POTENTIAL ISOLATES OF THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* FOR GLASSY-WINGED SHARPSHOOTER CONTROL

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ABSTRACT
Two California isolates and a Texas isolate of the entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, demonstrated their potential as effective pathogens of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (formerly *H. coagulata*). Virulence of these isolates evaluated in the laboratory assays at different conidial concentrations and in small caged tests was similar. Adult GWSS feeding on plants sprayed with fungal inoculum were infected and killed by the fungus in the caged tests. When conidia were exposed to sun light and assessed for their viability, the two California isolates appeared to be more tolerant of solar radiation.

INTRODUCTION
A collaborative project between UC Davis and USDA-ARS is aimed at identifying suitable entomopathogenic fungi for the control of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (formerly *H. coagulata*), a pest that threatens the grape industry in California as a vector of the Pierce's disease causing bacterium, *Xylella fastidiosa*. Entomopathogenic fungi, which enter the host through the cuticle, are ideal candidates for insects like GWSS with piercing and sucking mouthparts. Entomopatho-genic fungi were isolated from GWSS in the Southeast US (Mizell and Boucias 2002, Kanga et al. 2004). But no fungal pathogen has so far been reported in California GWSS populations. However, we recovered several isolates of two generalist fungi, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin, from GWSS habitats in California and tested them against GWSS (Dara et al. In Press, Kaya et al. 2004, 2005). We also isolated *B. bassiana* from California harvester ant, *Pogonomyrmex californicus* (Buckley), three-cornered alfalfa hopper, *Spissistilus festinus* (Say) and a darkling beetle from Kern, Fresno and Riverside counties, respectively. These isolates were evaluated, along with a Texas isolate and the commercial isolate, for their virulence to adult GWSS and ability to grow at different temperatures (Dara et al. In Press). Based on the results, two California isolates – recovered from the three-cornered alfalfa hopper and a soil sample from a citrus orchard in Riverside Co – and the Texas isolate were further evaluated for their virulence at different concentrations and efficacy to infect GWSS when sprayed on the plants. Viability of these isolates following exposure to solar radiation and pathogenicity to selected natural enemies were also evaluated.

OBJECTIVES
1. Conduct surveys to find fungal infections in GWSS populations or insects closely related to GWSS.
2. Culture and isolate the fungi and evaluate their pathogenicity against GWSS.
3. Assess environmental effects like temperature and sunlight on conidial survival and germination, fungal growth, and infectivity.
4. Evaluate the host range of fungi that infect GWSS.
5. Conduct small-scale caged tests to evaluate selected pathogens against GWSS.

RESULTS
Natural infections in GWSS populations
We continue to search for natural infections in GWSS populations in southern California. GWSS adults were periodically collected in the urban areas around Bakersfield on Chinese photinia, prostrate acacia, oleander and crepe myrtle. These insects were maintained in the laboratory for the bioassays. No entomopathogenic fungi have been found in these insects.

Virulence of entomopathogenic fungi to GWSS:
Beauveria bassiana
Laboratory-reared GWSS adults supplied by CDFA, Riverside were used for the bioassays. The two California isolates and the Texas isolate of B. bassiana were evaluated against adult GWSS at three fungal concentrations - 105, 107 and 109 conidia/ml. GWSS were anesthetized by exposing them to CO_2 for 20 sec and then inoculated by rolling them in a 10 µl drop of conidial suspension. Controls were treated with 0.01% of Silwet, an adjuvant used to prepare conidial suspensions. GWSS were incubated on potted cowpea plants covered with cylindrical cages and their mortality was recorded daily for two weeks. Cadavers were surface sterilized in 3% sodium hypochlorite solution and incubated on water agar for fungal emergence. These assays were repeated twice. There were significant differences ($P < 0.05$) in the infections caused at different concentrations within each isolate (Figs. 1 and 2). But there was no significant difference among the isolates.

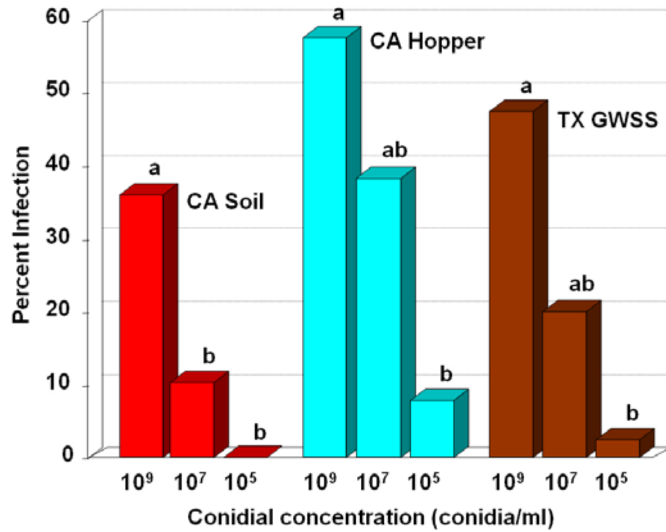

Figure 1. Virulence of selected *B. bassiana* isolates to GWSS at different concentrations

Figure 2. Sporulating cadaver of GWSS infected by a California isolate of *B. bassiana.*

Molecular characterization

Different fungal isolates used against GWSS in our study were characterized using molecular techniques for a better understanding of the isolate identity and the variation in their virulence (Fig. 3). Genetic relatedness of *B. bassiana* isolates from California, Texas and Mississippi were compared with the commercial isolate GHA using single sequence repeat (SSR) markers or microsatellites as described by McGuire et al. (2006). Fungal cultures were grown on Sabouraud dextrose agar enriched with yeast extract, and DNA was extracted using MagAttract 96 DNA Plant kit (Qiagen, Valencia, CA) and a Rectsch MM301 Mixer Mill (Retsch, Germany). Seven PCR primer pairs (Ba01, Ba02, Ba03, Ba05, Ba06, Ba08, and Ba12) which flank SSR markers were used for the molecular characterization of these fungal pathogens. To evaluate the pattern of genetic similarities among the selected isolates of the fungal pathogens, pair-wise genetic similarity coefficient was calculated based on Jaccard's similarity coefficient (Jaccard 1908). A dendrogram was constructed using the neighboring join (N-J) clustering analysis (Saitou and Nei 1987) with midpoint rooting method. All statistical analysis and the construction of the dendrogram were performed using the numerical taxonomy and multivariate analysis system (NTSYS-pc) version 2.1 (Rohlf 2002).

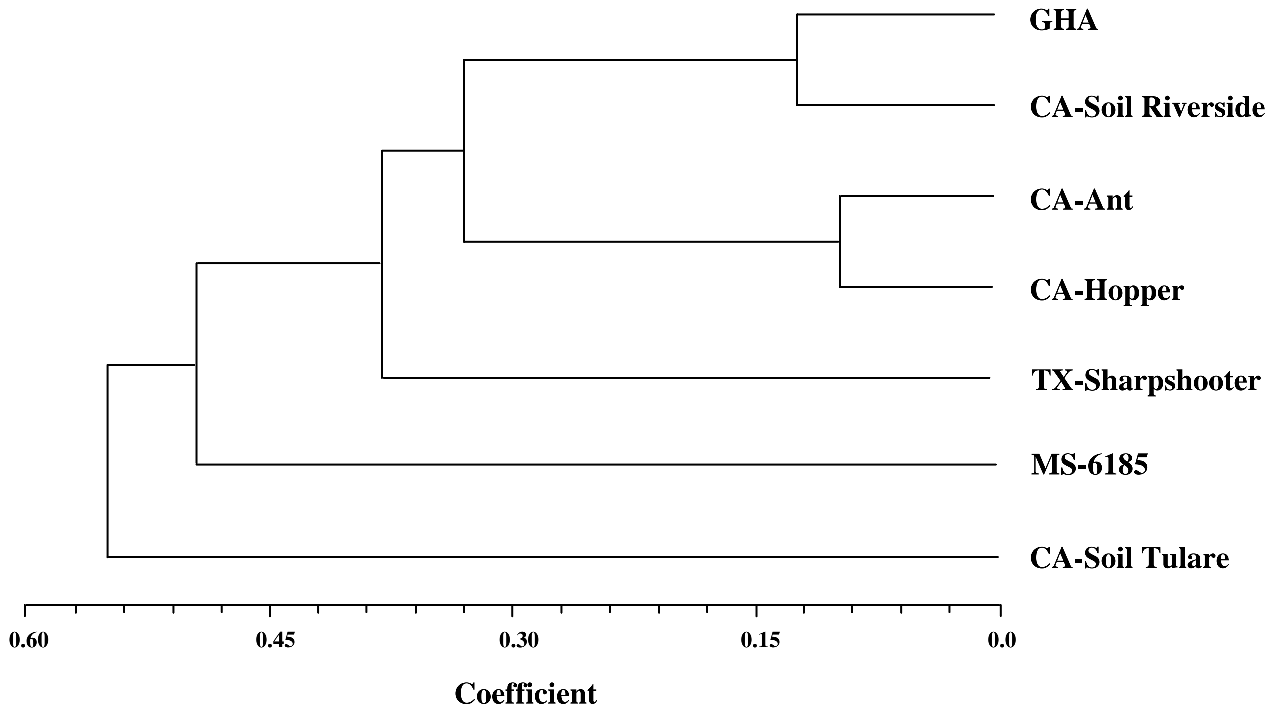

Figure 3. Genetic relatedness of *B. bassiana* isolates based on seven SSR markers

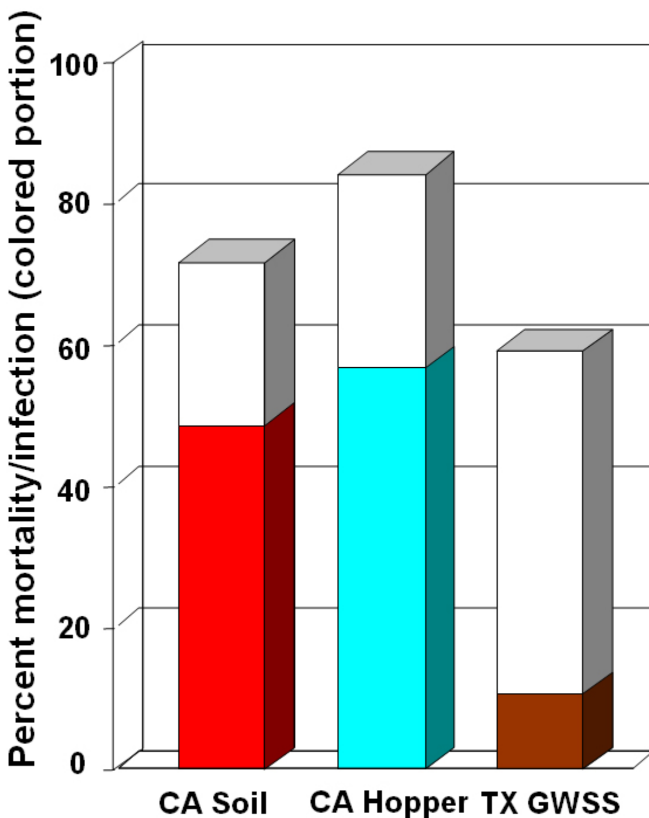

Figure 4. Mortality and infection caused by *B. bassiana* isolates in GWSS feeding on treated plants

Effect of solar radiation on the viability of selected *B. bassiana* isolates
The effect of temperature on the radial growth of different fungal isolates was previously reported (Dara et al. In Press). The effect of exposing the fungal inoculum to solar radiation for 1, 2, 4 and 6 h on the viability of the three selected isolates of *B. bassiana* was evaluated in this study. Treatments included $1X10^9$ conidia/ml suspensions of Texas, three-cornered alfalfa hopper and Riverside soil isolates in 0.01% Silwet. A 25 µL conidial suspension was dispensed on a 18X18 mm coverslip as several droplets and allowed to dry under the hood for 30 min. Each treatment had four coverslips placed on a 60 mm filter paper in a 90X15mm Petri plate bottom. Petri plates were arranged on a plastic tray in a radomized complete block design and were partially covered with the lids to prevent heat build up. Tray was placed about 3 m away from the pyranometer at 1 m height on a flat surface. Solar radiation was measured using an Eppley Black and White Pyranometer (645-48) (Eppley Laboratory, Inc., Newport, RI) located at the weather station at the Shafter Research and Extension Center. Pyranometer had a quartz dome which measures the wavelengths from 200 to 4500 nanometers. At designated time intervals, one plate of each treatment was brought back to the laboratory and conidia from one of the coverslips were washed into 1 ml 0.01% Silwet. This conidial suspension was added to 20 ml PDB with gantamicin (100 mg/L) and incubated on a rotary shaker at the room temperature. Germination of conidia was assessed after 24 and 48 h of incubation in PDB to determine the effect of solar radiation on fungal viability. All isolates withstood exposure to solar radiation for up to 4 hours. However, viability was higher for both California isolates compared to the Texas isolate at all time intervals. Between the two California isolates, the isolate from the three-cornered alfalfa hopper advanced more into successive stages of development compared to the soil isolate.

Pathogenicity of the isolates to natural enemies
Pathogenicity of the three isolates of *B. bassiana* to the convergent lady beetle, *Hippodamia convergence* Guérin-Méneville and the egg parasitoid, *Gonatocerus ashmeadi* Girault, was determined in two different assays. Adult lady beetles were purchased from InsectLore (Shafter, CA) and parasitoids were provided by CDFA, Riverside. Treatments included $1X10^9$ conidia/ml suspensions of the three selected isolates and Silwet 0.01% as a treated control along with an untreated control. Insects were anesthetized by exposing to CO_2 and inoculated by dipping in conidial suspension. Lady beetles were incubated in Petri plates (90X20 mm) with two screened vents and provided with soaked raisins and strands of paper. Parasitoids were also incubated in similar plates and provided with strips of tissue (Kimwipes) soaked in 50% honey solution. All three isolates were pathogenic to both the lady beetles and the parasitoids.

Efficacy of the selected *B. bassiana* isolates in the cage tests
The three selected isolates of *B. bassiana* were evaluated in cage tests that were repeated thrice. GWSS were collected on prostrate acacia and Chinese photinia in the Bakersfield area and maintained in the laboratory on euonymus plants until used in the test. About a month-old cowpea plants were individually sprayed iwth a 40 ml conidial suspension containing $1x10^{10}$ viable conidia in 0.01% Silwet, an adjuvant. Plants were dried under shade for 15-20 min before placing them in a cage (BugDorm from BioQuip). Fifty adult GWSS were placed in each cage. Each isolated had only one plant due to the limited availability of GWSS. A plant treated with Silwet was used as a control. Cages were maintained under the laboratory conditions where average temperature was $26.1 \pm 4.0\ ^oC$, relative humidity fluctuated between 36 and 62% with an average of 42%, and a 16L:8D photoperiod. Mortality of the insects was monitored for two weeks. Cadavers were surface sterilized in 3% sodium hypochlorite solution, followed by rinsing in deionized water, and incubated on 1% water agar at 28 ^oC for fungal emergence. Fungal growth on the cadavers was microscopically examined to determine infection.

Virulence of the three isolates was similar in the caged tests (Fig. 4). However, fungal emergence, in general, occurred more in insects exposed to the three-cornered alfalfa hopper isolate compared to the other isolates. Several of the GWSS feeding

on plants treated with the Texas and the Riverside soil isolate died within one day after the treatment in two tests. It usually takes 3-5 days for the fungus to infect and kill the insects. Suspecting a fungal toxin for this rapid mortality, an assay was conducted using cell free fungal extracts where treated insects did not die ruling out any toxins (results not shown). The exact cause of the rapid mortality in the cage tests was undetermined. However, this would be useful in controlling the insects.

Pseudogibellula formicarum

We previously described the non-pathogenicity of the fungus, *Pseudogibelluala formicarum* (Mains) Samson & Evans, to GWSS in an earlier CDFA report. Because the genus *Pseudogibellula* has species that are pathogenic to invertebrates and *P. formicarum* was isolated from GWSS cadavers in Mississippi, we tested this particular isolate against GWSS in Caifornia. Below, we provide the results of our findings again.

The fungus, *P. formicarum* recovered from GWSS cadavers in Mississippi, was tested in two assays. As this fungus could not be cultured on standard microbial culture media, conidia scraped from the cadavers were added to 0.01% Silwet to prepare the inoculum suspension. In the first assay, CO_2 anesthetized GWSS adults were rolled in 50 µL suspension (~10^6 conidia/ml) and individually incubated in clip cages attached to a euonymus plant. Untreated insects were used as controls. In the second assay, conidial suspensions were injected into adult GWSS at 0.5 µL/insect. Untreated insects and those treated with Silwet were used for comparison. Only five insects per treatment were used in both of these assays due to the limited numbers of insects available. In both assays, *P. formicarum* couldn't infect the treated GWSS. Another assay was also conducted to evaluate the pathogenicity of *P. formicarum* to daddy longlegs spiders (*Holocnemus pluchei*) where the fungus could not infect the spiders.

CONCLUSIONS

The Texas isolate and two California isolates - from the three-cornered alfalfa hopper and the Riverside soil – of *B. bassiana* have the potential to be microbial control agents of GWSS.

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GENETIC CHARACTERIZATION OF *GONATOCERUS TUBERCULIFEMUR* FROM SOUTH AMERICA UNCOVERS DIVERGENT CLADES: PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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ABSTRACT
In present study we genetically characterized the prospective South American egg parasitoid candidate, *Gonatocerus tuberculifemur* (Ogloblin) of the glassy-winged sharsphooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] for a neoclassical biological control program in California. Two molecular methods, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and a phylogeographic approach inferred by the mitochondrial cytochrome oxidase subunit I gene (COI). Five geographic populations from South America were analyzed; in addition, a phylogenetic analysis was performed with several named and two unnamed *Gonatocerus* Nees species. DNA fingerprinting uncovered a fixed geographic banding pattern difference in the population from San Rafael, Mendoza Province, Argentina. The COI analysis uncovered haplotype or geographic structure in *G. tuberculifemur*. A neighbor-joining distance tree clustered the populations into two well-supported distinct clades with very strong bootstrap values (96-100%) with the population from San Rafael clustering into a separate clade than the rest of the South American populations. No haplotype sharing was observed between individuals from the two clades. A phylogenetic analysis performed by the neighbor-joining method of 15 *Gonatocerus* Nees species confirmed species boundaries and again uncovered two distinct clades in *G. tuberculifemur* with very strong bootstrap support (96-100%). The two molecular methods were in accord and the evidence is suggestive of a species level divergence. Because *G. tuberculifemur* is under consideration as a potential biological control agent for the invasive GWSS in California, understanding possible cryptic variation of this species is critical.

INTRODUCTION
Uncertainty exists as to whether egg parasitoids native to California will be as effective against the glassy-winged sharpshooter (GWSS) *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)]as they are in their co-evolved native range (Jones 2001, Logarzo et al. 2003, 2004, Virla et al. 2005). As a consequence, beginning in 2000, egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2005). In surveys conducted in Argentina and Chile during 2000 through 2005, two prospective egg parasitoid candidate agents were identified among several *Gonatocerus* Nees species reared from *T. rubomarginata* (Jones et al. 2005, Logarzo et al. 2005, Virla et al. 2005). The egg parasitoid candidates from South America were identified by S. V. Triapitsyn (UC-Riverside) as *Gonatocerus tuberculifemur* and *G. metanotalis* (Ogloblin) (Hymenotpera: Mymaridae). *Gonatocerus tuberculifemur* is now being permitted for release in California (CDFA 2005). Mymarid wasps are the best-known egg parasitoids for controlling populations of leafhoppers (Huber 1986, Döbel and Denno 1993). Molecular studies of insects are becoming increasingly important in resolving taxonomic relationships critical to the success of biological control programs. Identifying the correct natural enemy is critical to the success of classical biological control programs. Lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVE
The aim of the present study was to survey molecular methods useful in egg parasitoid identification and discrimination and investigate the possibility that *G. tuberculifemur* (Ogloblin) could exist as a cryptic species complex. In addition, perform a phylogenetic analysis with several species within the genus *Gonatocerus* Nees to confirm species boundaries and to test the support for the species groups considered.

RESULTS AND CONCLUSIONS
ISSR-PCR DNA fingerprinting. Amplification reactions were performed with geographic populations from Argentina and Chile with 5-9 separate individuals from pooled egg masses per location. Locations included were: Argentina: Rio Colorado (RC) (Rio Negro Province), San Rafael (SR) (Mendoza), San Miguel de Tucumán (SMT) (Tucumán), and Chile: Jalsuri (CH). Previously, we have demonstrated a positive correlation between ISSR-PCR banding patterns and species distinction (de León and Jones 2004, de León et al. 2004a,b, 2006). In addition, we have utilized the method to distinguish about 8 *Gonatocerus* species. As a first approach, we asked whether the ISSR-PCR method was suitable to distinguish geographic populations of *G. tuberculifemur* from Argentina and Chile. The results of this analysis are shown on Figure 1. Three geographic- or population-specific bands were identified within the San Rafael population, as indicated by the arrows. Slight variation was seen within the rest of the populations, but in general, similar banding patterns were observed within these