12 fluorescent microscope (Leica Microsystems Inc., Heerbrugg, Switzerland). Positive samples were confirmed by QRT PCR.

A higher proportion of xylem fluid samples tested positive for the presence of *Axd* than phloem samples in both trials: in trial 1 xylem 8/20, phloem 2/20 (x^2=4.8, 1df, p=0.0284); in trial 2 xylem 15/20, phloem 8/20 (x^2=5.013, 1df, p=0.025). In all

cases, positive phloem samples were detected only when the corresponding xylem samples was positive, whereas, most xylem samples were positive when phloem samples were negative. This indicated that positive detection of *Axd* in the xylem was due to actual presence of the bacterium; detection in phloem may have been due to contamination. Of the samples that tested positive, xylem samples contained 10X more cells on average than phloem although these values were not significant at the p=0.05 level (Trial 1: F=0.911, 1df, p=0.368. Trail 2: F=3.123, 1df, *p*=0.092). All plant samples which tested positive by RT PCR were confirmed by culturing followed by visualization under fluorescent microscopy.

Movement of Axd into GWSS Populations

After being exposed to an artificial feeding system containing DsRed *Axd* for 48h [6], 2 GWSS were marked with paint and placed on an individually caged chrysanthemum with 10 naive GWSS for 2 weeks. At the end of this period, all GWSS were collected from the cage and analyzed for the presence of DsRed *Axd* by QRT PCR. In two trial, each with 10 replicates (10 individually caged plants), 81% of the test insects survived through the studies. In both trials, more than 57% of the surviving, previously "naïve", GWSS tested positive for the presence of *Axd* (Trial 1, 51.2%; Trial 2, 64.3%). Therefore, through passive delivery of the symbiont in a finite period of time, more then ½ of the insects acquired the bacterium

Effect of Axd or Xf on GWSS Biology

Colonies of GWSS which were orally inoculated with DsRed *Axd*, wild-type *Axd*, S1 *Axd* (bacterium expressing an antibody), *Xf*, or no introduced bacteria (control) were maintained under laboratory conditions. Feeding ability, natural mortality, and dry weight post-mortem were compared between groups to determine if the presence of bacterium influenced any of these biological factors. In preliminary studies, mean g consumed after 5 was not significantly different for any of the 5 groups (n=20, p<0.001). Mortality rates of GWSS maintained in the laboratory were plotted over an 18 day period (Figure 1). Decline of the colony was consistent over time and no significant differences in mortality rates were detected (p<0.001). Randomly, individual GWSS were collected from each colony, dried in a desicating oven for 48 h, and weighed. The average weight of a dried GWSS was 0.01g and no significant differences in dry weight were revealed (*p*=0.7). In two trials, 50 wild GWSS that were collected on the UCR campus were microinjected with different amounts either, DsRed *Axd* or H_2O. Bacteria were detected in the hemocoel; however, based on Chi-square analysis there were no significant differences in the mortality rates between the two groups.
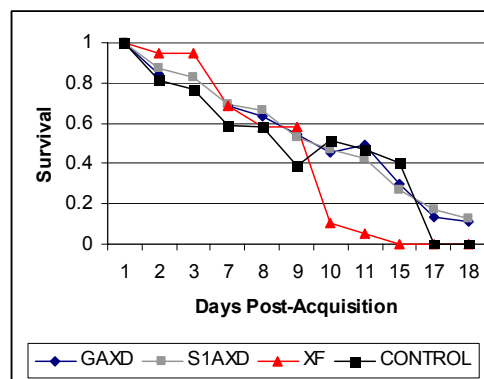

Figure 1. Mortality rate in four GWSS colonies maintained in the lab oratory.

Laboratory-Based Artificial Disease Cycle

A simple and efficient transmission cycle was developed for the study of *Xf* transmission by GWSS which allowed collection of sufficient transmission data in 1 wk. Specific numbers of cells were detected both in plant tissue and within the insect vector by QRT PCR. *Xf* cells were scraped from a PD3 plate and suspended in sterile ½ strength PBS (OD^{600}=2.0). Five cm sections of cut chrysanthemum stems were used for bacterial inoculations [6]. Five GWSS per 5 cm of stem were caged in snap cap vials for 48 h. After the acquisition access period (AAP), GWSS were placed on *Xf*-free chrysanthemums for 48 h, so that any detection of bacteria would be associated with transmission and not stylet contamination. Pairs of GWSS were transferred to sterile vials containing a fresh chrysanthemum stem cutting. The insects were exposed to a stem for an inoculation access period (IAP) of 48 or 96 h. DNA was extracted from the inoculation targets with the XNAR Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO) and PCR was run following a standard QRT-PCR protocol. Across 9 replicates using a 48h IAP, the mean transmission rate of *Xf* by GWSS was 0.508+0.122, while the mean rate when given a 96h IAP was 0.341+0.138. Using Chi-square analysis, these ratios were significantly different ($\aleph^2$=16.281, df=1, p≤0.001). The lower rate associated with the longer IAP is probably due to the non-hospitable environment of the test plant stems.

Interruption of PD Cycle

Transmission of *Xf* from infected grapevine to healthy grapevine by GWSS was blocked by feeding GWSS on the plant-based AFS containing an *Xf*-specific antibody fragment (scFV S1) expressed in the coat of a M13 bacteriophage, between a 5 d AAP and the 5 d inoculation access period (IAP). At two concentrations of phage/antibody (10^{14} and 10^{15}) transmission of *Xf* was 0% (n=10 and n=13, respectively), compared to 50% transmission in the control group (n=8). Transmission of *Xf* was reduced when GWSS were fed Indolicidin (American Peptide Company, Inc., Sunnyvale, CA) between the AAP and IAP from 50% in the control group to 35% (n=14) at 100µg/ml and 7% (n=14) at 500µg/mL. These experiments are currently being replicated. While the rate of *Xf* transmission was higher than previously reported [1, 2, 9], we feel this is a fair assessment of the insects' ability to transmit.

CONCLUSIONS

Several major biological associations were found which support the feasibility of symbiotic control to reduce transmission of *Xf* by GWSS:

1. Natural populations of GWSS are commonly found thriving on several citrus varieties.
2. *Axd* colonized and grew best in the citrus varieties tested.
3. *Axd* colonized the xylem vessels of test plants, the same tissue from which GWSS feed.
4. *Axd* passively moved through populations of GWSS.
5. *Axd* did **not** negatively affect GWSS.

Interestingly, *Axd* appears to mirror the host range of GWSS. Genetically marked *Axd* colonizes several host plants. This suggests that genetic modification does not interfere with the biology of *Axd*, which should enter into the insect-plant cycle and be transmitted along with the pathogenic bacteria target. While GWSS is the vector of greatest interest in California, two other native sharpshooters also transmit the vehicle bacterium, *Axd*, and several plants can serve as hosts.

In the laboratory, inhibition of *Xf*-transmission by GWSS was demonstrated using two different categories of reagents, a surface antibody fragment and an antibiotic peptide (Indolicidin). The antibody fragment was specific to *Xf*. In our trials the antibody fragment was being expressed in the coat of a phage, so the effects on transmission might be greater when the antibody fragment is expressed on the surface of *Axd*. Indolicidin inhibited *Xf* growth *in vitro*, but did not affect growth of *Axd*. Transformation of *Axd* to produce each/or both of these reagents is currently under way.

We concluded that *Axd* will be an effective delivery agent of a symbiont control strategy for combating *Xf*. GWSS readily acquired *Axd* from a plant source and this bacterium translocated and colonized a variety of plants tested. We have yet to determine the effect of the reagents on *Xf* in infected grapevines.

Previously, plant symptoms confirmed by ELISA or PCR detection were used to determine if transmission had occurred. Unfortunately, these systems require the bacterium to colonize and infect the host plant to determine transmission. If an infected plant is asymptomatic, important but less obvious transmission events may be missed. Our system removes the plant "unknowns" from the equation. However, we recognize the importance of actual plant infection as a measure of transmission importance, but suggest using the artificial disease cycle as an initial rapid measure of vector competence.

REFERENCES

1. Almeida RPP, Purcell AH (2003) *Homalodisca coagulata* (Hemiptera, Cicadellidae) transmission of *Xylella fastidiosa* to almond. Plant Dis. 87:1255-1259
2. Almeida RPP, Purcell AH (2003) Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). J. Econ. Entomol. 96:264-271
3. Beard CB, Cordon-Rosales C, Durvasula RV (2002) Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. Annu. Rev. Entomol. 47:123-141
4. Beninati C (2000) Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria. Nat. Biotechnol. 18:1060-1064
5. Bextine B, Lampe D, Lauzon C, Jackson B, Miller TA (2004) Establishment of a genetically marked insect-derived symbiont in multiple host plants. Curr. Microbiol. *In Press*.
6. Bextine B, Lauzon C, Potter S, Lampe D, Miller TA (2004) Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Curr. Microbiol. 48:327-331
7. Bextine B, Miller TA (2004) Comparison of whole-tissue and xylem fluid collection techniques to detect *Xylella fastidiosa* in grapevine and oleander. Plant Dis. 88:600-604
8. Chang TL-Y, Chang CH, Simpson DA, Xu Q, Martin PK, Lagenaur LA, Schoolnik GK, Ho DD, Hillier SL, Holodniy M, Lewicki JA, Lee PP (2003) Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. Proc. Nat. Acad. Sci. 100:11672-11677
9. Costa HS, Blua MS, Bethke JA, Redak RA (2000) Transmission of *Xylella fastidiosa* to oleander by the glassy-winged sharpshooter, *Homalodisca coagulata*. Hortsci. 35:1265-1267
10. Meade MJ, Waddell RL, Callahan TM (2001) Soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. *denitrificans* inactivate triclosan in liquid and solid substrates. Microbiol. Let. 204:45-48
11. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E (2000) Treatment of Murine Colitis by *Lactococcus lactis* Secreting Interleukin-10. Science 289:1352-1354
12. Yang HL, Sun XL, Song W, Wang YS, Cai MY (1999) Screening, identification and distribution of endophytic associative diazotrophs isolated from rice plants. Acta Bot. Sinica. 41:927-931

EXPLOITING *XYLELLA FASTIDIOSA* PROTEINS FOR PIERCE'S DISEASE CONTROL

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ABSTRACT

The *Xylella fastidiosa* (*Xf*) is the causal agent of Pierce's disease of grape. In previous work, we discovered, partially purified, and investigated the processing of the *Xf* protein MopB, which previously had been known only from the nucleotide sequence of its gene. The amino acid sequence of MopB, the uniform staining of *Xf* cells with fluorescent anti-MopB antibody and the abundance of MopB in total protein extracts of *Xf* cells suggest that MopB is the major outer membrane protein of *Xf*. As such, MopB is expected to participate in *Xf* colonization of grape xylem elements. We previously demonstrated that partially purified MopB binds to (xylem-rich) balsa wood or cellulose (filter paper) disks under conditions in which other proteins do not adhere. Here we report improvements in our MopB purification procedure and observations on adherence of MopB in *Xf* cells to cellulose disks under conditions that eluted other *Xf* proteins. A high (0.25mM) concentration of the cellulose fragment cellotetraose did not interfere with the binding of MopB to cellulose, suggesting that the binding reaction of MopB is not specific for cellulose. We exposed *Xf* cells or MopB to each of three fibrous polymer disks and to cellulose disks and observed similar adherence of MopB from both sources to all four polymer disk types. Thus, MopB appears to associate with porous materials generally when it is exposed to such materials in purified form or as *Xf* cells. The abundance and exterior exposure of MopB makes MopB an ideal target for Pierce's disease control strategies. We seek to develop soluble proteins with high affinity for MopB. We will apply, as an anti-*Xf* agent, a selected MopB-binding protein alone or as a chimera with a bacterial cell-inactivating peptide or protein. Our expectation is that expression of the anti-*Xf* protein, targeted to the xylem in grape rootstock, may result in the anti-*Xf* protein moving into and protecting the grafted scion. In this reporting period, experiments were initiated with the objective of creating a protein having high affinity for MopB. As a first step towards this objective, Project Scientist Paul Feldstein developed *E. coli* strains expressing surface elements of MopB protein, so that the experimentally compliant *E. coli* can be used to select proteins with high affinity for *Xf* MopB.

INTRODUCTION

We have been investigating an abundant protein of *Xf*, MopB. We showed that MopB is the major outer membrane protein of *Xf* and is partly exposed on the outside of the bacterial cell. We purified MopB, prepared antibodies against it, and demonstrated an apparent affinity of MopB for cellulose. This last observation and the abundance of MopB suggested that MopB may participate in the initial attachment of *Xf* to the inner surface of the xylem vascular elements or in some other critical event in the initiation of infection leading to the development of Pierce's disease. Regardless of whether MopB is critical in this process, its location and prevalence support our contention that MopB is an ideal target for a *Xf*-specific bactericide or for a reagent that would coat and thereby inactivate *Xf* cells. Our strategy for creating a high-affinity MopB-binding protein is to begin with a protein that has evolved to bind tightly to the major outer membrane protein of *E. coli*, OmpA, and to convert the specificity of that protein from OmpA-binding to MopB-binding. The T2-like *E. coli* bacteriophage K3 has OmpA as its receptor. The K3 tail fiber adhesion gp38 is responsible for binding of bacteriophage K3 to OmpA in a reaction whose rate and irreversibility suggest a high-affinity association. Mutational conversion of gp38 from its natural receptor OmpA to other *E. coli* surface proteins has been demonstrated in several publications (Drexler et al., 1991, and references cited therein). In outline, our planned experimental steps for creating an anti-*Xf* protein are (i) replace the OmpA protein of *E. coli* with a protein that has MopB sequences displayed on the cell exterior, (ii) select variants of bacteriophage K3 that can infect the modified *E. coli* and also can bind to *Xf* cells, (iii) isolate the variant bacteriophage K3 gene *gp38* (expected to encode a MopB-binding gp38 protein), and (iv) genetically modify the MopB-binding gp38 to confer solubility and (in collaboration with the Gupta laboratory) possibly fuse the gp38 to a bactericidal peptide-encoding sequence. Step (v) will be the expression of a xylem-targeted version of the gp38 or gp38 fusion protein in rootstock and will be performed in collaboration with the Dandekar laboratory.
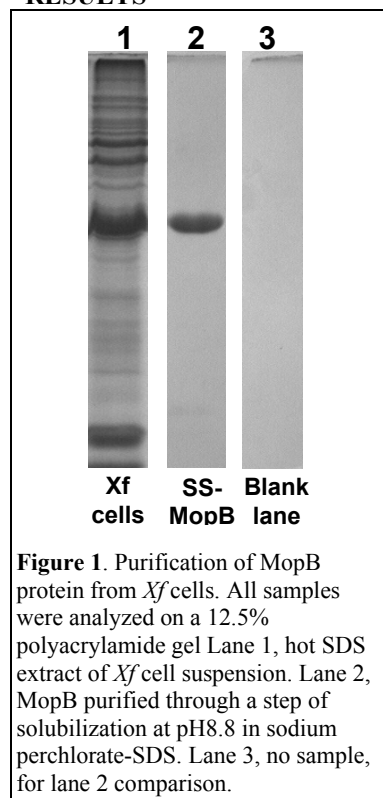
OBJECTIVES

For period 15 Oct 2003 through 30 June 2004, previous project title "Roles of *Xylella fastidiosa* Proteins in Virulence"
1. To identify specific *Xylella fastidiosa* (*Xf*) protein(s) and determine their roles in virulence, particularly major outer membrane protein MopB
2. To develop strategies for interfering with *Xf* infection of grape and/or with development of Pierce's disease

For period 1 July 2004 through 11 October 2004, new project title "Exploiting *Xylella fastidiosa* Proteins for Pierce's Disease Control"
1. Discover or develop low molecular weight proteins with high affinity for portions of the MopB protein that are displayed on the *Xf* cell exterior.
2. Test MopB-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity, and for interference with disease initiation following inoculation of grape with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins for their effects on *Xf* cells in culture.
4. In collaboration with the Dandekar laboratory, prepare transgenic grape expressing the candidate anti-*Xf* proteins; test the transgenics for resistance to infection by *Xf*

RESULTS

Figure 1. Purification of MopB protein from *Xf* cells. All samples were analyzed on a 12.5% polyacrylamide gel Lane 1, hot SDS extract of *Xf* cell suspension. Lane 2, MopB purified through a step of solubilization at pH8.8 in sodium perchlorate-SDS. Lane 3, no sample, for lane 2 comparison.

Purification of MopB from *Xf* cells. A dilute suspension of *Xf* cells scraped from plates is incubated at 30°C for 30 min in Tris-HCl-EDTA buffer pH 8.5 containing 8mg/mL SDS, 0.2µL/mL 2-mercaptoethanol. High speed centrifugation collects a precipitate (designated SP-MopB) that is highly enriched in MopB but includes substantial amounts of non-protein material from the *Xf* cells. The precipitate is dispersed into Tris-HCl-EDTA buffer, pH 8.8, containing 1.2M sodium perchlorate, 1mg/mL SDS, 10µL/mL 2-mercaptoethanol and is incubated at 30°C for 18hr. The supernatant after centrifugation at 50K rpm, 10°C for 20min is designated as the SS-MopB fraction. Sodium perchlorate reduces the solubilization of non-MopB proteins from SP-MopB preparations. The effective concentration of SDS is very low in SS-MopB due to the common ion effect with sodium perchlorate. SS-MopB, concentrated by centrifugal filtration, binds to porous polymer disks as described below.

Preponderance of MopB in the *Xf* outer membrane. *Xf* cells were washed with cold 1M perchloric acid to elute low molecular weight compounds. The cell suspension was assayed for DNA by the diphenylamine assay and for protein using the BCA reagent. The amount of DNA per stationary state cell is assumed to be 2.7×10^6 base pairs. MopB appears to be 10-15% of the *Xf* cell protein, based on analyses such as those in Fig. 1. From these results, *Xf* cells have at least 80,000 MopB molecules per cell. We assume that the packing volume of MopB is similar to the packing volume derived from x-ray crystallography for the amino-terminal domain (residues 1-171) for *E. coli* OmpA, which crystallized as a 2.6nm diameter cylinder (Pautsch and Schutz, 1998). The diameter of a *Xf* cell is about 400nm. 80,000 molecules of hexagonally packed MopB would form a cylinder 400nm in diameter and almost 400nm high, accounting for more than 10% of the surface area of the 1000 to 5000nm long *Xf* cell.

General association of MopB with porous substances. We reported previously on the spontaneous association of MopB from solution with balsa wood (composed largely of xylem) and cellulose disks (filter paper). Other proteins, mixed with the MopB, did not absorb to balsa wood or cellulose. Fig. 2 reports our extension of this work to other porous polymeric materials of diverse chemical character. Cellulose, polyamid, polyester, and a rayon-nylon blend provided in approximately the same mass, all became associated with MopB, whether the MopB was supplied as partially purified protein in solution or as MopB in the outer membrane of *Xf* cells. Quantitatively, there was little variation in the extent of association among the polymers, all of which were exposed to the same NP-40 (non-ionic detergent) solution. Bovine serum albumin (BSA) was not absorbed by any of the porous polymer disks. Elution of polymer disks exposed to *Xf* cells in the presence of excess BSA was carried out in two stages. A mild elution ("A1" under the lanes in Fig. 2B), with neutral-pH SDS solution at 30˚C, eluted most of the proteins not already removed from the polymer disks by the initial rinses with SCP buffer ("F" under lanes, Fig. 2B). Elution with hot, alkaline SDS-mercaptoethanol solution should remove all of the remaining proteins to the "A2" fractions. The A2 fractions contained about 40% of the MopB supplied to the disks in the initial incubation. However, only limited amounts of other *Xf* proteins remained after the A1 elution, i.e., to be eluted in the A2 fraction. We interpret these results as showing a tight association between MopB displayed on the outside of *Xf* cells and the polymers or a polymer-mediated precipitation of the MopB protein, which then could be released and/or solubilized only by exposure to hot, alkaline SDS solution. These results indicate no specificity of MopB for association with (or precipitation by) a specific polymer, so, unlike MopB itself, the polymer side of the MopB-polymer pair is not an attractive target for interfering with *Xf*-xylem interactions.
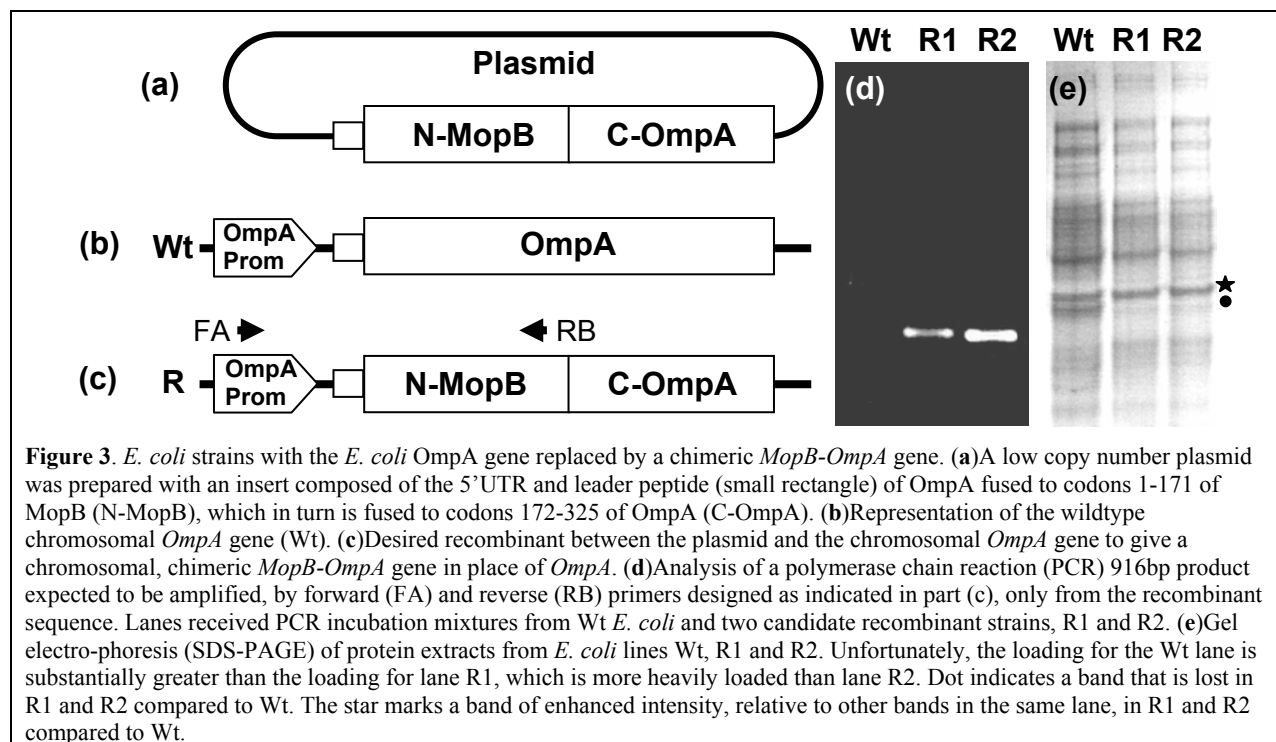
Figure 2. Polymer disk accumulation of *Xf* MopB from protein mixture and *Xf* cells. **(a)**. A solution of SP fraction MopB and BSA was dispersed in 1x SCP, 1mg/mL NP-40. 8mm diameter disks were prepared from filter paper (2 disks, 19mg), polyamid (3 disks, 21mg), polyester (5 disks, 20mg), and 30% nylon, 70% rayon (3 disks, 19mg). 0.25mL of the BSA-MopB dispersion was dispensed into an empty vial (lane 1, V) and into vials containing polymer disks as indicated. The vials were incubated at room temperature for 2hr with orbital shaking at 100rpm. Free, unassociated material rinsed off with SCP: lanes 2, 4, 6 and 8 (F below lanes). Material eluted from polymer disks with alkaline hot SDS-mercaptoethanol solution: lanes 3, 5, 7 and 9 (A below lanes). **(b)** *Xf* cells were dispersed into 1xSCP, 1mg/mL NP-40 containing a great excess of BSA (150μg/mL). 0.25mL of the suspension was dispensed to vials containing polymer disks as indicated. Elution was in two stages: A1, SDS in SCP at 30˚C and A2, hot SDS-mercaptoethanol at alkaline pH. Numbers under lanes indicate fraction of MopB band material in A and A2 fractions.
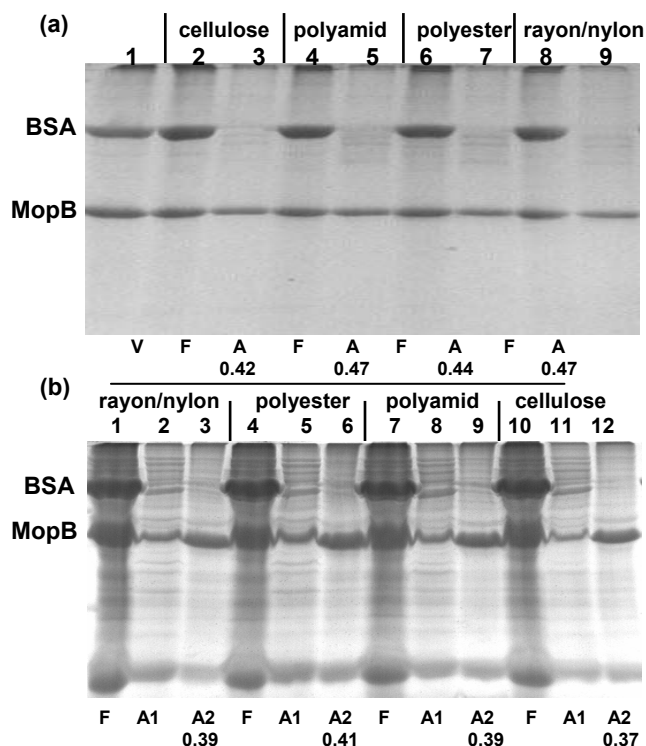
E. coli displaying MopB outer peptide loops. Attempted cloning and expression of the full *Xf mopB* gene in *E. coli*, including the *Xf MopB* promoter, were not successful. However, a system that included an inducible bacteriophage T7 RNA polymerase and T7 promoter driving the MopB-encoding sequence was adapted to create *E. coli* cultures generating low levels of MopB when induced with the gratuitous inducer IPTG. Intact *Xf* MopB accumulation may sicken *E. coli*, accounting for the low level accumulation. The Introduction describes in outline a strategy for creating a MopB-binding, anti-*Xf* protein. This strategy requires substitution of *E. coli* OmpA by a new outer membrane protein that portrays the characteristics of MopB on the surface of *Xf* cells. To this end, we created a chimeric MopB-OmpA construction in *E. coli* and subjected the cells to conditions designed to select cells in which recombination events resulted in the *E. coli* OmpA gene being replaced by the MopB-OmpA chimera (Fig. 3).

The predominant conformation of the OmpA protein as it resides in the outer membrane of *E. coli* probably has amino acid residues 1-171 inserted with 8 trans-membrane segments and four external loops (Singh et al., 2003). MopB can be cast in a similar conformation based on the crystallographic structure of OmpA and computer predictions of folding for OmpA and MopB. Our design for the chimeric MopB-OmpA gene retains the OmpA promoter and replaces only the 1-171 residue region of OmpA with the corresponding MopB sequence. Our rationale is that retaining the OmpA leader peptide, which targets the molecule to the outer membrane, and the OmpA carboxy-terminal portion, which includes the trans-periplasmic space sequences and the sequence that is inserted into the peptidoglycan layer, will result in a molecule that is more compatible with *E. coli* that an intact *MopB* gene would be.

The low-copy-number plasmid construction indicated in Fig. 3(a) encodes the desired chimeric molecule and the associated OmpA 5'UTR and leader peptide but lacks the OmpA promoter, so the chimeric protein should be expressed at a very low level, at the most, in transformed *E. coli*. The robust, highly recombination competent *E. coli* strain ER2738 was transformed with the Fig. 3(a) plasmid under the expectation that recombination events would replace the chromosomal *OmpA* gene [Fig. 3(b)] with sequences encoding the MopB amino-half molecule flanked by the OmpA leader peptide and carboxy-half OmpA sequences, creating the desired structure diagrammed in Fig. 3(c).

Figure 3. *E. coli* strains with the *E. coli* OmpA gene replaced by a chimeric *MopB-OmpA* gene. (**a**)A low copy number plasmid was prepared with an insert composed of the 5'UTR and leader peptide (small rectangle) of OmpA fused to codons 1-171 of MopB (N-MopB), which in turn is fused to codons 172-325 of OmpA (C-OmpA). (**b**)Representation of the wildtype chromosomal *OmpA* gene (Wt). (**c**)Desired recombinant between the plasmid and the chromosomal *OmpA* gene to give a chromosomal, chimeric *MopB-OmpA* gene in place of *OmpA*. (**d**)Analysis of a polymerase chain reaction (PCR) 916bp product expected to be amplified, by forward (FA) and reverse (RB) primers designed as indicated in part (c), only from the recombinant sequence. Lanes received PCR incubation mixtures from Wt *E. coli* and two candidate recombinant strains, R1 and R2. (**e**)Gel electro-phoresis (SDS-PAGE) of protein extracts from *E. coli* lines Wt, R1 and R2. Unfortunately, the loading for the Wt lane is substantially greater than the loading for lane R1, which is more heavily loaded than lane R2. Dot indicates a band that is lost in R1 and R2 compared to Wt. The star marks a band of enhanced intensity, relative to other bands in the same lane, in R1 and R2 compared to Wt.

E. coli transformants displaying MopB sequences were selected using magnetic beads covalently coupled to anti-MopB IgG. Beads were plated on agar medium to recover colonies growing up from bead-selected cells. Pooled colonies were cultured, and the cells were exposed to the OmpA-specific bacteriophage K3 at a multiplicity of infection of 15 to deplete the population in cells still bearing OmpA. Fig. 3 provides evidence for the occurrence of the expected recombination events and for the production of the chimeric MopB-OmpA protein in amounts visible on a coomassie brilliant blue-stained gel [Fig. 3(d) and (e)]. The cells derived by these approaches agglutinate beads displaying anti-MopB IgG, providing evidence that some part of the MopB portion of the chimera, presumably the MopB outer loops, is displayed on the exterior of the *E. coli* cell.

CONCLUSIONS

Based on results reported here and in previous progress reports, MopB is a highly suitable target for strategies designed to interfere with the ability of *Xf* to initiate infections leading to development of Pierce's disease. Our overall strategy for creating grape plants resistant to *Xf* is revealed by the four new objectives stated above in the Objectives section. Experimental steps (i), (ii) and (iii) outlined at the end of the Introduction reveal how we intend to satisfy new Objective 1. Results in Fig. 3 suggest that we have completed experimental step (i) and that we are ready to proceed to the selection of variant gp38 proteins capable of high affinity binding to MopB on the surface of *Xf* cells, i.e., experimental steps (ii) and (iii).

REFERENCES

Drexler, K., Dannull, J., Hindennach, I., Mutschler, B. & Henning, U. (1991). Single Mutations in a Gene for a Tail Fiber Component of an Escherichia-Coli Phage Can Cause an Extension from a Protein to a Carbohydrate as a Receptor. *Journal of Molecular Biology* **219**, 655-664.

Pautsch, A. & Schulz, G. E. (1998). Structure of the outer membrane protein A transmembrane domain. *Nature Structural Biology* **5**, 1013-1017.

Singh, S. P., Williams, Y. U., Miller, S. & Nikaido, H. (2003). The C-terminal domain of Salmonella enterica serovar Typhimurium OmpA is an immunodominant antigen in mice but appears to be only partially exposed on the bacterial cell surface. *Infection & Immunity* **71**, 3937-3946.

Funding

CHARACTERIZATION OF NEONICOTINOIDS AND THEIR PLANT METABOLITES IN CITRUS TREES AND GRAPEVINES, AND EVALUATION OF THEIR EFFICACY AGAINST THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
The toxicities of established and new members of the neonicotinoid insecticide class were assessed against the glassy-winged sharpshooter in topical application bioassays. All compounds were highly toxic to the insect. Clothianidin elicited its toxic response more rapidly than thiamethoxam and was 3-fold more toxic overall at the LD50 level. Clothianidin has been proposed as an active derivative of thiamethoxam, so it is important to establish the fate of these chemicals within plant systems that are likely to be treated for GWSS control.

INTRODUCTION
The primary means of controlling the spread of Pierce's disease (PD) in California vineyards is through the elimination of its vector using insecticides. The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* feeds directly from the plant xylem system and, therefore, systemic insecticides are currently being evaluated on both citrus and grapes. Of the various classes of insecticide under consideration, the neonicotinoids, especially imidacloprid, have proven to be the most effective at suppressing GWSS populations. Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-N-nitro-1H-imidazol-2-amine) is a nicotinic acetylcholine receptor agonist that combines high potency with low mammalian toxicity and favorable persistence. As a systemic, seed, soil or foliar treatment, it has proved to be especially effective against a wide range of homopterous insect pests, including the GWSS. The success of imidacloprid in controlling GWSS is due largely to its excellent systemic properties. Systemic applications exploit the xylophagous feeding behavior of the insect, and thereby disrupt the transmission of PD and other *X. fastidiosa*-related diseases.

This project is an extension of a one-year project that was funded by the UC Pierce's Disease Research Grant Program. It will focus on the fate of imidacloprid and other neonicotinoid insecticides in citrus and grapevines, and the impact of these chemicals on GWSS. In a previous study, imidacloprid and two of its derivatives were shown to be highly toxic to GWSS adults (Byrne and Toscano, 2003).

The aims of this study are to determine the extent to which metabolites of neonicotinoids are formed in citrus trees and grapevines, and to determine their toxicological significance towards GWSS. The presence of insecticidal metabolites in xylem sap could contribute to the excellent persistence of imidacloprid treatments against sharpshooters. As well as maintaining the toxic pressure of the initial application, the metabolism of neonicotinoids to yield equally or more toxic metabolites may also account for the stability of this chemical class to resistance.

Of particular interest to us are thiamethoxam and clothianidin, which are being evaluated for use against citrus and grape pests. During the past year, it has been established that thiamethoxam is converted into clothianidin by insects and cotton plants (Nauen et al., 2003). This is an important finding, as it could have ramifications for the use of these products on grapes and citrus. When several products from the same class become available for pest management, it is important that their use be carefully monitored in order to circumvent potential resistance problems. The possibility that thiamethoxam is converted into clothianidin is, therefore, of concern when formulating management strategies based around the neonicotinoids. Receptor binding studies have suggested that thiamethoxam does not bind to the same receptor site as imidacloprid and so it has been proposed as a suitable product for alternation with imidacloprid because of the reduced resistance risk (Weisner and Kayser, 2000). Now that thiamethoxam has been shown to be a potential pro-insecticide, and clothianidin has been shown to bind to the same receptors as imidacloprid, new issues are raised about its suitability as a product for rotation with other neonicotinoids. This is an important reason for determining the fate of thiamethoxam in citrus and grapes.

OBJECTIVES
1. Determine the metabolic fate of neonicotinoids within citrus trees and grapevines.
2. Determine the relative toxicities of neonicotinoids and their metabolites to the adult and egg stages of the GWSS.

RESULTS
The toxicity of four neonicotinoid insecticides has been assessed for GWSS adults using a topical application bioassay (Table 1). Thiamethoxam, clothianidin and acetamiprid were all more toxic than imidacloprid. Clothianidin was

approximately 3-fold more toxic than thiamethoxam, and the dose-response was steeper as indicated by the higher slope. It was evident during these bioassays that the toxic effects of thiamethoxam were delayed compared with the other insecticides, suggesting that thiamethoxam may require activation to a toxic derivative within the GWSS.

Table 1. Toxicity of neonicotinoids to the GWSS in topical application bioassays.

Compound	LD50 (ng a.i. per insect)	95% FL	Slope	No. of insects
Imidacloprid	4.8	2-8	1.5 ±0.4	100
Thiamethoxam	2.6	2.0-3.3	1.4 ±0.3	200
Clothianidin	0.7	0.6-0.9	5.2±0.9	125
Acetamiprid	0.7	0.6-0.9	3.7±0.6	125

CONCLUSIONS
In this study, we tested four neonicotinoids against the GWSS. Although there were differences in LD50s, all compounds were highly toxic. These results confirm that the newer neonicotinoids could have a place in GWSS management programs. We are currently investigating the fate of these chemicals in both citrus trees and grapevines. Establishing the potential for conversion of thiamethoxam into clothianidin is of particular importance if these chemicals are to be incorporated into management strategies.

REFERENCES

Byrne, F.J., and N.C. Toscano. 2003. Characterization of plant metabolites of imidacloprid in citrus trees and grapevines, and evaluation of their efficacy against the glassy-winged sharpshooter, *Homalodisca coagulata*.
Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V. L., and M. Kaussmann. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. Pesticide Biochemistry and Physiology 76: 55–69.
Wiesner, P. and H. Kayser. 2000. Characterization of nicotinic acetylcholine receptors from the insects *Aphis craccivora*, *Myzus persicae*, and *Locusta migratoria* by radioligand binding assays: relation to thiamethoxam action. Journal of Biochemical and Molecular Toxicology 14: 221.

FUNDING AGENCIES

EVALUATION OF RESISTANCE POTENTIAL IN THE GLASSY-WINGED SHARPSHOOTER USING TOXICOLOGICAL, BIOCHEMICAL, AND GENOMICS APPROACHES

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Reporting Period: The results reported here are from work conducted from July 2004 to October 2004.

ABSTRACT
Geographically distinct populations of GWSS differ in their toxicological responses to pyrethroid insecticides. We have shown that these different responses are unlikely to be caused by an esterase-mediated mechanism. The distributions of esterase activity in insects tested from Riverside and Redlands citrus orchards remained unchanged after selection with an LD50 dose of esfenvalerate.

INTRODUCTION
We are using a multi-disciplinary approach to understand the biological and genetic mechanisms contributing to the toxicological differences between GWSS populations. This will allow us to determine whether the basis for decreased tolerance is due to target site changes or due to the selection of detoxification mechanisms. Whereas target-site modifications will only impact the pyrethroid class of insecticides, the selection of detoxification mechanisms are more critical due to their potential to confer cross-resistance to chemical classes that differ in their modes of action. In this first report, we describe selection experiments designed to test the potential involvement of esterases in conferring pyrethroid tolerance (Objective 2).

OBJECTIVES
1. Monitor toxicological responses of geographically distinct populations of GWSS to pyrethroid insecticides
2. Measure biochemical activity of putative resistance-causing enzymes in these populations.
3. Clone and sequence the sodium-channel genes in GWSS populations differing in susceptibility to insecticides.
4. Perform microarray gene expression profiles in GWSS populations differing in susceptibility to insecticides to isolate novel genes involved in resistance.

RESULTS
Bioassays
Topical application bioassays (Byrne et al., 2003) have been conducted on Riverside GWSS adults to determine an LD50 for esfenvalerate. The LD50 was determined to be 0.75ng esfenvalerate per insect.

Selections
For selection experiments, insects were collected from the UC Agricultural Operations orchard in Riverside. Adults were treated with 0.75ng esfenvalerate by topical application. Esterase activity was measured in a subsample of insects taken before the bioassay, and in the survivors (at 48 hours) from the bioassay (Figure 1). Although there were differences in activities between males and females, there were no differences in activities attributable to selection by esfenvalerate.

In additional selection experiments, insects from Redlands and Riverside orchards were treated with 0 (controls), 0.075ng (sub-lethal) and 0.75ng (LD50) esfenvalerate per insect. Control and survivors at each treatment were used to prepare target RNA for gene expression profiling studies.

Microarrays
PCR amplified inserts from 1,536 normalized library clones were spotted onto amino-silane coated glass slides. Each clone was spotted in side by side duplicate spots and the entire array was duplicated on each slide. Total RNA was isolated from two individual insects from each treatment for target preparation. Each total RNA was reverse transcribed and PCR amplified separately with Cy3- and Cy5-tagged dUTP. Slides were hybridized for 16 hours at 42°C on a Genomics Solutions GEN TAC® hybridization station and washed twice at medium stringency for 40 seconds. Each hybridization was repeated as a target dye swap. Slides were scanned on an Applied Precision Array Worx fluorescence scanner. Data is being evaluated using the Silicon Genetics GeneSpring program.
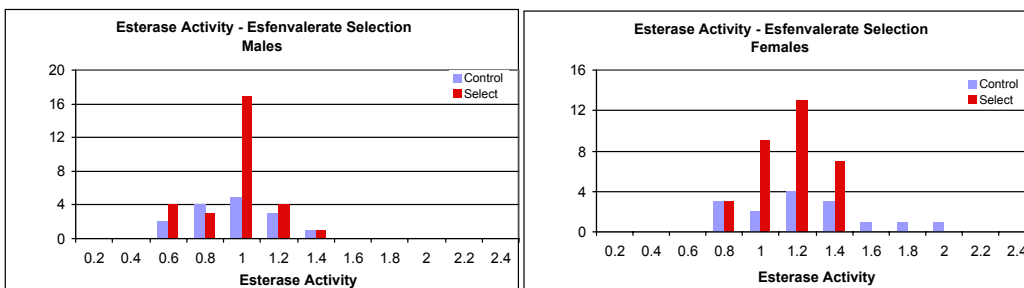
Figure 1. Distributions of esterase activity in adult male and female glassy-winged sharpshooters rom a Riverside citrus orchard. Insects were treated topically with either acetone (Control) or 0.75ng esfenvalerate (Select), and esterase activity measured in survivors.
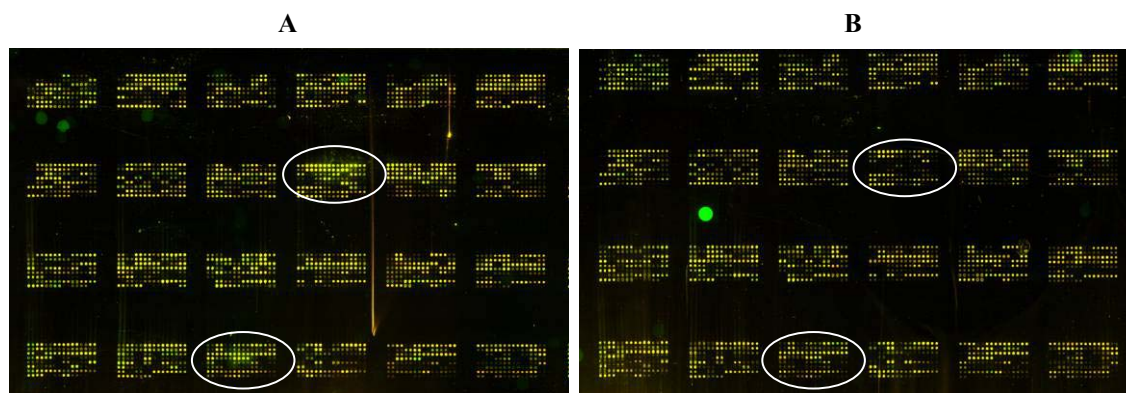
A   **B**

Figure 2. Scan data of microarrays hybridized to Cy3 labeled control target (green) and Cy5 labeled sub-lethal target (**A**) or LD50 target (**B**) (red). Circled results show obvious gene expression differences.

CONCLUSIONS

In this study, we tested populations of GWSS from Riverside citrus orchards with 0.75ng esfenvalerate. This dose of esfenvalerate is the LD50 for the Riverside population when topically applied to the insect abdomen. Distributions of esterase activity revealed that there were no differences between the untreated insects and the treated survivors. These results suggest that esterases do not contribute directly to the toxicological differences between these populations. In addition, many and different gene expression changes occur in GWSS in response to sub-lethal and LD50 doses of esfenvalerate.

REFERENCES

Byrne, F.J. and Toscano, N.C. 2003. Characterization of plant metabolites of imidacloprid in citrus trees and grapevines, and evaluation of their efficacy against the glassy-winged sharpshooter *Homalodisca coagulata*. Proceedings of the Pierce's Disease Research Symposium pp. 286-287, Coronado Island Marriott Resort, San Diego, California. Dec 8-11, 2003.

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FUNCTIONAL GENOMICS OF THE GRAPE-*XYLELLA* INTERACTION: TOWARDS THE IDENTIFICATION OF HOST RESISTANCE DETERMINANTS

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ABSTRACT

We have used *in silico* mining of EST data and Real Time PCR to identify a set of *Xylella*-induced grape genes. Controlled time course analyses demonstrate that the genes are induced prior to symptom development, in coincidence with pathogen colonization. Analysis of field samples from grapes under a variety of biotic and abiotic stresses demonstrate that these genes are up-regulated in response to *Xylella* but not in response to the other pathogens assayed, including common viral, nematode and fungal pathogens, or by *Phylloxera* infestation or herbicide damage. By contrast, transcriptional responses similar to those observed in *Xylella*-infected tissues were observed in grapes under severe drought stress (in excess of normal field drought) and in plants where the vascular system had been blocked by damage from the grape cane girdler insect. These results are consistent with transcriptional regulation in response to insult within the vascular tissue of grape, but not to pathogen infection generally.

INTRODUCTION

All organisms adapt to external stressors by activating the expression of genes that confer adaptation to the particular stress. For example, when exposed to conditions of heat or drought, genes for adaptation to heat and drought stress are up-regulated. Similarly, when a plant is exposed to a pathogen, numerous genes are induced including those that encode proteins involved in disease resistance. In the case of Pierce's disease, such genes are likely to include those coding for resistance to *Xylella* or to the insect vector.

Genomics technology offers an opportunity to monitor gene expression changes on a massive scale (so-called "transcriptional profiling"), with the parallel analysis of thousands of host genes conducted in a single experiment. In the case of Pierce's disease of grapes, the resulting data can reveal aspects of the host response that are inaccessible by other experimental strategies. Prior to carrying out transcriptional profiling, it is first necessary to (1) catalog the gene content of grapes by means of sequencing and bioinformatic analyses, and (2) develop gene-based arrays that allow the simultaneous monitoring of gene expression for >10,000 genes. Our research to date has contributed significantly in each of these areas. In May of 2004, the first Affymetrix gene chip was made available for public use, with ~15,000 *Vitis* genes represented. This gene chip has been developed based primarily on a collaboration between the Cook laboratory and researchers at the University of Nevada-Reno. With the arrival of the Affymetrix gene chip, we are poised to make a quantum leap in the identification of host gene expression in response to *Xylella fastidiosa*.

In addition to enumerating differences between susceptible and resistant genotypes of *Vitis*, the ongoing research will test a long-standing but largely untested hypothesis that pathogen-induced drought stress is one of the fundamental triggers of PD symptom development. The utility of this type of data will be to inform the PD research community about the genes and corresponding protein products that are produced in susceptible, tolerant and resistant interactions. Differences in the transcriptional profiles between these situations are expected to include host resistance and susceptibility genes, and thus provide the basis for new lines of experimental inquiry focused on testing the efficacy of specific host genes for PD resistance. It should be possible, for example, to determine the extent to which resistance responses in grapes are related to well-characterized defense responses in other plant species [e.g., Maleck et al., 2002; Tao et al., 2003; de Torres et al., 2003]. In addition to identifying candidate effectors of disease resistance, such knowledge would aid the development of testable hypotheses regarding susceptibility and resistance to *Xylella fastidiosa* in grapes.

Three co-lateral benefits from the identification of pathogen-induced genes are: (1) the promoters for such genes are candidates to control the expression of transgenes for resistance to Pierce's disease, (2) the protein products of induced genes may have roles in disease resistance, and (3) knowledge of host gene expression can be used to develop improved diagnostic assays for disease. In the first case, we are currently characterizing pathogen-responsive promoters, which would allow us to test candidate genes (the second case) for resistance phenotypes. In the third case, gene expression patterns can be used to develop so-called "molecular signatures" or "biomarkers" [MacNeil 2004] that are diagnostic of an organism's physiological status. Biomarkers are finding application in clinical medicine, where data on gene expression patterns are useful for characterizing disease states and improving clinical outcome [Alizadeh et al., 2001; Van't Veer et al., 2002; Ramaswamy et al., 2003]. In the case of Pierce's disease, the identification of early genes (i.e., genes expressed prior to the appearance of visible symptoms), and/or genes that are induced systemically in response to local infection, would greatly increase the reliability of disease diagnosis, which is currently prone to false negatives due to mis-sampling of locally-infected asymptomatic vines. At the same time, the identification of disease-related gene expression profiles would provide a novel measure of host response, and thus provide tools for basic Pierce's disease research applications.

OBJECTIVES AND PRODUCTS OF THE RESEARCH
Completed objectives
1. The public release of 61,203 EST sequences to the National Center for Biotechnology Information.
2. Development of a public, on-line relational database for analysis of the grape genome (http://cgf.ucdavis.edu).
3. Production of a public Affymetrix microarray, in collaboration with international researchers, available May 2004.

Ongoing Objectives
4. Identify genes and gene pathways in susceptible *Vitis vinifera* correlated with *Xylella* infection: (a) identify *Xylella*-responsive genes in *V. vinifera*, (b) distinguish early from late gene expression, and (c) determine the correlation between drought stress and Pierce's disease.
5. Determine host genotype affects on gene expression in response to *Xylella* infection: (a) susceptible *Vitis vinifera* compared to resistant genotypes of *Vitis arizonica* and *Vitis aestivalis*, (b) comparison of pathogen-induced gene expression with gene expression triggered by salicylic acid and ethylene, and (c) analysis of gene expression in resistant and susceptible bulked segregants of *Vitis arizonica* X *Vitis rupestris*.
6. Development of Real Time PCR assay for routine monitoring of *Xylella*-induced genes under field, greenhouse and laboratory settings.
7. Isolation and characterization of *Xylella*-responsive plant promoters.

RESULTS
Analysis of the Grape Transcriptional Response to Pathogen Challenge
The results described below are based on the analysis of combined data sets generated under this project and that of our collaborators at the University of Nevada-Reno, and other members of the grape genomics community. In total, 40% of the 135K *V. vinifera* ESTs and 100% of the sequencing focused on Pierce's disease originated from this project.

In silico Identification of Xylella-induced Genes in Vitis vinifera
We have identified 31 genes that appear to be up-regulated in response to infection by *Xylella fastidiosa*. The analysis, which involved construction of a correlation matrix and 2-dimensional hierarchical clustering, was based on EST frequency in various tissues with or without *Xylella* infection. The most abundant contig (7061) shares homology with a stress-related RNA from Arabidopsis, although the function is unknown in any system. Interestingly, this gene is up-regulated in infected plants, prior to symptom development, making it a top candidate for an early and sensitive marker of Pierce's disease. Other genes in the list have homology to proteins implicated in signaling during disease resistance, while others have been identified as pathogen responsive, or have been implicated in plant-insect interactions. After confirmation of the *Xylella*-specific transcription of such contigs (see Real Time PCR assays, below) we initiated the isolation of the promoters from these genes from genomic DNA libraries. The potential application of such promoters to drive *Xylella*-induced and/or tissue specific expression of transgenes is planned as a topic of a future grant proposal.

Development of Real-Time PCR for Gene Expression Analyses and Disease Diagnosis
Detailed analysis of transcriptional responses will require methodical analysis by means of microarray gene expression studies, which we initiated in July 2004 under a one-year renewal to this project. At the same time, the current list of putatively *Xylella*-induced genes may provide leads for further analysis by means of Real Time PCR.

Real Time PCR has three primary uses for Pierce's disease research: (1) It can be used as an alternative to pathogen-based assays for disease diagnosis. For example, the identification of host genes that are expressed early and systemically could provide a significantly more reliable test for PD infection. This "biomarker" strategy is gaining increasing use for human medicine. (2) Real Time PCR assays offer a useful point of comparison for data from *in silico* analysis of gene expression (i.e., from statistical analysis of EST data) and for confirming results for key genes identified in Affymetrix microarray experiments. (3) Real Time PCR of differentially expressed host genes can provide a convenient research tool for investigators in need of a sensitive measure of host response.

Based on the *in silico* analysis, described above, four *Xylella*-induced genes, a constitutively expressed control *Vitis* gene, and a bacterial gene, were selected to develop a multiplex PCR assay. This "dual-diagnosis" system may have potential as a tool for disease diagnosis.

Isolation of Pathogen-induced Promoters

DNA probes were developed based on the *Xylella*-induced genes and used to screen high-density filters of *Vitis vinifera* genomic DNA libraries. Clones were isolated, fingerprinted to confirm relatedness, and analyzed by PCR and sequencing to verify that they contained the genes of interest. A shotgun sequencing strategy is being used to obtain the complete sequence of each clone and promoter constructs are being made to test in transient and stable transformation assays. Gene fusions will include reporter proteins to monitor temporal and spatial patterns of transcription (e.g., green fluorescent protein and ß-glucuronidase) and candidate pathogen resistance proteins that may protect grapes against *Xylella* infection.

CONCLUSIONS

To date we have identified several genes of *Vitis vinifera* that are up-regulated in response to *Xylella* infection. Ongoing research will identify larger sets of grape genes expressed in response to this pathogen and provide the basis for biotechnological approaches to dealing with Pierce's disease.

How will these technologies help in solving Pierce's disease? *In the short term* they will (1) yield improved genetic tools for breeding resistance to Pierce's disease (for example single nucleotide polymorphism "SNP" and simple sequence repeat "SSR" genetic markers currently available from our web site "http://cgf.ucdavis.edu), (2) provide gene-promoters that are an essential, but currently unavailable, tool for effective genetic engineering in grapes, and (3) potentially provide the basis for more reliable detection of the pathogen based on Real Time PCR using a "biomarker" strategy. (4) *In the long term*, transcriptional profiling will identify candidate genes and gene pathways that may confer resistance to the pathogen (*Xylella fastidiosa*) and/or to the insect vector (Sharpshooter leaf hopper) and it will allow testing of long-standing hypotheses such as the relationship between host response to drought and host response to *Xylella*. Other strategies, such as reverse genetics and analysis of natural genetic variation for host responses, will be required to establish a causal role for candidate genes.

REFERENCES

Alizadeh, A.A., et al. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403:503-511.

MacNeil, J.S. (2004) Better Biomarkers for the Diagnostics Labyrinth. Genome Technology July/August, 24-33.

Maleck, K., et al. (2001) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. Nature Genetics 26:403-410.

Ramaswamy, S., Ross, K., Lander, E.S. and Golub, T.R. (2003) A molecular signature of metastasis in primary solid tumors. Nature Genetics 33:49-54.

Tao, Y., et al. (2003) Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. The Plant Cell 15:317-330.

de Torres, M., Sanchez, P., Fernandez-Delmond, I., and Grant, M. (2003) Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in *RPM1*-mediated resistance. The Plant Journal 33:665-676.

Van't Veer, L.J., et al. (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530-536.
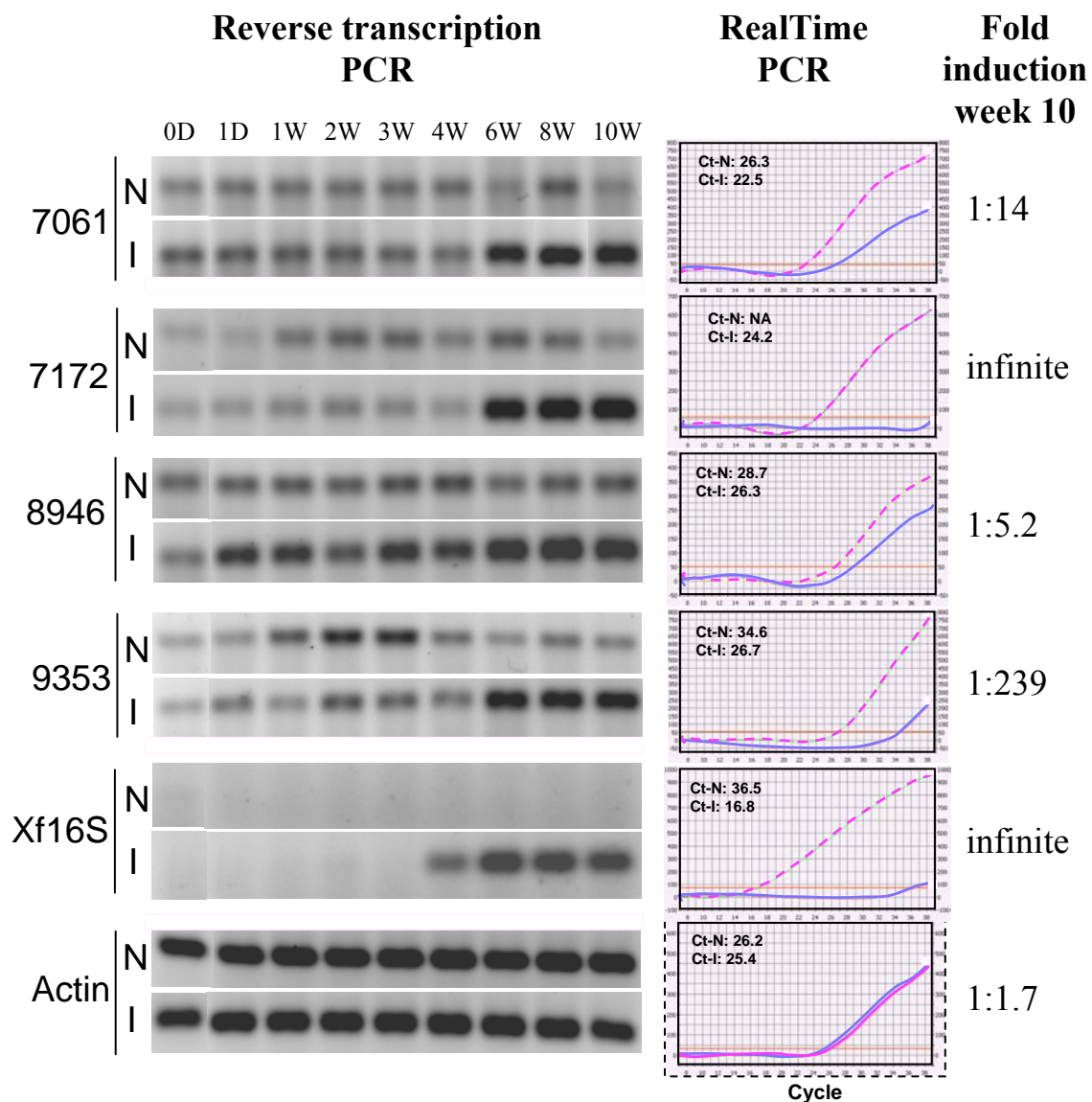
FUNDING AGENCIES

Figure 1. **Monitoring of PD-induced genes using conventional reverse transcriptase-PCR and Real Time PCR.** Leaf tissue was sampled from growth chamber-grown plants at nine time points (0, 1d, 1w, 2w, 3w, 4w, 6w, 8w, 10w: d-day, w-week) after inoculation. *Xylella* up-regulated genes identified from in silico analysis are 7061, 7172, 8946, and 9353. Actin serves as a constitutively expressed control. *Xf*16S = *Xylella fastidiosa* 16S gene. N; Non-inoculated, I; Inoculated with *X. fastidiosa*.

CONTROL OF PIERCE'S DISEASE THROUGH DEGRADATION OF XANTHAN GUM

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Reporting Period: The results reported here are from work conducted between October 2003 and October 2004.

ABSTRACT
Acinetobacter johnsonii GX123, a *Xylella* gum-degrading endophyte was co-inoculated with *Xylella fastidiosa* strain Texas in oleander plants to determine its efficacy as a biocontrol agent in preliminary experiments. Symptoms appeared in both plants inoculated with *X. fastidiosa* alone and plants co-inoculated with the endophyte. However, symptoms were more severe and appeared earlier in plants inoculated with *X. fastidiosa* than in those co-inoculated with the endophyte. *A. johnsonii* GX123 seems to be a promising candidate to control *X. fastidiosa*. Experiments using a sequential strategy of inoculating the *Xylella* gum-degrader endophyte prior to *X. fastidiosa* are ongoing and its effects on symptom expression are still under investigation.

INTRODUCTION
Pierce's disease (PD) of grapevine and other leaf scorch diseases caused by *Xylella fastidiosa* (*Xf*) are associated with aggregation of bacteria in xylem vessels, formation of a gummy matrix, and subsequent blockage of water uptake. In the closely-related pathogen, *Xanthomonas campestris* (Xc), xanthan gum is known to be an important virulence factor (Katzen et al, 1998), probably contributing to bacterial adhesion, aggregation, and plugging of xylem. The published genome sequence of *Xf* (Simpson et al, 2000; Bhattacharyya et al, 2002; Van Sluys et al, 2003) revealed that this pathogen also has genes for producing an exopolysaccharide with a very similar structure to that of xanthan gum. In PD, this *Xylella* gum is likely to contribute to plugging of the grapevine xylem (Keen et al, 2000) and possibly to the aggregation of the bacterium in the mouthparts of the glassy-winged sharpshooter. Because of its importance as an industrial thickener and emulsifier, xanthan gum synthesis and degradation have been extensively studied (Becker et al, 1998). Bacteria that produce xanthan-degrading enzymes have been isolated from soils using enrichment techniques with xanthan gum as the sole carbon source (Sutherland 1987; Ruijssenaars et al, 2000).

The purpose of this project is to identify bacteria that produce xanthan-degrading enzymes to target this specific virulence factor of *Xf*. This approach has the potential to significantly reduce the damage caused by PD in grapes and potentially in other hosts of *Xf* such as almond and oleander. If the gum is important in the aggregation of the pathogen in the insect vector, then our approach may also reduce the efficiency of transmission of PD. Our first approach will be to develop endophytic bacteria that produce these enzymes in the xylem of grapevines, but another approach is to engineer grape plants to produce these enzymes. Through the cloning and characterization of genes encoding xanthanases and xanthan lyases we will facilitate possible efforts to transform grapevines to produce these enzymes.

Previously, we used modified xanthan gum that mimics *Xylella* gum from a Xc mutant as the sole carbon source for enrichment culture from infected grapevines and oleanders. The *Xylella* gum biosynthetic operon in the *Xf* genome is different than the one in Xc from which the commercial xanthan gum is obtained. Since it is not feasible to produce *Xylella* gum for our studies from the slow-growing *Xf*, we genetically modified a strain of Xc to produce a modified xanthan gum that is predicted to have the same chemical structure as that from *Xf*. This was accomplished by deleting the *gumI* gene from the biosynthetic operon. Over 100 bacterial strains were initially recovered from enrichment experiments, and 11 were subsequently confirmed to effectively degrade *Xylella* gum. These strains were then tested for cellulase activity. Degradation of the cellulosic backbone of the gum polymer would be desirable, but we do not want enzymes that recognize and degrade plant cellulose. One particular strain (GX123) with high gum-degrading activity but no cellulase activity isolated from oleander was identified as *Acinetobacter johnsonii* (Aj), and characterized in more detail. In vitro, growth and biofilm production by GX123 were enhanced by *Xylella* gum as a substrate and by cells of *Xf* added to a minimal medium. The gum was degraded rapidly during log-phase growth of this endophyte, and viscosity was reduced almost to non-detectable levels. GX123 colonized stems and leaves of oleander systemically (10^4-10^5 cfu/g of plant tissue 20 days after inoculation), and systemic colonization was enhanced by co-inoculation with *Xf*. The effect of using GX123 as an endophyte to reduce the ability of *Xf* to produce disease symptoms in oleander was studied.

OBJECTIVES

1. Characterize xanthan-degrading enzymes from endophytic bacteria isolated from grape
2. Explore applications of naturally-occurring endophytic bacteria that produce xanthan-degrading enzymes for reduction of Pierce's disease and insect transmission
3. Clone and characterize genes encoding xanthan-degrading enzymes for enzyme overproduction and construction of transgenic endophytes and plants

RESULTS

Co-inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants

GX123 was co-inoculated with *Xf* strain Texas in 3 different cultivars of oleander in the green house: White, Single Red and Betty. At the same time, controls were inoculated with GX123 alone, *Xf* alone or PBS buffer. Four plants were used per inoculation condition and per cultivar, totaling 48 plants obtained commercially. The appearance of symptoms was checked at approximately monthly intervals. Chlorotic mottling along the edges of leaves (Purcell et al, 1999) started to appear approximately in the eighth month after the inoculations, slowly developing into generalized chlorotic mottling and dried tissue (Table 1). The oleander cultivars White and Single Red were the first ones to show symptoms, while the cultivar Betty started to show symptoms 12 months after the inoculations. For all the cultivars, symptoms appeared in both plants inoculated with *Xf* and plants co-inoculated with the endophyte. However, the severity of the symptoms was less for the plants co-inoculated with the endophyte than for the plants not co-inoculated (Figures 1-3). Symptoms were more severe and appeared earlier in plants inoculated with *Xf* than in those co-inoculated with GX123 (Table 1 and 2). One year after being inoculated with *Xf* alone all the plants infected by *Xf* (positive result in ELISA test) showed symptoms, while one year after co-inoculations only 75% of the plants infected by *Xf* showed symptoms (Table 3). On the other hand, one year after inoculations *Xf* was detected in infected plants (10^5-10^6 ufc/g of plant tissue), while GX123 was not detected, showing a probable need for re-inoculation of the endophyte for a long term survival or a different strategy of introducing the biocontrol endophyte.

Table 1. Severity of the symptoms in oleander plants, regardless of the cultivar, inoculated with *X. fastidiosa* strain Texas alone or co-inoculated with GX123; 12 plants total per inoculation condition per month sampling.

Months	*X. fastidiosa* strain Texas				*X. fastidiosa* strain Texas/GX123			
	8	10	12	14	8	10	12	14
(+)	2	0	2	3	3	1	2	3
+	3	1	0	0	2	2	3	0
++	2	3	4	1	0	4	4	2
+++	0	3	3	4	0	0	0	5
AD	0	0	0	1	0	0	0	0
D	0	0	0	2	0	0	0	0

(+) chlorotic mottling along the edges of a few leaves; + chlorotic mottling along the edges of many leaves evolving into a uniform chlorotic mottling; ++ chlorotic mottling of many leaves, starting to wrinkle and dry; +++ chlorotic mottling of many leaves and zones of dead tissue (dried, straw color), smaller leaves; AD many dried leaves, plant almost dead; D plant dead.

Table 2. Number of symptomatic plants after inoculation with *X. fastidiosa* strain Texas alone, co-inoculated with GX123, GX123 alone or PBS buffer; 12 plants total per inoculation condition per month sampling.

Months	*X. fastidiosa* strain Texas	*X. fastidiosa* strain Texas/GX123	GX123	PBS
8	7	5	0	0
10	7	7	0	0
12	9	9	0	1
14	11	10	0	1

Table 3. Symptomatic plants and ELISA results after 1 year of inoculation; 12 plants total per inoculation condition.

Inoculations	*X. fastidiosa* strain Texas	*X. fastidiosa* strain Texas/GX123
Symptomatic plants	9	9
Positive ELISA for *X. fastidiosa*	9	12

Figure 1. Oleander 'White' after 1 year of inoculation with *X. fastidiosa* strain Texas.

Figure 2. Oleander 'White' after 1 year of co-inoculation with *X. fastidiosa* strain Texas and GX123.

Figure 3. Oleander 'White' after 1 year of inoculation with GX123.

Sequential Inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants

To examine the effect of different strategies to introduce the *Xylella* gum-degrader endophyte to control *Xf* in plants, GX123 was inoculated in oleander plants (cultivar white) prior to *Xf*. Sequential inoculation of *Xf* was done 20 days after GX123 was inoculated in the same point when the titers of GX123 were already around 10^4-10^5 cfu/g of plant tissue. This experiment is still ongoing and symptoms have not developed yet, consequently the effect on disease expression is still unknown.

CONCLUSIONS

The *Xylella* gum-degrader endophyte *Acinetobacter johnsonii* GX123 colonized plants and delayed symptoms of infected oleander plants in preliminary experiments. It is a potential candidate as a biocontrol agent for *Xylella fastidiosa,* and therefore a promising tool to fight Pierce's disease.

REFERENCES

Becker, A., Katzen, F., Pühler, A., Ielpi, L. 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Appl. Microbiol. Biotechnol. 50:145-152.

Bhattacharyya, A., et al. 2002. Whole-genome comparative analysis of three phytopathogenic *Xylella fastidiosa* strains. Proc. Natl. Acad. Sci. USA 99:12403-12408.

Katzen, F., Ferreiro, D. U., Oddo, C. G., Ielmini, M. V., Becker, A., Pühler, A., Ielpi, L. 1998. *Xanthomonas campestris* pv. campestris *gum* mutants: effects on xanthan biosynthesis and plant virulence. J. Bacteriol. 180:1607-1617.

Keen, N. T., Dumenyo, C. K., Yang, C.-H., Cooksey, D. A. 2000. From rags to riches: insights from the first genomic sequence of a plant pathogenic bacterium. Genome Biology. 1:1019.1-1019.3.

Purcell, A. H., Saunders, S. R., Hendson, M., Grebus, M. E., Henry, M. J. 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. Phytopathol. 89:53-58.

Ruijssenaars, H. J., Hartmans, S., Verdoes, J. C. 2000. A novel gene encoding xanthan lyase of *Paenibacillus alginolyticus* strain XL-1. Appl. Environ. Microbiol. 66:3945-3950.

Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa.* Nature 406:151-159.

Sutherland, I. W. 1987. Xanthan lyases – novel enzymes found in various bacterial species. J. Gen. Microbiol. 133:3129-3134.

Van Sluys, M. A., de Oliveira, M. C., et al. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185:1018-1026.

FUNDING AGENCIES

PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: TOXIC PEPTIDES AGAINST *XYLELLA*

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ABSTRACT

The use of symbiotic bacteria in insects to disrupt pathogen transmission is a new approach to disease control. *Alcaligenes xylosoxidans denitrificans* bacterium was isolated from the mouthparts of wild glassy-winged sharpshooter and was chosen to be the first candidate for delivery products that inhibit *X. fastidiosa.* To find an appropriate agent for control of Pierce's disease, 90 antimicrobial peptides (AMPs) derived from a combinatorial peptide library (in addition to 59 screened previously from different sources) were tested for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. Forty four peptides showed potent antimicrobial activity against all strains studied. Six antimicrobial peptides (in addition to 4 found last year) were selected with toxicity to *X. fastidiosa* but not against *Alcaligenes* as a candidates for engineering of the sharpshooter's symbiont. More detailed studies of minimum inhibitory concentrations of these peptides were conducted. The Glutathione s-transferase gene fusion and *trc* expression systems are being developed to express individual AMPs *in vitro*.

INTRODUCTION

Xylella fastidiosa causes of Pierce's disease (PD), an important disease of grapevines in the United States. Because of the mobility and vector capacity of glassy-winged sharpshooter (GWSS), PD has become a great concern to grape production in California. One promising method for long-term *X. fastidiosa* control is limiting pathogen spread by rendering GWSS vector-incompetent. Paratransgenesis (Beard et al. 2001), which is the genetic alteration of bacteria carried by insect is currently being developed to deliver pathogen toxic substances that would inhibit *X. fastidiosa* and reduce disease transmission.

Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop growth of bacteria. A second type of antibiotics called antimicrobial peptides (AMPs) are produced by organisms including bacteria, plants, insects, birds, amphibians, and mammals (Cammue et al. 1992, Casteells et al. 1993, Nayler et al. 1989, Schroder 1999). These compounds interact directly with target bacterial membranes, but can do so with a receptor-like specificity, and can act via both membrane ion pore formation and by preventing cell wall formation (Maloy and Kari 1995). Because AMPs are "gene-based", they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters. For example, the antimicrobial peptide MSI-99, an analog of Magainin 2, was expressed via the chloroplast genome to provide inhibition of growth against *Pseudomonas syringae* pv *tabaci*, a major plant pathogen (DeGray 2001). A combinatorial libraries represent a vast new source of molecular diversity for the identification of potential lead antimicrobial and antifungal compounds (Blonde and Lohner 2000, Jing et al. 2003). A combinatorial peptides are significantly shorter than other AMPs isolated from various biological sources. An amphipathic structure may allow this peptide to penetrate deeper into the interfacial region of membranes, leading to local membrane destabilization (Jing et al. 2003).

Use of symbiotic bacteria to deliver gene-based product is a new strategy of disease control. We demonstrated previously the expression of *Bacillus thuringiensis* toxin Cyt1A in the symbiotic bacterium *Enterobacter gergoviae* isolated from the gut of the pink bollworm (Kuzina et al. 2002). Bextine et al. (2004) used the expression of a red fluorescent protein (dsRed) by *Alcaligenes* (*Axd*) to study the colonization of the cibarial region of the GWSS. Genetically transformed symbiotic bacteria have been used to control the pathogen that caused Chagas disease (Beard et al. 1992, Beard et al. 2001, Durvasula et al. 1997).

OBJECTIVES

The overall goal of this project is to genetically transform symbiotic bacterium of the glassy-winged sharpshooter to produce toxic substances that would inhibit or kill *X. fastidiosa* and reduce disease transmission.

1. Identify toxic peptides effective against *X. fastidiosa* but non-toxic to *Alcaligenes*, selected symbiotic bacterium.
2. Design and construct genes encoding indolicidin and other peptides.
3. Develop a transformation system for expression of indolocidin .
4. Construct a transport cassette for secretion of indolicidin into *Alcaligenes*.

RESULTS

During the reporting period, we have screened an additional 90 antimicrobial peptides derived from a combinatorial library for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. *Axd* was isolated from the mouthpart of wild captured GWSS by Carol Lauzon. We found that 44 AMPs showed potent antimicrobial toxicity against all strains studied. Six AMPs were found with activity toward *X. fastidiosa* and non-toxic to *Alcaligenes*. These 6 peptides (along with 4 these screened last year) were more extensive examined for effective inhibitory concentration to *Xylella* and toxicity to *Alcaligenes* and *E. coli* as a target organism (Table 1). Blake Bextine studied the ability of GWSS to transmit *X. fastidiosa* to naive grapevine seedlings by oral delivery one of several antimicrobial peptide - indolicidin at 2 concentration: 100 µg/ml and 500 µg/ml. *X. fastidiosa* transmission rates were reduced from 50% in the control group, to 35% with the 100 µg/ml concentration and 7% with the 500 µg/ml concentration when GWSS were exposed to indolicidin prior to inoculation access. Therefore, indolicidin was chosen to be the first candidate for the development of gene-cassette. Artificial gene(s) to code indolicidin were designed and constructed for expression in *E. coli*. cDNA-encoding this peptide was amplified by PCR with incorporation of a *Sal*1 restriction site and/or *BamH*1 and *EcoR*1 restriction sites. We are using the Glutathione s-transferase gene fusion system (GST) (Pharmacia Biotech. Inc) and *trc* expression system (Invitrogene Co.) to express individual peptides. The GST gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. A pTrcHisTOPO expression kit provides a highly efficient, rapid cloning strategy for direct insertion of *Taq* polymerase-amplified PCR product into a plasmid vector for expression in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing specific sequences were required. We transformed competent cells of *E. coli* DH5λ and TOPO by pGEX and pTrcHisTOPO vectors containing indolicidin gene. Several transformants were selected using LB medium containing ampicillin at 50 µg/ml (Sigma) and currently are being examined for production of indolicidin with and without IPTG.

Table 1. Toxicity of antimicrobial peptides to X. fastidiosa, Alcaligenes, and E. coli strains

Peptide	Range of MICs (µg/ml) to *X. fastidiosa*[a]	*Alcaligenes sp.*[b]	*E. coli*[c]	Source
1. Indolicidin	16-64	-	-	APS[d]
2. PA2	32-128	-	-	NCSU[e]
3. PA6	32-64	-	-	NCSU
4. PA7	32-64	-	-	NCSU
5. DCR1	16-32	-	-	TPIMS[f]
6. DCR2	8-16	-	-	TPIMS
7. DCR3	32-64	-	-	TPIMS
8. DCR4	16-32	-	-	TPIMS
9. DCR5	16-32	-	-	TPIMS
10.DCR6	8-16	-	-	TPIMS

[a] – *MICs of the antimicrobial peptides to eleven* X. fastidiosa *strains studied*
[b] – *Activity of AMPS to* Alcaligenes xylosoxidans denitrificans *134, 135, and 136 is negative*
[c] – *Activity of AMPs to* E. coli *DH5λ and TOPO is negative*
[d] – *American Peptide Company, Sunnyvale, CA*
[e] – *North Carolina State University, Raleigh, NC*
[f] – *Torrey Pines Institute for Molecular Studies, San Diego, CA*

CONCLUSIONS

The 10 antimicrobial peptides were found with toxicity to 11 *X. fastidiosa* strains isolated from grape, oleander and almond, but not against the glassy-winged sharpshooter gut bacterium *Alcaligenes xylosoxidans denitrificans*. We consider these AMPs as a candidates for use as reagents in delivery vehicle for paratransgenesis: Indolicidin, a 13-residue peptide-amide, isolated from the cytoplasmic granules of bovine neutrophils (Selsted 1992); 3 pescidins, isolated from the mast cells of aquacultured fish (Silphaduang and Noga 2001); and 6 peptides derived from a combinatorial peptide library (Blonde and Lohner 2000) (Table 1). *Alcaligenes* will be engineered to produce a peptide(s) toxic substance that would inhibit *X. fastidiosa* and reduce disease transmission. To develop a transformation system to express peptide(s) in *E. coli* first, we are using the Glutathione s-transferase gene fusion and *trc* expression systems. We got several ampicillin resistant transformants which are being studied for production of indolicidin. Artificial genes of other peptides are being designed for expression and secretion by *E. coli* and *Alcaligenes* as well.

REFERENCES

Beard, C.B., Mason, P.W., Aksoy, S., Tesh, R.B., Richards, F.F. 1992. Transformation of an insect symbiont and expression of a foreign gene in the Chagas disease vector *Rhodnius prolixus*. Am. J. Trop. Med. Hyg. 46:195-200

Beard, C.B., Dotson, E.M., Pennington, P.M., Eichler, S., Cordon-Rosales, C., and Durvasula, R.V. 2001. Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. Int. J. Parasitol. 31:621-627

Bextine, B., Lauzon, C., Potter, S., Lampe, D and Miller, T. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Curr. Microbiol. 48:327-331

Blonde, S.E. and Lohner, K. 2000. Combinatorial libraries: a tool to design antimicrobial and antifungal peptide analogues having lytic specificities for structure-activity relationship studies. Biopolymers 55:74-87

Cammue, B.P.A., De Bolle, M.F.C., Terras, F.R.G., Proost, P., Van Damme, J., Rees, S.B., Vanderleyden, J., Broekaert, W.F. 1992. Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J. Biolog. Chemistry 267:2228-2233

Casteells, P., Ampe, C., Jacobs, T. and Tempst, P. 1993. Functional and chemical characterization of Hymenoptaecin, an antimicrobial polypeptide that is infection-inducible in the Honeybee (*Apis mellifera*). J. Biolog. Chemistry 268: 7044-7054

DeGray, G., Rajasekaran, K., Smith, F., Sanford, J. and Daniell, H. 2001. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiology 227:852-862

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Asoy, S., Merrifield, R.D., et al. 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc. Natl. Acad. Sci. USA 94:3274-3278

Jing, W. et al. 2003. The structure of the antimicrobial peptide Ac-RRWWRF-NH$_2$ bound to micelles and its interactions with phosfolipid bilayers. J. Pept. Research 61:219-229.

Kuzina, L.V., Miller, E.D., Ge, B and Miller, T.A. 2002. Transformation of *Enterobacter gergoviae* isolated from pink bollworm (Lepidoptera: Gelechiidae) gut with *Bacillus thuringiensis* toxin. Curr. Microbiol. 44:1-4

Maloy, W.L. and Kari, U.P. 1995. Structure-activity studies on magainins and other host defense peptides. Biopolymers 37: 105-122

Nayler, W.G., Gu, X.H., Casley, D.J. 1989. Sarafotoxin S6c is a relatively weak displacer of specifically bound. Biochem. Biophys. Res. Commun. 161:89-94

Schroder, J.M. 1999. Epithelial peptide antibiotics. Biochem. Pharmacology 57:121-134

Selsted, M.E., Novotny, M.J., Morris, W.L., Tang, Y-Q, Smith, W., and Cullor, J.S. 1992. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. J. Biol. Chem. 267: 4292-4295

Silphaduang, U and Noga, E.J. 2001. Peptide antibiotics in mast cells of fish. Nature. 414:268-269

DEVELOPMENT OF AN ARTIFICIAL DIET AND EVALUATION OF ARTIFICIAL OVIPOSITIONAL SUBSTRATES FOR THE *IN VITRO* REARING OF *GONATOCERUS* SPP. PARASITOIDS OF THE EGGS OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
The intent of this project is to develop an *in vitro* rearing system for one or more of the three mymarid species of *Gonatocerus* currently being reared and released in California to control GWSS. A complete *in vitro* rearing system will include both a growth-enhancing artificial diet for larval and pupal development as well as a suitable oviposition substrate, or "artificial egg". Initial studies will formulate artificial diets based on those developed previously for hymenopteran parasitoids, with an emphasis being placed on diets for other egg parasitoids. To accomplish this, *Gonatocerus spp.* eggs and/or larvae will be dissected from host eggs and placed in cell culture plates containing selected diets. Comparisons will be made between the development of parasitoids on these artificial diets, and those developing on the natural host. Developmental parameters measured will include extent of development, developmental time per stage, and weight. Once a promising diet is formulated, the reproductive rate and reproductive fitness of adults reared from these diets will be compared by using ovarian scoring and by assessing differences in fecundity and egg viability from crosses of diet-reared and host-reared adult wasps (Wittmeyer et al., 2001; Wittmeyer and Coudron, 2001). Refinement of the diet will be performed by modifying the diet based on its ability to meet the nutritional, phagostimulatory, and endocrine requirements of the parasitoid, and may include the additional of undefined components such as insect or cell-culture derived components. The suitability of artificial eggs, composed of different combinations of membranes and cupule sizes, will be evaluated statistically using pairwise comparisons of the proportion of "artificial eggs" and natural host eggs successfully parasitized by the same number of female *Gonatocerus* parasitoids (SAS, 2002).

INTRODUCTION
Surveys of potential biological control agents in Texas (where GWSS is endemic and under natural control) and California revealed that *Gonatocerus* spp. parasitoids are the predominant natural enemy of GWSS in the field, parasitizing between 75-90% of GWSS egg masses (Phillips, 2000; Jones, 2002; Hoddle 2003a). In California, over 90% of the eggs laid by the second generation of GWSS in late summer and early fall are parasitized by *Gonatocerus* spp., however, only 10 – 50% of the eggs laid by the first generation in the early spring are parasitized (Phillips et al., 2004; Hoddle 2003b). This suggests that survival of overwintering adult parasitoids is low, or that the current cohort of species of *Gonatocerus* are not effective in parasitizing GWSS eggs early in the season (Hoddle, 2003b; Jones, pers. comm.). However, augmentation of *Gonatocerus* spp. populations in early spring may be able to significantly reduce the population of GWSS that vector the disease later in the season and could be used to reduce pesticide use thereby aiding in the development of a classical biological control program. The current list of species being considered for biocontrol of GWSS in CA include the solitary egg parasitoids *Gonatocerus ashmeadi* (which accounts for 80-95% observed GWSS egg parasitization in California) and *G. triguttatus* (the primary GWSS egg parasitoid in Texas), as well as the gregarious egg parasitoid *G. fasciatus* (which may have a greater host finding efficiency than the other two) (Hoddle 2003a).

The implementation of current classical and augmentative biological control programs against GWSS has been complicated by a number of factors. Currently, no artificial diet exists for GWSS, and high costs are associated with rearing the sharpshooters in sufficient numbers to provide the necessary quantity of host eggs (Lauziere et al., 2002; Jones, pers. comm.). Long-term stockpiling of host eggs is not feasible at this time because host acceptance declines after refrigeration for 20 days at 13°C, and parasitized eggs only remain viable for 7 days at 2°C (Leopold, 2003). Consequently, augmentation of *Gonatocerus* spp. in many areas of California relies on the labor-intensive process of rearing the parasitoid on host eggs collected from the field (Jones, pers. comm.). Thus, the development of an artificial diet and ovipositional substrate as part of an *in vitro* mass rearing system for *Gonatocerus* spp. has a number of potential advantages over current rearing techniques. Additionally, *in vitro* rearing would also be more easily automated, reducing labor costs (Li-Ying, 1992; Qin, Beijing Univ., pers. comm.) and would provide an easier means for studying the reproductive and nutritional physiology of *Gonatocerus* spp.

Efforts to develop an artificial diet capable of supporting larval and pupal development will initially focus on testing established diets formulated for the *in vitro* rearing of other egg parasitoids, e.g., those used for rearing lepidopteran egg parasitoids including several *Trichogramma* spp. (Hoffman et al., 1975; Li-Ying 1992; Consoli and Parra, 1997; Xie et al., 1997; Grenier et al., 1998; Qin, Beijing Univ. pers. comm.;), *Telenomus heliothidis* (Strand et al., 1988), and *Ooencyrtus* spp. (Masutti et al., 1994; Lee and Lee, 1994); a coleopteran egg parasitoid, *Edovum puttleri* (Hu et al., 1999; Hu et al., 2001) , and a pentatomid egg parasitoid, *Trissolcus basalis* (Volkoff et al., 1992*).* For studies on the development of an artificial ovipositional substrate, membranes that will be derived from a variety of sources will be tested, such as: oxygen-permeable films used for mass rearing *Trichogramma* spp. (Qin, Beijing University, pers. comm.), parafilm (Wittmeyer et al., 2001; Cooperband and Vinson, 2001), and polycarbonate, polyvinylchloride, polyethylene, and/or polypropylene membranes (Masutti et al., 1994; Morrison et al., 1983; Consoli and Parra 1999).

OBJECTIVES
1. Formulate an artificial diet capable of supporting the development and reproduction of *Gonatocerus* spp. parasitoids of the eggs of glassy-winged sharpshooter, *Homalodisca coagulata.*
2. Screen, modify, and evaluate existing materials for their suitability as ovipositional substrates for these egg parasitoids.
3. Develop and optimize an *in vitro* rearing unit, consisting of an artificial diet and ovipositional substrate, that can be utilized for *Gonatocerus* spp. oviposition, parasitoid development, and release.

RESULTS AND CONCLUSIONS
This project has just been funded. Preparation of quarantine facilities is complete and the identification of insect cultures to be used in our studies is underway. The process to hire an additional researcher has been initiated. Preliminary experiments have been conducted in collaboration with Leopold at ARS in Fargo that indicate cold-storage processes should offer suitable method(s) to preserve the natural host of the parasitoid for these studies.

REFERENCES
Consoli FL, Parra JRP, 1997. Biological Control 8: 172-176.
Cooperband MF, Vinson SB, 2001. Biol. Control 17(1): 23-28.
Grenier S, Han SC, Chapelle L, Liu WH, Guillaud J, 1998. Biocontrol Science & Technology. 8(4): 589-596.
Hoffman JD, Ignoffo CM, Dickerson WA, 1975. Ann. Entomol. Soc. Am. 68: 335-336.
Hoddle M, 2003a. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
Hoddle M , 2003b. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
Hu JS, Gelman DB, Bell RA, 2001. BioControl 46: 43-60.
Hu JS, Gelman DB, Bell RA, Lynn DE, 1999. Arch. Insect Biochem. Physiol. 40: 173-182.
Jones WA, 2002. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
Lauziere I, Ciomperlik M, Wendell L, 2002. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
Lee HP, and Lee KS, 1994. Korean J. of Entomol. 24: 311-316.
Leopold RA, 2003. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
Li-Ying L, 1992. Korean J. Applied Entomol. 31: 241-246.
Masutti L, Battisti A, Milani N, Zanata M, Zanazzo G, 1994. Entomophaga 38: 327-333.
Morrison RK, Nettles WC, Jr., Ball D, Vinson SB. 1983. Southwestern Entomol. 8: 248-251.
Phillips PA, Wilen CA, Varela LG, 2004. URL: www.axp.ipm.ucdavis.edu/PMG/PESTNOTES/pn7492.html
Phillips PA, 2000. KAC Plant Protect Quarterly 10: 6-7.
Strand MR, Vinson SB, Nettles WC, Jr., Xie ZN, 1988. Entomologia Experimentalis et Applicata 46: 71-78.
Volkoff N, Vinson SB, Wu ZX, Nettles WC Jr., 1992. Entomophaga 37: 141-148.
Wittmeyer JL, Coudron TA, Adams TS, 2001. Invertebrate Reproduction & Development 39: 9-20.
Wittmeyer JL, Coudron TA, 2001. J. Econ. Entomol. 94: 1344-1352.
Xie ZN, Wu ZX, Nettles WC Jr., Saldana G, Nordlund DA, 1997. Biological Control 8: 107-110.

DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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ABSTRACT
Xylella fastidiosa (*Xf*), is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (3). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. Traditionally, antibiotics are prescribed as a preferred therapy; however, a pathogen often develops antibiotic resistance and escapes their anti-microbial action (4). In this UC/LANL project, we propose a novel protein-based therapy that circumvents the shortcomings of an antibiotic. We have designed a chimeric anti-microbial protein with two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, Elastase is the SRD that recognizes mopB, the newly discovered *Xf* outer membrane protein (5). The second domain is Cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (Elastase and Cecropin B) display activity against *Xf*, which is increased when both proteins are combined. We have tested Elastase against purified mopB and intact *Xf* cells and found that mopB is degraded in both cases, suggesting that it is potentially a target for Elastase. The HNE-GSTA-Cecropin B chimera gene has been synthesized and is currently being cloned into vectors for overexpression in insect and grapevine cells in order to test its activity against *Xf in vitro*. We have also initiated transgenic grapevine cultures expressing a pear polygalacturonase inhibiting protein that is secreted into the medium using a CELLline 350 bioreactor. In the future, we plan to use this system to test secretion and anti-*Xf* of the chimeric protein.

INTRODUCTION
Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf*) is a devastating bacterial pathogen that causes PD in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorch disease in numerous other agriculturally significant plants including almonds in California (http://danr.ucop.edu/news/speeches). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the availability of the whole genome sequences of PD and CVC strains of *Xf* offer new avenues to directly target and inactivate the pathogen. In this project, we propose a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (1-2). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several short and long term impacts.

OBJECTIVES

Objective 1:   a) Utilize literature data and computer modeling to identify an SRD that specifically targets mopB (Elastase)

                    b) Utilize literature data and computer modeling to identify a useful Cecropin (i.e., Cecropin B)

                    c) *In vitro* testing of anti-*Xylella* activity of the mopB-specific SRD (Elastase) and *Xyllela*-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of Elastase and Cecropin B.

Objective 2:   a) Design and construction of synthetic gene encoding Elastase-Linker-Cecropin B Chimeric protein

                    b) Expression Elastase-Linker-Cecropin B in insect and plant cells and testing activity *in vitro*.

Objective 3:   a) Expression in transgenic plants

                    b) Testing for anti-*Xylella* activity *in planta* and testing for graft transmissibility.

RESULTS AND CONCLUSION

Human Neutrophil Elastase (HNE) (6) was chosen as our first SRD. Neutrophils contain a variety of proteins that enable the cells to migrate toward and eliminate microbial pathogens (7). Until 1991, no specific antibacterial activity had been ascribed to HNE (8). However recent research has established that HNE is the only human neutrophil protein, which is capable of individually killing *Borrelia burgdorferi*, the causative agent of Lyme disease (9, 10). Furthermore, it is known that HNE can augment the cidal properties of other active proteins (11). Sequence-structure analysis of mopB revealed that it contained an specific cleavage site for HNE that is exposed on the surface. We have studied the efficacy of HNE in combination with the antibacterial peptide Cecropin B, that inserts preferentially into the lipid bilayer of gram-negative bacteria, in killing *Xf*. Measuring the number of colony forming units remaining after the bacterium was exposed to HNE, Cecropin B and the combination of both, we found that HNE greatly stimulates the lysis induced by Cecropin B. In addition, we found that Mop B was partially digested by HNE after incubating either purified Mop B or *Xf* cells with HNE for an hour. Based on these preliminary results, we have designed a chimeric protein of Cecropin B and HNE; in order to stabilize the Cecropin B peptide and enhance the overall affinity of the ligands for the bacterial surface. The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

Our strategy began with the generation of a 3-D model of the chimera. The modeling was based on published protein data bank (PDB) structures of HNE and nuclear magnetic resonance structures of peptides homologous to Cecropin B. A short G-S-T-A peptide linker was inserted between the C-terminus of HNE and the N-terminus of Cecropin B to allow both functional domains to make contact with the bacterial surface simultaneously without steric interference. Energy minimization and molecular dynamics analysis using the AMBER 7.0 force field indicated that the chimera forms a stable structure. The HNE-GSTA-Cecropin B chimera gene was synthesized and is currently being cloned into a baculovirus vector for overexpression in insect cells. The chimera will be purified from insect cells and tested for its activity against *Xf in vitro*. The chimera will be also cloned into a plant vector for transformation of grape embryogenic callus growing in a CELLline 350 bioreactor where they will be analyzed for the production and anti-*Xf* activity of the secreted protein. We will choose the most promising embryogenic lines for plant regeneration. The plant expression vector will have necessary regulatory sequences to facilitate transcription and extracellular delivery of the protein product. Currently we are investigating grapevine embryogenic callus for the extracellular production of the pear polygalacturonase inhibiting protein (pPGIP). This protein has been found in the xylem exudate of transgenic grapes expressing the pPGIP gene and will be used to modify delivery of the chimeric protein to grapevine xylem tissues.

IVGGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHCVANVNVRAVRVVLGAHNLSRREPTR
QVFAVQRIFEDGYDPVNLLNDIVILQLNGSATINANVQVAQLPAQGRRLGNGVQCLAMGWGLLGRNRG
IASVLQELNVTVVTSLCRRSNVCTLVRGRQAGVCFGDSGSPLVCNGLIHGIASFVRGGCASGLYPDAFAP
VAQFVNWIDSIIQ<u>GSTA</u>**KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL**

Figure 1. HNE-cecropin B chimeric amino acid sequence. HNE is attached to cecropin B (shown in bold) by the GSTA linker, which is underlined.
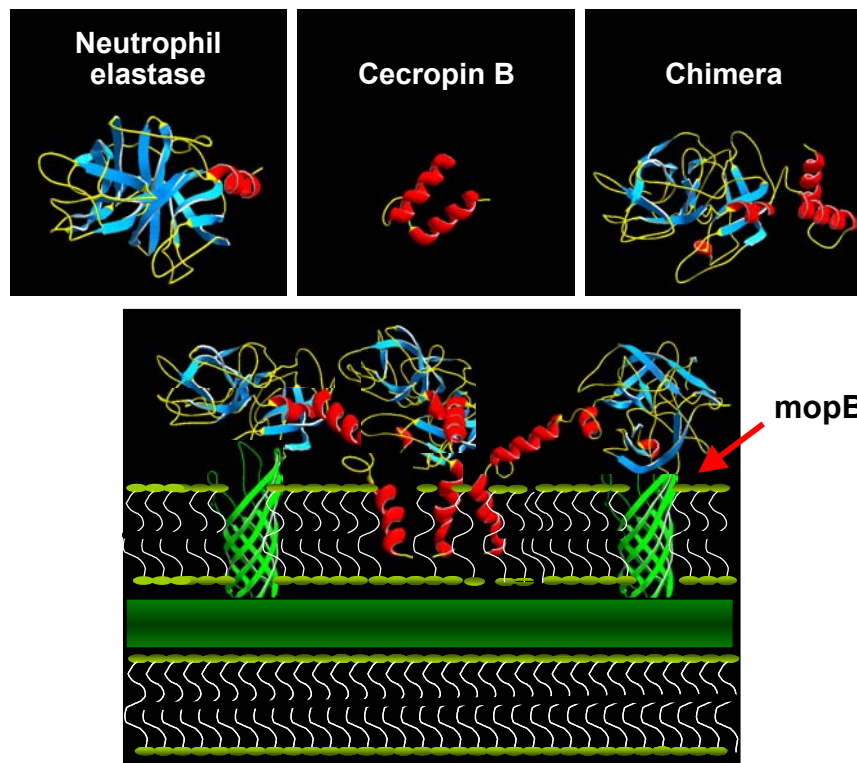
Figure 2. Design and mechanism of chimeric protein targeted to *X. fastidiosa.* The top panel shows the two domains of the chimera in separate planes: neutrophil elastase (1HNE from PDB) is on the left. A homology model of ceropin B is shown in the middle. The right plane shows the energy minimized model of the elastase-cecropin B chimera. The bottom panel is a schematic of the hypothetic mechanism of the chimeric protein. Elastase binds to and cleaves a specific loop on the *X. fastidiosa* outer membrane protein mopB. This action brings cecropin B in close contact with the membrane, where is associates with other cecropin molecules and disrupts the membrane by forming a pore, thereby disabling the bacterium.

REFERENCES
1. Cohn, J., Sessa, G., and Martin, G.B. (2001). Innate immunity in plants. Curr. Opin. Immunol. 13:55-62.
2. Magor, B.G. and Magor, K.E. (2001). Evolution of effectors and receptor of innate immunity. Dev. Comp. Immunol. 25:651-682.
3. Pieters, J. (2001). Evasion of host cell defense by pathogenic bacteria. Curr. Opin. Immunol. 13:37-44.
4. Baquero, F. and Blazquez, J. (1997). Evolution of antibiotic resistance. Trends Ecol. Evol.12:482-487.
5. Bruening, G., Civerelo, E., Kirkpatrick, B., and Gilchrist, D. (2002). Virulence analysis of The Pierces Disease Agent *X. fastidiosa*. PD research symposium Dec 15-18 2002, San Diego, CA.
6. Sinha, S., W. Watorek, et al. (1987). Primary structure of human neutrophil Elastase. Proc. Natl. Acad. Sci.USA 84: 2228-2232.
7. Elsbach, P., and Weiss, J. (1988). Phagocytic cells: oxygen-independent anti-microbal systems. *In* Inflammation: basic principles and clinical correlates. Gallin, J.L., Goldstein, I.M. and Snuderman, R. (Ed.). Raven Press, New York, USA, pp 445-470.
8. Wasiluk, K. R., K. M. Skubitz, et al. (1991). Comparison of granule proteins from human polymorphonuclear leukocytes which are bactericidal toward *Pseudomonas aeruginosa*. Infection and Immunity 59: 4193-4200.
9. Garcia, R., L. Gusmani, et al. (1998). Elastase is the only human neutrophil granule protein that alone is responsible for in vitro killing of *Borrelia burgdorferi*. Infection and Immunity 66(4): 1408-1412.
10. Lusitani, D., S. E. Malawista, et al. (2002).*Borrelia burgdorferi* are susceptible to killing by a variety of human polymorphonuclear leukocyte components. The Journal of Infectious Diseases 185: 797-804.
11. Miyasaki, K. T. and Bodeau, A.L. (1991). In vitro killing *of Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. by human neutrophil cathepsin G and Elastase. Infection and Immunity 59: 3015-3020.

EXTENSIVE SEQUENCE DIVERGENCE IN THE ITS2 RDNA FRAGMENT IN A POPULATION OF *GONATOCERUS ASHMEADI* FROM FLORIDA: PHYLOGENETIC RELATIONSHIPS OF *GONATOCERUS* SPECIES

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ABSTRACT

The aim of the present study was to resolve the genetic relationships of geographic populations of *Gonatocerus ashmeadi*, a primary egg parasitoid of the glassy-winged sharpshooter. A phylogenetic approach was implemented by sequencing the Internal Transcribed Spacer-2 (ITS2) region of several individuals per population. In addition, the phylogenetic relationships of several *Gonatocerus* species were also determined. Six geographic populations of *G. ashmeadi* were analyszed: Quincy, FL (QFL), two populations from Weslaco, TX (WTXa and WTXb), Louisiana (LA), San Antonio, TX (SATX), and California (CA). The percentage divergence (%D) of the ITS2 sequences, as measured by genetic distance, was small among LA, SATX, and CA (0.10-1.10%); whereas, the %D for QFL vs these populations was extremely high (65.9-69.8%). A Nieghbor-Joining distance tree separated the QFL population into a separate clade supported by very high bootstrap values (100%). When the Weslaco populations were included in the anaylsis, they clustered into two distinctive clades, WTXb clustered with QFL and WTXa clusterd with the rest of the populations; again very high bootstrap values (100%) supported the topology of the distance tree. These results indicate the present of sympatric strains in Weslaco. The phylogenetic analysis of several *Gonatocerus* species clustered the respective species into North and South American clades. The %D of the QFL population fell within the range (75.4-87.2%) of the South American *Gonatocerus* species and clustered within the South American clade. The present molecular phylogenetics results provide strong evidence that *G. ashmeadi* from Florida may be a different species. In addition, the data is suggestive that the origin of *G. ashmeadi* in California is the Texas region, including the closely located Louisiana. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's Disease biological control program in California.

INTRODUCTION

Gonatocerus ashmeadi (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Huber 1998). A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding leafhopper is a serious economic pest that vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's Disease in grapevines. Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990). The Internal Transcribed Spacer regions (ITS-1 and –2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, and success with this approach has been reviewed by Collins and Paskewitz (1996).

OBJECTIVES

1. Determine the phylogenetic relationships of geographic populations of *G. ashmeadi*.
2. Determine the phylogenetic relationships of several *Gonatocerus* species, including candidate species from South America (Argentina).

RESULTS AND CONCLUSIONS

Genetic Relatedness Among Geographic Populations Of G. ashmeadi

Levels of genetic divergence in the ITS2 rDNA fragment among populations were determined by calculating the pairwise estimates for genetic distance (Table 1). Recently, we determined by ISSR-PCR DNA fingerprinting that *G. ashmeadi* geographic populations were highly differentiated (de León and Jones 2004). The data demonstrated that the Quincy, FL (QFL) population had the highest gene diversity value. In addition, the data indicated that two Welsaco, TX populations collected at different times of the year were divergence or differentiated from each other and gave a first clue as to the presence of sympatric strains in Weslaco. As seen on Table 1, the sequence percentage divergence (%D) between the QFL population and the rest of the *G. ashmeadi* geographic populations (LA, SATX, WTXa, and CA) was extremely high, ranging from 65.9 to 69.8%. The %D between QFL and the outgroup population (*G. morrilli*) ranged from 77.8-81.2%, whereas LA, SATX, WTXa, and CA ranged from 31.4 to 37.0% compared to the outgroup. The %D among LA, SATX, WTXa,,and CA populations was extremely low, 0.10 to 1.10%, indicating the very close genetic similarity among these geographic populations. This range is within the intra-populational variation found within each of these populations. A phylogenetic anaylsis (Fig. 1A) demonstrated that the QFL and the LA, SATX, WTXa, and CA populations formed two distinct clades supported by extremely high bootstrap support values; in most case they were at 100%. Our second goal was to confirm whether sympatric strains of *G. ashmeadi* indeed existed in Weslaco. Table 1 shows that the %D between QLF

and WTXb is very low (0.00-0.40%) and falls within the range of the inra-populational variation. In contrast, the %D between WTXb and the rest of the populations falls within the same range that the QFL population (65.9-69.8%) fell in. The phylogenetic analysis of all populations (Fig. 1B), including the two Weslaco populations (WTXa and WTXb) demonstrated that these two populations fell on separate clades, confirming the existence of sympatric strains in Weslaco. WTXb clustered with QFL and WTXa clustered with the rest of the *G. ashmeadi* populations. Again, the distance tree is supported by extremely high bootstrap support values (100%). The very high %D values indicate that the QFL and WTXb complex diverged some time ago. The earliest record of *G. ashmeadi* in California was from 1979 (Vickerman et al. 2004) and recently, we showed that a subset of glassy-winged sharpshooters in California had their origin in central Texas (de León et al. 2004). The present results lend support to the idea that *G. ashmeadi* may have its origins in central Texas (SATX) (including the very closely located Louisiana). So it is possible that *G. ashmeadi* was transported to California along with the Glassy-winged Sharpshooter from central Texas.

Phylogenetic Relationships Among Gonatocerus Species

Resolution of relationships requires information about variability not only at the level of populations within a species but also between species (Narang et al. 1993; Unruh and Woolley 1999); therefore, a molecular systematic approach was undertaken with various *Gonatocerus* species, including candidates from South America (Argentina). For the pairwise sequence distance analyses, the *G. ashmeadi* populations (LA, SATX, WTXa, and CA) that formed one clade in fig. 1 were pooled (Ga*, Table 2) and compared to the rest of the *Gonatocerus* species. The %D values among these populations were very low (0.10-0.90%), falling within the range of the intra-specific variation seen within each individual species. The %D of *G. triguttutas* (Gt) and *G. morrilli* (Gm) vs Ga* is 15.8-17.9 and 35.0-38.9%, respectively. In contrast, the %D of *G. ashmeadi* from Florida [Ga(FL)] vs Ga* is 75.4-79.8%, these values fall within the %D range of all South American species (Table 2). This is demonstrated visually on the phenogram in Fig. 2 with very strong bootstrap values supporting the topology of the Nieghbor-Joining distance tree. As seen from the phenogram, the North and South American *Gonatocerus* species are separated into their perspective clades. It is interesting to note that Ga(FL) is more closely related to *G. metanotalis* (Gmet) (8.30-9.00%), a South American species than it is to any North America species (Fig. 2). The *Gonatocerus* species more closely related to Ga* is Gt (15.8-17.9%). The present results showing extensive sequence divergence at the ITS2 rDNA fragment in a population of *G. ashmeadi* from Florida lends strong support to the fact that these individuals may actually be another species or rather *G. ashmeadi* exists in nature as a species-complex. Our results are in contrast with those of Vickerman et al. (2004). In our studies we performed a phylogenetic analyses of the ITS2 rDNA sequences. In addition, Vickerman et al. (2004) demonstrated that populations of *G. ashmeadi* from Florida vs other geographic regions were able to hybridize. We have not yet performed these types of studies, but it may be necessary to extend these crossing studies to the F2 generation to seen a negative effect or as demonstrated by Wu et al. (2004) a negative effect was not seen until backcrosses were performed. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's Disease biological control program in California.

Table 1. Pairwise sequence distances (range) of ITS-2 rDNA fragments from geographic populations of *G. ashmeadi* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-4) were included. QFL, Quincy, Florida; WTXb, Weslaco, TX; LA, Louisiana; SATX, San Antonio, TX; WTXa, Weslaco, TX; CA, California; Gm, *G. morrilli* (outgroup). Relate to figure 1B.

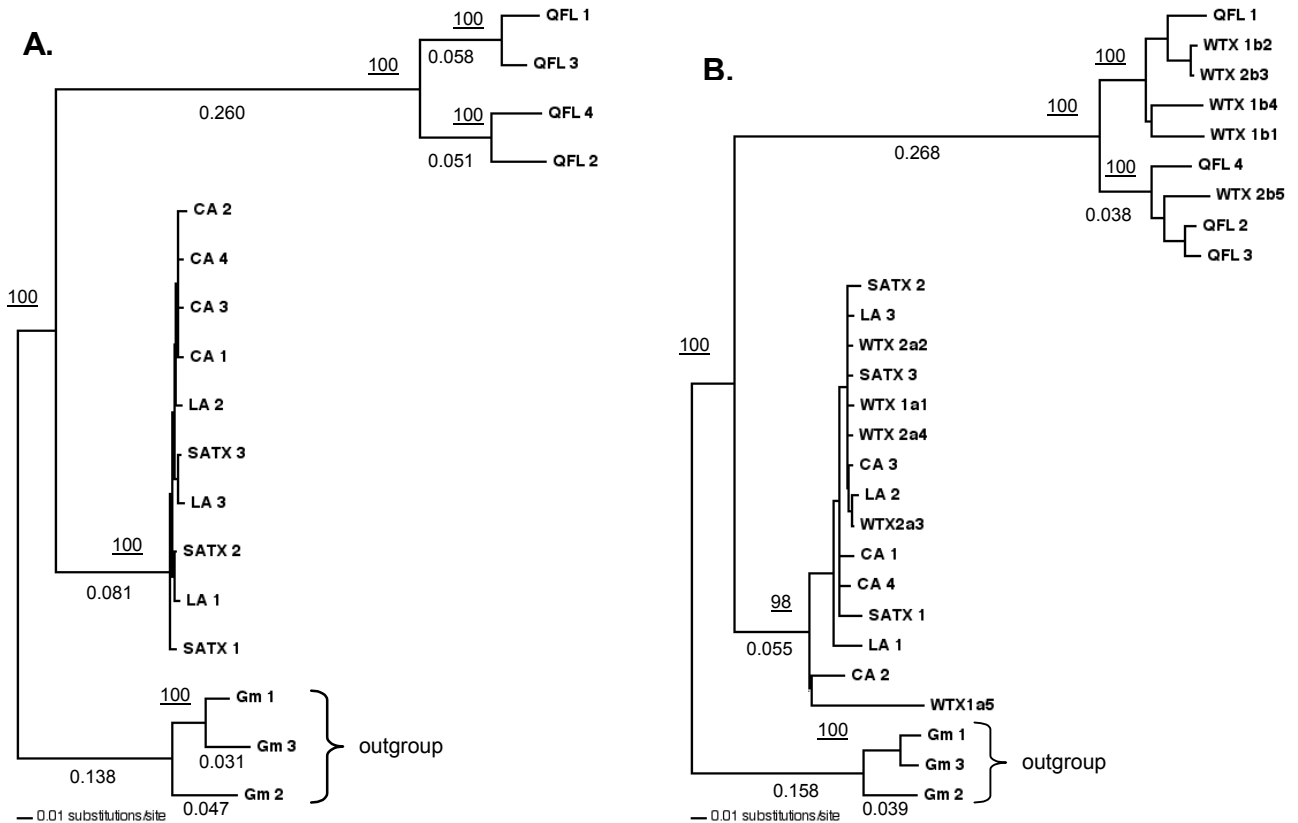| Pop	QFL	WTXb	LA	SATX	WTXa	CA	Gm
QFL	0.10-0.40						
WTXb	0.00-0.40	0.00-0.10					
LA	68.0-69.8	68.1-70.4	0.60-0.90				
SATX	68.2-69.8	67.9-70.8	0.30-0.80	0.20-0.90			
WTXa	67.1-69.5	66.6-70.1	0.20-0.70	0.20-0.90	0.10-0.90		
CA	65.9-67.6	66.0-67.9	0.80-1.00	0.60-1.10	0.30-1.00	0.20-0.80	
Gm	77.8-81.2	77.6-82.3	32.3-36.3	31.4-37.0	31.8-40.6	36.3-36.7	0.00-0.30

Figure 1. Phenograms of ITS2 rDNA sequence fragments from geographic populations of *G. ashmeadi*. Analyses were performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. morrilli* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. Trees are presented both without Weslaco, TX populations **(A)** and with Weslaco, TX populations **(B)**. To account for intra- and inter-populational variation, several randomly chosen individuals (3-4) were included.

Table 2. Pairwise sequence distances (range) of ITS-2 rDNA fragments from *Gonatocerus* species showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for this analysis. To account for intra- and inter-specific variation, several individuals (2-3) were included. Ga*, *G. ashmeadi* (California, San Antonio, TX, and Louisiana were pooled for a total of 10 individuals); Gt, *G. triguttutas* (TX); Gm, *G. morrilli* (TX); and candidate South American (Argentina) species: Gann, *G. annulicornis*; nGt, near *G. triguttutas*; Gtub, *G. tuberculifermur*; Ga(FL), *G. ashmeadi* (Quincy, FL USA); Gmet, *G. metanotalis*; and Tb, *Trichogramma bourarachae* (outgroup).

| G species	Ga*	Gt	Gm	Gann	nGt	Gtub	Ga(FL)	Gmet	Tb
Ga*	0.10-0.90								
Gt	15.8-17.9	0.10-0.20							
Gm	35.0-38.9	41.7-45.5	1.80-1.80						
Gann	82.4-87.2	97.5-101	87.0-88.1	0.00-0.10					
nGt	80.0-83.5	94.8-97.3	82.7-84.2	3.40-3.60	0.10-0.10				
Gtub	*78.0-82.0*	*90.8-92.0*	*81.4-84.1*	*11.5-12.1*	*11.6-11.8*	*0.10-0.50*			
Ga(FL)	75.4-79.8	88.4-90.2	84.3-87.0	37.7-39.3	36.7-38.1	35.9-36.4	0.10-1.00		
Gmet	76.2-80.4	87.6-89.4	85.5-88.2	35.4-36.4	34.7-35.3	34.9-36.1	8.30-9.00	0.10-.040	
Tb	84.8-92.5	87.2-91.5	88.4-90.4	66.1-67.6	69.0-70.3	68.5-70.5	77.3-79.6	74.2-76.2	0.20-0.90
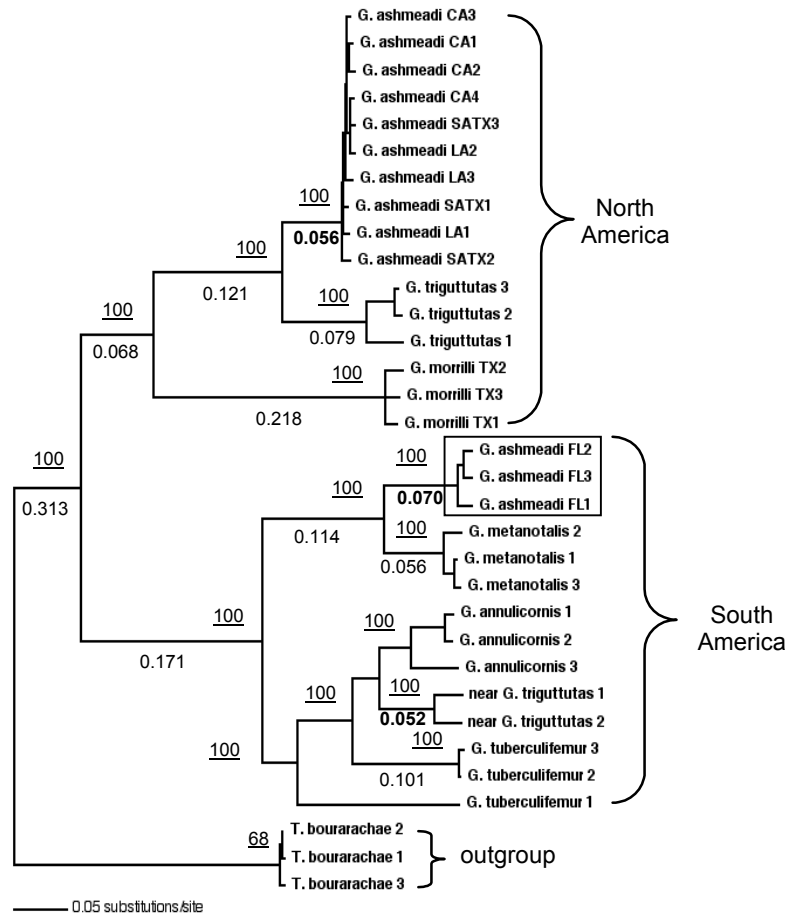
Figure 2. Phenograms of ITS2 rDNA sequence fragments from *Gonatocerus* egg parasitoid species, including candidate species from South America (Argentina). Analysis was performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *Trichogramma bourarachae* (1, AF043624; 2, AF043625; 3, AF043626) are included are an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-specific variation, several randomly chosen individuals (2-4) were included.

REFERENCES

de León JH, Jones WA. 2004. Genetic differentiation among geographic populations of *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae), the predominant egg parasitoid of *Homalodisca coagulata* (Homoptera: Cicadellidae). Journal of Insect Science (in press).

de León JH, Jones WA, Morgan DJW. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. *Annals of the Entomological Society of America* 97: 809-818.

Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* an *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.

Löhr BA, Varela M, Santos B. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. *Bulletin of Entomological Research* 80: 417-425.

Messing RH, Aliniazee MT. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphidiidae). *Annuals of the Entomological Society of America* 81: 6-9.

Narang SK, Tabachnick WJ, Faust RM. 1993. Complexities of population genetic structure and implications for biological control programs. In: Narang SK, Barlett AC, Faust RM, editors. Applications of Genetics to Arthropods of Biological Control Significance, 19-52. CRC Press Inc, Boca Raton, Florida.

Porter CH, Collins FH. 1991. Species-diagnostic difference in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene* 45, 271-279.

Thompson JD, Gibson TJ, Plewniak F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Swofford DL. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Vickerman DB, Hoddle MS, Triapitsyn S, Stouthamer R. 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: morphology, DNA sequences, and reproductive compatibility. Biological Control 31: 338-345.

Unruh TR, Woolley JB. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. Biological Control, 57-85. Chapman and Hall, NY.

Wu Z, Hopper KR, O'Neil RJ, Voegtlin DJ, Prokrym DR, Heimpel GE. 2004. Reproductive compatibility and genetic variation between two strains of *Aphelinus albipodus* (Hymenoptera: Aphelinidae), a parasitoid of the soybean aphid, *Aphis glycines* (Homoptera: Aphididae). Biological Control 31: 331-319.

FUNDING AGENCIES

GENETIC DIFFERENTIATION AMONG GEOGRAPHIC POPULATIONS OF *GONATOCERUS ASHMEADI*, A PRIMARY EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT

The aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released. In the present study, Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) was utilized to estimate the population genetic structure of *Gonatocerus ashmeadi*. Six populations from throughout the U. S. and a population from Argentina identified as near *G. ashmeadi* were analyzed. Four populations [California (CA), San Antonio, TX (SATX), Weslaco, TX (WTX-2), and Quincy, Florida (QFL)] were field collected and two [Louisiana (LA) and Weslaco, TX (WTX-1)] were reared. Three ISSR-PCR reactions were pooled to generate 41 polymorphic markers among the six U. S. populations. Nei's expected heterozygosity values (*h*), including the reared population from Louisiana were high (9.0-14.3%) for all populations, except for a reared population from WTX-1 (2.9%). The total genetic diversity value (Ht) for the field populations was high (23%). Interestingly, the Florida population that was collected from one egg mass generated the greatest number of polymorphic markers (20) and was observed with the highest gene diversity value (14.3%). All populations, except WTX-2 generated population-specific markers. Comparison of genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated good agreement between G_{ST} and θ values, 0.38 and 0.50, respectively for field populations, and 0.44 and 0.50, respectively for all populations. Average genetic divergence (D) indicated that the WTX-1 population was the most differentiated. Average D results from the Argentina population support the taxonomic data that it is a different species. The present results estimate the population genetic structure of *G. ashmeadi*, demonstrating extensive genetic divergence and restricted gene flow (Nm = 0.83) among populations. These results are of interest to the Pierce's Disease/Glassy-winged Sharpshooter biological control program because the key to successful biological control may not be in another species, but instead in different geographic races or biotypes.

INTRODUCTION

Gonatocerus ashmeadi (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the glassy-winged sharpshooter (Huber 1998). A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding sharpshooter is a serious economic pest that vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's Disease in grapevines. Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit in the selection and screening of biological control organisms, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. In addition, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination. Populations of parasitoids from distinct geographical regions may differ in relevant biological characteristics of importance to biological control (Powell and Walton 1989; Narang *et al*. 1993; Unruh and Woolley 1999). An aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released (Messenger and van den Bosch 1971); in other words, the key to successful biological control may not be in another species, but instead in different geographic races or biotypes (Diehl and Bush 1984). Reliable methods are needed for distinguishing various exotic strains of these biological control agents from those indigenous to the U. S., including parasitoids from different states within the U. S. Release of unidentified and uncharacterized strains can make it difficult to document their establishment and dispersal. Therefore, genetic typing of strains prior to their release in the field is highly desirable (Narang *et al*. 1993).

OBJECTIVES

1. Estimate genetic variation or gene diversity within and among populations.
2. Estimate the population genetic structure.
3. Determine whether ISSR-PCR was sensitive enough to identify diagnostic markers in geographic populations.
4. Confirm the species identification of a population of egg parasitoids from Argentina identified as near *G. ashmeadi*.

RESULTS AND CONCLUSIONS

ISSR-PCR Marker Heterozygosity and Genetic Diversity

A total of 41 polymorphic markers were generated in the six populations of G. ashmeadi (163 individuals) from the U. S. with three pooled ISSR-PCR reactions. G^2-contingency tests indicated significant heterogeneity of marker frequency across all U. S. populations for 31 of 41 markers and for 25 of 34 markers for the field populations (not shown). All populations,

except the WTX-2, were associated with population-specific markers (data not shown). Within populations, gene diversity values (h) were observed ranging from 2.9 to 14.3% with WTX-1 having the lowest and QFL having the highest value (Table 1). In general, the two Weslaco populations (WTX-1 and -2) were found to have the lowest h values. No significant differences in h were seen between the two Weslaco populations (t = 1.49, df = 58, P > 0.05), but significant differences (P < 0.05) were observed between WTX-1 and the rest of the U. S. populations. Interestingly, no significant differences in h were observed between the reared LA and the rest of the field populations. The fact that QFL was associated with an h value of 14.3% was surprising since this population was from a single egg mass. Overall, the field populations and all the U. S. G. ashmeadi populations together had an h value of 23.0 and 20.8%, respectively. The number of polymorphic markers ranged from 12 to 20 with WTX-1 and -2 having the lowest and QFL the highest. Percentage of polymorphic markers (%P) ranged from 29.3 to 58.8%, but overall, 100% of the ISSR-PCR markers were polymorphic, including the field populations analyzed separately. The two Weslaco populations were associated with the lowest %P and QFL with the highest. It is interesting to note that even though both LA and WTX-1 were reared, WTX-1 is presented with a significantly (P < 0.05) lower h value. These results may indicate a real genetic difference between the two Weslaco populations, including the possibility of sympatric strains.

ISSR-PCR Differentiation Among US G. ashmeadi Populations

Table 2 presents the results from the different approaches used to apportion variation into within- and among-populations levels. Simultaneous exact tests for population differentiation indicated that highly significant differences in marker frequencies exist among the six U.S. populations (All: χ^2 = 676.2; df = 82; P = 0.0000, and fc: χ^2 = 485.2; df = 68; P = 0.0000). These statistically significant tests suggest that discrete subpopulations exist. The average genetic diversity within populations (Hs) value for the field populations is 14.4%. Table 2 also shows a comparison of other genetic differentiation estimates, G_{ST} and θ. Good agreement was seen between G_{ST} and θ values, respectively for field and for all populations. The G_{ST} values for field and all populations indicate that about 38 and 44% of the variance is distributed among populations, and 62 and 56% is distributed within populations, respectively. The θ values show that about 50% of the variance is seen among populations in both field and all populations. The indirect estimate of gene flow, Nm base on G_{ST}, demonstrated low values for both field and all U. S. populations. These values indicate restricted gene flow among the populations.

Genetic Relatedness among G. ashmeadi Populations from the US

Average genetic divergence (D) among both field [Nei = 0.1702 (0.1021-0.2230); Reynolds = 0.6208 (0.4069-0.8138)] and all populations [Nei = 0.1304 (0.0715-0.2024); Reynolds = 0.6512 (0.3705-0.8890)] was high (Table 3). We compared the level of genetic divergence between the field populations and the WTX-1 and LA reared populations and found mean D values of 0.1806 (Nei) and 0.8589 (Reynolds) and 0.1065 (Nei) and 0.5371 (Reynolds), respectively. These results indicate that WTX-1 is more diverged than LA. A comparison of Nei's genetic distance within the Texas populations, WTX-2 vs WTX-1 (0.1391) and WTX-2 *vs* SATX (0.1286), showed that divergence is slightly higher between the Weslaco populations. Sympatric species tend to have higher levels of genetic differentiation; more work is needed to confirm this possibility. The divergence between ARG and all U. S. *G. ashmeadi* populations was very high, 0.3633 (Nei) and 1.6093 (Reynolds), respectively. These results support the taxonomic data that ARG is another species. Dendrograms based on Nei's genetic distance are shown on Fig. 1 with all populations including ARG (Fig. 1A) and the field populations analyzed separately (Fig. 1B). At least two main clusters are identified on the dendrogram with ARG clustered as an outlier (Fig. 1A). Within a second cluster or all *G. ashmeadi* from the U. S., WTX-1 appears to be the most differentiated (Fig. 1A). The CA population appears to form a second subcluster and the two southeastern populations, LA and QFL form a single cluster. The WTX-1 and –2 populations are distributed in different clusters. Also within Texas (Fig. 1B), WTX-2 and SATX show divergence as they appear on a separate cluster. It is interesting to note that this same pattern of differentiation is seen with *H. coagulata* within Texas (de León *et al.* 2004).

In summary, the major observations of this study were that 1) among *G. ashmeadi* populations, based on genetic differentiation measurements (exact test, G_{ST}, θ), extensive genetic structure was identified; 2) the mean expected gene diversity value for LA did not differ from field populations, whereas WTX-1 was observed with a significantly lower mean expected gene diversity value as compared to field populations (except WTX-2); 3) QFL generated the most polymorphic markers (20) with only 13 individuals, even though they were all siblings or from one egg mass. This is an interesting result since it may be assumed that siblings are not associated with high variability or have isofemale line characteristics. These results indicate that *G. ashmeadi* parasitoid siblings somehow manage to maintain their genetic diversity. Further studies are required to confirm this observation in this species and other *Gonatocerus* species. Variation within 10 male individuals (*Anaphes* sp.nov.) was demonstrated with RAPD markers by Landry *et al.* (1993), but they were not from the same egg mass; 4) based on genetic distance or average divergence, WTX-1 appeared to be the most differentiated population. Within Texas, field populations WTX-2 and SATX appeared on separate clusters, indicating that these populations are differentiated even though they are within the same state; and 5) The ARG population is confirmed to be a different species. More research is required to confirm these results, sequencing of standard genes [e. g., mitochrondia cytochrome oxidase (COI)] and ITS-2 fragments are in progress.

Table 1. Single-populations descriptive statistics for *G. ashmeadi* from the U. S. and genetic variation statistics for all loci. Genetic variation was analyzed using the POPGENE 3.2 genetic software program and the program Tools for Population Genetic Analyses (TPFGA). No. M, number of monomorphic markers; No. P., number of polymorphic markers; %P, percentage of polymorphic loci; Poym. ratio, number of polymorphic markers per number of insects; h, gene diversity (SD). One-tailed unpaired t test performed for h values.

Pop.	No. Insects	No. M	No. P	Total# markers	%P	Polym. ratio	h (SD)
CA	30	5	16	21	39.2	0.53	0.1329 (0.182)[a]
WTX-1	30	7	12	19	29.3	0.40	0.0290 (0.158)
WTX-2	30	6	13	19	31.7	0.43	0.0901 (0.160)
SATX	30	5	16	21	39.0	0.53	0.1123 (0.170)[a]
LA	30	5	17	22	41.5	0.57	0.1252 (0.182)[a]
QFL	13	1	20	21	58.8	1.54	0.1431 (0.199)[a]
Fc	103	0	34	34	100.0	0.33	0.2300 (0.184)
All	163	0	41	41	100.0	0.25	0.2082 (0.187)
ARG	30	11	8	19	16.7	0.27	0.0434 (0.127)

[a]Significantly different from WTX-1, $P < 0.05$; df = 58

Table 2. Nei's analysis of gene diversity in populations of *G. ashmeadi* from the US (fc, field collected; Ht, total genetic diversity (SD); Hs, average genetic diversity within populations (SD); G_{ST} (mean), coefficient of gene differentiation; θ (mean), theta (SD) is analogous to F_{ST}; and Nm, gene fow).

Ht	Hs	G_{ST}	θ	Nm
fc				
0.2312	0.1442	0.3761	0.4957	0.8295
(0.032)	(0.016)		(0.077)	
All				
0.2087	0.1161	0.4438	0.4927	0.6267
(0.034)	(0.013)		(0.057)	

Table 3. Nei's unbiased (1987) genetic distance (below diagonal) and Reynolds et al. (1983) genetic distance (above diagonal). Six populations *of G. ashmeadi* from the US field populations were also analyzed separately (bottom portion of table).

Pop.	CA	WTX-1	WTX-2	SATX	LA	QFL
CA	*****	0.8682	0.6818	0.6441	0.6275	0.4227
WTX-1	0.2024	*****	0.8080	0.8703	0.6871	0.8890
WTX-2	0.1341	0.1391	*****	0.7213	0.6663	0.5322
SATX	0.1384	0.1789	0.1286	*****	0.4842	0.4956
LA	0.1422	0.1335	0.1233	0.0890	*****	0.3705
QFL	0.0896	0.2020	0.0890	0.0951	0.0715	*****

Pop.	CA	WTX-2	SATX	QFL
CA	*****	0.8138	0.8075	0.4559
WTX-2	0.2215	*****	0.7741	0.4069
SATX	0.2230	0.2015	*****	0.4666
QFL	0.1308	0.1021	0.1328	*****

REFERENCES

Collins FH, Paskewitz SM. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anophels* species. *Insect Molecular Biology* 5: 1-9.

de León JH, Jones WA, Morgan DJW. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. *Annals of the Entomological Society of America* 97: 809-818.

Diehl, SR, Bush GL. 1984. An evolutionary and applied perspective of insect biotypes. *Annual Review of Entomology* 29: 471-504.

Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* an *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.

Landry BS, Dextraze L, Biovin G. 1993. Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasp species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. *Genome* 36: 580-587.

Messenger PS, van den Bosch R. 1971. The adaptability of introduced biological control agents. In: Huffaker, CB editor. Biological Control, 68-92. Plenum Press, New York.

Narang SK, Leopold RA, Krueger CM, DeVault JD. 1993. Dichomotomus RAPD-PCR key for identification of four species of parasitic hymenoptera. In: Narang SK, Barlett AC, Faust RM, editors. Applications of Genetics to Arthropods of Biological Control Significance, 53-67. CRC Press Inc, Boca Raton, Florida.

Powell W, Walton MP. 1989. The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pest. In: Loxdale HD, den Hollander J, editors. Electrophoretic Studies on Agricultural Pests, 443-65. Oxford, Clarendon.

Unruh TR, Woolley JB. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. Biological Control, 57-85. Chapman and Hall, NY.

FUNDING AGENCIES

MOLECULAR DISTINCTION BETWEEN POPULATIONS OF *GONATOCERUS MORRILLI*, EGG PARASITOIDS OF THE GLASSY-WINGED SHARPSHOOTER, FROM TEXAS AND CALIFORNIA

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ABSTRACT
Two molecular methods were utilized to distinguish geographic populations of *Gonatocerus morrilli* (Howard) from Texas and California and to test the possibility that this species could exist as a species-complex. Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) was performed with a 5'-anchored ISSR primer. Twenty-five markers were generated with four populations (40 individuals) of *G. morrilli*, 23 were polymorphic and percentage of polymorphic loci was 92%. Most markers could be considered diagnostic since there was no band sharing between the Texas and California populations. Such differences typically are not found unless the populations are reproductively isolated. Exact tests for population differentiation indicated significant differences in markers frequencies among the populations. Comparison of other genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated excellent agreement between G_{ST} and θ values, 0.92 and 0.94, respectively; indicating that about 92 to 94% of the variance was distributed among populations. Average genetic divergence (D), as measured by genetic distance, was extremely high (Nei = 0.82 and Reynolds = 2.79). A dendrogram based on Nei's genetic distance, separated the Texas and California populations into two clusters, respectively. Amplification of the Internal Transcribed Spacer-1 (ITS-1) region showed no size differences, whereas the ITS-2 DNA fragments varied in size between the two geographic populations. The ITS-2 fragment sizes were about 865 and 1099 base pairs for the California and Texas populations, respectively. The present study using the two molecular methods provides novel data critical to the glassy-winged sharpshooter/Pierce's disease biological control program in California.

INTRODUCTION
Gonatocerus morrilli (Howard) (Hymenoptera: Mymaridae) is an egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Turner and Pollard 1959; Triapitsyn *et al*. 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper, which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* L. and *V. labrusca* L.) (Hopkins and Mollenhauer 1973). Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990). There is a need for molecular markers for natural enemies to provide new characters for studies of phylogenetic relatedness, for identification of cryptic species and biotypes, and for the assessment of heritable variation for population genetics and ecological investigations (Unruh and Woolley 1999). Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. Furthermore, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination (Powell and Walton 1989; Narang *et al*. 1993; Unruh and Woolley 1999).

OBJECTIVES
1. Survey molecular methods useful in egg parasitoid identification and discrimination
2. Investigate the possibility that *G. morrilli* could exist as a species-complex in nature

RESULTS AND CONCLUSIONS
ISSR-PCR DNA Fingerprinting.
Figure 1 shows an example of ISSR-PCR DNA fingerprinting demonstrating the banding pattern differences between the geographic populations of *G. morrilli* from California (OrCo) and Texas (Wes-2) performed with a 5'-anchored ISSR primer. Markers ranged in size from about 200 to 900 base pairs. Overall, a total of 25 markers were generated among all four populations with a total of 40 individuals. Twenty-three were polymorphic and percentage of polymorphic loci was 92%. Within individual populations, no diversity was seen within the California populations and only slight diversity was observed in the Texas populations. For the Texas populations, Wes-2 and Wes-3, 5 polymorphic markers each were generated and 20% of the markers were polymorphic. Most markers are geographic-specific and can therefore be considered diagnostic since there is no band sharing between the Texas and California populations.

ISSR-PCR Differentiation Among Four G. morrilli Populations.
Exact tests (simultaneous analysis) for population differentiation indicated that highly significant differences in marker frequencies existed among the *G. morrilli* populations (Table 1). Total genetic diversity (Ht) was high (35%), whereas the average genetic diversity within populations was low (3%). Table 1 also shows a comparison of other genetic differentiation estimates, G_{ST} and θ, which evaluate the degree of genetic subdivision among populations. Excellent agreement was seen between G_{ST} and θ values, 0.92 and 0.94, respectively. Theses values indicate that about 92 to 94% of the variance is distributed among populations. The indirect estimate of gene flow, Nm base on G_{ST}, demonstrated a low value (0.04) among the geographic populations; this value indicates highly restrictive gene flow. Overall, genetic differentiation measurements (exact tests, G_{ST}, θ, and Nm) indicate profound genetic divergence/structuring between *G. morrilli* populations from Texas and California.

Genetic Relatedness Among G. morrilli Populations.
Levels of genetic divergence among populations were also determined by calculating pairwise estimates for genetic distance by the procedures of Nei (1978) and Reynolds *et al*. (1983) (Table 2). Average genetic divergence (D) among populations was extremely high [Nei = 0.82 (0.89-1.07) and Reynolds = 2.79 (1.4-3.4)]. A dendrogram based on Nei's genetic distance is shown on Fig. 2 with all *G. morrilli* geographic populations. Two clades are identified on the dendrogram with the California and Texas populations appearing on separate clusters. These two clusters are supported by strong bootstrap support values, 68 and 64%, respectively for the California and Texas populations.

Amplification of the ITS-1 and –2 regions in G. morrilli Geographic Populations.
Monomorphic patterns were demonstrated with amplification of the ITS-1 region in all of the populations from California and Texas (~850 bp) (Fig. 3); whereas, polymorphic or different DNA fragment sizes were detected within the ITS-2 region. The California populations were observed with an ITS-2 fragment size of about 865 base pairs and the Texas populations with a size of about 1099 base pairs.

Good agreement is seen between the two molecular methods and they both suggest that cryptic species may exist. The results with ISSR-PCR demonstrating distinct banding patterns (no band sharing) between geographic populations typically is not found unless the populations are reproductively isolated. Similar results were obtained by Hoy *et al*. (2000) with two populations of *Ageniaspis citrocola* performed by RAPD-PCR. The following genetic differentiation parameters, extract test, G_{ST}, θ, genetic distances, and gene flow (Nm) lend support to this observation. The extremely low value for gene flow between the populations from California and Texas lend support that these populations are isolated reproductively. Restricted gene flow usually leads to increased differentiation among populations as seen from the G_{ST} and θ values (92 to 94% of the variance is seen among populations). In addition, the divergence (D) between these populations is also high.

Methods incorporating SSR appear to be sensitive at detecting DNA polymorphisms in natural populations. Previously, we utilized ISSR-PCR to distinguish three species of *Homalodisca* sharpshooters (*H. coagulata*, *H. liturata*, and *H. insolita*) (de León and Jones 1994). Even though this method is sensitive, there are not many reports in the literature utilizing ISSR-PCR to study insect population genetics and phylogenetics. We have also had success determining the population genetic structure of *H. coagulata* representing 19 populations from through the U. S. (de León *et al*. 2004). The Internal Transcribed Spacer regions (ITS-1 and –2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, and success with this approach has been reviewed by Collins and Paskewitz (1996). Stouthamer *et al*. (1999) used ITS-2 DNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the genus *Trichogramma*. In cases where species were observed with similar sized ITS fragments these authors suggested amplification, sequencing, and restriction digestion.
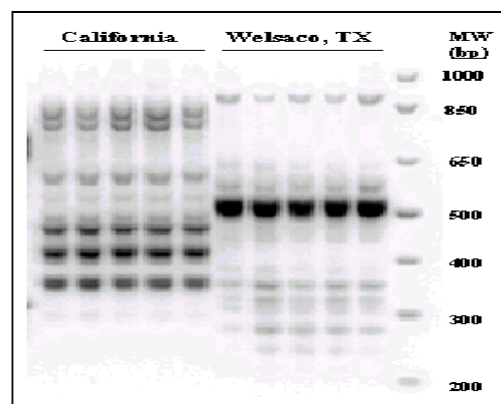

Figure 1. Representative example of ISSR-PCR DNA fingerprinting of *G. morrilli* populations from California and Texas. Reactions were performed with genomic DNA from separate individuals and the 5'-anchored ISSR primer HVH(TG)₇T (Zietkiewicz *et al*. 1994) as describe in the Materials and Methods. M: 1.0 Kb Plus DNA Ladder.

These novel observations strongly suggest that *G. morrilli* may exist in nature as a species-complex. Results from our recent study with *H. coagulata* suggest that a subset of these insects have their origin in Texas (de León *et al*. 2004). Those results together with our present results with *G. morrilli* may suggest that this egg parasitoid from Texas may be a good candidate for the biological control efforts in California against *H. coagulata,* the causative agent of Pierce's disease.

Table 1. Nei's analysis of gene diversity in populations of *G. morrilli* from Texas and California. Ten individuals per population (40 total) were subjected to ISSR-PCR DNA fingerprinting. Genetic variation was analyzed using the POPGENE 3.2 genetic software program and the program Tools for Population Genetic Analyses (TPFGA). X^2, exact tests (simultaneous analysis) for population differentiation, df = degrees of freedom; Ht, total genetic diversity (SD), Hs, average genetic diversity within populations (SD); G_{ST} (mean), coefficient of gene differentiation; θ theta (analogous to F_{ST}), and Nm, gene flow. [***]$P = 0000$.

X^2 (df)	Ht	Hs	G_{ST}	θ	Nm
400.8 (50)[***]	0.35	0.03 (0.04)	0.92 (0.00)	0.94	0.04 (0.02)

Table 2. Nei's unbiased (1978) genetic distance (below diagonal) and Reynolds et al. (1983) genetic distance (above) diagonal. Four geographic populations of *G. morrilli*, two from Texas (Hidalgo Co, Wes-2 and Wes-3) and two from California (OrCo, Orange county and SDCo, San Diego county).

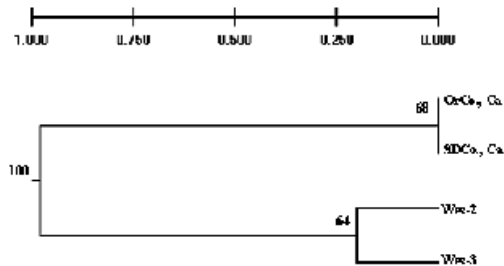| Pop	OrCo	SDCo	Wes-2	Wes-3
OrCo	***	undef	3.40	2.88
SDCo	0.00	***	3.40	2.88
Wes-2	1.07	1.07	***	1.40
Wes-3	0.89	0.89	0.20	***

Figure 2. Dendrogram based on Nei's genetic distance (1978) by the method of UPGMA. Relationships among the four geographic populations of *G. morrilli* performed by ISSR-PCR DNA fingerprinting. Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.
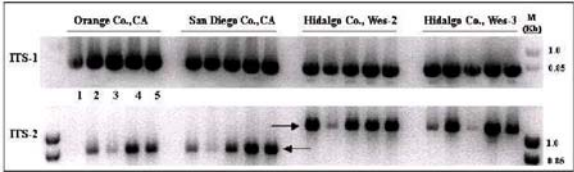

Figure 3. Amplification of the Internal Transcribed Spacer regions (ITS). The ITS-1 and –2 regions were amplified with standard ITS-specific primers with genomic DNA from five separate individuals from each geographic population. Arrows indicate different ITS fragment sizes. M: 1.0 Kb Plus DNA Ladder.

REFERENCES

Collins FH, Paskewitz SM. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anophels* species. *Insect Molecular Biology* 5: 1-9.

de León JH, Jones WA. 2004. Detection of DNA polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by polymerase chain reaction-based DNA fingerprinting methods. *Annals of the Entomological Society of America* 97: 574-585.

de León JH, Jones WA, Morgan DJW. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. *Annals of the Entomological Society of America* 97: 809-818.

Hopkins DL, Mollenhauer HH. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179: 298-300.

Hoy MA, Ayyamperumal J, Morakete R, Lo MKC, Nguyen R. 2000. Genomic analyses of two populations of *Ageniaspis citricola* (Hymenoptera: Encyrtidea) suggest that a cryptic species may exist. *Biological Control* 17: 1-10.

Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* an *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.

Löhr BA, Varela M, Santos B. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. *Bulletin of Entomological Research* 80: 417-425.

Messing RH, Aliniazee MT. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphidiidae). *Annuals of the Entomological Society of America* 81: 6-9.

Narang SK, Leopold RA, Krueger CM, DeVault JD. 1993. Dichomotomus RAPD-PCR key for identification of four species of parasitic hymenoptera. In: Narang SK, Barlett AC, Faust RM, editors. Applications of Genetics to Arthropods of Biological Control Significance, 53-67. CRC Press Inc, Boca Raton, Florida.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590.

Powell W, Walton MP. 1989. The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pest. In: Loxdale HD, den Hollander J, editors. Electrophoretic Studies on Agricultural Pests, 443-65. Oxford, Clarendon.

Reynolds J, Weir BS, Cockerham CC. 1983. Estimation of the coancestry coeffient: basis for a short-term genetic distance. *Genetics* 105: 767-779.

Stouthamer R, Hu J, Van Kan FJPM, Platner GR, Pinto JD. 1999. The utility in internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *BioControl* 43: 421-440.

Triapitsyn SV, Mizell RF III, Bossart JL, Carlton CE. 1998. Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist* 81: 241-243.

Turner WF, Pollard HN. 1959. Life histories and behavior of five insect vectors of phony peach disease. *USDA Technical Bulletin* 1188: 28 pp.

Unruh TR, Woolley JB. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. Biological Control, 57-85. Chapman and Hall, NY.

Zietkiewicz E, Rafalski A, Labuda D. 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176-183.

SEQUENCE DIVERGENCE IN TWO MITOCHONDRIAL GENES (COI AND COII) AND IN THE ITS2 RDNA FRAGMENT IN GEOGRAPHIC POPULATIONS OF *GONATOCERUS MORRILLI*, A PRIMARY EGG PARASITIOD OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
The aim of the present study was to resolve the genetic relationships of geographic populations of *Gonatocerus morrilli*, a primary egg parasitoid of the Glassy-winged Sharpshooter. A phylogenetic approach was implemented by sequencing two mitochondrial genes (COI and COII) and the Internal Transcribed Spacer-2 (ITS2) region of several individuals per population. Two populations from Weslaco, TX (WTX) (collected at different times), one from Quincy, FL (QFL), two from California (CA) (Orange and San Diego counties), and an outgroup (*G. ashmeadi*) were analyzed. For all three sequence fragments, percentage sequence divergence (%D) (as measured by genetic distance), the results demonstrated that both the WTX and QFL populations were closely related; in constrast, the %D between WTX and CA fell within the range of the outgroup, *G. ashmeadi*. For all three sequence fragments, Nieghbor-Joining distance trees separated the CA and WTX and QFL populations into two distinctive clades (A and B). The topology of the clades in each case was supported by very strong bootstrap values, 100% in the three sequence fragments (COI, COII, and ITS2). The present molecular phylogenetics results provide strong evidence that *G. morrilli* from California may be a different species. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's disease biological control program in California.

INTRODUCTION
Gonatocerus morrilli (Howard) (Hymenoptera: Mymaridae) is an egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Turner and Pollard 1959; Triapitsyn *et al*. 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper, which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* L. and *V. labrusca* L.) (Hopkins and Mollenhauer 1973). Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990).

OBJECTIVES
Determine the phylogenetic relationships of geographic populations of *G. morrilli* by sequencing two mitochondrial genes (COI and COII) and one rDNA spacer region (ITS2).

RESULTS AND CONCLUSIONS
Sequence divergence in the mitochondrial COI gene in G. morrilli geographic populations. Levels of genetic divergence in the mtCOI gene among populations were determined by calculating the pairwise estimates for genetic distance. Recently, we determined that populations of *G. morrilli* from California and Texas shared no ISSR-PCR banding patterns, indicating that these populations were reproductively isolated. In addition, we demonstrated that the ITS2 rDNA fragments varied in size between these geographic populations (de León *et al*. 2004). The percentage sequence divergence (%D) for mtCOI is shown on Table 1. In general, the intra-populational variation (0.0-0.6%) was small within each population and species, with the exception of the Quincy, FL population (QFL) (2.0-4.8%). The %D between Weslaco, TX (WTX) and QFL is 0.0-4.8%, which falls within the intra-populational variation of these populations; these results indicate that these geographic populations are genetically similar. In constrast, the %D of WTX and CA is 5.4-5.6%, falling within the range (5.4-6.9%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 1 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

Sequence divergence in the mitochondrial COII gene in G. morrilli geographic populations. The percentage sequence divergence (%D) for mtCOII is shown on Table 2. Intra-populational variation is seen in both the WTX (0.0-4.5%) and QFL (0.0-3.2%) populations. The %D between WTX and QFL is 0.3-4.7%, these values fall within the intra-populational variation range and therefore these populations would be considered closely related. On the other hand, the %D between WTX and CA is 7.4-11.1%, these values fall within the range (7.4-11.5%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 2 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

Sequence divergence in ITS rDNA fragment in G. morrilli geographic populations. The percentage sequence divergence (%D) for ITS2 is shown on Table 3. The %D between WTX and QFL is 0.0-1.40%, this falls within the intra-populational range of both populations and therefore shows that these populations are closely related. In contrast, the %D between WTX and CA is 6.2-10.7%, falling within the range (7.9-13.3%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 3 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

Table 1 (COI) and Table 2 (COII). Pairwise sequence distances (range) of mitochondrial COI and II genes from geographic populations of *G. morrili* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for these analyses. To account for intra- and inter-populational variation, several individuals (3-6) were included. WTX, Weslaco, TX (two populations from Hidalgo Co; 5-6 total individuals); QFL, Quincy, FL (3 individuals); CA, California (two populations, Orange Co. and San Diego Co.; 6 total individuals); Ga, *G. ashmeadi* (outgroup) (3 individuals).

Table 1. COI.

Pop	WTX	QFL	CA	Ga
WTX	0.0-0.6			
QFL	0.0-4.8	2.0-4.8		
CA	5.4-5.6	5.4-8.6	0.0-0.2	
Ga	5.4-6.9	5.4-10.8	6.7-7.1	0.0-0.2

Table 2. COII.

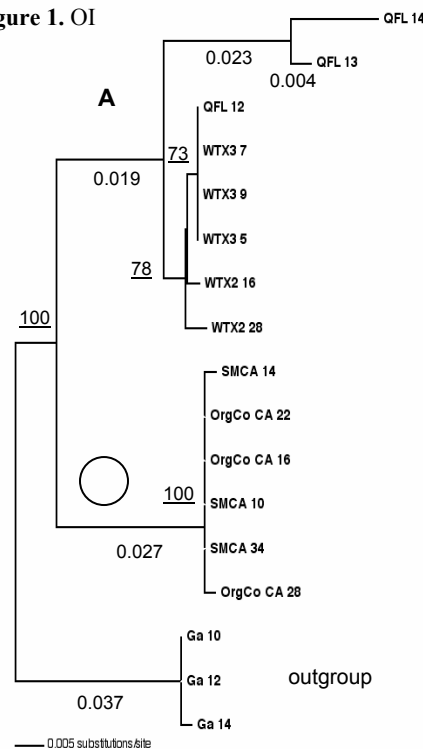| Pop	WTX	QFL	CA	Ga
WTX	0.3-4.50			
QFL	0.3-4.70	0.2-0.6		
CA	7.4-11.1	7.6-8.9	0.0-3.2	
Ga	7.4-10.5	7.1-7.8	6.9-8.0	0.0-0.2

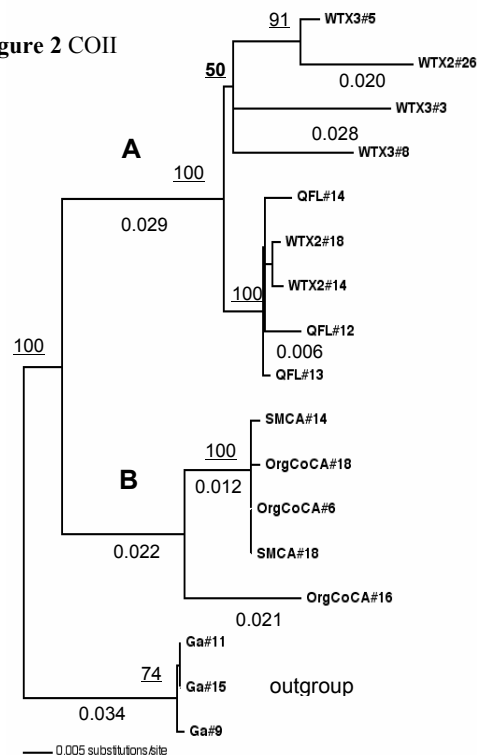**Figure 1. OI**

Figure 2 COII

Figure 1 (COI) and Figure 2 (COII). Phenograms of mitochondrial COI and COII genes from geographic populations of *G. morrilli*. Analyses were performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. ashmeadi* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches) underlined), as percentage of 1000 replications. To account for intra- and inter-populational variation, several randomly chosen individuals (3-6) were included. SMCA, San Marcos, CA; OrgCo CA; Orange county, California.

Table 3. Pairwise sequence distances (range) of ITS-2 rDNA fragments from geographic populations of *G. morrilli* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (2-7) were included. WTX, Weslaco, TX (two populations from Hidalgo Co; 7 total individuals); QFL, Quincy, Florida (2 individuals); CA, California (two populations, Orange Co. and San Diego Co; 5 total individuals) Ga, *G. ashmeadi* (outgroup) (4 individuals).

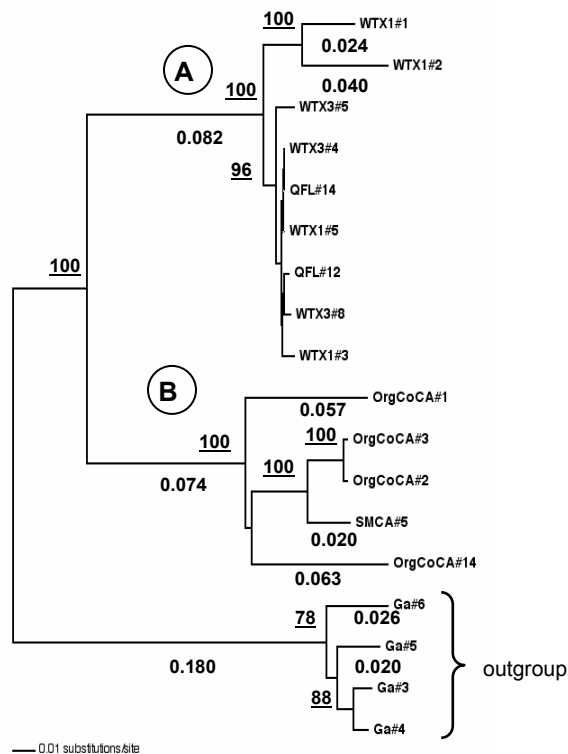| Pop	WTX	QFL	CA	Ga
WTX	0.0-1.70			
QFL	0.0-1.40	0.0-0.30		
CA	6.2-10.7	6.3-7.70	0.0-0.60	
Ga	7.9-13.3	8.4-12.4	7.8-12.0	0.5-0.9

Figure 3. Phenogram of ITS2 rDNA sequence fragment from geographic populations of *G. morrilli*. Analysis was performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining tree was created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. ashmeadi* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-populational variation, several randomly chosen individuals (2-7) were included. SMCA, San Marcos, CA; OrgCo CA; Orange county, California.

REFERENCES

de León JH, Jones WA, Morgan DJW. 2004. Molecular distinction between populations of *Gonatocerus morrilli*, egg parasitoids of the glassy-winged sharpshooter *Homalodisca coagulata*, form Texas and California: Do cryptic species exist? Journal of Insect Science (in press).

Hopkins DL, Mollenhauer HH. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179: 298-300.

Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* an *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.

Löhr BA, Varela M, Santos B. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. *Bulletin of Entomological Research* 80: 417-425.

Messing RH, Aliniazee MT. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphidiidae). *Annuals of the Entomological Society of America* 81: 6-9.

Thompson JD, Gibson TJ, Plewniak F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Triapitsyn SV, Mizell RF III, Bossart JL, Carlton CE. 1998. Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist* 81: 241-243.

Turner WF, Pollard HN. 1959. Life histories and behavior of five insect vectors of phony peach disease. *USDA Technical Bulletin* 1188: 28 pp.

Swofford DL. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

FUNDING AGENCIES

DEVELOPMENT OF MOLECULAR DIAGNOSTIC MARKERS FOR *HOMALODISCA* SHARPSHOOTERS PRESENT IN CALIFORNIA TO AID IN THE IDENTIFICATION OF KEY PREDATORS

Project Leaders: **Cooperator:**
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ABSTRACT
The aim of the present study was to develop molecular diagnostic markers to identify key predators of *Homalodisca* sharpshooter species present in California, *H. coagulata* (Glassy-winged Sharpshooter, GWSS) and *H. liturata* (Smoke-tree Sharpshooter, STSS). RAPD-PCR DNA fingerprinting of several sharpshooter species identified specific bands that were excised, sequenced, and SCAR (Sequenced Characterized Amplified Region) markers were designed. The results demonstrated that both GWSS- and *Homalodisca*-specific markers were specific toward their targets. The GWSS-specific markers amplified only GWSS and the *Homalodisca*-specific markers amplified only GWSS and STSS. The sensitivity limits for both marker sets was at 50 pg of DNA. The mitochondrial cytochrome oxidase subunit gene II (COII)-specific markers that were developed were each specific for GWSS and *Homalodisca* sharpshooters. The development of diagnostic markers designed toward *Homadisca* sharpshooters present in California should aid in finding key predators and therefore enhance biological control efforts against these sharpshooters.

INTRODUCTION
The Glassy-winged Sharpshooter, *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), is a large xylem feeding leafhopper that is a serious pest because it vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* and *V. labrusca*) (Hopkins and Mollenbauer 1973). A biological control program is currently in progress in California against *H. coagulata*. Effective control of GWSS will require an area-wide pest management approach. A major component of such an approach is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. Unfortunately, very little is known about GWSS natural enemies, this is especially true for their predators (Triapitsyn *et al*. 1998). Direct visual field observations of predation are difficult to obtain and historically, the study of insect predation has relied mainly on inexact and indirect techniques for measurement and analysis. Presently, Hagler and Naranjo (1997) and Hagler *et al*. (1991) have had success in developing monoclonal antibodies and detecting prey in predator gut contents by enzyme linked immunoassays (ELISA). Recently, other methods have been developed that allow for the detection of prey in predator gut contents. These molecular methods include, Sequence Characterized Amplified Region (SCAR), where RAPD-PCR species-specific bands are excised from gels, sequenced, and primers are designed toward those DNA fragments (Agusti *et al*. 1999; Agusti *et al*. 2000) and targeting genes that are present in the cell in high copy number, such as, mitochondrial genes (COI and COII) and Internal Transcribed Spacer regions (ITS1) (Agusti *et al*. 2003; Chen *et al*. 2000; Symondson 2002).

OBJECTIVE
Develop molecular diagnostic markers for *Homalodisca* sharpshooter species (GWSS and STSS) found in California in order to identify key predators.

RESULTS AND CONCLUSIONS
GWSS-specific SCAR (5/7) Markers
RAPD-PCR DNA fingerprinting was performed with several sharpshooter species and *Homalodisca*-specific bands were excised, sequenced, and primers designed (SCAR markers). Figure 1A demonstrates that GWSS-specific SCAR (5/7) markers were highly specific with no amplification of any other sharpshooter species or predators. The GWSS-specific markers were also able to detect GWSS eggs in predator gut contents (Figure 1B). The sensitivity of the SCAR marker set was tested by varying the amount GWSS DNA (0.1 to 3.2 ng) (Figure 2). In this experiment, the limit of sensitivity was at 100 pg, but later experiments showed the detection limit at 50 pg (not shown).
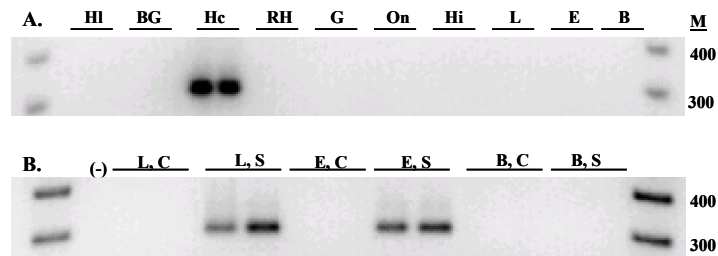
Figure 1. RAPD-PCR DNA fingerprinting was performed with the following sharpshooters: *Homalodisca liturata* (**Hl**); *Graphocephala atropuncta* [blue-green (**BG**)]; *H. coagulata* (**Hc**); *Carneocephala fulgida* [red-headed (**RH**)]; *Draeculacephala minerva* [green (**G**)]; *Oncometopia nigricans* (**On**); and *H. insolita* (**Hi**). Amplification products/bands unique to GWSS were excised, sequenced, and primers (SCAR markers) were designed to amplify a 302-bp fragment. **A).** Specificity of GWSS-specific SCAR-5/7 markers. **L**, lacewing larvae (*Chrysoperla carnea*); **E**, earwig (*Forficula auricularia*); and **B**, ground beetle (*Calosoma sp.*). **B).** Detection of GWSS in predator gut contents by SCAR-PCR assays. (-), negative control (no template); C, control (not fed on GWSS); S, sample (fed on GWSS). Lacewing and earwig fed on GWSS eggs and ground beetle fed on a GWSS adult.
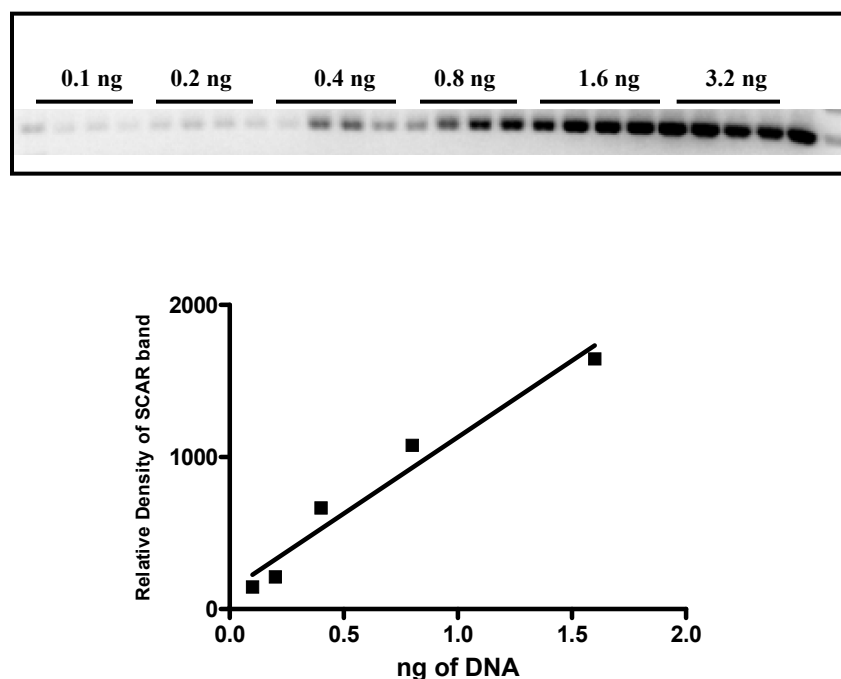

Figure 2. Sensitivity assay with GWSS-specific SCAR 5/7. GWSS DNA was varied from 0.1 to 3.2 ng, each point in quadruplicate (inset). The four determinations per point were averaged and plotted vs relative density of the SCAR bands. Since the highest amount of DNA (3.2 ng) did not fall within the linear portion of the curve (saturated) it was eliminated.
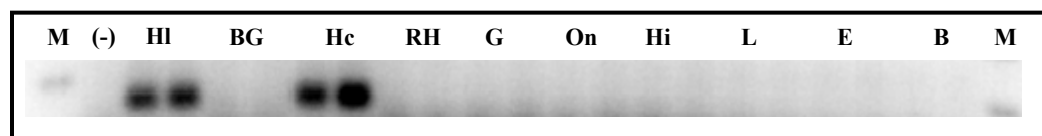

Figure 3. California *Homalodisca* (GWSS/STSS)-specific SCAR 6/9 specificity assay. California *Homalodisca*-specific primers were designed toward a RAPD-PCR fragment. Refer to Figure 1 for assignments.
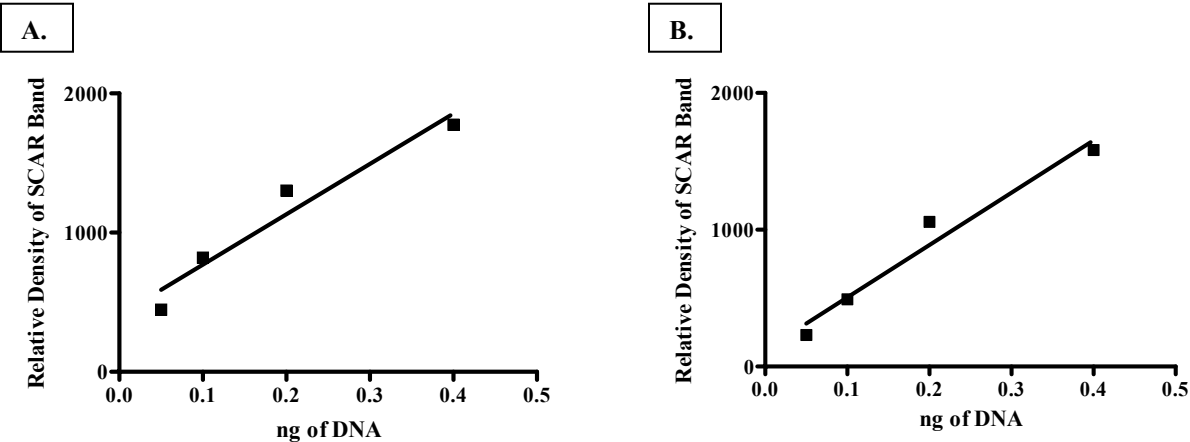
Figure 4. SCAR 6/9 sensitivity assays with GWSS DNA (A) and STSS DNA (B). DNA ranged from 0.05 to 0.80 ng with each point in triplicate. The three determinations per point were averaged and plotted vs relative density of the SCAR bands. The highest amount of DNA (0.80 ng) was not in the linear portion of the curve (saturated), so it was eliminated from the analysis.
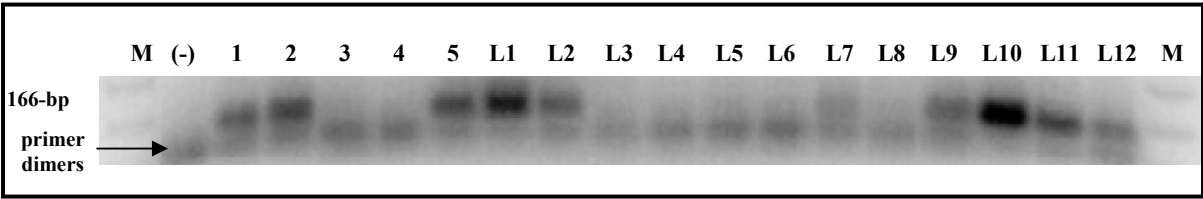

Figure 5. SCAR-PCR (6/9) assays with predators (Lacewing, L1-10) that fed on GWSS eggs. Lanes: 1, Qiagen prep control plus GWSS DNA; 2, crude extract control plus GWSS DNA; 3, crude extract negative control (not fed); 4, Qiagen prep negative control (not fed); 5, GWSS DNA positive control.
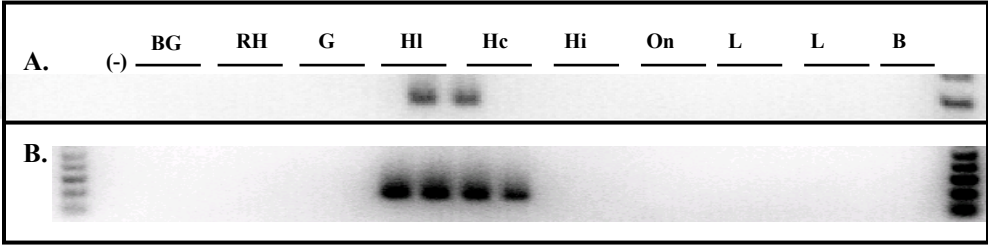

Figure 6 (below). California *Homalodisca* mitochondrial COII-specific primers. The mitochondrial COII genes of both GWSS and STSS were sequenced and both *Homalodisca*- and GWSS-specific primers were designed. Refer to fig. 1 for assignments.

Homalodisca (GWSS/STSS)-Specific SCAR (6/9) Markers

Figure 3 shows the specificity of the *Homalodisca* markers, as seen only GWSS and STSS DNA is amplified with this marker set and no other sharpshooters or predators amplified. The sensitivity of this SCAR (6/9) marker set was tested with

both GWSS (Figure 4A) and STSS (Figure 4B) DNA individually. The amount of DNA was varied from 0.05 to 0.80 ng. These experiments show the sensitivity limits with both GWSS and STSS DNA to be at 50 pg. The SCAR (6/9) marker set was tested with predators (Lacewings L1-12) that fed on GWSS eggs (Figure 5). At least 7 of the 12 specimens tested positive with this marker set. The assay system was tested for competition or interference of predator DNA with both Qiagen preps and crude DNA extracts. The DNA crude extract procedure was developed as a rapid method to assay hundreds of samples more efficiently. The results show that predator DNA does not compete or interfere with the SCAR-PCR assays.

Homalodisca and GWSS-specific Mitochondrial COII primers
Mitochondrial DNA is present in hundreds or multiple copies within each cell (Chen *et al*. 2000; Symondson 2002). In order to increase the sensitivity of our diagnostic assays, the mtCOII genes of both GWSS and STSS were sequenced and both *Homalodisca*- and GWSS-specific primers were designed. Figure 6 demonstrates that both GWSS- (Figure 6A) and *Homalodisca*- (Figure 6B) specific primers were successful without amplifying any other sharpshooters or predators.

REFERENCES
Agusti N, De Vicente MC, Gabarra R. 1999. Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. Molecular Ecology 8: 1467-1474.
Agusti N, De Vicente MC, Gabarra R. 2000. Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. Insect Molecular Biology 9: 263-268.
Agusti N, Shayler SP, Harwood JD, Vaughan IP, Sunderland KD, Symondson WOC. 2003. Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection withing predators using molecular markers. Molecular Ecology 12: 3467-3475.
Chen Y, Giles KL, Payton ME, Greenstone MH. 2000. Identifying key cereal aphid predators by molecular gut analysis. Molecular Ecology 9: 1887-1898.
de León JH, Jones WA. 2004. Detection of DNA polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by polymerase chain reaction-based DNA fingerprinting methods. *Annals of the Entomological Society of America* 97: 574-585.
Hagler JR, Naranjo SE. 1997. Measuring the sensitivity of an indirect predator gut content ELISA: Detectability of prey remains in relation to predator species, temperature, time, and meal size. Biological Control 9:112-119.
Hagler JR, Cohen AC, Enriquez FJ, Bradley-Dunlop D. 1991. An egg-specific monoclonal antibody to *Lygus hesperus*. Biological Control 1:75-80.
Hopkin, D L, Mollenhauer HH. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. Science 179: 298-300.
Symondson WOC. 2002. Molecular identification of prey in predator diets. Molecular Ecology 11: 627-641.
Triapitsy S., Mizell RF, Bossart JL, Carlton CE . 1998. Egg parasitoids of *Homolodisca coagulata* (Homoptera: Cicadellidae). Florida Entomologist. 81: 241-243.

THE ALIMENTARY TRACK OF GLASSY-WINGED SHARPSHOOTER AS A TARGET FOR CONTROL OF PIERCE'S DISEASE, AND DEVELOPMENT OF MIMETIC INSECTICIDAL PEPTIDES FOR GLASSY-WINGED SHARPSHOOTER CONTROL

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ABSTRACT
Transgenic insecticidal crops expressing *Bacillus thuringiensis* (*Bt*) toxins have been successfully developed to control major chewing insect pests of agriculture, such as caterpillars and beetles. The same *Bt* toxin technology also has been used with *Bacillus sphaericus* for the control of mosquito species such as *Aedes aegypti* and *Culex quinquefasciatus*, important vectors of human diseases. However, this transgenic technology has not yet been applied to economically important xylem-feeding sucking insect pests such as the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS). Our goal is to use a genomics approach to develop novel, highly specific mimetic insecticidal proteins derived from the variable binding domains of immunoglobulin molecules. "Mimetic" peptides mimic the normal substrates of key components of essential processes to block the activities of these proteins. Our research is targeting the exposed active domains of transport proteins on the surface of the GWSS midgut microvillar membrane and enzymes found in GWSS saliva. Degenerate PCR amplification of genes characterized in other insect species encoding proteins involved in gut transport and saliva activity and screening a cDNA microarray to identify novel gut and saliva protein encoding genes are the approaches being used to identify GWSS target proteins. Due to the target specificity, mimetic peptide technology can provide an environmentally sound approach to the control of vasculature feeding insect pests and could thereby provide a means of controlling Pierce's disease and crop losses due to GWSS feeding.

INTRODUCTION
Mimetic technology is new to agriculture, but has been used extensively and successfully in medicine (Clemens, 1996). Examples of medical uses include the inactivation of disease-related enzymes (Burke et al., 2001), blockage of metabolic receptors important to disease (Berezov et al., 2000), and the use of antibodies developed against disease constituents (Moe et al., 1999). Human cancers (Monzayi-Karbassi and Keiber-Emmons, 2001), diabetes (Deghenghi, 1998), and heart disease (Lincoff et al., 2000) all have been treated successfully through these applications of mimetic technology. In spite of lacking a history of application of mimetics to agriculture problems, its development should be straight forward. Antibody proteins have been synthesized successfully in plants for the production of antibodies to be used in medical applications (Larrick et al. 2001; Stoger et al., 2002), and the production of transformed lines of crop plants in which promoters that have been isolated by other researchers (Shi et al., 1994; Springer, 2000), which direct expression to the cell wall and vascular structures of plants, will assure that our antibody peptides are synthesized in a tissue-specific manner. Last year we succeeded in isolating portions of five GWSS genes by degenerate PCR: the A and c V-ATPase subunits, genes encoding trypsin-like and maltase-like saliva proteins, and a membrane transporter. This year we have added another membrane transporter gene clone, most closely related to the potassium coupled amino acid transporter isolated from Manduca sexta, KAAT1 (Castagna et al., 1998). These clones and others isolated from our normalized cDNA are being analyzed using bioinformatics tools to identify functional domains which will be effective and specific targets. The identified target peptides will be synthesized in a *Baculovirus* expression system. Peptides produced will be used as antigens for polyclonal antibody production, the products of which will be cloned into phage display libraries. Screening the phage display antibody libraries will identify the mimetic peptides that bind most efficiently to the targeted GWSS proteins. Ultimately these peptides will be used in feeding studies to identify those which are the best candidates for GWSS control.

OBJECTIVES
1. Determine the structure and cell types in the midgut epithelium and salivary glands of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*;
2. Prepare a normalized cDNA microarray of GWSS using pooled cDNAs isolated from each developmental stage.
3. Screen the microarray using cDNA probes derived from midgut and salivary gland tissue-specific probes to determine the tissue-specific expression of key midgut microvillar and saliva proteins;
4. Clone and sequence genes encoding one or more key midgut microvillar and saliva proteins and determine their suitability as targets for a molecular biological approach to GWSS and Pierce's disease control.
5. Predict functional domains of key GWSS midgut epithelium- and salivary gland-specific proteins based on sequences of genes using bioinformatics;
6. Express functional domain peptides for antibody production;
7. Clone single-chain fragment variable antibody genes into recombinant phage libraries and screen the libraries;
8. Conduct feeding studies to identify efficacious mimetic peptides effective in killing or deterring GWSS.

RESULTS

We have had a normalized cDNA library constructed by Evrogen JSC from total RNA isolated from whole GWSS of both sexes and all life stages, as well as from GWSS that have fed on grape infected with *X. fastidiosa*. We've had 10,752 clones isolated, glycerol stocks prepared, and PCR products of all inserts amplified and purified for microarray spotting. This August three members of our laboratory were trained at the Custom Microarray Facility at the University of Arizona and we are currently repeating the results obtained there at the Core Instrumentation Facility in the Institute for Integrative Genome Biology on the Riverside campus. A subset of 1,536 clones was spotted in duplicate (side by side spots) and the entire array duplicated on the same slide. These arrays were hybridized to Cy3 labeled control cDNA and Cy5 labeled cDNA reverse transcribed and amplified from total RNA isolated from GWSS treated with a sub-lethal dose or an LD50 dose of esfenvalerate. Dye swap experiments were performed. These experiments are part of a collaborative related project funded by CDFA with Frank Byrne as Project Leader. Our results are presented in his report for the project entitled "Evaluation of resistance potential in the glassy-winged sharpshooter (GWSS) using toxicological, biochemical and genomics approaches." The arrays detected obvious differences in gene expression levels between the two treatments. These experiments were chosen for our test study because it is known that several genes encoding cytochrome P450 proteins are up-regulated dramatically in response to pesticide treatment. We have succeeded in cloning the entire GWSS V-ATPase A gene (Figure 1) by RLM-RACE. Differences in both the 5'- and 3'- sequences between the clones obtained indicate more than one copy of the V-ATPase A gene exists in the GWSS genome
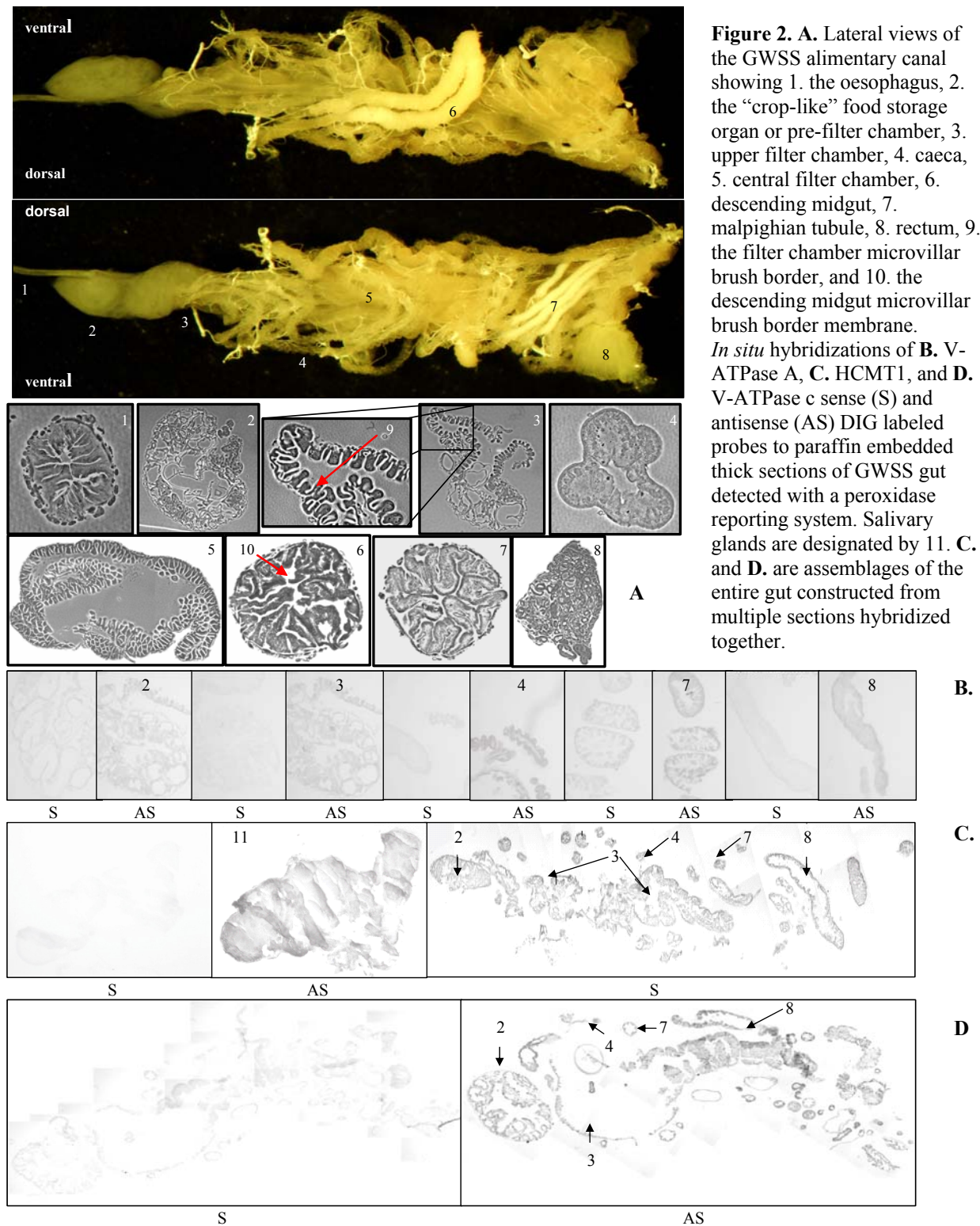
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   1  ctcgtgatatcagctggtgactgggtgaggtagctgcttcgtctgatcattacagtaagggatagctgaagatgcctttgcagaagattaaagatgag
                                                                          M  P  L  Q  K  I  K  D  E

  99  gacaaggagtccaagtttggatatgtgtacgctgtatccggaccggtcgttacggccgagaagatgtccgggtcagctatgtacgagttggtgcgtgtc
       D  K  E  S  K  F  G  Y  V  Y  A  V  S  G  P  V  V  T  A  E  K  M  S  G  S  A  M  Y  E  L  V  R  V

 198  ggctactttgagctggtaggagagatcattcgtctggaaggtgacatggcaactattcaggtatacgaagagacgtcaggtgtgacggttggtgaccct
       G  Y  F  E  L  V  G  E  I  I  R  L  E  G  D  M  A  T  I  Q  V  Y  E  E  T  S  G  V  T  V  G  D  P

 297  gtattgcggactggcaagcctctgtctgtggaactcggtcccgggatcctgggcgctattttttgatggtatccagcgacccttgaaggacatcaatgaa
                                   t  t     a     aag  c        c
       V  L  R  T  G  K  P  L  S  V  E  L  G  P  G  I  L  G  A  I  F  D  G  I  Q  R  P  L  K  D  I  N  E
                                                        M     S

 396  ctgtctcacagcatctacatccctaagggagtcaacgttcccgcactctccagaacagccacttgggagtttaacccccttaacatcaagactggaagc
                                   t                                 c
       L  S  H  S  I  Y  I  P  K  G  V  N  V  P  A  L  S  R  T  A  T  W  E  F  N  P  L  N  I  K  T  G  S

 495  cacattaccggaggagatatttatggaatcgtccacgagaataccctggtgaaacacaagatgctgctgcctcctcgagctaagggaacagtgacgtac
                                                               g
       H  I  T  G  G  D  I  Y  G  I  V  H  E  N  T  L  V  K  H  K  M  L  L  P  P  R  A  K  G  T  V  T  Y

 594  atcgcatcccctggcaactacactgttgatgatgttgttctggaaacggagtttgacggtgagaagtctaagtatacaatgttgcaagtgtggcctgta
       I  A  S  P  G  N  Y  T  V  D  D  V  V  L  E  T  E  F  D  G  E  K  S  K  Y  T  M  L  Q  V  W  P  V

 693  cgtcagcccagacctgtgaccgagaagctgccagccaatcatcccactgctcactggtcaacgtgtgctcgactctctattcccttgtgtgcaaggtggt
       R  Q  P  R  P  V  T  E  K  L  P  A  N  H  P  L  L  T  G  Q  R  V  L  D  S  L  F  P  C  V  Q  G  G

 792  accacggccatccctggagccttcggttgcggtaaaaactgtcatctctcaggccctgtccaaatactccaactcggatgtcatcatttacgtaggatgt
       T  T  A  I  P  G  A  F  G  C  G  K  T  V  I  S  Q  A  L  S  K  Y  S  N  S  D  V  I  I  Y  V  G  C

 891  ggagagcgaggaaatgaaatgtctgaggtattgcaagacttccctgagctgtctgtagagattgatggagtcacggagagtataatgaagcgaactgct
       G  E  R  G  N  E  M  S  E  V  L  Q  D  F  P  E  L  S  V  E  I  D  G  V  T  E  S  I  M  K  R  T  A

 990  ctggtagccaacacctccaacatgcctgtcgccgcccgagaagcttccatttatacaggaatcacactgtctgaatatttccgagacatgggctacaat
       L  V  A  N  T  S  N  M  P  V  A  A  R  E  A  S  I  Y  T  G  I  T  L  S  E  Y  F  R  D  M  G  Y  N

1089  gtttctatgatggctgactctacatcccgttgggccgaagccctgagagaaatttctggacgattggcagaaatgcctgctgacagtggttaccccgca
       V  S  M  M  A  D  S  T  S  R  W  A  E  A  L  R  E  I  S  G  R  L  A  E  M  P  A  D  S  G  Y  P  A

1188  tacctgggtgcccgtctagcctccttctacgagagagagccggtcgagtcaagtgcctgggaaatcccgatcgtgaaggctctgtcagtattgtcggtgct
       Y  L  G  C  P  S  S  L  L  L  R  X  S  R  S  S  Q  V  P  G  N  P  D  R  E  G  S  V  S  I  V  G  A

1287  gtgtcacctcctggtggtgacttctcagatcccgtcacctccgccacccttggtatcgtacaggtcttctggggtcttgacaagaaattggcacaaagg
       V  S  P  P  G  G  D  F  S  D  P  V  T  S  A  T  L  G  I  V  Q  V  F  W  G  L  D  K  K  L  A  Q  R

1386  aaacactttccctccattaactggctcatatcatacagtaaatacatgagagcactagatgacttctatgacaagagcttcccagagtttgtaccctg
       K  H  F  P  S  I  N  W  L  I  S  Y  S  K  Y  M  R  A  L  D  D  F  Y  D  K  S  F  P  E  F  V  P  L

1485  aggaccaaggtaaaggagattctgcaggaggaagaagatttgtcagaaattgtacaactggttggcaaagcatcacttgccgaaactgacaaaatcacc
                                                                                gagtcc
       R  T  K  V  K  E  I  L  Q  E  E  E  D  L  S  E  I  V  Q  L  V  G  K  A  S  L  A  E  T  D  K  I  T
                                                                                   S

1584  ctcgaggtcaataggctactgaaagaagattttctgcaacagaacagctactctccgtatgatcgtttctgtcccttctacaagacagtgggcatgctc
       L  E  V  A  R  L  L  K  E  D  F  L  Q  Q  N  S  Y  S  P  Y  D  R  F  C  P  F  Y  K  T  V  G  M  L

1683  cgcaacatgattgcattctttgacatgtcaaggcatgccgtcgagtcgacagcacaaagtgaaaacaagatcacctggagtgtcatcaaggacggcatg
       R  N  M  I  A  F  F  D  M  S  R  H  A  V  E  S  T  A  Q  S  E  N  K  I  T  W  S  V  I  K  D  G  M

1782  ggcaacattctgtaccaactgtcgtcaatgaaattcaaggatcccgtgaaagatgaaaaaaaaaaaa
       G  N  I  L  Y  Q  L  S  S  M  K  F  K  D  P  V  K  D  E
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Figure 1. The complete cDNA and translated protein of GWSS V-ATPase A. The atg indicates the translational start site. Nucleic acid and protein sequence variations are indicated in bold. Sequence variations were determined from both sense and antisense sequences.

We have dissected and identified all of the components of the GWSS alimentary canal, performed ultrastructural studies of these tissues, and developed *in situ* hybridization techniques for the localization of gene expression (Figure 2). As expected the genes encoding the V-ATPase A and c subunits and that expressing HcMT1 are all expressed throughout the GWSS gut. HcMT1 clearly also is expressed in the salivary glands. The studies localizing the expression of the trypsin-like and maltase-like genes are in progress.

Figure 2. A. Lateral views of the GWSS alimentary canal showing 1. the oesophagus, 2. the "crop-like" food storage organ or pre-filter chamber, 3. upper filter chamber, 4. caeca, 5. central filter chamber, 6. descending midgut, 7. malpighian tubule, 8. rectum, 9. the filter chamber microvillar brush border, and 10. the descending midgut microvillar brush border membrane. *In situ* hybridizations of **B.** V-ATPase A, **C.** HCMT1, and **D.** V-ATPase c sense (S) and antisense (AS) DIG labeled probes to paraffin embedded thick sections of GWSS gut detected with a peroxidase reporting system. Salivary glands are designated by 11. **C.** and **D.** are assemblages of the entire gut constructed from multiple sections hybridized together.

Transcript sizes for each of the genes partially cloned have been determined by RNA blot hybridization (Figure 3). The transcript sizes were determined as: ~1,900 bp for V-ATPase A, which corresponds well with that determined from the cDNA sequence of 1,849 bp, ~1,200bp for V-ATPase c, and ~875 bp for HCMT1 and the trypsin-like gene. These values

correspond to those in the literature for each of these genes (van Hille *et al.* 1993; Pietrantonio and Gill, 1993; Zeng *et al.*, 2002; Liu *et al.,* unpublished data).

CONCLUSIONS

The presence of more than one GWSS V-ATPase A subunit gene will be confirmed by DNA blot hybridization. We have developed a clone capture technique which will allow us to isolate all gene clones with sequence similarity from our cDNA library in a single experiment. This procedure involves the formation of a RecA-mediated triple-stranded molecule between our biotinylated partial clone and full length cDNA clones with sequence similarity. Triple-stranded molecules are then removed from the reaction using streptavidin magnetic beads. This approach will allow us to much more quickly analyze all the members of specific gene families already partially cloned. Thus far we have succeeded in isolating clones similar to the KAAT-like gene clone recently obtained (data presented in the report of a related project: Development of Glassy-winged Sharpshooter Mimetic Insecticidal Peptides, and an Endophytic Bacterial System For Their Delivery to Mature Grape.). The clones isolated are being analyzed to identify the regions best suited for antibody targeting using bioinformatics tools. We anticipate that this approach also will allow us to isolate gene families of genes identified by microarray screening as being tissue-specifically expressed. This will be important in determining that a potential target does not have similarity to genes expressed other than in the organs we want to target.
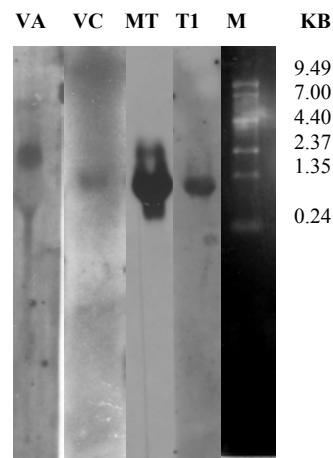

Figure 3. RNA blot hybridizations of 10µg GWSS total RNA hybridized to V-ATPase A (VA), V-ATPase c (VC) and the trypsin-like gene (T1) clones labeled with DIG and detected with chemiluminecsence and the HCMT1 (MT) gene clone labeled with 32P.

REFERENCES

Berezov, A., H-T. Zhang, M. Greene, and R. Murali. 2001. Disabling ErbB receptors with rationally designed exocyclic mimetics of antibodies: structure-function analysis. J. Med. Chem. 44: 2565-2574.

Burke, T., Z-J. Yao, D-G. Liu, J. Voigt, and Y. Gao. 2001. Phosphoryltyrosyl mimetics in the design of peptide-based signal transduction inhibitors. Biopolymers. 60: 32-44.

Castagna, M., C. Shayakul, D. Trotti, V. Sacchi, W. Harvey, and M. Hediger. 1998. Cloning and characterization of a potassium-coupled acid transporter. Proc. Natl. Acad. Sci. USA. 95: 5395-5400

Clemens, U. 1996. Current concepts of treatment in medical oncology: New anticancer drugs. J. of Cancer Res. Clin. Oncol. 122: 189-198.

Deghenghi, R. 1998. Synthetic peptides and their non-peptidyl mimetics in endocrinology: From synthesis to clinical perspectives. J. Endocrin. Invest. 21: 787-793.

Larrick , J., L. Yu, C. Naftzger, S. Jaiswal, and K. Wycoff. 2001. Production of secretory IgA antibodies in plants. Biomolecular Engineering. 18. 87-94.

Lincoff, A., R. Califf, and E. Topol. 2000. Platelet glycoprotein IIb/IIIa receptor blockade in coronary artery disease. J. Amer. Coll. Card. 35: 1103-1115.

Liu, J., J. Zhang, R. Zhou, and C. Jin. Molecular cloning and preliminary study on biological function of a novel gene overexpressed in human hepatocellular carcinoma. unpublished . accession number CAB81951.1

Moe, G., S. Tan, and D. Granoff. 1999. Molecular mimetics of polysaccharide epitopes as vaccine candidates for prevention of *Neisseria meningitidis* serogroup B disease. FEMS Immun. Med. Micro. 26: 209-226.

Monzayi-Karbassi, B., and T. Kieber-Emmons. 2001. Current concepts in cancer vaccine strategies. Biotechniques. 30: 170-189.

Pietrantonio, P.V., and S.S. Gill. 1993. Sequence of a 17 kDa vacuolar H(+)-ATPase proteolipid subunit from insect midgut and Malpighian tubules. Insect Biochem Mol Biol. 6: 675-680.

Shi, Y., M. Wang, K. Powell, E. Van Damme, V. Hilder, A. Gatehouse, D. Boulter, and J. Gatehouse. 1994. Use of the rice sucrose synthase-1 promotor to direct phloem-specific expression of β-glucuronidase and snowdrop lectin genes in transgenic tobacco plants. J. Exp. Botany. 45: 623-631.

Springer, P. 2000. Gene traps: Tools for plant development and genomics. Plant Cell. 12: 1007-1020.

Stoger, E., M. Sack, R. Fischer, and P. Christou. 2002. Plantibodies: Applications, advantages and bottlenecks. Curr. Opinion Biotech. 13. 161-166.

Van Hille B., H. Richener, D.B. Evans, J.R. Green, and G. Bilbe. 1993. Identification of two subunit A isoforms of the vacuolar H(+)-ATPase in human osteoclastoma. J Biol. Chem. 268: 7075-7080

Zeng F., Y.C. Zhu, A.C. Cohen . 2002. Molecular cloning and partial characterization of a trypsin-like protein in salivary glands of *Lygus hesperus* (Hemiptera: Miridae). Insect Biochem. Mol. Biol. 4: 455-464.

REALIZED LIFETIME PARASITISM AND THE INFLUENCE OF BROCHOSOMES ON FIELD PARASITISM RATES OF GLASSY-WINGED SHARPSHOOTER EGG MASSES BY *GONATOCERUS ASHMEADI*

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Reporting period: The results reported here are from work conducted from July 2004 to October 2004.

ABSTRACT

INTRODUCTION
GWSS is an exotic pest in California having invaded and established in this state in the late 1980's. One potential reason for the inordinate numbers of GWSS in California compared to population densities in the pest's home range in southeastern USA is a lack of an efficient natural enemy fauna that has evolved to use GWSS as a resource. As part of a classical biological control program against GWSS, scientists with the CDFA and UCR have been prospecting for, importing into quarantine, and clearing for release mymarid egg parasitoids from the home range of GWSS for establishment in California. To date, two new parasitoid species have been established in CA, *Gonatocerus triguttatus* and *G. fasciatus*. It is too early to ascertain the impact on GWSS population growth that these two parasitoids will have. The self introduced *G. ashmeadi* (Vickerman et al., 2004) is the key natural enemy of GWSS egg masses in CA at present (Blua et al., 1999). Over summer, parasitism levels of GWSS egg masses and individual eggs in masses by *G. ashmeadi* approaches 100% but parasitism levels of the spring generation of GWSS are substantially lower (Triapitsyn and Phillips, 2000). Naturally occurring populations of *G. ashmeadi* in CA have been augmented with mass reared individuals from populations found in the southeastern USA and northeastern Mexico which encompasses the home range of GWSS (D. Morgan - CDFA, pers. comm. 2003).

Substantial laboratory work with *G. ashmeadi* has been conducted in an attempt to understand and parameterize basic aspects of this parasitoid's reproductive biology, and host selection behaviors. Irvin and Hoddle (2001) have evaluated oviposition preferences of *G. ashmeadi* when presented GWSS eggs of various ages. Interspecific competition between *G. ashmeadi* with *G. triguttatus* and *G. fasciatus* for GWSS egg masses of different ages has been assessed along with factors influencing the sex ratio of offspring (Hoddle and Irvin, 2002; 2003). The effect of resource provisioning and nutrient procurement on the longevity of *G. ashmeadi* has also been determined (Irvin unpublished data). Furthermore, the foraging efficacy of *G. ashmeadi* in simple and complex environments for scarce and abundant GWSS egg masses has also been completed and compared to similar data collected for *G. triguttatus* (Irvin unpublished data).
The effect of brochosomes on the foraging efficacy of *G. ashmeadi* has also been evaluated in the laboratory. Brochosomes are a chalky material produced by the malpighian tubules in many xylophagous cicadellid species (Rakitov, 1999; 2000; 2004). Brochosomes are excreted from specialized openings on the posterior of the abdomen and are collected and deposited by mated females on the forewings. During oviposition, females rub brochosomes off the forewings and deposit them on the tops of eggs masses (Hix, 2001). The adaptive significance of covering egg masses with brochosomes is uncertain (Rakitov,1999). Hix (2001) has suggested that brochosomes may protect GWSS eggs from desiccation, UV light, natural enemies (parasitoids, predators and pathogens); or they provide a signal to other female GWSS that leaves have already been oviposited in. We have investigated the effect of brochosomes on the foraging efficacy of *G. ashmeadi* in the laboratory. Data clearly demonstrate that moderate to heavy brochosome coverage of GWSS eggs is a major impediment to oviposition to *G. ashmeadi* when compared to conspecific parasitization efficiency of GWSS eggs with light or no brochosome coverage (Velema et al., 2004).

Studies currently funded by the CDFA to by conducted by this lab will look at: (1) laboratory-level fecundity rates of *G. ashmeadi* under varying temperature regimens; (2) field cage studies assessing interspecific competition between parasitoids released for the classical biological control of GWSS; (3) factors affecting sex ratio allocation during mass production of mymarid parasitoids; and (4) the effect of resource provisioning on parasitization rates and overwintering longevity of key mymarid parasitoids under field conditions. The work proposed in this grant will complement and support completed studies and work in progress.

Many factors act in concert to affect successful biological control. The GWSS-*Gonatocerus* system has benefited from intensive laboratory study to generate a basic understanding of factors influencing host selection and parasitism success. The

next step that is now required is to test hypotheses generated from lab studies in the field. Field level assessments will evaluate our understanding of the system under investigation, and consolidate interpretations needed to determine the most important aspect of the GWSS biological control program: "How effective are egg parasitoids at controlling GWSS in California?" To get to the crux of this issue we are asking two questions in this proposal: (1) How big an impact do individual female parasitoids have on GWSS population growth via parasitization of eggs, and (2) do biotic impediments such as brochosomes affect parasitization efficacy in the field? When these two questions are addressed together we will begin to develop a comprehensive understanding of the impacts parasitoids have at the field level and factors affecting parasitization success. This will allow us to form a much better understanding of what levels of control we can expect from mymarid egg parasitoids when different ecological conditions are prevailing in the field.

OBJECTIVES
This is a new proposal that was officially funded in July 2004. This project has two objectives aimed at determining the field level impact individual female *Gonatocerus ashmeadi* have on glassy-winged sharpshooter (GWSS) egg masses. These two research objectives are complimentary:
1. Measure real life time contributions of individual female parasitoids to parasitism of GWSS egg masses under field conditions. This research objective is high priority.
2. Determine the ecological significance of brochosome deposition on GWSS egg masses and its effect on parasitism rates by *G. ashmeadi* under field conditions.

RESULTS
This project has not commenced. There are two major reasons for this: (1) Recruitment of Dr. Nic Irvin as the post-graduate researcher for this program has been held up by the excessive time it has taken to process the required visas to employ her in the USA given her alien status. (2) Dr. Irvin will start working on this project in early March 2005 when GWSS populations begin to build again. It made no sense to employ Dr. Irvin earlier than this time as at the time of notification of successful visa application GWSS populations were declining in the field and there would be few reproductive adults and parasitoids to work with. We will be formally requesting a no cost extension for this project.

REFERENCES
Blua, M.J., Phillips, P.A., and Redak, R.A. 1999. A new sharpshooter threatens both crops and ornamentals. Calif. Agric. 53: 22-25.

Irvin, N. and Hoddle, M.S. 2001. Egg age preference and "window of susceptibility" of *Homalodisca coagulata* eggs to attack by *Gonatocerus ashmeadi* and *G. triguttatus*. Pierce's Disease Control Program. Symposium Proceedings, Pierce's Disaese Research Symposium, December 5-7, 2001. Coronado Island Marriott Resort. pp. 135-136.

Hix, R.L. 2001. Egg-laying and brochosome production observed in glassy-winged sharpshooter. Calif. Agric. 55: 19-22.

Hoddle M.S. and Irvin, N. 2002. Interspecific competition between *Gonatocerus ashmeadi* and *G. triguttatus* for glassy-winged sharpshooter egg masses. Pierce's Disease Control Program. Symposium Proceedings, Pierce's Disaese Research Symposium, December 15-18, 2002. Coronado Island Marriott Resort. pp. 86-87.

Hoddle, M.S. and Irvin, N. 2003. Interspecific competition between *Gonatocerus ashmeadi*, *G. triguttatus*, and *G. fasciatus* for glassy-winged sharpshooter egg masses. Pierce's Disease Control Program. Symposium Proceedings, Pierce's Disaese Research Symposium, December 8-11, 2003. Coronado Island Marriott Resort. pp. 250-254.

Rakitov, R.A. 1999. Secretory products of the malpighian tubules of Cicadellidae (Hemiptera: Membracoidea): an ultrastructural study. International Journal of Insect Morphol. Embryol. 28: 179-193.

Rakitov, R.A. 2000. Secretion of brochosomes during the ontogenesis of a leafhooper. *Oncometopia orbona* (F.) (Insecta, Homoptera, Cicadellidae). Tissue & Cell 32: 28-39.

Rakitov, R.A. 2004. Powdering of egg nests with brochosomes and related sexual dimorphism in leafhoppers (Hemiptera: Cicadellidae). Zool. J. Linn. Soc. 104: 353-381.

Triapitsyn, S.V. and Phillips, P.A. 2000. First record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of Homalodisca coagulata (Homoptera: Cicadellidae) with notes on the distribution of the host. Florida Entomologist **83:** 200-203.

Velema H.P., Hoddle, M.S. Hemerik, L. and Luck, R.F. 2004. The influence of brochosomes on parasitisation efficiency of *Gonatocerus ashmeadi* (Girault) (Hymenoptera: Mymaridae), parasitising *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae) egg-masses. Ecol. Entom. *Submitted*

Vickerman, D., Hoddle, M.S., Triapitsyn S. and Stouthamer, R. 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: Morphology, DNA sequences and reproductive compatibility. Biol. Cont. *In Press.*

FUNDING AGENCIES

REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF *GONATOCERUS ASHMEADI*, AN EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
The reproductive and developmental biology of *Gonatocerus ashmeadi* Girault, a self-introduced parasitoid of the glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* Say, was determined at five constant temperatures in the laboratory; 15; 20; 25; 30; and 33°C. Wasps at each experimental temperature were given, on average, between 10 and 15 GWSS eggs per day for its natural life for oviposition. At 30°C, immature *G. ashmeadi* sustained the highest mortality rates as adult emergence was lowest at this temperature. The largest proportion of female offspring was produced at 25°C and lifetime fecundity was greatest at 25°C. The development time was greatest at 15°C and lowest at 30°C. Mean adult longevity was inversely related to temperature with a maximum of approximately 30 days at 15°C to a minimum of approximately two days at 33°C.

INTRODUCTION
The mymarid wasp species *Gonatocerus ashmeadi* Girault, *G. triguttatus* Girault, *G. morrilli* Howard, and *G. fasciatus* Girault are the most common natural enemies associated with the insect pest *Homalodisca coagulata* in it's home range of southeastern USA and northeastern Mexico (Triapitsyn and Phillips, 2000). The wasp *G. ashmeadi* is a self-introduced resident of California and most likely came into the state in parasitized *Homalodisca coagulata* eggs (Vickerman et al., 2004) and has established widely in association with *H. coagulata*.

One factor that can limit the success of the establishment of natural enemies is mismatching the environmental conditions favored by the introduced agent with those that predominate in the receiving range (Hoddle, 2004). Quantification of the reproductive and developmental biology of a natural enemy is paramount to predicting, planning, and promoting the establishment and population growth of introduced agents. This can be enhanced by determining demographic characteristics such as day-degree requirements for immature development, population doubling times and lifetime fecundity for estimating population growth rates at various temperatures and for comparison with the target pest and other species of biological control agents. Determining the introduced control agent's reproductive and developmental biology and environmental requirements with that of the host will allow for a greater understanding of factors affecting biological control of GWSS.

The following work was undertaken to provide information on the reproductive and developmental biology of the parasitoid wasp *G. ashmeadi*. These data will provide knowledge of the insect's life cycle, in particular in relation to GWSS, and will improve the understanding of optimal timings of its release for biological control purposes in many agricultural systems as well as improve the efficiency of laboratory rearing of these insects. In addition to improving release and rearing strategies, this information will target foreign exploration of strains of *G. ashmeadi* for possible introduction into California and also identify geographical areas that will be conducive to the use of this species as biological control agent following GWSS establishment in various parts of California and in areas such as Tahiti and Hawaii where GWSS has recently invaded.

OBJECTIVES
1. Examine the developmental and reproductive biology of *G. ashmeadi* in order to determine its day-degree requirements, and demographic statistics.

RESULTS
The rates for oviposition that led to successful reproduction of offspring were highest at 30°C (Figure 1). Each wasp at each temperature, on average, had the same number of GWSS eggs made available to them for oviposition. At 30°C, approximately 42% of eggs presented to wasps produced into viable parasitoid offspring. Conversely, this rate decreased with temperature to 1% at 15°C. Higher temperatures similarly lowered the production of viable offspring with approximately 13% surviving to adult stages at 33°C. These results suggest that *G. ashmeadi* progeny survivorship was most successful when oviposition occurred at 30°C, intermediate at 20-25°C and lowest at 15°C.
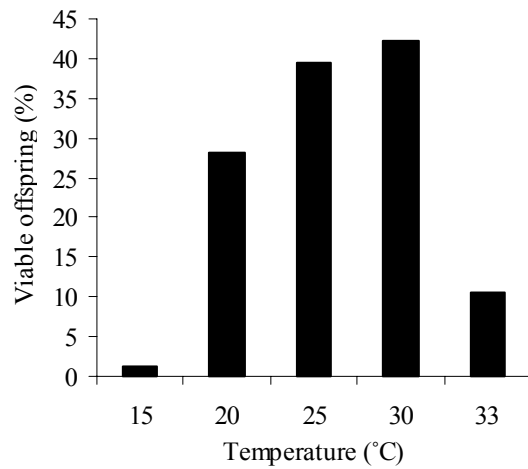
Figure 1. Mortality rates fell as temperatures rose until 30°C. Few viable offspring were produced at 33°C. The highest percentage of viable offspring from available eggs was at 30°C.
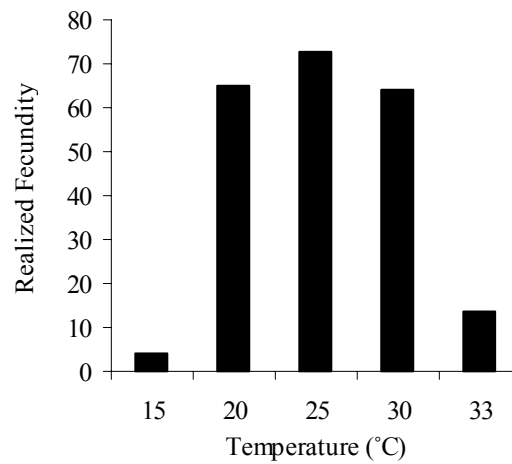
Figure 2. The average number of offspring emerging from parasitized eggs at each temperature. Parasitized eggs that did not yield viable offspring are not represented here.

The number of offspring produced by individual wasps over their lifetime was greatest at 25°C and fell sharply as temperature either increased of decreased (Figure 2). Approximately 73 offspring were produced by wasps at 25°C down to an average of around 4 and 14 at 15°C and 33°C, respectively. These data show that at constant high or low temperatures wasps fail to produce many offspring and may have little or no impact on GWSS population growth as a consequence.

There appeared to be no trends to the ratios of females produced at each experimental temperature (Figure 3). The highest percentage of females was produced at 25°C with approximately 70% of offspring being female. All other temperatures were, with the exception of 20°C, were within 10% of this temperature. These results indicate that temperature may not play an important role in the sex selection of *G. ashmeadi* offspring.

The time between eggs being made available to individual wasps and the emergence of offspring, fell from a high of approximately 39 days at 15°C to approximately 10 days for 30 – 33°C (Figure 4). As temperature rose, the time required for the development of wasp larvae was reduced. This faster development time at higher temperatures suggests that wasps will cycle through several generations in comparison to GWSS.
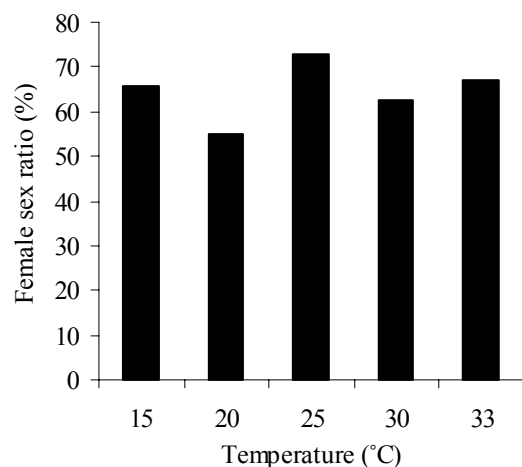

Figure 3. The percentage of *G. ashmeadi* offspring that was identified as female at each temperature.
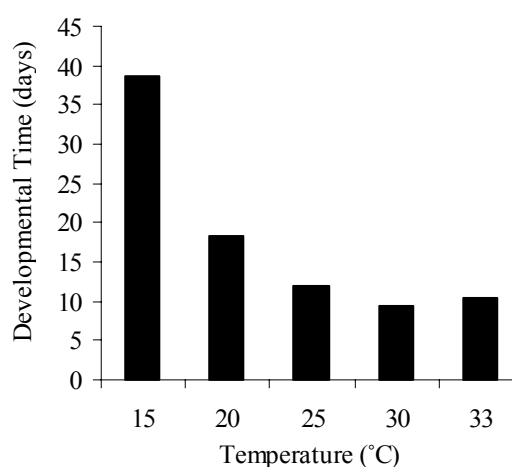
Figure 4. The period of time between oviposition by *G. ashmeadi* and the emergence of wasp offspring represented in days.

Mean adult longevity for individual mated female *G. ashmeadi* used in this study fell from an average of approximately 20 days at 15°C to approximately eight days at 33°C (Figure 5).
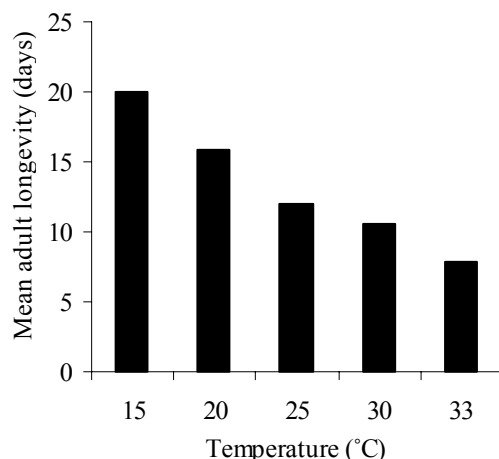
Figure 5. The average time, in days, from when mated females used in the study first emerged to when they died of natural causes.

CONCLUSIONS
The wasps at 30°C died quicker (figure 5) and laid fewer eggs (figure 3) than wasps at 25°C. This difference was offset by the findings that the individuals at 30°C successfully utilized a higher percentage of the eggs that were made available to them than those at 25°C. Whilst individuals at 30°C produced fewer viable offspring, it is possible that as a population effect greater numbers of offspring may be produced due to a faster generation turnover and higher rate of parasitism overall. Wasps at 30°C will cause a population to grow at a much faster rate due to the wasp ovipositing in, largely, an equal number of eggs. The success of the wasp at this temperature is indicative of the much shorter developmental times whereby the wasp will produce offspring that develop at much faster rates. Individual wasps surviving for extended periods of time were observed at 15°C and declining in a linear manner as temperature rose. Whilst wasps at 15°C, for example, survived considerably longer than at other temperatures their efficacy was affected by the temperature and made very little impact on the number of offspring produced.

The success of a biological control agent is measured by the mortality it inflicts on its target which is in part a function of its reproductive and developmental activity across a range of temperatures (Nahrung and Murphy, 2002). The results from this study suggest that *G. ashmeadi* operates most effectively at moderate to high temperatures. Identifying the optimal temperature for reproduction and developmental of *G. ashmeadi*, will greatly aid mass-rearing efforts, using day-degree models to predict geographic range, to assess generational turnover in various locales in comparison to GWSS and to optimize releases of natural enemies into a field environment.

REFERENCES
Hoddle M.S., 2004. The potential adventive geographic range of glassy-winged sharpshooter, *Homalodisca coagulata* and the grape pathogen *xylella fastidiosa*: Implications for California and other grape growing regions of the world. Crop Protect. 23, 691-699.

Nahrung H.F., Murphy, B.D., 2002. Differences in egg parasitism of *Chrysophtharta agricola* (Chapuis) (Coleoptera : Chrysomelidae) by *Enoggera nassaui* Girault (Hymenoptera : Pteromalidae) in relation to host and parasitoid origin. Austral. J. Entomol. 41, 267-271.

Triapitsyn S.V., Phillips, P.A., 2000. First record of *Gonatocerus triguttatus* (Hymenoptera : Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera : Cicadellidae) with notes on the distribution of the host. Florida Entomologist 83, 200-203.

Vickerman D.B., Hoddle, M.S., Triapitysn, S.V., Stouthamer, R., 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: Morphology, DNA sequences and reproductive compatibility. Biol. Cont. *In Press*.

FUNDING AGENCIES

SEARCHING FOR AND COLLECTING EGG PARASITOIDS OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER *HOMALODISCA* SPECIES IN SOUTHEASTERN AND SOUTHWESTERN MEXICO

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Reporting Period: The results reported here are from work conducted from July 1, 2004 to October 6, 2004.

ABSTRACT
According to the proposed (and approved) research timetable, work on this project will commence as early as in January 2005, when we may have the first chance to collect parasitized egg masses of *Homalodisca* spp. in Mexico. This report is only for information purposes about this project.

INTRODUCTION
Egg parasitoids of the Glassy-winged Sharpshooter (GWSS), *Homalodisca coagulata* (Say), were discovered through survey activities conducted throughout USA and northeastern Mexican states of Nuevo Leon and Tamaulipas, which resulted in collection, introduction, and release in California of several species of mymarid eggs parasitoids (*Gonatocerus* spp.) (Morgan et al. 2000; Triapitsyn et al., 2002; Triapitsyn & Hoddle, 2001, 2002). During 2003 and 2004, we conducted a survey of egg parasitoids of GWSS in central and eastern USA (Hoddle & Triapitsyn, 2003, 2004). According to McKamey (2002), the native host range of GWSS also includes central and southern Mexico, well beyond the currently known range mapped by Triapitsyn & Phillips (2000). McKamey's (2002) report is supported by the CLIMEX-predicted distribution range of GWSS (Hoddle 2004; also Map below).

Here we propose the final step in the development of a classical biological control program against GWSS in California: to search new climatically suitable areas in Mexico for GWSS parasitoids. Additionally, our previous exploratory work in Mexico (in the States of San Luis Potosí, Tamaulipas and Veracruz) during 1999-2003 resulted in the discovery of at least two new, undescribed species of *Homalodisca* egg parasitoids, which are related to *G. ashmeadi* Girault and *G. morrilli* (Howard) but differ from those both morphologically (Triapitsyn et al. 2002) and genetically (D. Vickerman, unpubl. data). These parasitoids need to be recollected in Mexico and tested as potential biological control agents against GWSS in California.

OBJECTIVES
This project has two main objectives:
1. Search for and collect egg parasitoids in southern-most home range of GWSS and other *Homalodisca* species in southeastern and southwestern Mexico; and
2. Introduction and establishment of quarantine cultures of the selected species and their following initial evaluation for potential establishment in California.

RESULTS
There are no results to be reported at this time. The following experimental procedures will be used to accomplish the above objectives:

Exploratory Work.
Search for and collect egg parasitoids of southern-most home range of GWSS and other *Homalodisca* species (in the Mexican states of Tamaulipas (southern part), Veracruz, San Luis Potosí, Campeche, Oaxaca, Yucatán, and Quintana Roo) for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation. Several short exploratory trips will be made to those states during winter and spring 2005 and parasitized egg masses of *Homalodisca* will be collected there and sent to UCR quarantine facility under the existing permit. The two already known egg parasitoids of GWSS from Tamaulipas and adjacent Mexican states (*G.* near *ashmeadi* and *G.* near *morrilli*) will be recollected from known localities.

Quarantine and Identification Work
Colonies of the selected parasitoids will be established in UCR quarantine using GWSS as a host (fresh egg masses will be supplied by David Morgan). Voucher specimens of the collected parasitoids will require appropriate curation as a result of

the proposed exploratory work; these will need to be critically point-dried from ethanol, point- or card-mounted, labeled, slide-mounted, and identified to genera and species. DNA analyses will be conducted if necessary.

Figure 1. Current and CLIMEX-predicted geographical range of GWSS. Large blue dots indicate good climatic conditions for GWSS. Small blue dots are marginal habitats. x on map indicate unsuitable areas.
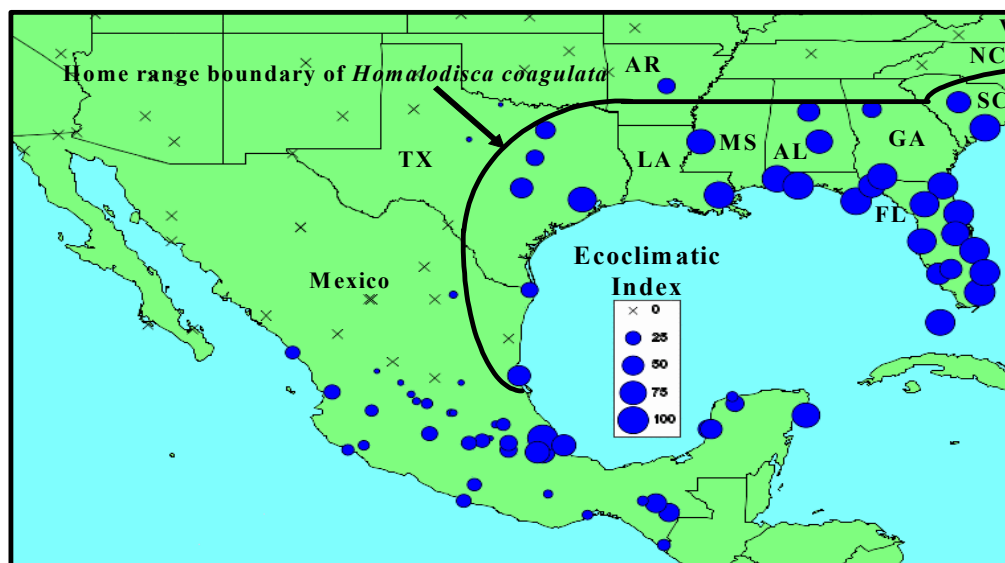
CONCLUSIONS
Research to be conducted in the course of this project will be of benefit primarily to the CDFA GWSS Biological Control Program as well as to other biocontrol specialists and agencies conducting projects against GWSS in California such as the USDA-APHIS. Ultimately, we hope that this project will be beneficial to California's agriculture.

REFERENCES
Hoddle, M. S. 2004. The potential adventive geographic range of glassy-winged sharpshooter, *Homalodisca coagulata*, and the grape pathogen *Xylella fastidiosa*: implications for California and other grape growing regions of the world. Crop Protect. *In press.*

Hoddle, M. S. and S. V. Triapitsyn. 2003. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA, pp. 261-262. *In*: Proceedings of the Pierce's Disease Research Symposium, December 8-11, 2003, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, P. Blincoe, R. Spencer, L. Houser, A. Ba and T. Esser), Copeland Printing, Sacramento, California, 323 p.

Hoddle, M. S. and S. V. Triapitsyn. 2004. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA. In: Proceedings of the Pierce's Disease Research Symposium, December 7-10, 2004.

McKamey, S. H. 2002. Developing a stable classification of the glassy-winged sharpshooter genus *Homalodisca*, pp. 122-123. In: Proceedings of the Pierce's Disease Research Symposium, December 15-18, 2002, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, and T. Esser), Digital Logistix, Sacramento.

Morgan, D. J. W., S. V. Triapitsyn, R. A. Redak, L. G. Bezark, and M. S. Hoddle. 2000. Biological control of the glassy-winged sharpshooter: current status and future potential, pp.167-171. *In*: [Proceedings] California Conference on Biological Control, held July 11-12, 2000 at the historic Mission Inn, Riverside, California (M. S. Hoddle, Ed.), 205 pp.

Triapitsyn, S. V., L. G. Bezark and D. J. W. Morgan. 2002. Redescription of *Gonatocerus atriclavus* Girault (Hymenoptera: Mymaridae), with notes on other egg parasitoids of sharpshooters (Homoptera: Cicadellidae: Proconiini) in northeastern Mexico. Pan-Pacific Entomologist 78: 34-42.

Triapitsyn, S. V. and M. S. Hoddle. 2001. Search for and collect egg parasitoids of glassy-winged sharpshooter in southeastern USA and northeastern Mexico, pp. 133-134. In: Proceedings of the Pierce's Disease Research Symposium, December 5-7, 2001, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt & T. Esser), Copeland Printing, Sacramento, California, 141 pp.

Triapitsyn, S. V. and M. S. Hoddle. 2002. Search for and collect egg parasitoids of glassy-winged sharpshooter in southeastern USA and northeastern Mexico, pp. 94-95. In: Proceedings of the Pierce's Disease Research Symposium, December 15-18, 2002, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, P. Blincoe and T. Esser), Digital Logistix, Sacramento, California, 177 p.

Triapitsyn, S. V. and P. A. Phillips. 2000. First host record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae), with notes on the distribution of the host. Florida Entomologist 83: 200-203.

FUNDING AGENCIES

SEARCHING FOR AND COLLECTING EGG PARASITOIDS OF GLASSY-WINGED SHARPSHOOTER IN THE CENTRAL AND EASTERN USA

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ABSTRACT
Search for egg parasitoids of proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Proconiini) in central and eastern USA during 2003 and 2004 resulted in rearings of several species of Mymaridae and Trichogrammatidae (Hymenoptera) (Table 1). Cultures of some species, notably of *Anagrus epos* Girault, were established at UCR quarantine.

INTRODUCTION
Presence of the proconiine sharpshooters *Homalodisca coagulata* (Say) (GWSS - the Glassy-winged Sharpshooter) and its close relative *Oncometopia orbona* (Fabricius) (the Broad-headed Sharpshooter) in central and eastern United States justified conducting a survey of their principal natural enemies, egg parasitoids in the families Mymaridae and Trichogrammatidae. No such surveys have ever been conducted North of central Georgia, Mississippi, Louisiana, and Texas. Prior research showed presence of the mymarid *Gonatocerus fasciatus* Girault there (Triapitsyn et al. 2003). A number of trichogrammatid genera and species were recognized in southeastern USA from eggs of a grass-inhabiting *Cuerna costalis* (Fabricius), also a proconiine sharpshooter, as well as from *H. coagulata* and *O. orbona* (Triapitsyn 2003).

OBJECTIVES
1. **Exploratory work** - Search for and collect egg parasitoids of proconiine sharpshooters in the northern- and eastern-most home range of GWSS, *Oncometopia* spp., and *Cuerna* spp. for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation.
2. **Curatorial work** – Curate the collected voucher specimens of mymarid and trichogrammatid egg parasitoids.

RESULTS
Objective 1.
The first exploratory trip was made to Kentucky and Tennessee by S. Triapitsyn in July 2003 (Hoddle & Triapitsyn 2003). The second trip to Illinois (the northernmost distribution range of *Oncometopia orbona* and *Cuerna costalis*), eastern Kentucky, and south-central Tennessee was made by S. Triapitsyn in April 2004, in an attempt to locate and collect the overwintered and egg-laying adults of *C. costalis*. Part of the trip (in southern Illinois) was made together with Roman Rakitov, who showed his methods of collecting *C. costalis* in known localities where this species had been collected in the past (occurrence of proconiine sharpshooters there is spotty). We were able to collect several adults of *C. costalis* in one locality in Shawnee National Forest, on a private meadow. Yellow pan traps were placed in this locality and we managed to collect a specimen of *Gonatocerus novifasciatus* Girault (Mymaridae), a known parasitoid of *H. coagulata* elsewhere. There it most probably is parasitoid of *Cuerna costalis*, the only proconiine sharpshooter occurring on that meadow. This gave us a hint what species of egg parasitoids occur there despite the fact that it is practically impossible to find egg masses of this proconiine sharpshooter when its density is so low. Also parasitoids and leafhoppers were collected there using vacuum. In several locations in southern Illinois, both methods revealed frequent presence of *Gonatocerus rivalis* Girault and its likely host, *Draculacephala antica* (Walker) (determined by Roman Rakitov). *Draculacephala* is a cicadelline (tribe Cicadellini) sharpshooter genus, which members were the most abundant leafhoppers of the subfamily Cicadellinae in all three states visited. This could be an apparent new host association for this species of *Gonatocerus*, which is a member of the *sulphuripes* species group.

Subsequent trips to Georgia, North Carolina, and South Carolina in June and August 2004 by S. Triapitsyn resulted in collections of several mymarid and trichogrammatid species, listed in Table 1, which were reared from egg masses of proconiine sharpshooters. Quarantine colonies of *Gonatocerus ashmeadi* Girault from Georgia and South Carolina were discontinued several generations following their establishment because it was shown that this species is morphologically, biologically, and genetically homogenic throughout its range (Vickerman et al. 2004). Both GWSS and to some degree *O. orbona* were found to be abundant almost everywhere in the lowlands (especially coastal) in Georgia, North Carolina, and

South Carolina whereas GWSS could not be found in the forested hills and mountains of northern Georgia, eastern North Carolina, Kentucky, and Tennessee, where only a few adult *O. orbona* as well as its old egg masses (all with evidence of parasitization) were collected.

Our survey also benefited greatly from the exploratory work by Roman Rakitov, who reared mymarid and trichogrammatid egg parasitoids of several species of the genus *Cuerna* (other than *C. costalis*). Particularly, the mymarid *Anagrus epos* Girault was reared by Roman Rakitov near Glyndon, Clay Coounty, Minnesota, from egg masses of a *Cuerna* sp. and sent to UCR quarantine facility under a permit. This is the first representative of the genus *Anagrus* ever reared from eggs of a proconiine sharpshooter. We were able to establish a quarantine colony of this species on eggs of GWSS, which is a fictitious host for *A. epos* (GWSS does not occur in Minnesota). *Anagrus epos* is a gregarious species: 3-5 adult wasps emerged from smaller eggs of the original host, *Cuerna* sp., whereas up to 10-12 adult wasps emerged from larger eggs of GWSS. Under quarantine laboratory conditions (temperature 24°C, RH ca. 50%), the first two generations of *A. epos* developed from egg to adult within 20-21 days; for unknown reasons, it took the next two generations much longer (more than 30 days) to develop under the same conditions. Currently, this species is under quarantine evaluation as a potential biocontrol agent against GWSS in California.

Table 1. Species of egg parasitoids collected during 2004 and sent to University of California, Riverside quarantine.

Genus and species of egg parasitoid	Originally from: (State: locality)	Original or probable sharpshooter host	Propagated on GWSS at UCR quarantine (Yes/No)
Acmopolynema sema Schauff (Mymaridae)	GA: nr. Centerville	*?Homalodisca insolita* (Walker)	No
Gonatocerus ashmeadi Girault (Mymaridae)	GA: nr. Centerville	*H. coagulata / O. orbona*	No
	GA: Byron	*H. coagulata / O. orbona*	Yes
	NC: Garner	*H. coagulata*	No
	NC: North Myrtle Beach	*? H. coagulata*	No
	NC: nr. Warsaw	*H. coagulata*	No
	SC: Charleston	*H. coagulata*	Yes
	SC: nr. Yemassee	*H. coagulata / O. orbona*	Yes
Gonatocerus fasciatus Girault (Mymaridae)	GA: nr. Centerville	*H. coagulata / O. orbona*	No
	GA: Byron	*H. coagulata / O. orbona*	No
	NC: Garner	*H. coagulata*	No
	NC: nr. Greensboro	*?O. orbona*	No
	NC: nr. Warsaw	*H. coagulata*	No
Zagella spirita (Girault) (Trichogrammatidae)	GA: Byron	*H. coagulata / O. orbona*	No (failed)
Ufens new species (Trichogrammatidae)	GA: Byron	*H. coagulata / O. orbona*	No (failed)
Paracentrobia acuminata (Ashmead) (Trichogrammatidae)	GA: nr. Centerville	*?H. insolita/ ?Cuerna costalis*	No

Objective 2
As a result of the exploratory work conducted during the reported period, numerous specimens of proconiine sharpshooters and of their egg parasitoids were collected and preserved in ethanol with appropriate labels; many of these were critically point-dried from ethanol, point- or card-mounted, labeled, and identified to genera and species. Representatives of some species (of both sexes) were selected, dissected, and slide-mounted. The specimens were deposited in the collections of Entomology Research Museum, UC Riverside.

CONCLUSIONS
This is the next step in the development of a "classical" biological control program for the reduction of glassy-winged sharpshooter (GWSS) densities in California as a cornerstone for an IPM program to manage GWSS. As the result of our surveys conducted during 1997-2004, several previously unknown proconiine sharpshooter host associations were discovered for various species of Mymaridae and Trichogrammatidae. We concluded searching for egg parasitoids of GWSS in the Nearctic part of its distribution range. Next year, our exploratory efforts will focus on the southernmost part of the distribution range of GWSS in southern Mexico, which is in the Neotropical region.

REFERENCES

Hoddle, M. S. and S. V. Triapitsyn. 2003. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA, pp. 261-262. In: Proceedings of the Pierce's Disease Research Symposium, December 8-11, 2003, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, P. Blincoe, R. Spencer, L. Houser, A. Ba & T. Esser), Copeland Printing, Sacramento, California, 323 p.

Triapitsyn, S. V. 2003. Taxonomic notes on the genera and species of Trichogrammatidae (Hymenoptera) - egg parasitoids of the proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Proconiini) in southeastern USA. Trans. Amer. Entomol. Soc. 129: 245-265.

Triapitsyn, S. V., D. J. W. Morgan, M. S. Hoddle and V. V. Berezovskiy. 2003. Observations on the biology of *Gonatocerus fasciatus* Girault (Hymenoptera: Mymaridae), egg parasitoid of *Homalodisca coagulata* (Say) and *Oncometopia orbona* (Fabricius) (Hemiptera: Clypeorrhyncha: Cicadellidae). Pan-Pacific Entomologist 79 (1): 75-76.

Vickerman, D. B., M. S. Hoddle, S. Triapitsyn, and R. Stouthamer. 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: morphology, DNA sequences, and reproductive compatibility. Biol. Cont. 31: 338-345.

DEVELOPMENT OF PEPTIDE ANTIBIOTIC-BASED CONTROL STRATEGIES FOR *XYLELLA FASTIDIOSA*

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ABSTRACT

Peptide antibiotics are short (generally less than 70 amino acid residue-long), pore forming peptides encoded by single genes. Because peptide antibiotics are 'gene-based' they can be produced directly at the target location where they are needed (e.g., grape stock). In this project, we are testing the hypothesis that peptide antibiotics such as cecropins A, B, and/or P1 can be used as an effective means to control or reduce the spread of *Xylella fastidiosa*-induced disease. During the reporting period, we have established the optimal growth and assay conditions for the *X. fastidiosa* bacterium. Under these optimal conditions, we found that cecropin A can effectively inhibit *X. fastidiosa* growth over a two-week period (initial concentration of 0.05µM). Longer-term growth inhibition was seen only when higher concentrations of cecropin A were used suggesting that the cecropin A is being degraded under the conditions of our assay. On the basis of the effectiveness of cecropin A against *X. fastidiosa*, a synthetic plant codon (i.e., *Arabidopsis thaliana*) optimized, cecropin A gene was synthesized. The product of this synthetic cecropin A gene was expressed using the baculovirus expression vector system (BEVS) in insect cells. In insect cells roughly 90 mg/liter of culture of biologically active cecropin A was produced by the recombinant baculovirus. Following confirmation of biological activity of the insect cell produced cecropin A, the synthetic cecropin A gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* and eventually grape stock. Four different recombinant pCAMBIA1305 vectors were generated (carrying either the pro or mature cecropin A gene fused to either an authentic insect- or plant- (rice glycine-rich protein) derived signal peptide sequence). We are currently in the process of generating transgenic *A. thaliana* using these pCAMBIA vectors. We believe that continuous expression (although potentially at relatively low levels) of cecropin A will be effective for reducing or inhibiting the growth of *X. fastidiosa* within the plant.

INTRODUCTION

Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Antibiotic inhibition of *X. fastidiosa* (at least 17 isolates tested) has been analyzed for six different antibiotics (ampicillin, kanamycin, neomycin, penicillin, streptomycin, and tetracycline) [1, 2]. These studies demonstrate that antibiotic treatment is potentially an effective method for the control of *X. fastidiosa*. Under field conditions, however, barriers between the antibiotic and bacterium, and degradation effects will require significantly higher application doses than those found effective in the laboratory. Such doses may be impractical especially for broad-spectrum antibiotics due to secondary effects (e.g., toxicity against mammalian red blood cells) and the risk of increasing resistance. Thus, although traditional antibiotics such as tetracycline are highly active, an effective delivery system to bring them in contact with *X. fastidiosa* in the plant or insect vector is not available.

Recently, a great deal of scientific effort is being put into the study of a second type of antimicrobial agent called peptide antibiotics. Peptide antibiotics have been identified from a wide range of organisms including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals. In general, peptide antibiotics are small (less than 50 amino acids), have a net positive charge, and are composed of 50% or more of hydrophobic amino acids [3, 4]. One class of peptide antibiotic is composed of so-called ribosomally synthesized peptides [5]. These peptides are encoded by single genes and synthesized by a protein complex (ribosome) that is found in all cells and processed following synthesis via common pathways [3, 6]. In other words, unlike traditional antibiotics, peptide antibiotics have the potential to be easily produced by common protein expression systems or in transgenic organisms (e.g., plants). Furthermore, because peptide antibiotics are "gene-based", they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters.

Some of the best-characterized peptide antibiotics are the cecropins. Cecropins were the first peptide antibiotics to be identified in an animal, the giant silkmoth *Hyalophora cecropia* [7, 8]. At least ten different cecropins have been isolated from lepidopteran (moths and butterflies) and dipteran (flies) insects [9, 10]. Cecropins are composed of a single chain of 35-39 common L-amino acids and do not contain disulfide bonds [10]. Cecropins are active against many Gram(-) bacteria and

some Gram(+) bacteria, but are inactive against eukaryotic cells at concentrations that are antimicrobial [4, 9, 11] and possibly at concentrations up to 300 times higher [8]. *X. fastidiosa* is a Gram(-) bacterium [12]. In Gram(-) bacteria, the antibacterial activities of cecropins A, B, and P1 are up to ten-times greater than tetracycline [9, 13]. Cecropins have a unique combination of characteristics (specificity, gene basis, small size, potency against Gram(-) bacteria, etc.) that may make them potentially ideal substances for the control of *X. fastidiosa* in GWSS.

OBJECTIVES
I.   Identify peptide antibiotics (cecropins) that are effective against *Xylella fastidiosa*
   i.   Determine the antibiotic sensitivity of *X. fastidiosa* to chemically synthesized cecropins
   ii.   Produce recombinant cecropins using baculovirus expression vectors
   iii. Determine the toxicity of cecropins against GWSS cells grown in culture
II.   Analyze the effectiveness of cecropins produced in transgenic *Arabidopsis*
   i.   Generate transgenic *Arabidopsis* expressing cecropin that is active against *X. fastidiosa*
   ii.   Determine the localization, yield, activity, and stability of plant-expressed cecropin
   iii. Analyze the effect of cecropin expression on the transgenic *Arabidopsis*
   iv.   Analyze the effectiveness of plant-expressed cecropin for the control of *X. fastidiosa* transmission

RESULTS AND CONCLUSIONS
In order to establish the optimal conditions for the growth, storage, and assay of *X. fastidiosa* (Temecula strain) in our laboratory, we tested three different media (PD3, PW, and GYE; see [14]) and various inoculation routines. In general, our procedures were modified from protocols established in the Bruce Kirkpatrick laboratory at U.C. Davis. Optimal conditions for the generation of bacterial (*X. fastidiosa*) lawns for agar disc diffusion assays were also determined. Of the three media that were tested, PD3 gave the fastest growth of *X. fastidiosa* in liquid medium (roughly 20-and 135-fold increases in the OD_{600} at 7 and 14 days post inoculation, respectively) and on agar plates (formation of a lawn by 7-10 days post seeding). In order to generate a lawn, 150 µL of a 14 day-old culture (OD_{600}=0.48-0.5) was spread onto a 10 cm-diameter plate containing PD3 agar medium.

Using the optimal growth conditions with PD3 medium, we examined the minimal inhibitory concentration (MIC assay) at which cecropins A, B, and P1 (commercially purified peptides) were effective in inhibiting the growth of *X. fastidiosa*. We found that cecropins A, B, and P1 were effective at partially inhibiting the growth of *X. fastidiosa* at concentrations that were equal to or greater than 0.05, 0.25, and 0.5 µM, respectively, at two weeks post inoculation (Table 1). In general, cecropin A was the most effective against *X. fastidiosa*. The effectiveness of the cecropins as well as kanamycin was reduced by three weeks post inoculation. This was speculated to be the result of antibiotic degradation.

Once the sensitivity of *X. fastidiosa* to the various cecropins was established, a codon-optimized (for *A. thaliana*) cecropin A gene (pro gene including the insect-derived signal peptide sequence) was synthesized using commercially synthesized oligomers. A comparison of the *A. thaliana*-optimized (upper) and authentic (lower) cecropin A gene sequences is as follows:

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ATGAACTTCTCTAGAATCTTCTTCTTCGTGTTCGCTTGCCTCACTGCTCTCGCTATGGTGAACGCTGCTCCTGAGCCTAAGTGGAAGCTCTTCAAGAAGA 100
ATGAACTTTTCGAGGATCTTTTTCTTCGTCTTCGCTTGCCTGACGGCTCTAGCAATGGTCAATGCGGCGCCGGAACCTAAATGGAAGTTATTCAAGAAGA 100
******** ** ** ***** ******** *********** ** ***** ** ***** ** ** ** ** ** ***** ****** * **********

TCGAGAAAGTGGGTCAGAACATCAGAGATGGAATCATCAAGGCTGGACCAGCTGTGGCTGTGGTGGGACAGGCTACACAGATCGCTAAGGGTTGA 195
TTGAGAAAGTCGGTCAGAACATTCGAGATGGCATCATCAAAGCTGGCCCAGCCGTCGCTGTTGTAGGCCAGGCAACACAGATTGCTAAGGGTTAA 195
** ******* ******** ***** ***** ** ***** ** ** ***** ******* ******** *********** ********** *
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Of the 195 nucleotides that encode the pro gene, 33 nucleotides were mutated for optimal expression in *A. thaliana* (and putatively in grape stock). The synthesized gene was directionally cloned into the baculovirus transfer vector pAcUW21 at the *Bgl*II and *Eco*RI sites. Subsequently, the recombinant transfer vector was used to generate a recombinant baculovirus (vAcCecA) expressing the cecropin A gene using standard procedures. Expression of biologically active cecropin A was confirmed by minimal inhibitory concentration assays using *E. coli* by comparison of vAcCecA- or wildtype AcMNPV-infected insect Sf-21 cell culture supernatants or cell extracts (Table 2). These experiments confirmed that the synthetic gene encoded a functional peptide and that this peptide was correctly processed in insect-derived cells. vAcCecA expressed high levels (roughly 90 mg/liter of insect cell culture (2×10^6 cells/mL)) of cecropin A.

Following confirmation that the synthetic gene produces biologically active cecropin A, the synthetic gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* (and eventually grape stock). Four different recombinant pCAMBIA1305 vectors were generated by PCR-amplification as follows:

1. pro cecropin A sequence with authentic insect signal peptide sequence
2. pro cecropin A sequence with rice glycine rich protein and authentic insect signal peptide sequences
3. mature cecropin A sequence with rice glycine rich protein signal peptide sequence

4. mature cecropin A with no signal peptide sequence

The authenticity of the PCR-amplified sequences was confirmed by nucleotide sequencing in both directions and the constructs are currently being used to generate transgenic *A. thaliana* by standard procedures.

Table 1. Effect of cecropins and kanamycin against the growth of *X. fastidiosa*

	Concentration (μM)	Increase in bacterial concentration in comparison to cultures lacking antibiotic		
		Week 1 (% ± s.d.)	Week 2 (% ± s.d.)	Week 3 (% ± s.d.)
cecropin A	0.5	69 ± 3	47 ± 47	64 ± 42
	0.25	72 ± 10	80 ± 21	117 ± 5
	0.1	103 ± 13	68 ± 2	87 ± 25
	0.05	110 ± 46	50 ± 1	91 ± 22
cecropin B	0.5	69 ± nd	114 ± 6	87 ± 45
	0.25	63 ± 31	75 ± nd	110 ± 15
	0.1	72 ± 101	128 ± 63	90 ± nd
	0.05	93 ± 17	101 ± 18	74 ± 10
cecropin P1	0.5	98 ± 18	70 ± 40	70 ± 62
	0.25	82 ± 18	98 ± nd	120 ± 17
	0.1	111 ± 52	93 ± 24	72 ± 24
	0.05	93 ± 10	99 ± 22	73 ± 18
kanamycin	2	11 ± 3	9 ± 8	16 ± 2
	1	19 ± 8	32 ± 39	33 ± 22
	0.5	42 ± 16	77 ± 9	103 ± 16
	0.25	60 ± 13	72 ± 17	105 ± 12

nd = not determined

Table 2. Effect of recombinant cecropin A on the growth of *E. coli*

Source of recombinant cecropin A	Inoculum dose (bacteria/mL)	Inhibition (%)
Sf21 cell pellet (1 x 10^5 cells)	1.1 x 10^3	3.1 ± 13.2
Sf21 cell supernatant (undiluted)	1.1 x 10^3	99.7 ± 0.1
Sf21 cell supernatant (undiluted)	1.0 x 10^4	57.9 ± 1.6
Sf21 cell supernatant (undiluted)	8.5 x 10^4	51.6 ± 0.2
Sf21 cell supernatant (undiluted)	7.3 x 10^5	13.1 ± 0.1
Sf21 cell supernatant (1:5 diluted)	7.0 x 10^5	11.1 ± 0.2
Sf21 cell supernatant (1:10 diluted)	7.0 x 10^5	2.5 ± 0.1

REFERENCES
1. Darjean, D.H., E.L. Civerolo, and B.C. Kirkpatrick, *In vitro growth inhibition of Xylella fastidiosa by selected metallic plant micronutrients and antibiotics.* Phytopathology, 2000. **90**(6 Supplement): p. S17-S18.
2. Lacava, P.T., et al., *RAPD profile and antibiotic susceptibility of Xylella fastidiosa, causal agent of citrus variegated chlorosis.* Letters in Applied Microbiology, 2001. **33**(4): p. 302-306.
3. Hancock, R.E.W. and G. Diamond, *The role of cationic antimicrobial peptides in innate host defences.* Trends in Microbiology, 2000. **8**(9): p. 402-410.
4. Tossi, A., L. Sandri, and A. Giangaspero, *Amphipathic, alpha-helical antimicrobial peptides.* Biopolymers, 2000. **55**(1): p. 4-30.
5. Hancock, R.E.W. and D.S. Chapple, *Peptide antibiotics.* Antimicrobial Agents and Chemotherapy, 1999. **43**(6): p. 1317-1323.
6. Mor, A., *Peptide-based antibiotics: A potential answer to raging antimicrobial resistance.* Drug Development Research, 2000. **50**(3-4): p. 440-447.

7. Hultmark, D., et al., *Insect Immunity: Purification and properties of three inducible bacterial proteins from hemolymph of immunized pupae of Hyalophora cecropia.* European Journal of Biochemistry, 1980. **106**: p. 7-16.
8. Steiner, H., et al., *Sequence and specificity of two antibacterial proteins involved in insect immunity.* Nature (London), 1981. **292**(16): p. 246-248.
9. Boman, H.G., *Cecropins: antibacterial peptides from insects and pigs*, in *Phylogenetic Perspectives in Immunity*, C. Janeway, Editor. 1994, Lanes Biomed.: Austin.
10. McDonald, C., *Cecropins: A Class of Lytic Peptides.* 1997, Pacific West Cancer Fund: Seattle. p. 1-24.
11. Hancock, R.E.W. and R. Lehrer, *Cationic peptides: A new source of antibiotics.* Trends in Biotechnology, 1998. **16**(2): p. 82-88.
12. Hendson, M., et al., *Genetic diversity of Pierce's disease strains and other pathotypes of Xylella fastidiosa.* Applied and Environmental Microbiology, 2001. **67**(2): p. 895-903.
13. Boman, H.G., *Peptide antibiotics and their role in innate immunity.* 1995. p. 61-92.
14. Campanharo, J.C., M.V.F. Lemos, and E.G.D. Lemos, *Growth optimization procedures for the phytopathogen Xylella fastidiosa.* Current Microbiology, 2003. **46**(2): p. 99-102.

FUNDING AGENCIES

MICROBIAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER
WITH ENTOMOPATHOGENIC FUNGI

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Reporting Period: The results reported here are from work conducted from April 2004 to September 2004.

ABSTRACT
Objectives of our study were to search for fungal pathogens of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) and evaluate their potential against the host. Searches within citrus orchards in Tulare and Riverside counties revealed no natural infections of entomopathogenic fungi in GWSS populations. Entomopathogenic fungi were also absent in cadavers of GWSS periodically collected from Riverside citrus orchards (courtesy CDFA) when incubated in the laboratory under ideal conditions for fungal emergence. However, about 140 isolates of *Beauveria bassiana* (Balsamo) Vuillemin and four isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin, both hyphomycetous fungi, were isolated from soil in GWSS habitats and other insect hosts. Some of these isolates along with a Weslaco isolate of *B. bassiana* from GWSS and a commercial *B. bassiana* isolate have been tested against GWSS. Preliminary results indicate that GWSS is susceptible to high concentrations of these fungi.

INTRODUCTION
The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), native to the southeastern United States, is a serious pest of the California grape industry because it vectors *Xylella fastidiosa* (Wells et al. 1987), a xylem-limited bacterium that causes Pierce's disease (PD). Although PD has been in California for a long time, the introduction and rapid spread of GWSS made the situation worse. In addition to grapes, GWSS has a wide host range and spreads various diseases in those hosts caused by *X. fastidiosa*. Vector control or avoidance has been a key tactic in controlling PD. Widely practiced chemical control with imidacloprid and application of kaolin particles have their limitations. While kaolin particles, although non-toxic, can leave unwanted deposits on the harvested grape bunches, chemical insecticides have undesirable effects including human health, impact on non-target organisms, and environmental concerns. Moreover, use of chemical insecticides in citrus disrupts the successful, long-term control afforded by IPM of many different citrus pests (Grafton-Cardwell and Kallsen 2001). Use of microbial agents, such as entomopathogenic fungi, can be a viable alternative that is compatible with IPM practices. Entomopathogenic fungi invade the host by penetrating through the integument and are appropriate candidates for GWSS that has piercing and sucking mouthparts.

Entomopathogenic fungi have been isolated from GWSS (Mizell and Boucias 2002, Jones - personal communication) and other cicadellids (Galaini-Wraight et al. 1991, Hywel-Jones et al. 1997, Magalhaes et al. 1991, Matsui et al. 1998, McGuire et al. 1987). The purpose of our study is to discover additional isolates of entomopathogenic fungi active against GWSS.

OBJECTIVES
1. Conduct surveys to find fungal infections in GWSS populations or insects closely related to GWSS and isolate soilborne entomopathogens from GWSS habitats.
2. Culture and isolate the fungi and evaluate their pathogenicity against GWSS.
3. Evaluate the host range of fungi that infect GWSS.
4. Conduct small-scale field tests to evaluate selected pathogens against GWSS on citrus in fall and winter.

RESULTS
Natural Infections in GWSS Populations
Citrus orchards in Tulare and Riverside counties were surveyed, in vain, for infected GWSS. GWSS cadavers from CDFA collections in the Riverside area were periodically obtained and incubated in the laboratory for fungal development. No entomopathogenic fungus has so far been found from these cadavers. However, cultures of *Beauveria bassiana* (Balsamo) Vuillemin from infected GWSS collected in Texas by Jones and *Hirsutella* spp collected in Florida by Mizell and Boucias were received in the past two months for testing against California GWSS.

Isolation of Fungal Pathogens

Soil samples were collected from an organic citrus orchard and a conventional pomegranate orchard in Tulare Co, CA and a citrus orchard at AgOps at UC Riverside. Fungal pathogens were isolated using larvae of the greater wax moth, *Galleria mellonella* L. and by soil plating on selective media. Waxworms were incubated in Petri plates with moist soil samples and fungal pathogens were isolated from cadavers. Alternatively, aliquots of soil suspensions were plated on media selective for *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin. So far, 140 *B. bassiana* isolates and 4 *M. anisopliae* isolates have been isolated (Table 1). Additionally, *B. bassiana* was also isolated from the California harvester ant, *Pogonomyrmex californicus* Buckley, collected in Shafter, CA and the three-cornered alfalfa hopper, *Spissistilus festinus* (Say), collected in Parlier, California. Fungal isolates were cultured on selective and non-selective media to multiply the inoculum.

Table 1. Fungal pathogens isolated from citrus and pomegranate orchards and infected insects

Source	Method	*B. bassiana*	*M. anisopliae*
Organic citrus in Tulare Co	Waxworm bait	37	-
Pomegranate in Tulare Co	Waxworm bait	3	4
Riverside citrus	Waxworm bait	78	-
Riverside citrus	Selective media	22	-
California harvester ant	Selective medium	1	N/A
Three-cornered alfalfa hopper	Selective medium	1	N/A

Pathogenicity of Entomopathogenic Fungi to GWSS

Laboratory-reared or field-collected GWSS adults supplied by CDFA, Arvin were used for the bioassays. GWSS were either placed at -5° C for 5 min or exposed to CO_2 for 15 sec to immobilize them and were inoculated by rolling them in a 10 μL drop of conidial suspension. Controls were treated with 0.01% of SilWet, an adjuvant used for preparing conidial suspensions. GWSS were individually incubated in a Petri plate with an excised citrus leaf and a moist filter paper. Petri plates were placed in a plastic box with moist paper towels and incubated at 27° C and 16:8 L:D photophase. GWSS were observed daily for mortality. Dead GWSS were surface sterilized in 3% sodium hypochlorite solution followed by rinsing in deionized water and incubated in sealed Petri plates on water agar or moist filter paper at 27° C in the dark.

Bioassay 1

The isolate of *B. bassiana* from *P. californicus* (PcBb1) was tested against laboratory-reared GWSS at four concentrations 10^1, 10^3, 10^5, and 10^7 conidia/ml in comparison with controls. Each treatment and control had 10 adult GWSS. Infections were observed only at higher concentrations with 50% infection in GWSS treated with 10^7 conidia/ml and 10% in those treated with 10^5 conidia/ml.

Bioassay 2

Five *B. bassiana* isolates and a *M. anisopliae* isolate were tested against field-collected GWSS at four concentrations of 10^3, 10^5, 10^7, and 10^9 (or 10^8 in case of *M. anisopliae*) conidia/ml along with untreated and SilWet (0.01%) treated controls. Isolates of *B. bassiana* included one from *P. californicus* (PcBb1), two from soil samples from citrus orchards in Tulare (GmBb25) and Riverside (GmBb41) counties, CA, one from *H. coagulata* in Weslaco, TX (TxBb) and a commercial isolate (designated GHA). The isolate of *M. anisopliae* (GmMa1) was from a soil sample from the pomegranate orchard in Tulare Co, CA. Each treatment and controls had 20 GWSS. Although all tested isolates were infective (Figures 1 and 2), all GWSS in this bioassay, including controls, suffered from a high mortality.
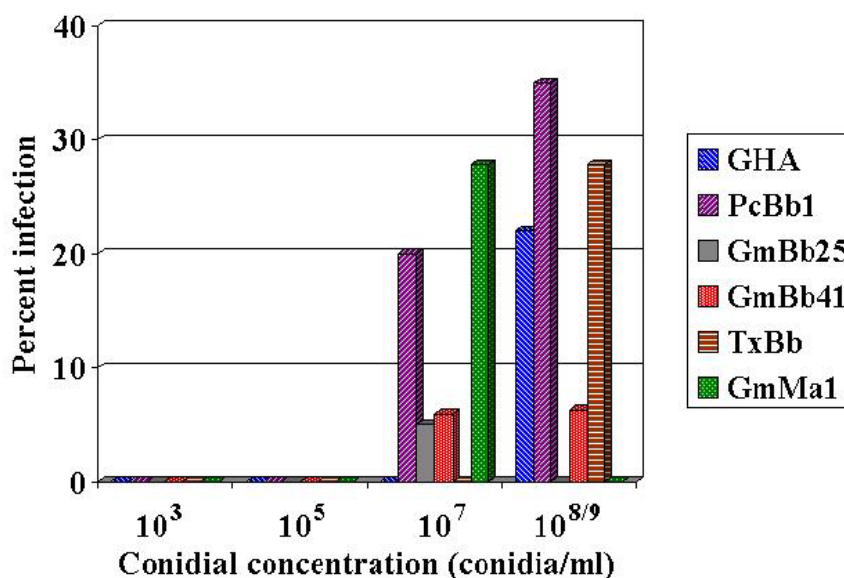

Figure 1. Pathogenicity of *B. bassiana* and *M. anisopliae* to GWSS

Figure 2. GWSS killed by *B. bassiana* and *M. anisopliae.*

Bioassay 3

This assay was conducted using only 10^9 conidia/mL concentration and 10 laboratory-reared GWSS per isolate. All the isolates from the previous bioassay were used in this assay except for PcBb1, which was replaced by the *B. bassiana* isolate from *S. festinus* (SfBb1). This assay had also suffered from very high mortality and all the insects died within 5 days after the treatment. Fungal infection was seen in only one GWSS cadaver treated with SfBb1.

CONCLUSIONS

The fact that GWSS is susceptible to entomopathogenic fungi such as *B. bassiana* is promising. Although infections occurred only at relatively high concentrations, there is enough variability in *B. bassiana* as a species to suggest other isolates may be more virulent. Efforts will continue to obtain isolates from collaborators and from likely GWSS host habitat in California for further laboratory evaluation and eventual field application.

REFERENCES

Galaini-Wraight,S., S.P. Wraight, R.I. Carruthers, B.P. Magalhaes, and D.W. Roberts. 1991. Description of a *Zoophthora radicans* (Zygomycetes, Entomophthoraceae) epizootic in a population of *Empoasca kraemeri* (Homoptera, Cicadellidae) on beans in central Brazil. J. Invertebr. Pathol. 58: 311-326.

Grafton-Cardwell, E.E. and C. Kallsen. 2001. Efficacy of insecticides used for glassy-winged sharpshooter control in citrus. Pierce's Disease Research Symposium, Dec 5-7, 2001. pp. 32-34.

Hywel-Jones, N.L., H.C. Evans, and Y. Jun. 1997. A re-evaluation of the leafhopper pathogen *Torrubiella hemipterigena*, its anamorph *Verticillium hemipterigenum* and *V. pseudohemipterigenum* sp. nov. Mycol. Res. 101: 1242-1246.

Magalhaes, B.P., R.A. Humber, E.J. Shields, and D.W. Roberts. 1991. Effects of environment and nutrition on conidium germination and appressorium formation by *Zoophthora radicans* (Zygomycetes, Entomophthorales): a pathogen of the potato leafhopper (Homoptera, Cicadellidae). Environ. Entomol. 20: 1460-1468.

Matsui, T., H. Sato, and M. Shimazu. 1998. Isolation of an entomogenous fungus, *Erynia delphacis* (Entomophthorales: Entomophthoraceae), from migratory planthoppers collected over the Pacific Ocean. Appl. Entomol. Zool. 33: 545-549.

McGuire, M.R., M.J. Morris, E.J. Amburst, and J.V. Maddox. 1987. An epizootic caused by *Erynia radicans* (Zygomycetes: Entomophthoraceae) in an Illinois *Empoasca fabae* (Homoptera: Cicadellidae) population. J. Invertebr. Pathol. 50: 78-80.

Mizell, R.F. and D.G. Boucias. 2002. Mycopathogens and their exotoxins infecting glassy-winged sharpshooter: survey, evaluation, and storage. Pierce's Disease Research Symposium, Dec 15-18, 2002. pp. 161-162.

Wells, J.M., B.C. Raju, H.Y. Hung, W.G. Weisburg, L. Mandelco-Paul and D.J. Brenner. 1987. *Xylella fastidiosa* gen. nov., sp. nov.: gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. 37: 136-143.

a

b

IDENTIFICATION OF MECHANISMS MEDIATING COLD THERAPY OF
XYLELLA FASTIDIOSA-INFECTED GRAPEVINES

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ABSTRACT

Preliminary xylem sap composition studies were conducted in February 2004 using Cabernet sauvignon and Pinot noir grapevines growing in Placerville (cold winter temperature) and UC Davis (warmer temperatures). The pH of xylem sap from both varieties was almost a full unit lower in vines grown in cold temperatures versus warm. A similar trend also occurred with sap osmolarity, however the differences were not as great. Because these vines were grown under different management practices and on different rootstocks these results must be considered preliminary. In 2004 we established four field sites in Shasta, Placer, Mendocino and Yolo counties to repeat these measurements on clonal vines that were grown in 5-gallon pots at University of California, Davis. One-half of the vines were inoculated with *Xf* while the other half is un-inoculated controls. Sap will be collected from the vines during the late winter and pH, osomolarity, carbohydrates, organic acids and abscisic acid (ABA) will be measured and compared. The vines will be returned to University of California, Davis at bud break and observed for the development of PD symptoms and tested by PCR to determine if any of the vines were "cold cured" of their infection. Similar experiments using potted vines that will be exposed to defined cold temperature regimes in cold storage facilities located at University of California, Davis will be conducted in 2005. Proteins present in the collected xylem sap will be analyzed by PAGE and the identity of major or unique xylem sap proteins will be determined by sequencing them. *Xf* viability studies using buffers of various pHs, xylem sap from warm- and cold-treated vines will also be studied. The goal of this research is to understand the physiological/biochemical basis of cold therapy that was first documented by A.H. Purcell.

INTRODUCTION

The geographical distribution of Pierce's disease (PD) in North America is strongly associated with the severity of winter temperatures, i.e. PD does not occur in New York, the Pacific Northwest nor at high altitudes in S. Carolina, Texas and even California (Hopkins and Purcell, 2002). Sandy Purcell demonstrated that relatively brief exposures to sub-freezing temperatures can eliminate *Xylella fastidiosa* in some percentage of cold treated *V. vinifera* grapevines, however some of the coldest temperatures he used killed the vines (Purcell 1977, 1980). He also found that a higher percentage of vines that were moderately susceptible to PD such as Cabernet sauvignon, were cured by cold therapy treatments compared to susceptible varieties such as Pinot noir. Purcell's group also showed that whole, potted vines exposed to low temperatures had a higher rate of recovery than PD-affected, detached bud sticks exposed to the same cold temperatures (Feil, 2002). Clearly, some factor(s) that were expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate *Xf*. If such factor(s) are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

OBJECTIVES

1. Develop an experimental, growth chamber temperature regime that can consistently cure Pierce's disease affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osomolarity, total organic acids, proteins and other constituents that occur in the xylem sap of cold-treated versus non-treated susceptible and less susceptible *Vitis vinifera* varieties.
3. Assess the viability of cultured *X. fastidiosa* cells growing in media with varying pH and osomolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

RESULTS AND CONCLUSIONS
Objective 1

The same varieties used by Purcell (1977, 1980) and Feil (2002) in previous cold therapy studies, Pinot noir (PD-susceptible) and Cabernet sauvignon (moderately resistant to PD) grapevines grafted on 101-14 rootstock were inoculated with *Xf* in the spring of 2004 using a pinprick inoculation procedure (Hill and Purcell, 1995; Purcell and Saunders, 1999). The vines were grown in five gallon pots in a greenhouse using a nutrient-supplemented irrigation regime. Treatment vines were inoculated with the Stagg's Leap strain of *Xylella fastidiosa*, whereas control vines were inoculated with water. During late summer and fall, the plants were moved into a screen house in order to acclimatize them to decreasing temperatures. While in the screen

house, plants were watered by drip irrigation and supplemental fertilizer application until the first week of October 2004. Twelve weeks after inoculation, the plants were rated for symptom development.

During October/November, 2004, 11 inoculated and 11controls of each variety (44 plants total) were transported to 3 sites that were selected because of their relatively cold winter temperatures, as well as University of California, Davis which was the control. Plot sites include: Fall River (Shasta County), University of California Hopland Research Station (Mendocino County), and University of California, Blodgett Forest Research Station (Placer County). Potted grapevines were planted in the ground to the top of the pot in order to maintain uniform soil type, prevent roots in the pots from exposure to abnormally cold temperatures, and to prevent the plants from falling over. Plants were irrigated as needed until rain provided adequate moisture for the vines. Vines will be allowed to undergo natural dormancy during the fall and experience ambient temperatures during the winter. Temperature, ETo, and other weather data for each plot are being monitored using CIMIS weather data (http://wwwcimis.water.ca.gov/cimis/data.jsp). This data, and previous temperature profiles at these sites, will be used to determine a growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality. Additional grapevines, using the same varieties and inoculated as described above, but grown in 6 inches standard pots will be exposed to different temperature regimes in cold rooms located at the Department of Pomology, University of California, Davis during the winter/spring of 2005.

Objective 2
Preliminary work from Pinot noir and Cabernet sauvingnon field materials collected from Placer and Yolo counties showed some differences in xylem sap pH and osmolarity. These results were obtained from Pinot noir and Cabernet sauvingnon vines growing in one Placerville vineyard and at a vineyard at University of California Davis. Both varieties were grown in the same manner at each site, however management practices at the two sites were not identical. It is also important to note that the University of California Davis vines were grown on 5C rootstocks while the Placerville vines were not grown on rootstocks and that these vines were not the same clones. Dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. Differences were noted in xylem sap pH, abscisic acid concentration, and osmolarity. These same parameters will be further examined in 2005 in the field sites and growth chamber experiments. Although only preliminary findings, we found that the pH of xylem sap collected in late February was lower, 5.37 for Pinot and 5.23 for Cabernet vines at the Placerville site (colder winter temperatures) than vines growing at University of California Davis, 6.35 and 6.06, respectively. Small differences in osmolarity were also noted in xylem sap from Placerville, 55.2 and 55.5, versus the osomolarity of xylem sap from Davis vines, 58.3 and 60.8 respectively. The significance and reproducibility of these differences needs to be confirmed this winter using the more controlled experimental units.

During the 2005 winter months, field grown and growth chamber plants will be sampled for potential changes in pH, osmolarity, total organic acids, proteins and other constituents that occur in xylem sap. Our hypothesis is that changes in xylem sap components in vines that undergo cold treatment may have significant effects on *Xf* viability. Previous research on several plant species has shown that a number of plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998). In some plants, these freeze-induced proteins are structurally related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related proteins (Hon, et al. 1995; Kuwabara, et al, 2002). Thus it maybe possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*. To investigate this possibility, xylem sap will be expressed from cold-stressed and control vines using the pressure bomb, concentrated by freeze drying, and protein profiles determined by 1 and 2 dimensional polyacrylamide gel electrophoresis (PAGE). If unique proteins are found in the cold stressed plants these proteins will be cut from the gel, end terminally sequenced by the University of California Molecular Structure Facility and their sequences compared to others in the database. The potential effect of these proteins on *Xf* viability will be assessed as described in Objective 3.

Objective 3
We have been assessing the effect of many of the physical, physiological and biochemical parameters we determined in Objective 1 and 2 on *Xf* viability. We have been assessing the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media such as PD3 and new chemically defined media (Leite, et al., 2004). The liquid solutions used for these viability experiments included: water, extracted xylem sap, PD3, the Leite medium, HEPES, sodium and potassium phosphate buffers. In order to further examine these parameters, cultures of *X. fastidiosa* Stagg's Leap strain were grown at 28°C on PD3 for 11 days. Cells were scraped from the culture plates and suspended at concentrations of 1.5×10^7 bacteria per mL of liquid medium. One mL of the suspension was then placed into each 1.5 mL microcentrifuge tubes and placed at various temperatures. Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells. Results of these experiments indicate that *X.f.* can survive at -5ºC for 8 weeks. At lower temperatures, our results were similar to those found by Feil (2002). *Xf* survived the best in HEPES and sodium phosphate buffers and the worse survival occurred in waters and xylem sap at –5ºC. At –10 and –20ºC *Xf* rapidly died in all liquid media tested.

We also adjusted the pH of potassium phosphate buffer to the values determined for cold-stressed and control xylem saps collected from Placerville and University of California, Davis vines described previously. Cultures of *X. fastidiosa* Stagg's Leap strain were again grown at 28°C on PD3 for 11 days. Cells were harvested from culture plates and suspended at

concentrations of 1.5 x 10^7 bacteria per mL of potassium phosphate buffer. One mL of suspension was then placed into each 1.5 mL microcentrifuge tubes and placed at -5ºC. Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days colonies were counted to determine the effect of pH on the viability of the *Xf* cells. *Xf* survived the best in potassium phosphate at pH 6.6 and 6.8 and the poorest survival occurred at pH 5.0. There was significant variation between reps of these experiments so they are now being repeated; however it is interesting that these initial trends are consistent with the pH values of xylem saps extracted from Placerville, where PD is not know to occur, and saps from vines growing at Davis where *Xf* can overwinter in grapevines.

Objective 4
Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as abscisic acid (ABA), which induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998). Preliminary studies, involving samples of Pinot noir and Cabernet sauvingnon field materials collected from Placer and Yolo counties in February, 2004, showed abscisic acid concentrations were lower in the Placerville, cold-exposed vines, that vines from Davis. ABA concentrations were lower in Pinot than Cabernet for both Placerville and Davis vines. Again, it will be important to verify these initial findings using vines grown under more controlled environments in growth chambers during 2005.

We will determine the concentration of ABA in cold-stressed and control vines growing both in the growth chamber using the temperature regimes determined in Objective 1 and in the field-grown plants in the four sites described in Objective 1. We will also determine the pH, osomolarity and protein profiles of xylem sap from ABA-treated vs. non-treated vines and assess the potential of this sap for anti-*Xf* activity.

During the spring, summer and fall, Cabernet and Pinot vines will be sprayed with 100uM solutions of ABA, a concentration that elicited cold-shock proteins at 23ºC in winter wheat (Kuwabara, et. al 2002). Additional concentrations up to 500uM may also be evaluated if no response is noted at 100uM. The pH and osmolarity of xylem sap from the treated vines will be determined as described above. The concentration of ABA in the sap will be determined using a commercially available immunoassay that has a sensitivity of 0.02-0.5 picomole/0.1 mL (Plant Growth Regulator Immunoassay Detection Kits, Sigma Chemical Co.). Preliminary work has shown that ABA concentrations in grapevine xylem sap are detectable using this kit. Xylem sap proteins will be collected, concentrated and analyzed by 1 and 2 dimensional PAGE as previously described. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced and analyzed as previously described.

REFERENCES
Bravo, L.A., Zuniga, G.E., Alberdi, M., and Carcuera, L.S. 1998. The role of ABA in freezing tolerance and cold acclimation in barley. Physiol. Plant. 103: 17-23.
Feil, H., 2002. Effect of sub-freezing temperature on the survival of *Xylella fastidiosa* in vitro and in plants. Ph.D. dissertation, University of California, Berkeley.
Hill, B.L. and Purcell, A.H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopathol. 85: 1368-1372.
Kuwabara, C., Takezawa, D., Shimada, T, Hamada, T., Fujikawa, S., and Arakawa, K. 2002. Abscisic acid- and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mold, *Microdochium nivali*. Physiol. Plant. 115: 101-110.
Purcell, A.H. 1977. Cold therapy of Pierce's disease grapevines. Plant Dis. Reptr. 61: 514-518.
Purcell, A.H. 1980. Environmental therapy for Pierce's disease of grapevines. Plant Dis. 64: 388-390.
Purcell, A.H. and Saunders, S.R. 1999. Fate of Pierce's disease strains of *Xylella fastidiosa* in common riparian plants in California. Plant Dis. 83: 825-830.
Thomashow, M.F. 1998. Role of cold responsive genes in plant freezing tolerance. Plant Physiol. 118:1-7.