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# Vibrational Communication and Incipient Speciation in Blue-Green Sharpshooters, *Graphocephala atropunctata*

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# DEDICATION

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# Introduction

Native pest systems are typically viewed as stable unchanging entities, and they are often overlooked in importance when a novel pest invades and attacks similar crops. Although exotic invaders can be highly disruptive in new areas, problems due to native pests continue and perhaps aggravate the situation even though their significance is seen as being less important than the recent invader. The blue-green sharpshooter, *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae) (BGSS), is native to the US, and has a range that includes most of California, and extends into Arizona, Mexico, and Nicaragua (McKamey 2007). BGSS has been a recognized pest of California grapes for well over one hundred years (Winkler 1949), yet relatively little basic research on behavior, ecology, and biology has been conducted on this insect. BGSS are leafhoppers that vary from six to seven millimeters in length with yellow markings on a primarily blue-green body color although their markings can be highly variable (Severin 1949a). They pass through five instars before molting to adults that overwinter and are capable of living for up to one year (Severin 1949a). BGSS are univoltine, and high density populations often inhabit moist riparian corridors composed of native weeds. This situation is especially common in the coastal fog belt region of California where vineyards are common (Severin 1949a). Much research effort has focused on developing efficient cost-effective ways to manage and kill BGSS in grapes (Purcell 1979).

Blue-green sharpshooters are capable of transmitting the xylem-dwelling bacterium *Xylella fastidiosa* Wells et al., the causative agent of Pierce's disease, a lethal malady of grape vines. Pierce's disease has been present in California since at least the 1880's where it was most likely introduced from the Southern United States or Mexico (Purcell and Feil 2001). Pierce's disease

is incurable in grapevines and causes defoliation and eventually vine death due to the bacterium's blockage of the water-conduction system in the plant (Hopkins and Purcell 2002). BGSS are more efficient vectors of this disease than other native sharpshooters such as the green sharpshooter, *Draeculacephala minerva* Ball, or the redheaded sharpshooter, *Carneocephala fulgida* Nottingham (Hill and Purcell 1995, Severin 1949a), and the exotic glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (all are Hemiptera: Cicadellidae) (Almeida and Purcell 2003). GWSS established in California in the late 1980's most likely originating from the Southeastern US, its native range (Hopkins and Purcell 2002). *Homalodisca vitripennis* has become the focus of major research campaign in California because of the threat it poses to grapes due to its ability to engage in vine to vine transmission of *X. fastidiosa*, which has greatly exacerbated disease severity. BGSS is not capable of vine to vine transmission of *X. fastidiosa* which has meant that Pierce's disease outbreaks associated with BGSS have been more easily manageable (Almeida 2007, Purcell and Saunders 1999). BGSS infected with *X. fastidiosa* are reported to pass this bacterium to grapevines with a 92% transmission rate (Hill and Purcell 1995), which is significantly greater than GWSS which has a transmission efficiency of just 50% (Almeida and Purcell 2003). BGSS nymphs are infective almost immediately upon acquiring the bacterium, and adults can remain so for the remainder of their lives, a period of approximately one year (Hill and Purcell 1995, Severin 1949b). This high rate of bacterial transmission in grape vines is especially problematic because the spatial distribution of BGSS often coincides with the distribution of vineyards as large numbers of BGSS are commonly seen in the coastal fog-belt area which is heavily planted with grapes (Purcell 1975). Despite the relatively recent invasion of GWSS and the massive research effort being directed against GWSS for Pierce's disease control, BGSS are arguably the most important vector of *X. fastidiosa* and

threat to grape production due to their high transmission rate, longevity, and wide spatial distribution that coincides closely with the premier wine producing areas of California such as Napa, Sonoma, and Mendocino Counties, areas that GWSS has failed to establish in.

The current method of controlling BGSS in California vineyards is early season spraying of pesticides, especially in areas adjacent to riparian corridors where BGSS's native host plants are abundant (Purcell 1979). BGSS move from their native hosts which include mugwort (*Artemisia vulgaris* L., [Asterales: Asteraceae]), stinging nettle (*Urtica dioica* L., [Rosales: Urticaceae]), and wild grape (*Vitis californica* Benth., [Vitales: Vitaceae]) (Purcell 1976), to commercial vineyards in spring (late March to mid-April) which coincides with the beginning of leaf growth of commercial grape vines (Purcell 1979). A standard pesticide-oriented control practice that may be employed by grapes growers is to spray as soon as yellow sticky traps indicate BGSS presence in vineyards, or once daytime maximum temperatures exceed 16°C in the spring as flight from riparian areas into vineyards is likely to occur (Purcell 1979). This control recommendation for BGSS is based on research conducted in the Napa Valley in Northern California, and it is not known whether these control recommendations also are efficacious for BGSS in the warmer areas of Southern California.

Proximity of vineyards to riparian areas with BGSS native host plants substantially increases the risk of BGSS movement and subsequent transfer of Pierce's disease into adjacent vineyards (Purcell 1975). Consequently, one recommended cultural control practice for BGSS is to remove native riparian plants that are hosts for this pest, and replace them with non-host plants (Purcell

et al. 1999). It is unknown if this destructive practice in sensitive wetland areas has been widely adopted by grape growers in Northern California.

In attempts to better understand BGSS biology and to develop ecologically-based management programs, studies have examined host plant preferences (Purcell 1976), BGSS flight activity (Boyd and Hoddle 2006, Feil et al. 2000), oviposition behaviors (Boyd and Hoddle 2006), and natural enemy complexes associated with eggs (Boyd and Hoddle 2006). These studies have all had limited geographic scope because they were conducted in a single region of California, but results have been assumed to be applicable to all areas with BGSS populations. For example, one study examined BGSS flight activity with the intention of developing a degree-day model so that growers can more accurately predict and treat vineyards prior to the arrival of BGSS. Feil et al. (2000) found the daily minimum flight threshold for BGSS to be 14.5°C, but this study was conducted in Berkeley (Alameda County) which has much cooler temperatures than most of Southern California where grapes are also grown commercially and BGSS is endemic (Feil et al. 2000). It is possible that BGSS from Southern California may have different flight activity thresholds because they have adapted to warmer temperatures which exist for longer periods of time in Southern California. These warmer temperatures could simultaneously promote longer windows of reproductive and feeding activity and subsequent disease related problems. Indeed, because temperatures in Southern California rarely drop below the daily minimum 14.5°C flight activity threshold for prolonged periods, it is difficult to determine if the results and subsequent control recommendations from the Feil et al. (2000) study are applicable to managing BGSS populations in Southern California (Boyd and Hoddle 2006).

Interestingly, BGSS populations seem to be strongly affected by temperature, rainfall, and especially drought conditions because these sharpshooters rely on native annuals such as wild grape, stinging nettle, and mugwort which require moist, humid conditions to grow (Purcell 1979). BGSS populations have notably decreased in drought years (Purcell 1979) and while searching for BGSS for research presented in this thesis, many areas from which BGSS populations had been reported during the 1970s were devoid of BGSS, possibly due to events that altered their fragile riparian habitat such as changes in temperature, rainfall, and incursion by invasive plants. As California climate continues to change because of global warming, temperature and annual rainfall patterns, vegetative communities will be altered (Lenihan et al. 2003) and presumably the insect faunas associated with them. BGSS populations may become more limited or isolated, especially in Southern California as riparian systems are threatened by predicted changes in rainfall and increased temperatures (Lenihan et al. 2003).

Although BGSS continue to be problematic pests in California vineyards, especially in Northern California (Purcell and Feil 2001), relatively little research has been conducted on the pest in comparison to GWSS, which is significantly less efficient at transmitting *X. fasitidiosa* than BGSS and has only been a significant Pierce's disease threat in Southern California and the more southerly areas of the Central Valley. Research that has been conducted on BGSS deals almost exclusively with populations in Northern California, despite the fact that BGSS are found throughout the length of California and has potentially caused significant economic damage to grapes in Southern California in the past. In fact, BGSS may have been responsible, in part, for the devastating outbreak of "Anaheim Disease" in the early 1880's that destroyed the incipient grape industry in Southern California (Pierce 1892). It is typically assumed that there are no

significant differences among populations of BGSS throughout California, and observations from research conducted out in Northern California have universal applicability for managing BGSS throughout California. Such an assumption may be incorrect, especially for some of the most fundamental aspects of BGSS biology. For example, significant differences may exist in the ability of Northern and Southern California populations of BGSS to acquire and spread *X. fastidiosa*, their natural enemy complexes may differ, which could affect population dynamics and phenology. If differences in *X. fastidiosa* transmission efficiencies exist, and flight phenology patterns differ significantly because of temperature, then management plans for north and south BGSS populations may need to be fundamentally different.

One of the few studies conducted on Southern California populations of BGSS determined that like many leafhoppers, BGSS communicate through substrate-borne vibrations (Percy et al 2008). Claridge (1985) recognized that most cicadellids used a tymbal mechanism to produce calls not unlike those used by cicadas but lack the characteristic cicada airsac which in turn dampens their calls so they are limited to substrate transmission. The BGSS acoustic study by Percy et al. (2008) revealed two distinct calls, one produced by males and one by females (Percy et al. 2008). Because BGSS populations in California are widely distributed, and in many instances, highly isolated, BGSS populations may exhibit significant differences in various aspects of their sexual behavior, such as, mating calls and the subsequent ability for populations to interbreed. Further, differences between widespread BGSS populations, should they exist, may also be quantifiable at the molecular level.

Consequently, research conducted as part of this thesis had three main objections which sought to determine if detectable differences existed between widely separated populations of BGSS. The first objective was to study key molecular markers typically used to identify groups in population genetics studies. The second objective examined the acoustic calling structure of males and females across Northern and Southern California. Finally, the third objective of this research was to examine cross-breeding potential between widely separated and highly isolated California populations. The collective goal of these research objectives was to improve basic understanding of an extremely important native California pest system and to demonstrate that some native pests, like BGSS, are not the static systems they are often perceived to be. The implications of this research is that effective management plans should consider the possibility for plasticity when native pest populations span vast areas and consequently recognize that control programs may need to be customized accordingly.

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# Genetic Analysis of Blue-Green Sharpshooter (*Graphocephala atropunctata*) Populations Across California

## Introduction

The blue-green sharpshooter, (BGSS), *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae: Cicadellinae) is a xylem feeding leafhopper that vectors the bacterium *Xylella fastidiosa* Wells et al., the causative agent of Pierce's disease in grape vines. BGSS is native to the Western United States and is common throughout California, with a range that extends as far south as Nicaragua in Central America (McKamey 2007). In California, BGSS is highly polyphagous and can feed on a wide variety of native and exotic plants (Severin 1949), but is found primarily on wild grape and blackberry, in humid riparian areas. Polyphagy, wide distribution, and transmission of *X. fastidiosa* combine to make BGSS a perennial threat to the California grape industry, especially in the Napa Valley area, where it is the primary vector of Pierce's disease.

Much of the research conducted on BGSS regarding life history, disease transmission, and control strategies has focused on populations from Northern California (Feil et al. 2000, Purcell 1976, Severin 1949) with the assumption that results apply to all populations found throughout California. However, because BGSS occupies a north-south longitudinal range of 1,000 km across California and has limited dispersal abilities, interbreeding between highly isolated populations across this vast range may be limited. Geographic isolation of BGSS populations may result in limited gene flow and the development of significant differences in reproductive behavior, biology, and ecology between populations of BGSS, and hence the potential for

incipient speciation. Such population differences, should they exist, could translate into differences in host plant and habitat preferences, disease transmission rates, associated natural enemy faunas, and subsequent population suppression by biological control agents (Brunner et al. 2004).

One known and readily observable difference between widely disparate populations of BGSS is morphology, in particular color, which varies dramatically from Southern to Northern California (Figure I.1). Typically, BGSS from Northern California are dark green with faint markings on the body, whereas Southern California BGSS are bright blue with very pronounced markings on the head, scutellum, and wings (Severin 1949). Differences in morphology may be indicative of incipient speciation in BGSS, which could have important implications for the direction of current research and control tactics presently used throughout California for control of this vineyard pest.

*Graphocephala atropunctata*'s large range, phenotypic plasticity, disjunct population distributions, occupation of isolated habitats, and significant pest status suggest that BGSS in California may have quantifiable molecular level differences that could be very useful for distinguishing populations. Consequently, the purpose of the work presented in this study was to determine whether genetic differences exist between Californian BGSS populations that could be indicative of groups undergoing incipient speciation. Using DNA sequence data from nuclear and mitochondrial genes, we examined differences among Californian populations of BGSS along a latitudinal cline, and assessed gene flow between these populations. Several criteria can be used to assess whether or not populations are different species such as inter-population

breeding, offspring viability and sex ratio, or molecular differences in 28sD2 or C oxidase subunit I sequences. Consequently the purpose of this work was to use molecular analyses of 28sD2 and COI sequences to indicate possible population differences in BGSS that could be indicative of incipient speciation. This work had three main objectives. The first objective was to examine the COI and 28sD2 sequences from different BGSS populations for differences that could be indicative of the existence of different species. Secondly, we sought to examine molecular level differences in BGSS populations across the state to ascertain the likely level of inter-population movement and subsequent gene flow across California. Lastly, we compared BGSS to two other species of *Graphocephala* to determine molecular similarity between these species with BGSS.

## Methods

### Specimen Collections

BGSS were collected from 1 July 2007 to 13 January 2008 from locations throughout California across a range encompassing over 900 kilometers. Coastal and inland Southern California, the central coast, the central valley, the San Francisco Bay Area, Napa and Sonoma Valleys, and coastal and inland Northern California were surveyed for populations of BGSS, from which specimens were collected for analyses (Figure I.3). Individual BGSS from 23 locations were collected, with sample populations used in analyses being separated by at least three km. Two additional species of *Graphocephala* were collected for DNA comparison to BGSS. Two samples of *Graphocephala cythura* Baker were collected in Fullerton, California, and three samples of *Graphocephala flavovittata* Metcalf were collected in Uruapan, Michoacán, Mexico (Figure I.2). All specimens collected for study were preserved in 95% ethanol, in 2 ml centrifuge

vials (Fisher Scientific, Pittsburgh, PA) and kept at -20°C until used for DNA analyses. Voucher specimens of *Graphocephala* species collected were deposited in the University of California-Riverside Entomology Museum (See table I.1 for museum voucher numbers).

**DNA Extraction**

DNA was extracted from a single tibia of individual specimens using a chelex extraction method (Walsh et al. 1991). Individual BGSS specimens were removed from alcohol and allowed to dry briefly on a tissue. The middle tibia was dissected out, transferred to a 0.5 µL microcentrifuge tube containing 2 µL proteinase-K, and ground up using a micro-pestle. 100 µL of a 5% chelex-100 suspension (in water) was added and the tubes were incubated at 55°C for 1hr, and then for a further 10 min at 99°C to inactivate the proteinase-K. Tubes were then spun in a microcentrifuge at 14,000 RPM for 4 min, pelleting the chelex and insect debris, and the supernatant was transferred to a new 5 µL microcentrifuge tube and stored at -20°C until used for amplification.

**Amplification of Extracted *G. atropunctata* DNA**

The polymerase chain reaction (PCR) was used to amplify a 532 bp section of the D2 region of 28s ribosomal RNA using the PCR primers; 28sF3633 (5'-TACCGTGAGGGAAAGTTGAAA-3'), and 28sR4076 (5'-AGACTCCTTGGTCCGTGTTT-3') (Choudhury & Werren 2006). PCR was performed in 25 µL reactions containing 2 µL DNA template from extraction (concentration not determined), 1 X Thermopol PCR buffer (New England BioLabs, Ipswich, MA), 200 µM of dNTP, 0.2 µM of each primer, and 1 U *Taq* polymerase (NEB). PCR was performed in a Mastercycler® 5331 or Mastercycler® ep gradient S thermocycler (Eppendorf North America

Inc., New York, NY) programmed for 94°C for 2 min, followed by 38 cycles of: 94°C for 30 sec, 58°C for 50 sec, 72°C for 90 sec, and a final extension for 10 min at 72°C.

A section of the cytochrome C oxidase subunit I (COI) of mitochondrial DNA was also amplified for each specimen. Initially, the PCR primer pair LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; (Folmer et al. 1994) was used. PCR was performed in 25 µL reactions containing 2-4 µL DNA template (concentration not determined), 2.5 µL 1 X Thermopol PCR buffer, 5.0 µL of 1mM dNTP, 0.2 µM of each primer, and 1 U *Taq* polymerase (NEB). PCR was performed in an Eppendorf thermocycler programmed for 94°C for 2 min, 3 cycles of: 94°C- 30 sec, 45°C- 50 sec, 72°C- 40 sec, 35 cycles of: 94°C- 30 sec, 51°C- 30 sec, 72°C- 40 sec, and a final extension at 72°C for 2 min.

As our study progressed, the initial COI primer set became increasingly problematic and amplifications deteriorated to the point where sequencing became impossible. The LCO/HCO primers are commonly employed in the laboratory and this observed deterioration may have been an example of "genotyping crash", which has been reported from laboratories that amplify DNA with the same pair of primers over an extended period (e.g., Han et al. 2006). To overcome this problem, specimens that could not be successfully amplified and sequenced with the original primers, were instead amplified using a set of internal primers BGSS COI-F2 (5'-TCGAATTGAAYTWGCWCAGC-3') and BGSS COI-R2 (5'-AGCTCCTGCYAAWACWGGTA-3'), under identical PCR conditions.

**Cleaning and Sequencing**

PCR products were electrophoresed on 1% agarose gel that was stained with ethidium bromide to verify amplification visually and then purified using the Wizard PCR Preps DNA Purification System (Promega, Madison WI). DNA was sequenced at the University of California-Riverside Genomics Institute Core Instrumentation Facility. Sequences were aligned manually using BioEdit version 7.0.9.0 (Hall 1999) and deposited in GenBank® (Benson et al. 2000) (Table I.1).

**Statistical analyses**

The number of BGSS mitochondrial haplotypes and haplotype diversity were calculated using DnaSp software Ver. 4.10.7 (Rozas et al. 2003) and a haplotype network was constructed using TCS version 1.21 (Clement 2000). COI sequences were arbitrarily grouped according to collection latitude, creating six BGSS population groups (Table I.2; Figure I.3). Genetic differences between these groups were approximated by calculating estimates of $F_{st}$ for each pair of populations using the program ARLEQUIN (Schneider et al. 2000). The significance of the fixation indices ($F_{st)}$ values was evaluated by permuting the haplotypes (1000 permutations) between groups. Partitioning of genetic variation was estimated using the analysis of molecular variance (AMOVA) implemented in ARLEQUIN. Genetic variation was partitioned into 3 levels: within populations of BGSS; among populations of BGSS; and among species. The significance of population differentiation was evaluated using the permutation method (1000 permutations) invoked in ARLEQUIN. To test for isolation by distance, a correlation between geographical and genetic distances of the six BGSS population groups was sought using a Mantel test in ARLEQUIN. Fst Values were linearized following a Slatkin correction (Slatkin 1995).

## Results

28sD2 sequences were trimmed at the primer ends and were 532 base pairs long for all sharpshooters sequenced, and were identical for all BGSS populations sampled in California. For the three *G. flavovittata* specimens collected in Mexico, one had an identical 28sD2 sequence to the California BGSS and the additional two had one base pair difference located at the 252$^{nd}$ base pair, which had a thymine substituted for a cytosine. *Graphocephala cythura* had nine base pair differences in 28sD2 when compared to BGSS.

COI sequence alignments were trimmed to match the length of the internal primer amplifications (465bp). Nineteen different mitochondrial haplotypes were found among the 75 sharpshooter specimens (all species) used in this study. *Graphocephala flavovittata* possessed two distinct haplotypes, *G. cythura* one, whereas BGSS possessed sixteen different haplotypes. Northern California (N37°52' to N40°55') possessed six distinct haplotypes, whereas Central and Southern California (N32°52' to N36°00') possessed ten distinct haplotypes (See Figure I.4 for COI haplotype network and Figure I.3 for distribution of haplotypes [$F_{st}$] across California).

Based on mitochondrial sequences of all species studied, the proportion of the total molecular variance was as followed: within populations was 50.32%; among populations and within species molecular variance was 27.25%; and differences between species was 22.43%. This result indicates that there are large differences between individuals even within the same species because over half of the total molecular variation in COI is attributable to within population variation.

Estimates of $F_{st}$ values ranged from 0.011 to 0.89 and those values linearized were 0.0016 to 8.25. $F_{st}$ values between populations were significant for 22 out of 28 population comparisons and those without significant differences (i.e., six samples) were typically populations of BGSS that were relatively close to each other (i.e., < 20 km). Within BGSS the highest pairwise $F_{st}$ differences were those that were geographically furthest from one another (division 1 versus division 6 in Figure I.3). $F_{st}$ values were highest between different species (Table I.3).

$F_{st}$ values were plotted against geographical distances for BGSS. A significant positive correlation between geographic distance and genetic difference was found based on COI sequences ($r^2 = 0.782$, $P = 0.004$) (Figure I.5). As suggested by BGSS extended range across California, a pattern of molecular separation for populations based on isolation by distance is strongly supported by the data.

## Discussion

Analysis of BGSS haplotypes resulted in distinct molecular groupings based on COI data that corresponded to geographic locations, indicating that geographic separation and subsequent isolation of BGSS populations in California has resulted in genetic differences. Pairwise $F_{st}$ values were mostly greater than 0.25 (three out of 15 were below 0.09 for BGSS) which indicates substantial genetic differentiation. According to $F_{st}$ definition, diversity is higher the closer the $F_{st}$ value is to 1.0 and diversity is lower as the $F_{st}$ value approaches zero. For intraspecific comparisons, 80% of pairwise BGSS population comparisons were considered significantly different, indicating that there is little gene flow between BGSS populations. This is likely the result of populations being isolated because of habitat preferences across a

heterogeneous landscape, though other barriers such as behavioral differences may also play a role. The three pairwise comparisons that were not significantly different represented near neighboring populations (division one and two, division two and four, division three and four) indicating that there is still significant gene flow between some neighboring populations of BGSS that are separated by less than 400 km. In contrast, other neighboring populations (division two and three, division four and five) with a minimum separation distance of 90 km were significantly different from each other. This implies that certain populations are capable of inter-mixing more easily than others, and this may be a result of differences in terrain, habitat availability, weather patterns (especially wind), and the human movement of infested plants across the state. Beirne (1956) noted that different populations of the leafhopper species *Macrosteles fascifrons* (Stal) (Hemiptera: Cicadellidae), which also has an extended range across the west coast of the United States, were biologically isolated from one another as a result of their low dispersal rates and large geographic range. A similar situation may exist in California for BGSS due to small size and relatively weak flying abilities.

The genetic structure as defined by the $F_{st}$ analysis of BGSS across California is best defined as isolation by distance. Generally, gene flow between geographically isolated BGSS populations does not appear to be common, despite potential widespread movement throughout California due to the nursery trade or home owner movement of plants such as roses that can be hosts for BGSS. BGSS populations often were difficult to locate even in habitat that looked suitable. This situation for BGSS contrasts markedly with the exotic cicadellid pest, *Homalodisca vitripennis* (Germar), the glassy-winged sharpshooter, a serious vector of *X. fastidiosa* in California. *Homolodisca vitripennis* is spread regularly from Southern California on ornamental

plants into uninfested areas in Northern California, despite quarantine efforts to curtail movement of this nature (Smith 2005).

Pairwise $F_{st}$ value comparisons among the three sharpshooter species examined in this study revealed significant differences in all but one comparison (BGSS populations from division five compared with *G. flavovittata*). However, due to BGSS and *G. flavovittata's* large differences in their COI regions, they do not appear to be cross-breeding despite their overlapping ranges. Data from cross-breeding studies conducted in the laboratory would be needed to confirm this. This contrasts with *G. cythura*-BGSS comparisons, which had $F_{st}$ values which were all significantly different from BGSS compared to differences between *G. flavovittata* with BGSS. *G. cythura* does not appear to hybridize with BGSS despite their close proximity to one another in Southern California as indicated by their higher $F_{st}$ values and statistically significant differences when compared to BGSS.

The differences in the 28sD2 sequences contrast markedly with the results of the COI analysis. Despite the clear morphological differences between BGSS populations from different areas, the 28sD2 sequences for all BGSS in California were identical. Mutations in ribosomal DNA evolve slowly and consequently identical 28sD2 sequences are expected within a species. However, for BGSS, COI sequences suggest that there is geographic isolation between populations, which over a long time could result in genetic incompatibility between different populations which would eventually be reflected by measurable differences in 28sD2.

BGSS and *G. cythura's* 28sD2 sequences were significantly different from one another, which supports the conclusions drawn from the COI $F_{st}$ values. Although BGSS and *G. cythura* are sympatric in Southern California, cross-breeding does not appear to be occurring.

The 28sD2 sequences of *G. flavovittata* were nearly identical to BGSS sequences, providing further evidence of their similarity. *Graphocephala flavovittata* and BGSS have partial overlapping geographic regions in Mexico and their close molecular similarity may be indicative of recent speciation. Morphologically, *G. flavovittata* and BGSS are very similar in color and body shape. Adults from both species are blue-green in color with obvious markings on the head, and elongate bodies with long and slender forewings. The nymphs also look remarkably similar in both body type and color (Oman 1949). Larger genetic samples and cross-breeding experiments could provide further data on possible hybridization, and whether BGSS and *G. flavovittata* are truly different species.

This work had three main objectives: the first objective was to determine whether BGSS are the same species by examining their COI and 28sD2 sequences; secondly, we sought to examine the differences in BGSS populations as a measure of population movement across California; and lastly, we compared BGSS to two other species of *Graphocephala* to determine molecular similarity between these three species. Based on the results of genetic analyses, it is concluded that BGSS appear to be the same species across California, though inter-breeding seems limited between many populations of BGSS which may eventually lead to behavioral and mating differences between these populations. Secondly, BGSS movement through California seems limited as indicated by COI groupings and a pattern of haplotype isolation that occurred with increasing geographic distance between populations. Lastly, BGSS exhibited large

differences between both other *Graphocephala* species, based on their COI sequences, but BGSS and *G. flavovittata* were nearly identical in their 28sD2 sequences. Further research is needed to verify the existence of a species complex involving BGSS and *G. flavovittata* and the ability of these sharpshooters to inter-breed and hybridize.

**Figure I.1** Photograph of three *Graphocephala atropunctata* specimens illustrating the differences in morphology among populations in California. A: Southern California, Laguna Beach, B: Central California, Berkeley, and C: Northern California, Redding

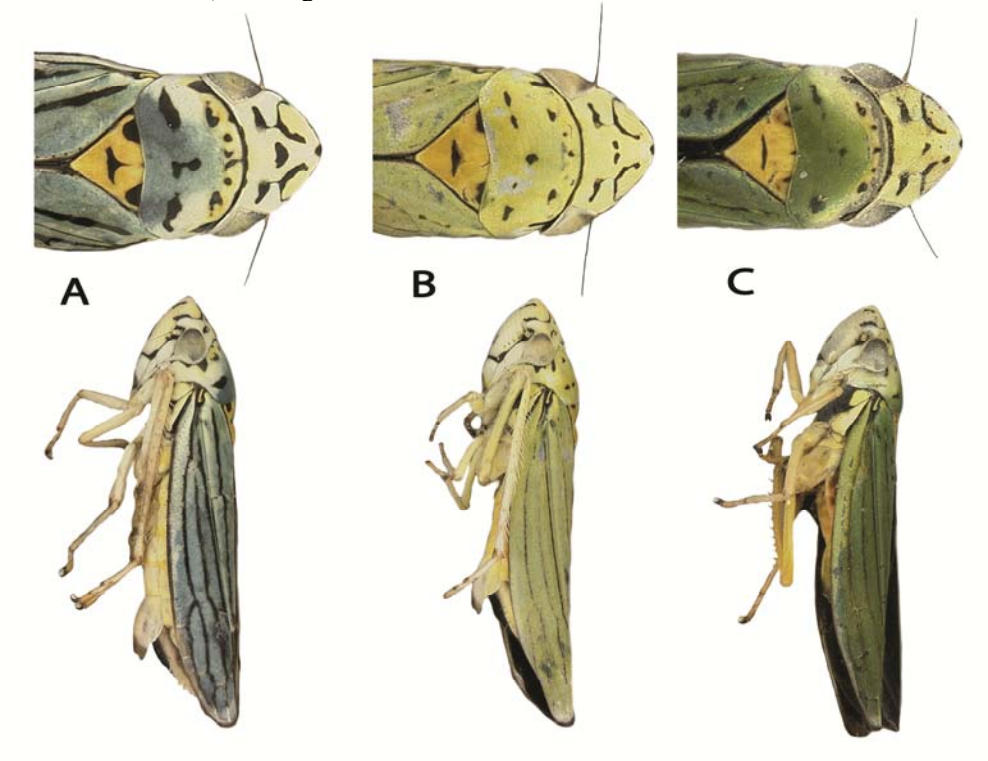



**Figure I.2** Photograph of A: *Graphocephala cythura*, and B: *Graphocephala flavovittata*

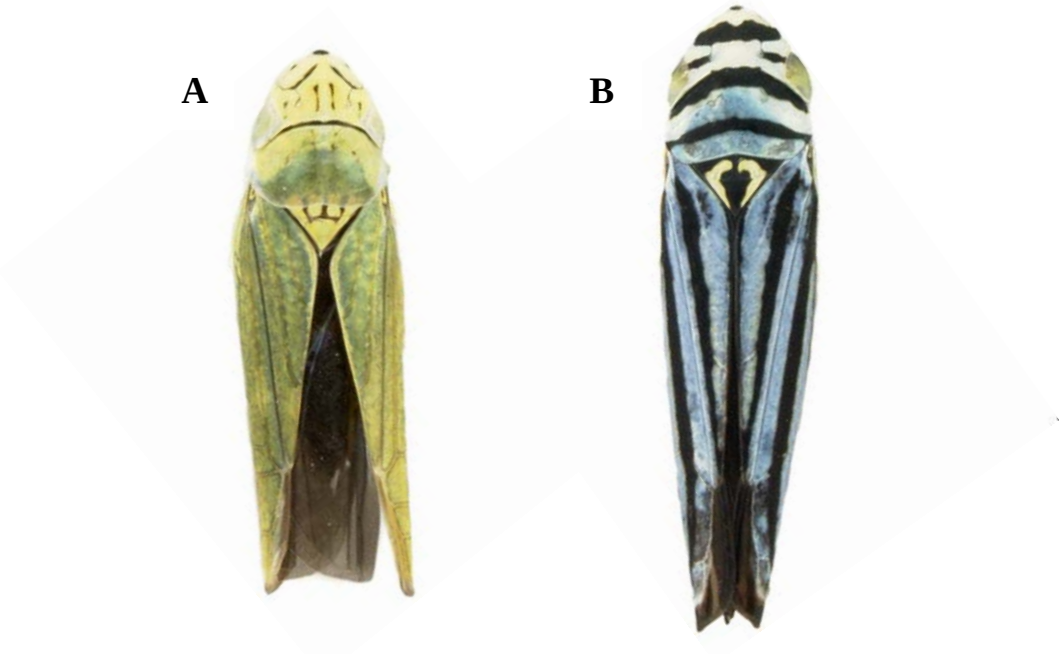


**Table I.1** Collection information for all *Graphocephala* species collected and used for genetic analyses.

| Collection Date | GPS Coordinates | Elevation (m) | County | Species | Map | UCR Accession Number |
|---|---|---|---|---|---|---|
| 7/15/2007 | 33°32’33”N 117°47’07”W | 0 | Orange | *G. atropunctata* | A | UCRC ENT-223957, 223956, 223955 |
| 7/17/2007 | 33°29’29”N, 117°15’09”W | 306 | Riverside | *G. atropunctata* | B | UCRC ENT- 223954, 223953 |
| 7/30/2007 | 37°52'39"N, 122°14'40"W | 309 | Alameda | *G. atropunctata* | C | UCRC ENT- 223946, 223945, 223944 |
| 7/30/2007 | 37°53'08”N, 122°15’42” | 146 | Alameda | *G. atropunctata* | D | UCRC ENT- 223943, 223942, 223941 |
| 7/30/2007 | 38°30'29”N, 122 53'09”W | 30 | Sonoma | *G. atropunctata* | E | UCRC ENT- 223937, 223936, 223935 |
| 7/30/2007 | 38°35'39N, 122° 55'0”W | 155 | Sonoma | *G. atropunctata* | F | UCRC ENT- 223937, 223936, 223935 |
| 7/1/2007 | 40°40’60”N, 122°39’00”W | 767 | Shasta | *G. atropunctata* | G | UCRC ENT- 223930, 223929, 223928 |
| 7/31/2007 | 38°53'48"N, 123°12'46"W | 280 | Mendocino | *G. atropunctata* | H | UCRC ENT-223933 |
| 7/31/2007 | 39°11'08"N, 123°01'35"W | 419 | Lake | *G. atropunctata* | I | UCRC ENT- 223952, 223951, 223950 |
| 8/1/2007 | 40°42'53”N, 122°38’04”W | 430 | Shasta | *G. atropunctata* | J | UCRC ENT- 223949, 223948, 223947 |
| 8/1/2007 | 40°40'10”N, 122°55'11”W | 516 | Trinity | *G. atropunctata* | K | UCRC ENT- 223927, 223926, 223925 |
| 8/1/2007 | 40°55’01”N, 122°23'33”W | 360 | Shasta | *G. atropunctata* | L | UCRC ENT- 223922, 23923, 223924 |
| 8/11/2007 | 32°52’44”N, 116°54’16”W | 197 | San Diego | *G. atropunctata* | M | UCRC ENT- 223932, 223931 |
| 8/13/2007 | 34°24'52"N, 119°44'12"W | 42 | Santa Barbara | *G. atropunctata* | N | UCRC ENT- 223964, 223965 |
| 8/15/2007 | 35°12'08"N, 120°42'56"W | 31 | San Luis Obispo | *G. atropunctata* | O | UCRC ENT- 223961 |
| 8/22/2007 | 33°04'54"N, 117°03'37"W | 123 | San Diego | *G. atropunctata* | P | UCRC ENT- 223934 |
| 8/23/2007 | 33°00'35"N, 117°14'23"W | 5 | San Diego | *G. atropunctat* | Q | UCRC ENT- 223921, 223920, 223919 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | *a* | | |
| 8/23/2007 | 33°21'40”N, 117°12'17"W | 162 | San Diego | *G. atropunctata* | R | UCRC ENT- 223918, 223917 |
| 8/26/2007 | 34°02'15"N, 118°44'59"W | 40 | Los Angeles | *G. atropunctata* | S | |
| 8/29/2007 | 33°41'06”N, 117°39'41”W | 260 | Orange | *G. atropunctata* | T | UCRC ENT- 223962, 223963 |
| 9/1/2007 | 35°15’10”N, 120°52’30”W | 54 | San Luis Obispo | *G. atropunctata* | U | UCRC ENT- 223958 |
| 8/31/2007 | 35°28’28”N, 120°51’01”W | 89 | San Luis Obispo | *G. atropunctata* | V | UCRC ENT- 223960, 223959 |
| 10/28/2007 | 36°00’36”N 121°31’05”W | 23 | Montere y | *G. atropunctata* | W | UCRC ENT- 223916, 223915, 223914 |
| 11/24/2007 | 33°53’16”N, 117°53'03”W | 80 | Orange | *G. cythura* | X | UCRC ENT- 223910 |
| 1/13/2008 | 19°28’18”N 102°26’43”W | 1675 | Michoacán Mexico | *G. flavovittata* | | UCRC ENT- 223913, 223912, 223911 |

**Table I.2** Division of *Graphocephala atropunctata* into six populations in California (see also Fig. I.1).

| Population | Latitude | Collection # | Map Legend | Species |
|---|---|---|---|---|
| 1 | 41°-40° | BGSS-CA- 5, 8, 9, 10 | A, B, C, D | *G. atropunctata* |
| 2 | 39°-38° | BGSS-CA- 3,4,6,7 | E, F, G, H, | *G. atropunctata* |
| 3 | 38°-37° | BGSS-CA- 1,2 | I, J | *G. atropunctata* |
| 4 | 36°-34° | BGSS-CA-25, 15, 23, 24 | K, L, M, N | *G. atropunctata* |
| 5 | 34°-33° | BGSS-CA- 14, 26, 22, 11, 20 | O, P Q, R | *G. atropunctata* |
| 6 | 33°-32° | BGSS-CA- 12, 19, 18, 16, 13 | S, T, U V, W | *G. atropunctata* |
| 7 | | BGSS-CA-26 | X | *G. cythura* |
| 8 | | BGSS-MX-1 | | *G. flavovittata* |

**Table I.3** $F_{st}$ values with corresponding P-values for the six populations of *Graphocephala atropunctata*[I], *G. cythura*[II], and *G. flavovittata*[III]. Bold $F_{st}$ Values have been linearized following Slatkin correction (Slatkin 1995).
* = P < .05; ** = P < .01; *** = P < .001

| | $1^{I}$ | $2^{I}$ | $3^{I}$ | $4^{I}$ | $5^{I}$ | $6^{I}$ | $7^{II}$ | $8^{III}$ |
|---|---|---|---|---|---|---|---|---|
| $1^{I}$ | **.00000** | 0.0016 | 0.43118 | 0.26041 | 0.57306 | 0.59895 | 0.89191 | 0.79372 |
| $2^{I}$ | **.00161** | **.00000** | 0.25100 | 0.08762 | 0.43182 | 0.46898 | 0.72504 | 0.59862 |
| $3^{I}$ | **.75804*** ** | **.33512** ** | **.00000** | 0.03132 | 0.21311 | 0.26385 | 0.41565 | 0.27826 |
| $4^{I}$ | **. 35210*** | **.09603** | **.03234** | **.00000** | 0.24845 | 0.29925 | 0.29925 | 0.33333 |
| $5^{I}$ | **1.34224*** ** | **.76000*** ** | **.27083*** ** | **.33059*** ** | **.00000** | 0.01109 | 0.48148 | 0.22981 |
| $6^{I}$ | **1.49347*** ** | **.88316*** ** | **.35842*** ** | **.42705*** ** | **.01121** | **.00000** | 0.41146 | 0.29196 |
| $7^{II}$ | **8.25184*** ** | **2.6390*** | **. 71131*** | **. 92857*** | **.54167*** | **.69913** ** | **.00000** | 0.57143 |
| $8^{III}$ | **3.84768** ** | **1.49138** ** | **.38555*** | **. 50000*** | **.29839** | **. 41235*** | **1.33333** | **.00000** |

**Figure I.3** Map of collection sites in California for *Graphocephala atropunctata* and *G. cythura*. See Table I.1 for map legend with corresponding collection information.

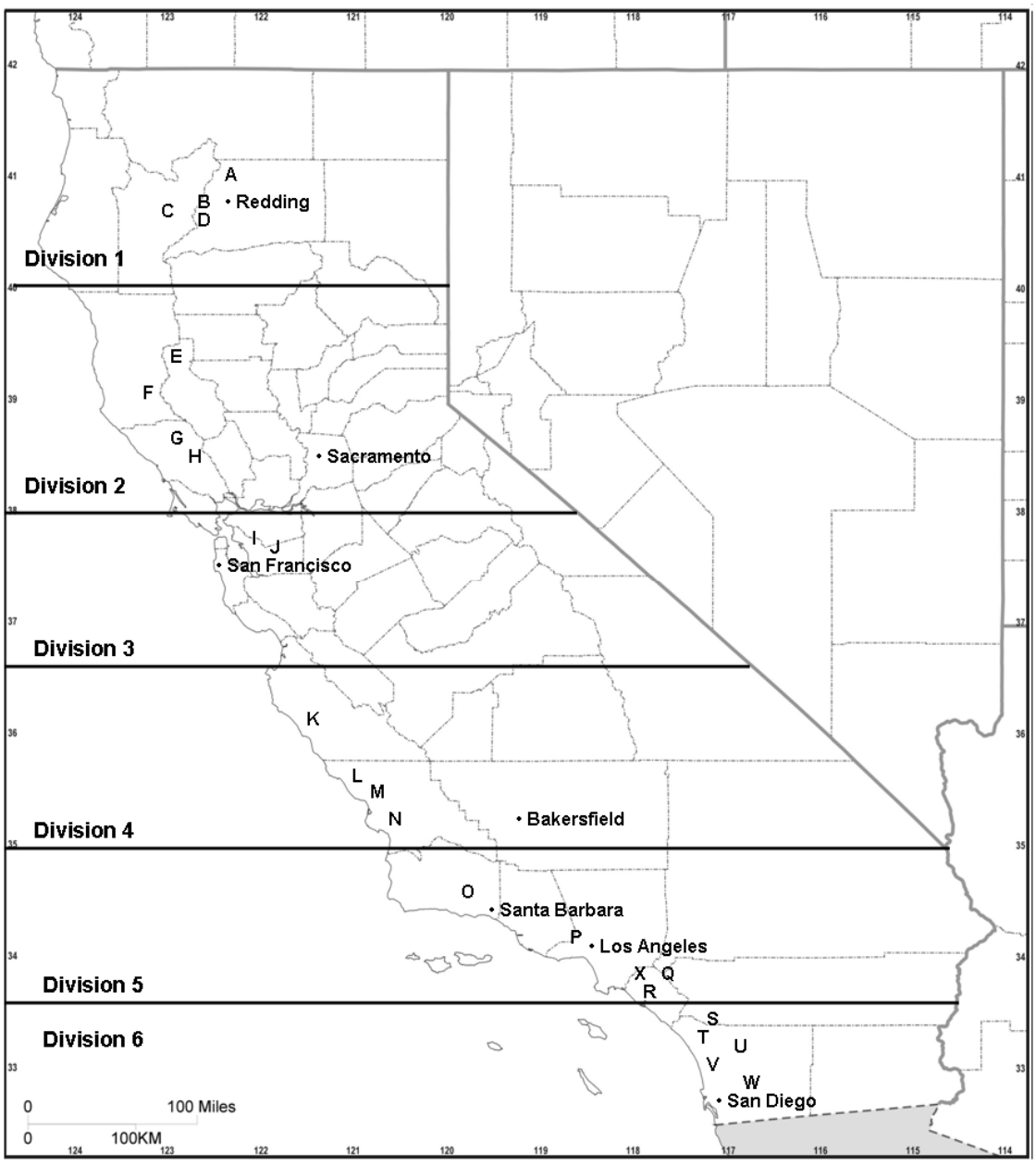


**Figure I.4** COI Haplotype diversity network for *Graphocephala atropunctata* specimens. Size of each haplotype circle represents the number of samples sharing this haplotype. Lines between haplotypes and hollow dots represent a mutation in a single base pair. Letters correspond to *G. atropunctata* collection sites (see Table I.1 and Figure I.3 for information on collection sites).

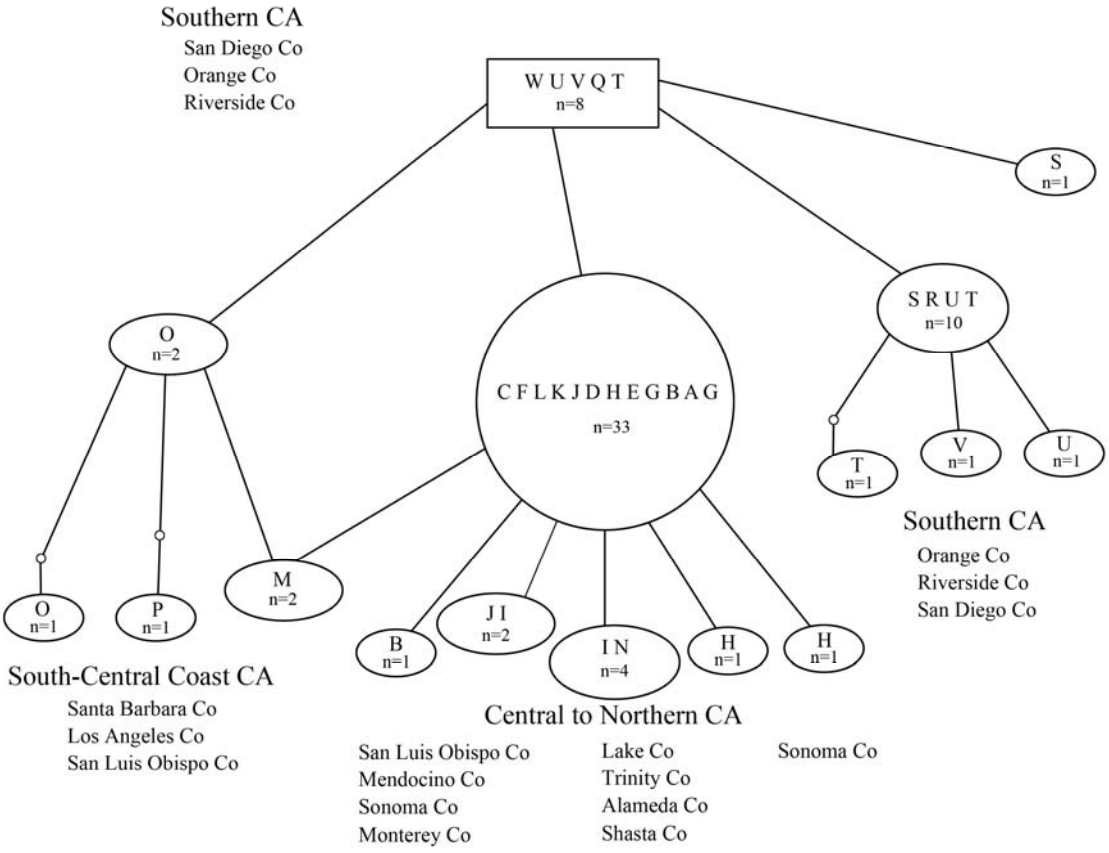


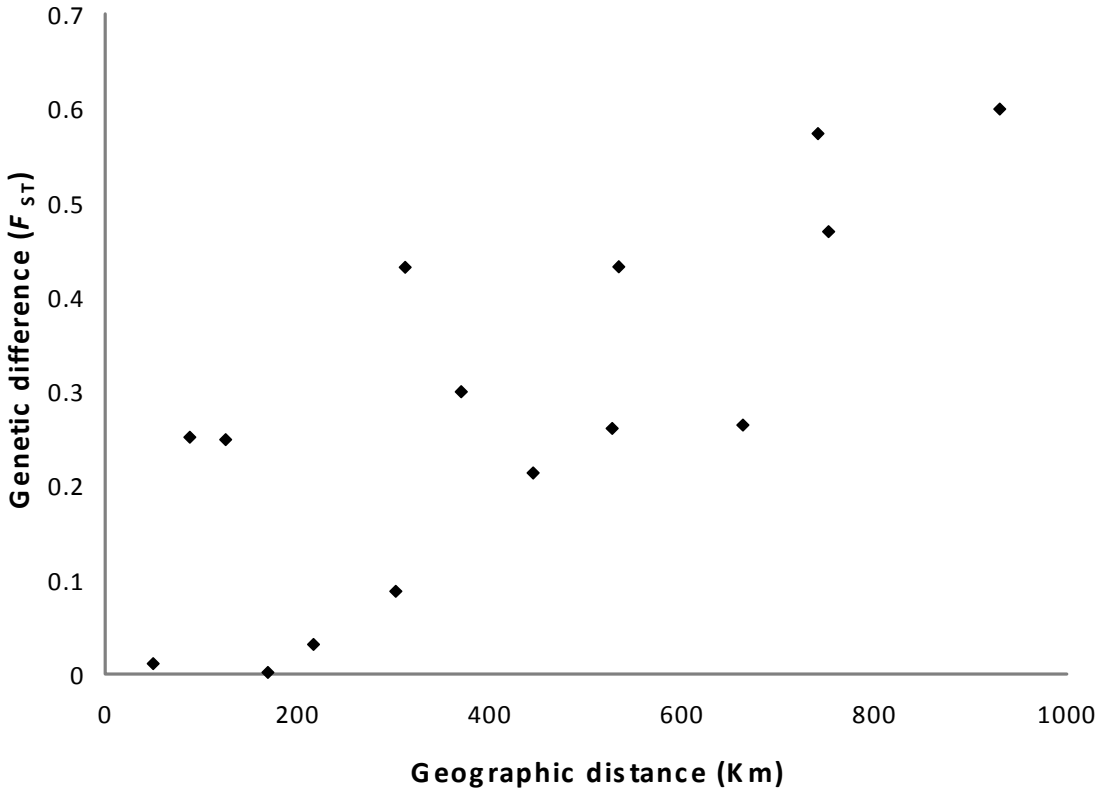
**Table I.4** Nineteen haplotypes based on COI sequences of three *Graphocephala sp.* and their Genbank accession numbers.

| Haplotype Number | Map | GPS | Species | Genbank Accession Number | UCR Accession Number |
|---|---|---|---|---|---|
| 1 | S | 34°02'15"N, 118°44'59"W | *G. atropunctata* | FJ890820 | UCRC ENT 015808 |
| 2 | S | 34°02'15"N, 118°44'59"W | *G. atropunctata* | FJ890821 | UCRC ENT 015809 |
| 3 | J | 39°11'08"N, 123°01'35"W | *G. atropunctata* | FJ890822 | UCRC ENT 015811 |
| 4 | J | 39°11'08"N, 123°01'35"W | *G. atropunctata* | FJ890823 | UCRC ENT 015810 |
| 5 | H | 38°53'48"N, 123°12'46"W | *G. atropunctata* | FJ890824 | UCRC ENT 015812 |
| 6 | H | 38°53'48"N, 123°12'46"W | *G. atropunctata* | FJ890825 | UCRC ENT 015813 |
| 7 | A | 33°32’33”N 117°47’07”W | *G. atropunctata* | FJ890826 | UCRC ENT 015814 |
| 8 | B | 33°29’29”N, 117°15’09”W | *G. atropunctata* | FJ890827 | UCRC ENT 015815 |
| 9 | O | 35°12'08"N, 120°42'56"W | *G. atropunctata* | FJ890828 | UCRC ENT 015816 |
| 10 | O | 35°12'08"N, 120°42'56"W | *G. atropunctata* | FJ890829 | |
| 11 | U | 35°15’10”N, 120°52’30”W | *G. atropunctata* | FJ890830 | UCRC ENT 015817 |
| 12 | V | 35°28’28”N, 120°51’01”W | *G. atropunctata* | FJ890831 | UCRC ENT 015818 |
| 13 | T | 33°41'06”N, 117°39'41”W | *G. atropunctata* | FJ890832 | UCRC ENT 015819 |
| 14 | Q | 33°00'35"N, 117°14'23"W | *G. atropunctata* | FJ890833 | |
| 15 | M | 32°52’44”N, 116°54’16”W | *G. atropunctata* | FJ890834 | |
| 16 | P | 33°04'54"N, 117°03'37"W | *G. atropunctata* | FJ890835 | UCRC ENT 015820 |
| 17 | X | 33°53’16”N, 117°53'03”W | *G. cythura* | FJ890836 | |
| 18 | | 19°28’18”N 102°26’43”W | *G. flavovittata* | FJ890837 | |
| 19 | | 19°28’18”N 102°26’43”W | *G. flavovittata* | FJ890838 | |

**Table I.5** Three haplotypes based on 28sD2 sequences of three *Graphocephala sp.* and their Genbank accession numbers.

| Haplotype Number | Map | GPS | Species | Genbank Accession Number | UCR Accession Number |
|---|---|---|---|---|---|
| 1 | S | 34°02'15"N, 118°44'59"W | *G. atropunctata* | FJ890817 | UCRC ENT 015808 |
| 2 | X | 19°28'18"N 102°26'43"W | *G. cythura* | FJ890818 | |
| 3 | | 19°28'18"N 102°26'43"W | *G. flavovittata* | FJ890819 | |

**Figure I.5** Correlation between genetic differences and geographical distance between populations of *Graphocephala atropunctata* in California. There is a highly significant relationship between geographic distance between populations and genetic differences in BGSS ($r^2$=0.782, P=0.004).



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# Vibrational Mating Calls and Cross-Breeding Abilities of Blue-Green Sharpshooters (*Graphocephala atropunctata*) from Two Geographically Distant Populations in California

## Introduction

Blue-green sharpshooters (BGSS), *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae), are xylophagous leafhoppers in the tribe Cicadellinini. BGSS are native to the western North America where they are found throughout California and have a range that extends north to Canada and south into Mexico and Nicaragua (Young 1977). BGSS are found primarily in riparian areas that have year round moisture and host plants such as wild grape (*Vitis californica* Benth., [Vitales: Vitaceae]), stinging nettle (*Urtica dioica* L., [Rosales: Urticaceae]), mugwort (*Artemisia vulgaris* L., [Asterales: Asteraceae]), and wild blackberry (*Rubus* sp. [Rosales: Rosaceae]) (Purcell 1976). Because California has extended areas of dry desert-like regions where BGSS cannot survive, BGSS distributions are highly fragmented and populations are often isolated from neighboring populations by inhospitable terrain that is not easily traversed.

BGSS are economically important because they are efficient vectors of the bacterium, *Xylella fastidiosa* Wells et al., the causative agent of Pierce's Disease, a lethal malady of grape vines (Severin 1949b). BGSS are especially problematic in the coastal fog belt area of California where they are the primary vector of this disease (Severin 1949a).

BGSS are approximately 6-7 mm long (DeLong and Severin 1949) and use substrate-borne vibrational mating calls produced with a tymbal mechanism to attract and find mates (Percy et al. 2008). The tymbal is an area on the abdominal cuticle with associated muscles that pull and distort the tymbal thereby producing vibrations (Claridge 1985). Vibrational signals can function as mating or warning calls, or serve as mechanisms for competition between individuals of the same species contesting a resource. Several authors argue that in order to properly distinguish species in the Auchenorrhyncha, vibrational mating calls must be studied because they can provide insight into the identities of species, or calls may suggest distinguishable differences between biotypes (Alexander 1967, Alexander and Moore 1958, Claridge 1965, Claridge 1983, Claridge and Reynolds 1973).

Vibrational mating calls can vary widely between different species of cicadellids in quantifiable characteristics such as frequency, amplitude, periodicity, and length. Differences in these attributes are thought to help receivers distinguish and locate individuals of their own species and therefore act as pre-mating isolating mechanisms (Claridge and Nixon 1985). Closely related cicadellid species often have very different call structures, (e. g., the leafhoppers *Oncopsis flavicollis* (L.) and O. *subandula* (Sahl) (Claridge and Nixon 1985). Species that appear morphologically identical can be recognized as distinct species based on differences in mating calls, which results in their inability to recognize and mate with their cryptic sibling species (e.g., green lacewings [Neuroptera: Chrysopidae] [Henry et al. 1996]).

To develop a better understanding of communication in BGSS, this study used multiple recordings of several BGSS individuals over 24 hr periods to establish BGSS calling periodicity,

and calls between widely separated and highly isolated BGSS were also studied. Because BGSS are known to communicate via substrate-borne calls (Percy et al. 2008), we sought to investigate whether BGSS in California potentially form a species complex, rather than a single species, by examining the vibrational calls and cross-mating potential of two widely separated populations. A previous study examined the call structure of one population of BGSS from southern California and found that they readily called and mated in the laboratory (Percy et al. 2008). However, this study was based on one recording dataset of only 10 hr. Cross-breeding studies were employed for the two BGSS populations studied here to determine if differences observed in calling between these two populations had a significant effect on their inter-breeding abilities.

Consequently, this study had three main objectives. The first objective was to characterize the types of calls produced by BGSS populations from northern and southern California. The second objective was to determine any differences in call characteristics (i.e., types and number of calls) given by individuals from by the two populations. The final objective was to determine whether the two populations were capable of cross-breeding and if offspring production rates differed when compared to intra-breeding individuals from the same population. The results of these acoustic and inter-breeding studies are presented here.

## Materials and Methods

### Insect Colonies and Host Plant Maintenance

Two laboratory colonies of BGSS were initiated from two different BGSS populations and maintained at the University of California-Riverside. Individuals from population one were collected in northern California (Shasta Co,; French Gulch, 40°42'53”N, 122°38’04”W 419m),

and population two was from southern California (Orange Co,; Laguna Beach, 33°32'33"N 117°47'07"W -2.4m). The two study populations were separated by 900 km. Adults and nymphs were collected six times from June 25 through August 5 2008 and were transported inside bottle cages (Boyd et al. 2007) with live sweet basil plants *Ocimum basilicum* L. (Lamiales: Lamiaceae) as hosts. BGSS from northern California were collected from a riparian area on wild grape (*Vitis californica*) and wild blackberry (*Rubus* sp.). BGSS from southern California were collected from an urban park located within 300 m of the ocean from *Rhaphiolepis* sp. (Rosales: Rosaceae). Once transported from their collection sites, BGSS were housed in a temperature controlled greenhouse at 26.2°C ± 5.7, 21.3% ± 7.8 RH, 16:8 (L:D) at the Agricultural Operations Facility, University of California-Riverside, California. BGSS colonies were maintained year round on sweet basil in 75 x 75 x 75 cm cages enclosed with mesh for ventilation and clear polyethylene panels for viewing cage contents. New basil plants grown from seeds were added as necessary, approximately every three wk.

Basil seeds were acquired from Harris Seed Company (Rochester, NY) (Sweet Dani Basil 00958-00-02) and planted individually into Jiffy Peat Pellets (Lorain, OH). As the basil approached three to four wk of age, plants were transferred to one quart plastic pots and planted in a mixture of 50% Kellogg Amend and 50% Kellogg Garden Soil (Carson, CA). Basil was watered daily and moved into BGSS colonies at approximately six wk of age.

**Preparation of BGSS for Experiments**

For all acoustic and cross-breeding experiments, individual fifth instar nymphs were removed from their respective colonies and placed individually onto an isolated sweet basil plant that was

approximately six wk of age, 15 cm tall, and contained within a transparent plastic 2 l bottle cage (Boyd et al. 2007) until their final molt to the adult stage. Fifth instars were easily identifiable by their darker color, and pronounced wing pads. Isolated individual adult BGSS were sexed upon adult emergence and these virgin adults were subsequently used for acoustic and cross breeding experiments. Although exact teneral periods are not known for this species, previous studies indicate that BGSS will readily call and mate within four days of the final molt (Percy et al. 2008). Consequently, all virgin adults used in these studies were between four and seven days of their final molt. All sweet basil plants used as host plants or calling substrates in experiments were previously unexposed to BGSS, and were used only once for experiments before being discarded.

**Recording Vibrational Signals Emitted by BGSS**

The acoustic signals for BGSS were recorded from adults placed on previously unexposed basil plants approximately six wk of age, 15 cm in height, and with ten medium to large-sized leaves. Plants were enclosed by plastic transparent 2 l bottle cages to contain BGSS on the plant and allow visual observations (Boyd et al. 2007). Basil leaves did not touch the sides of the enclosure during recordings. A 100 mV/G piezoelectric ICP® accelerometer model #35A24 (PCB Piezotronics, Buffalo, NY) was placed halfway up the main stem of the plant, secured with mounting wax, and was connected to a ICP® sensor signal conditioner model # 480E09 (PCB, Buffalo, NY) that amplified the outgoing signal 100 times before the signal was digitally recorded on a computer with Adobe Audition 3.0 software (Adobe Systems, San Jose, CA).

Recordings were conducted in a quiet laboratory maintained at an average of 24.4 ± 0.69°C, 45.5 ± 0.07% RH, with overhead florescent lighting on a 16:8 (L:D) (0500 to 2100 hr Pacific Time ) light cycle supplemented by ambient light from nearby windows. The recording apparatus was set up on a marble table top (91 x 61 x 12 cm) with sand-filled legs (76.2 x 33.52 x 15.24 cm) to reduce background ambient vibrations.

Two virgin females and three virgin males from the same population were placed within bottle cages with host plants. Vibrations from within the bottle cage were recorded for 24 hr at a sampling rate of 44,100 samples per sec (44.1 kHz). Each individual was used only once for acoustic recordings and then returned to its respective colony for breeding. Recordings began as soon as BGSS were placed inside the recording chamber and recordings were terminated 24 hr later and were analyzed using the software program Adobe Audition 3.0. Preliminary recordings suggested that sharpshooters called very little from 4 to 9 PM. Therefore recording sessions with groups of BGSS were set up and started each day between 4 and 9 PM to prevent interruption of peak calling periods. A total of forty 24 hr recordings were made, with twenty recordings made per population of BGSS during their breeding season from July 3 2008 through September 21 2008. Two recordings of all female, all male, and plants lacking BGSS were made to serve as experimental controls for mating calls and other types of inter-sex communications.

To avoid possible errors generated by decreased calling as the sharpshooters ended their breeding season, four recording periods were established and analyzed separately from one another. Each calling period, or block, consisted of all 24 hr recordings made during an approximate two wk period. All recordings were made during a twelve wk period from July 8

through September 21 2008. The first block was July 8 through July 30 2008, and had three recordings from southern California insects and seven recordings from northern California insects. The second block was August 6 through August 15 2008, and had six recordings from southern California and four recordings from northern California. The third recording block was August 17 through August 27 2008, and had three recordings from southern California and four recordings from northern California. The fourth and final block, was September 2 through September 21 2008, and had eight recordings from southern California and five recordings from northern California.

**Cross-Breeding Studies**

All four possible crosses between southern and northern California were performed to ascertain mating compatibility between the different BGSS populations that were maintained in colonies. Trials with individual virgin females from both locations were conducted to verify the absence of parthenogenesis in northern and southern California BGSS source populations. To determine inter-population breeding competence, one virgin male and one virgin female BGSS were placed onto a previously unexposed basil plant inside a bottle cage and were left together for one wk, after which both individuals were removed. Two months later, basil plants within cages were examined for offspring that would have resulted from mating. The number of offspring produced per cross-mating study was recorded. Trials that had sharpshooters die during the one wk mating period, or had plants that died before they could be checked for offspring, were discarded, and not factored into the final datasets used in analyses.

Cross-breeding trials were conducted from June 30 2008 through March 17 2009. Thirty-four southern California female controls were run July 14 through September 24 2008. Forty-one

northern California female controls were run July 12 through August 31 2008. Thirty-six crosses of southern California females with southern California males were run June 28 through January 30 2008. Twenty-eight crosses of northern California females with northern California males were run July 2 through March 14 2008. Forty-two crosses of southern California males with northern California females were conducted July 3 through March 17 2008. Thirty-eight crosses of northern California males with southern California females were conducted July 25 through March 17 2008.

**Statistical Analyses**

Recordings of BGSS-produced sounds were grouped together by two wk periods for each BGSS location for statistical analyses. Each two wk block contained between three and eight recordings that were averaged and analyzed for comparison. The different types of calls from the recording were averaged separately (i.e. the calls were sorted and counted according to type [see below for call type description]). A total of four blocks spanning a 12 wk interval of acoustic activity were analyzed for BGSS populations that originated from southern and northern California.

Calls from each entire 24 hr recording were analyzed for comparison between locations. To analyze and compare individual BGSS calls of different types (e.g., mating or accessory calls [see below for details on call classifications]) over a 24 hr time interval by population, each calling bout was numbered. Sets of five random numbers were generated in SAS and used to randomly select five calls of the same type for analysis. Consequently, a maximum of five randomly selected calls of the same type were analyzed from each 24 hr recording session. When fewer than five calls of one type were given in a 24 hr recording period, all calls of that

particular type were used for analyses. Statistical analyses comparing calls were made using Student's t-test at the 0.05 level of significance, Shapiro-Wilk's test was used to check for data normality, and Bartlett's test for assessing equal variance were conducted using SAS software, Version 9.2 of the SAS System for Windows (SAS 2007 Cary, NC). All data was normal with equal variance, so no transformations were necessary.

Calling periodicity and number of calls were calculated based on the number of individuals in the calling chamber. Because each individual was not observed for the full 24 hr, it was impossible to determine which individuals called and which did not. Therefore, the numbers of recorded calls were divided by the number of individual sharpshooters in the experimental arena to obtain a value which represented the average number of calls per male or female. Average call numbers estimated in this manner may be lower than what can be observed in nature because of the inclusion of non-calling individuals in analyses.

The average number of male mating calls per block was calculated as the total number of calls divided by the number of males in a bottle cage from which recordings were made. The maximum calling rate represents the average greatest number of calls given in a 24 hr period.

Cross-breeding trial data were analyzed using chi-square tests when the numbers of trials that produced offspring were greater than five. For trials with less than five competent breeders, Fisher's Exact Test was used for analyses. Tests were conducted at the 0.05 level of significance.

# Results

## Identification of Call Types

BGSS produced four types of distinct calls during this study (Fig. II.1) of varying lengths and frequencies (Table II.1). Females had a single call, labeled "female mating call" which was only given in response to male mating calls and was previously reported by Percy et al. (2008). The female mating call consisted of a single buzz that increased in frequency. The female calls from the two locations were not significantly different from each other in frequency ($t = -0.96$, $df = 36$, $p = 0.34$), or length ($t = -1.80$, $df = 36$, $p = 0.08$).

Males from northern and southern California had three types of calls: (1) "male mating call," (previously reported in Percy et al. 2008) (2) "gulping call," and (3) "chirping call." The male mating call was the most complex, and consisted of a series of pulses followed by a buzz that increased in frequency. The average frequencies of male mating calls from northern and southern California were significantly different from one another ($t=6.25$, $df=86$, $P<0.0001$), but the average durations were not significantly different ($t=0.07$, $df=86$, $P=0.94$). The differences in pulse frequencies (the first half of the male mating call) between males from northern and southern California were significantly different ($t=2.29$, $df=86$, $P=0.025$). The differences in the buzz frequencies (the second half of the male mating call) were also significantly different between males from northern and southern California ($t=3.16$, $df=86$, $P=0.002$).

The male BGSS gulping call was characterized as a single rising buzz, similar to the second half of the male mating call. Male gulping calls from northern and southern California were not significantly different in frequency ($t=0.70$, $df=80$, $P=0.49$) or length ($t=-0.76$, $df=80$, $P=0.45$).

The male BGSS chirping call is characterized by a single pulse which is typically repeated multiple times in rapid succession. Northern and southern California BGSS chirping calls were not significantly different in frequency ($t=0.43$, $df=58$, $P=0.67$) or length ($t=-0.22$, $df=58$, $P=0.82$).

**Calling Periodicity**

Peak acoustic activity occurred between 0600 and 0900 hr and within one hr of artificial and natural lighting illuminating bottle cages. BGSS from southern California consistently displayed a second calling period between 1200 to 1500 hr. This second period of calling activity in the afternoon was less pronounced for BGSS from northern California with just 25% of the activity exhibited by southern California BGSS. Very little calling was recorded during the night in the absence of artificial and natural light 2100 to 0500 hr. There were two instances of a male calling after dark for northern California and five records for southern California males. Females were not recorded calling at night.

Female BGSS from southern California called in only seven out of 20 trials, and acoustically active females only responded to male mating calls between 0600 and 1000 hr. Only one female from northern California out of 20 trials during the course of this study emitted calls, and did so between 1200 to 1500 hr in response to a northern California male mating call. Across all four recording interval blocks with female calling activity, females from southern California produced an average of 18.9 (± 6.7) mating calls in a 24 hr period. The one female from northern California that called produced four calls in a 24 hr period (Fig. II.2). Too few females called

during this study to determine if statistically significant differences in mating call frequency and duration existed between BGSS populations from northern and southern California.

A clear trend of decreased calling during the course of this study was observed for male BGSS from both locations with northern California calling decreasing rapidly after the first two wk recording block (Fig. II.3). During every two wk period, males from southern California called more often than males from northern California (Fig. II.4). The number of male mating calls was significantly different for the two locations for all four two-wk periods they were recorded period one (July 8 through July 30 2008): $t=4.87$, $df=238$, $P=<0.0001$, period two (August 8 through August 15 2008): $df=238$, $t=3.81$, $P=0.0002$, period three (August 17 through August 27 2008): $df=166$, $t=4.08$, $P=<0.0001$, period four (August 31 through September 21 2008): $df=310$, $t=3.13$, $P=0.002$). Male BGSS from southern California consistently displayed greater calling activity when compared to conspecific males from northern California.

The accessory male calls, previously referred to as chirping and gulping calls, were generally given during the same time periods as the male mating calls. These calls were given either during the male mating calls from conspecific males, or in alteration with the mating calls emitted by the signaler but never during a duet. Males from southern California gave these accessory calls, including the gulping call much more often than males from northern California (Fig. II.5). The mean number of gulping calls produced by males from southern California was significantly greater than that produced by males from northern California over period one ($t=6.71$, $df=262$, $P<0.0001$), period two ($t=3.42$, $df=216$, $P=0.0007$), period three ($t=3.52$, $df=190$, $P=0.0005$), and period four ($t=2.46$, $df=291$, $P=0.0143$).

The second accessory call produced by male BGSS, the chirping call, had a similar calling periodicity as the gulping calls for northern and southern California populations. Males from southern California gave the chirping call much more frequently than conspecific males from northern California (Fig. II.6). Males from southern California had 16 out of 20 recording trials (80%) with five or more chirps in 24 hr, compared to northern California that had males that chirped in seven out of twenty trials (35%) with five or more chirps in 24 hr (Fig. II.6). The average number of chirping calls produced by males from northern and southern California differed significantly across all four recording intervals. Males from southern California chirped significantly more often across period one ($t=3.86$, $df=238$, $P=0.0001$), period two, ($t=4.89$, $df=166$, $P=<0.0001$), period three ($t=4.89$, $df=166$, $P<0.0001$), and period four ($t=3.08$, $df=310$, $P=0.0023$).

**Control Recordings**

Four female-only recordings were made and found that female BGSS from northern ($n = 2$) and southern California ($n = 2$) did not call over the 24 hr recording periods. For male only recordings made over a 24 hr period for northern and southern California populations, males produced male mating, chirping, and gulping calls indicating that only male BGSS produced those calls because females were absent in these cages. Additionally, the mating call attributed to females was not recorded in cages containing only male BGSS, indicating it was a female specific call. During the two blank plant recordings, where no BGSS were present on basil plants inside bottle cages, no calls were recorded. The blank plant recordings ensured that calls

recorded were from BGSS and not other potential insects on host plants, such as whiteflies, which are known to communicate through vibrational calls (Kanmiya 2006).

**Cross-Breeding Studies**

All breeding trials between male and female conspecifics from northern and southern California had low success (see table II.4 and figure II.9). Crosses of southern California males with southern California females had the highest offspring success with 16 out of 36 (44%) pairs producing offspring. Northern California males crossed with northern California females had the next highest offspring production with eight out of 28 (28%) pairs producing offspring. The crosses between different populations had much lower breeding success than crosses made between members of the same population. Crosses of northern California males with southern California females resulted in 6 out of 32 (16%) trials producing offspring. Crosses of southern California males with northern California females had the lowest success rate with just three out of 39 (7%) trials generating offspring.

Offspring production resulting from crosses between different populations were significantly different from one another. Comparing progeny production outcomes between the same location populations (i.e., intra-population crosses for northern and southern BGSS) against crosses between BGSS populations yielded significant differences in offspring production rates ($\chi^2$ =98.7, df=2, P<0.0001). This result indicates that BGSS populations from the same location have higher breeding success when compared to offspring production that results from inter-population crosses. When comparing southern California intra-population crosses against inter-population crosses, significant differences in offspring production were observed ($\chi^2$ =16.2, df=1,

P<0.0001). Offspring production for intra-population southern California crosses compared to each inter-population cross was statistically significant for northern California males crossed with southern California females ($\chi^2$ =7.3, df=1, P=0.007) and southern California males crossed with northern California females ( P<0.0001, Fisher's exact test). Offspring production for intra-population crosses of northern California BGSS compared to the inter-population crosses were significantly different ($\chi^2$=4.7, df=1, P=0.03). Offspring production for intra-population northern California crosses compared to inter-population crosses were not statistically different for northern California males crossed with southern California females ($\chi^2$ =1.6, df=1, P=0.2) but was significantly different when compared to southern California males crossed with northern California females: (P=0.02, Fisher's exact test).

## Discussion

### Periodicity

The first and second objectives of this study were to document all call types given by BGSS from northern and southern California populations and to determine if differences in calls existed between populations. Four types of calls were found in both locations and all differed in the number given over 24 hr. One call type, the male mating call, differed in frequency between the two populations. Our results showed that acoustic signaling by BGSS is diurnal, which is common in the auchenorrhynchous Hemiptera (Virant-Doberlet and Zezlina 2007). Males were the most active and frequent callers, and appeared to initiate all mating duets regardless of whether males were from northern or southern California. Females only responded to male mating calls, and not to male accessory calls (i.e., chirps and gulps). The production of male associated accessory calls was significantly higher by males from southern California when

compared to northern California conspecifics. Northern California males rarely gave accessory calls, contrary to males from southern California which frequently gave accessory calls during periods of acoustic activity.

**Female Mating Calls**

Mating calls of females from both locations were similar and were nearly identical to those from a previous study conducted on BGSS mating calls (Percy et al. 2008). The study by Percy et al. (2008) reported female call frequencies and duration to be on average 203 ± 64 Hz and 0.52 ± 0.13 sec in duration. The study reported here revealed females from southern California to have, on average, calls at 219 ± 24 Hz, of 0.85 ± 0.05 sec in duration. The northern California female mating call was not significantly different to the southern California call, being on average, 174 ± 7 Hz and 0.63 ± 0.05 sec in length. This result suggests there is little variation among female calls between populations of BGSS in northern and southern California.

The proposed function of the female call in auchenorenchous hemipterans is to direct males towards females for mating and is often in the form of male-female duets (Claridge 1985). Consequently, it is possible that BGSS males are unable to recognize and mate with females which have songs that exhibit significant variation in frequency and structure, and male discrimination may have greatly constrained the characteristics of female BGSS calling patterns.

**Male Mating Calls**

Male BGSS mating calls appear to vary widely between northern and southern California. In the Percy et al. (2008) study, the mating call of BGSS males collected from southern California had

a frequency of 235 ± 68 Hz, with 1.17 ± 0.23 sec duration. Our recordings indicate that male mating calls from southern California average 205 ± 10 Hz and 2.15 ± 0.13 sec duration, and males from northern California call at 357 ± 29 Hz and 2.16 ± 0.13 sec duration. The experimental setup for both studies were very similar as BGSS from both studies were on basil plants of the same height, enclosed in identical plastic bottle cages, and were recorded in the same light and temperature conditions. Both studies used virgin adults of the same age and same number of males and females per cage. Male mating calls recorded by Percy et al. (2008) study differ from results presented here by 30 Hz and calls recorded in this study were about twice as long in duration. The southern California BGSS colony used by Percy et al. (2008) was sourced 50 km from the population used to initiate colonies used in these studies. The quantified differences in male calls suggest that even when BGSS populations are close to one another, significant differences in male mating calls may exist. Population differences between male BGSS mating calls probably become more extreme with increasing geographic distance as evidenced by comparisons presented here for populations from northern California and southern California. Further studies on the mating calls from the edges of the BGSS range (Canada and Nicaragua) could be useful to further quantify the relationship between distance between populations and differences between male mating calls.

**Male Accessory Calls**

This study found two additional call types that were previously undocumented in male BGSS. These accessory calls were described as gulping and chirping calls. The gulping and chirping calls were produced by BGSS males from northern and southern California, and these calls were made much more frequently by males from southern California. Males from northern California

only rarely emitted these calls, and gave them significantly less often than their mating calls. Accessory calls do not appear to have a direct function in mating because female BGSS never responded to accessory calls, yet males emitted these calls during times when they also gave their mating call though never in conjunction with mating duets. Because females did not respond to male accessory calls, these calls may not be directed at females as part of the acoustic repertoire used in mating. Male accessory calls may be warnings to rival males, or they could act as acoustic sabotage by jamming courtship calls by competitors when these calls are emitted during calling bouts dominated by mating calls that could be originating from conspecific males. Conspecific males were frequently heard giving the accessory calls during mating duets of other pairs, and these calls may serve to prevent successful mating between courting pairs.

Other species of Cicadellidae have been reported to use acoustic competition to thwart male competitors (Hunt and Morton 2001) and BGSS males may use accessory calls in a similar manner. The idea of courtship jamming by vibrational calls produced by competing males has been proposed for *Ennya chrysura* Fairmaire (Hemiptera: Membracidae) as a means to prevent females from adequately hearing and accurately judging male calls (Miranda 2006). Because male BGSS give their accessory calls during periods of high acoustic activity, these calls may also act as jamming signals.

Males from northern and southern California produced accessory calls at significantly different levels. Males from southern California gave accessory calls frequently during the window of calling activity 0600 to 0900 hr (Figure II.7). In contrast, males from northern California rarely gave either accessory call when mating calls were being made (Figure II.8). If accessory calls

are indeed components of courtship jamming, males from northern California do not seem to use this method as often as males from southern California.

Like most lab conducted studies, certain limitations and advantages arise from conducting experiments in an exclusively artificial laboratory setup. Recording BGSS in an indoor setting helps to eliminate background noise such as wind and other noises that cannot be held constant in field studies. It also allows control over the number of males and females that come into contact with one another. However, this study could benefit from field recordings under natural conditions. Because of the advantages of conducting acoustic studies in the lab, acoustic field studies of vibrational calls seem to have been overlooked up to this point. Field recordings may provide greater insight into the use of male accessory calls and the importance of calling for BGSS mating.

**Cross-Breeding Trials**

The final objective of this study was to determine mating capabilities between northern and southern BGSS. All mating trials had fairly low success as measured by offspring production, with three trials resulting in <30% of pairs producing nymphs. Low offspring production rates could be due to the small number of individuals set up in each breeding chamber (i.e., one male and one female were used). In preliminary acoustic studies, no acoustic signals were recorded when only one male and one female were confined in a bottle cage on a basil plant. Thus, BGSS may call only rarely in the absence of multiple conspecifics, for example because it may not be worth the cost of calling. Calling activity in BGSS may not be initiated unless individuals sense the presence of a critical number of conspecifics. Group calling of this nature, should it be

employed by BGSS, could result from the detection of vibrations produced by feeding, walking, and jumping. With only two sharpshooters per mating trial, it is possible that some males failed to detect the presence of females, resulting in a lack of male calling and consequently low mating rates even when intra-population crosses were made.

Northern California intra-population crosses produced fewer successful matings than southern California intra-population crosses (28% vs. 44%, respectively), though they were not statistically significantly different. This could indicate that the conditions in the laboratory in southern California may not have been as suitable for northern California BGSS to engage in successful mating behaviors. However, this seems unlikely because colonies of northern California BGSS performed equally well under identical environmental conditions that were used for rearing southern California populations. More likely, a key component in mating procedures for northern California populations, such as larger numbers of competing individuals on plants for mates, may have been responsible for low mating frequencies which resulted in the low numbers of northern California offspring produced from mating trials.

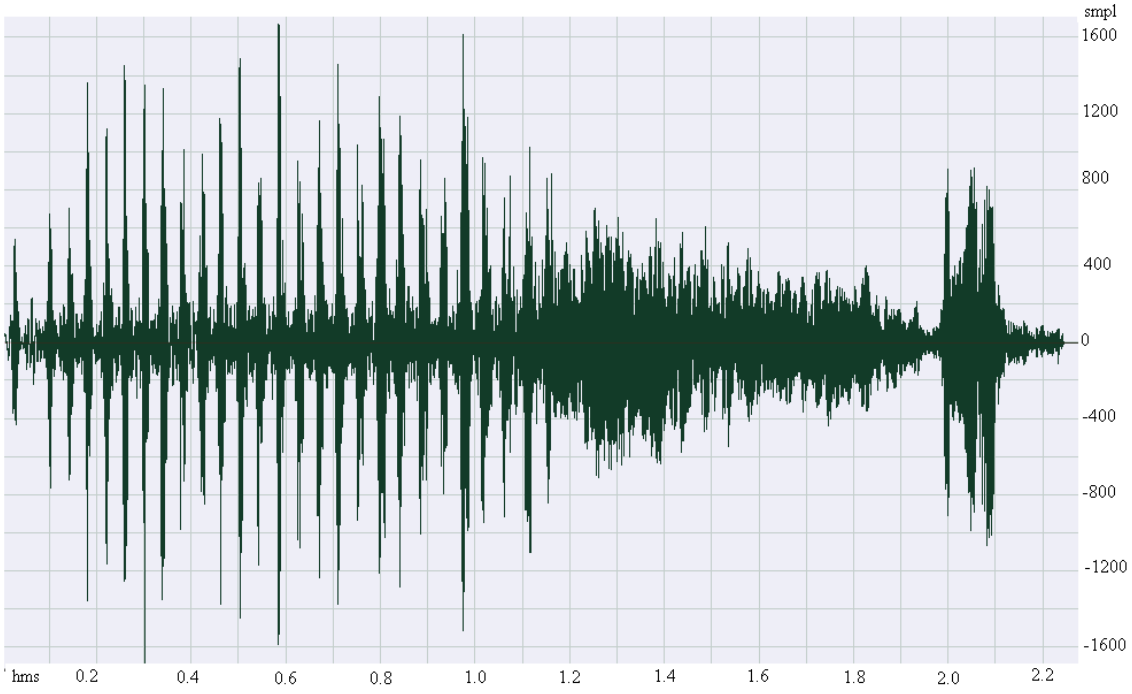
Intra-population crosses had significantly higher percentages of offspring production when compared to inter-population crosses. This suggests that BGSS prefer to mate with individuals from their own populations rather than conspecifics from a distant location. This seems especially true with individuals from southern California which had intra-population breeding success as high as 44%, vs. just 7% when presented with individuals from northern California for mating (southern California male and northern California female). As indicated earlier, male BGSS from southern California call more frequently and emit many more accessory calls than

conspecific males from northern California. Consequently, different responses by females BGSS to male accessory calls may be indicative of the development of a pre-mating isolation mechanism.

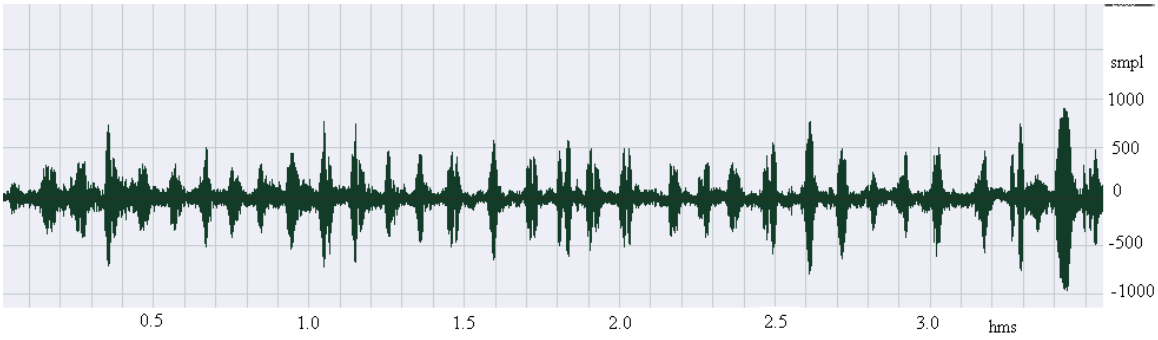
This study sought to determine differences in acoustic communication and cross-breeding abilities between widely separated populations of BGSS. Although the same types of calls were given by individuals from both locations, populations varied significantly in how often they gave these calls. Differences were observed in the frequency (Hz) of male mating calls between northern and southern California. Differences were also seen in cross-breeding capabilities, with individuals producing offspring more often with individuals from their own populations. Breeding success rates may be due, in part, to differences in male calls that were observed in this study. Increased understanding of BGSS ecology could benefit from research investigating whether additional differences exist between widely separated populations of BGSS. Additional studies could investigate transmission rates of *X. fastidiosa*, and the composition and impact of natural enemy complexes attacking BGSS eggs. Disease transmission capabilities and associated natural enemies may vary widely between BGSS populations that have large distances between them.

**Figure II.1.** Oscillographs of the four types of *Graphocephala atropunctata* calls, the X-axis represents time in seconds, and the Y-axis represents amplitude in decibels. A. Male mating call, B. Male chirping call, C. Male gulping call, D. Male and female mating duet.

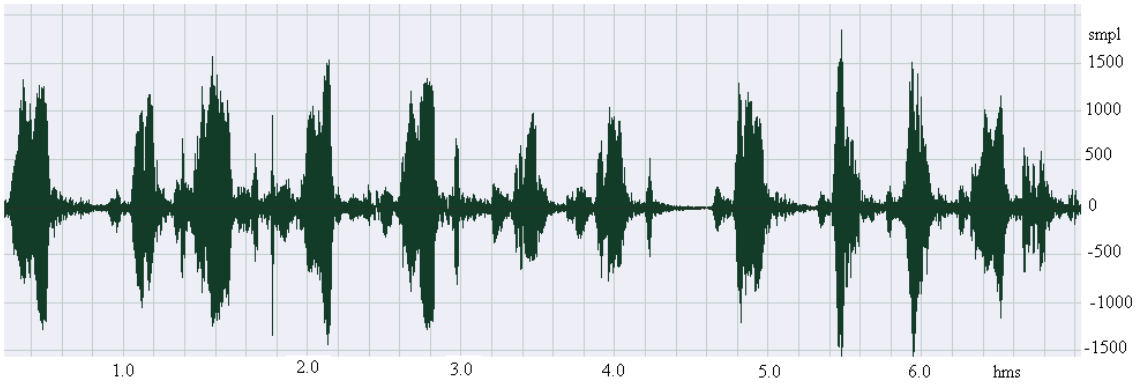
**A.**




**B.**



**C.**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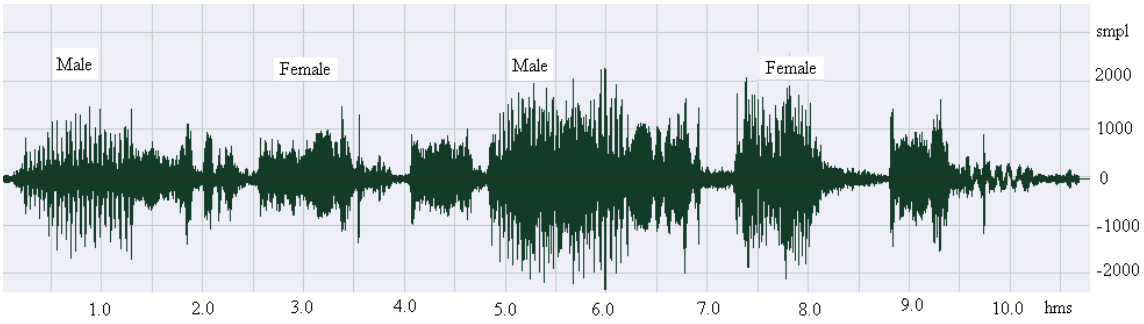
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**Table II.1** Summary of call qualities for individuals *of Graphocephala atropunctata* from northern California (N) and southern California (S). *=significant difference below the 0.05 level (Student's t-test).

| | N female mating call | S female mating call | N male Chirping call | S male Chirping call | N male gulping call | S male gulping call | N male mating call | S male mating call |
|---|---|---|---|---|---|---|---|---|
| Frequency | 174.2±6.7 | 219.1±23.8 | 421.7±25.5 | 408.2±17.1 | 207.7±9.2 | 197.5±8.0 | 356.7±28.9* | 205.0±10.3* |
| Length | 0.63±0.05 | 0.80±0.05 | 0.09±0.008 | .09±0.006 | 0.50±0.06 | 0.55±0.03 | 2.15±0.13 | 2.15±0.50 |

**Table II.2** Summary of *Graphocephala atropunctata* male mating call properties from individuals from northern California (N) and southern California (S). *=significant difference below the 0.05 level.

| | N male mating call | S male mating call |
|---|---|---|
| Overall Frequency* | 356.70 ± 28.9 | 204.98 ± 10.3 |
| Overall Length | 2.16 ± 1.06 | 2.15 ± 0.06 |
| Pulse Frequency* | 314.1 ± 27.4 | 239.39 ± 17.2 |
| Rising Buzz Frequency* | 300.7 ±33.5 | 200.55 ± 14.8 |

**Table II.3** Summary of calling periodicity over four two-wk intervals for individuals of *Graphocephala atropuncata* from northern California (N) and southern California (S).

| | N period 1 | S interval 1 | N interval 2 | S interval 2 | N interval 3 | S interval 3 | N interval 4 | S interval 4 |
|---|---|---|---|---|---|---|---|---|
| Total male mating calls | 27 | 94 | 0 | 61 | 2 | 24 | 2 | 17 |
| Max male mating calls per hr | 6 | 12 | 0 | 11 | 1 | 5 | 1 | 4 |
| Average male mating calling rate | 1.13±0.27 | 4.02± 0.64 | 0 | 2.53±0.54 | 0.09±0.03 | 2.5±0.54 | 0.075±0.02 | 0.7±0.16 |
| Total male gulping calls | 2 | 37 | 0 | 12 | 1 | 10 | 1 | 13 |
| Max male gulping calls per hr | 1 | 6 | 0 | 2 | 1 | 2 | 1 | 3 |
| Average male gulping calling rate | 0.09±0.03 | 1.53±0.28 | 0 | 0.51±0.14 | 0.03±0.02 | 0.4±0.1 | 0.16±0.13 | 0.53±0.18 |
| Total male chirping calls | 7 | 48 | 1 | 23 | 1 | 12 | 3 | 40 |
| Max male chirping calls per hr | 1 | 6 | 1 | 5 | 1 | 2 | 1 | 8 |
| Average male chirping calling rate | 0.29±0.06 | 2±0.33 | 0.02±0.008 | 0.97±0.2 | 0.04±0.01 | 0.51±0.1 | 0.12±0.03 | 1.66±0.39 |

**Figure II.2.** The total number of *Graphocephala atropunctata* female mating calls per hour for northern and southern California over 24 hr.

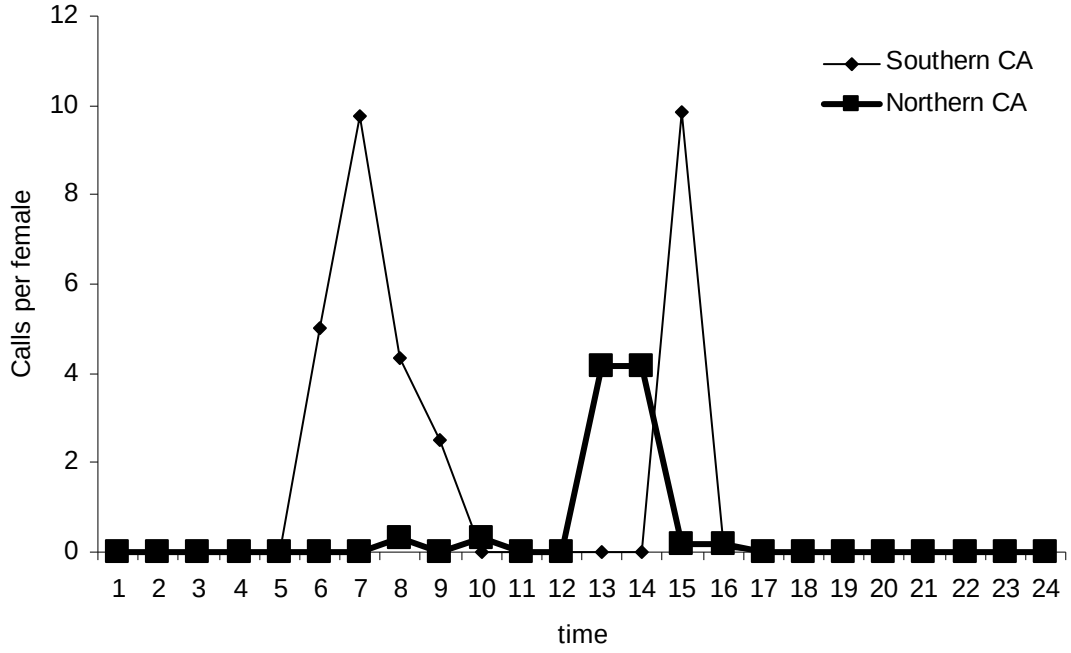



**Figure II.3.** Total number of male mating calls given by *Graphocephala atropunctata* males over four consecutive two-wk blocks of recordings. Block 1. July 8-30 2008 Block 2. August 8-15 2008, Block 3. August 17-27 2008, and Block 4. August 31-September 21 2008.

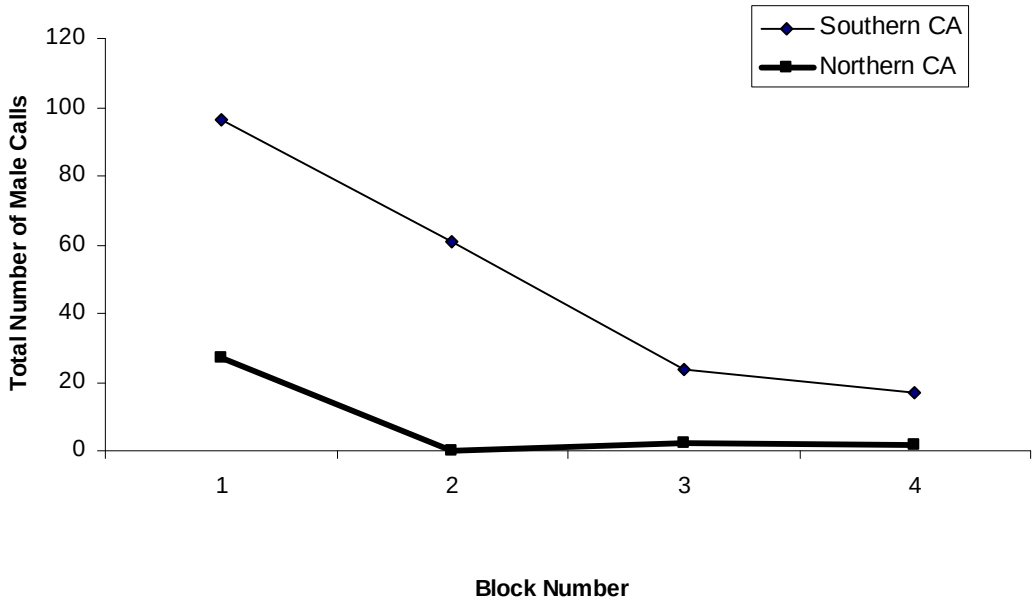


**Figure II.4.** Periodicity and frequency of *Graphocephala atropunctata* male mating calls over four consecutive two-wk periods. A. July 8-30 2008. B. August 8-August 15 2008. C. August 17-August 27 2008. D. August 31-September 21 2008.

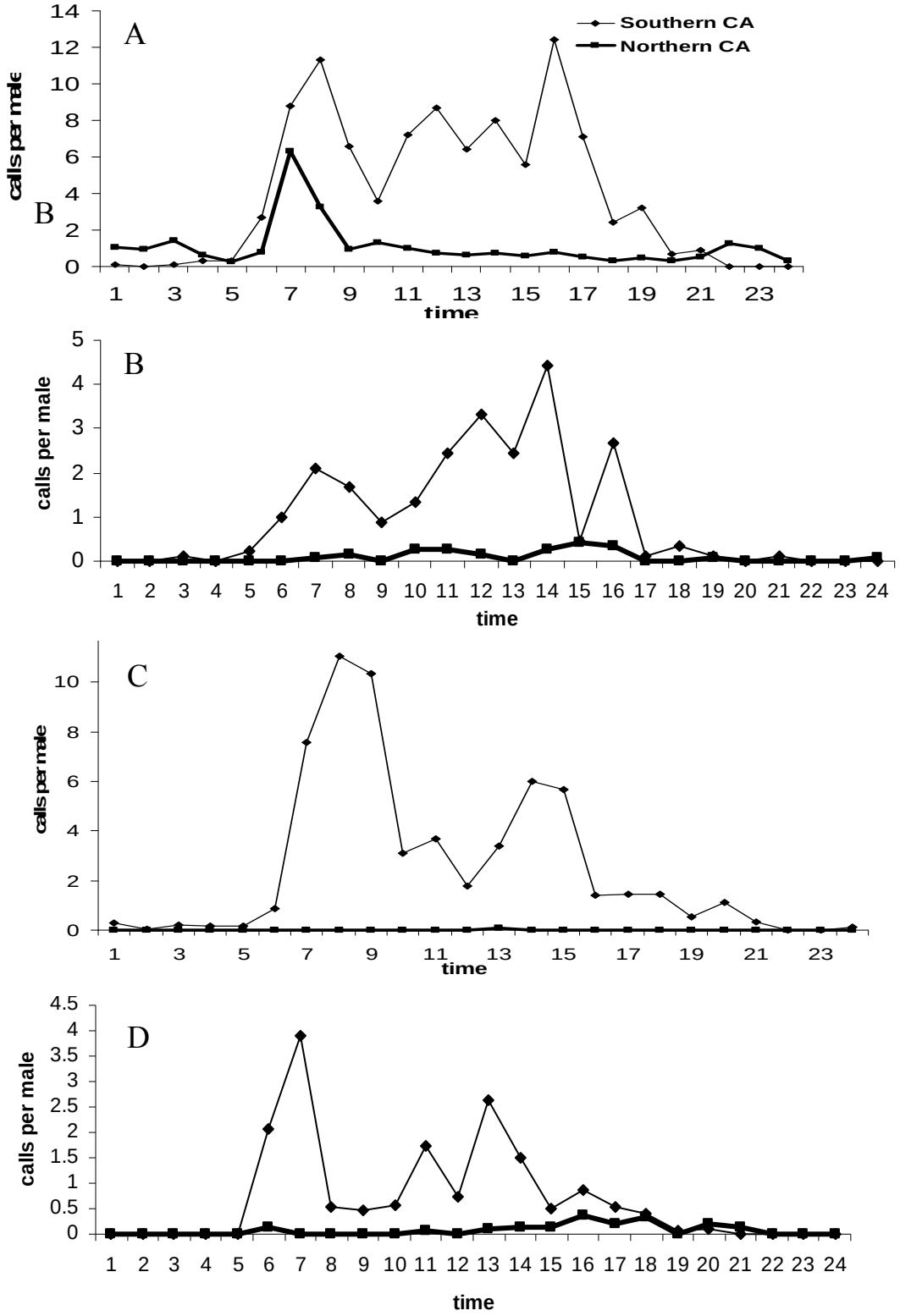


**Figure II.5.** Periodicity and frequency of *Graphocephala atropunctata* male gulping calls over four consecutive two-wk periods. A. July 8-30 2008. B. August 8-August 15 2008. C. August 17August 27 2008. D. August 31September 21 2008.

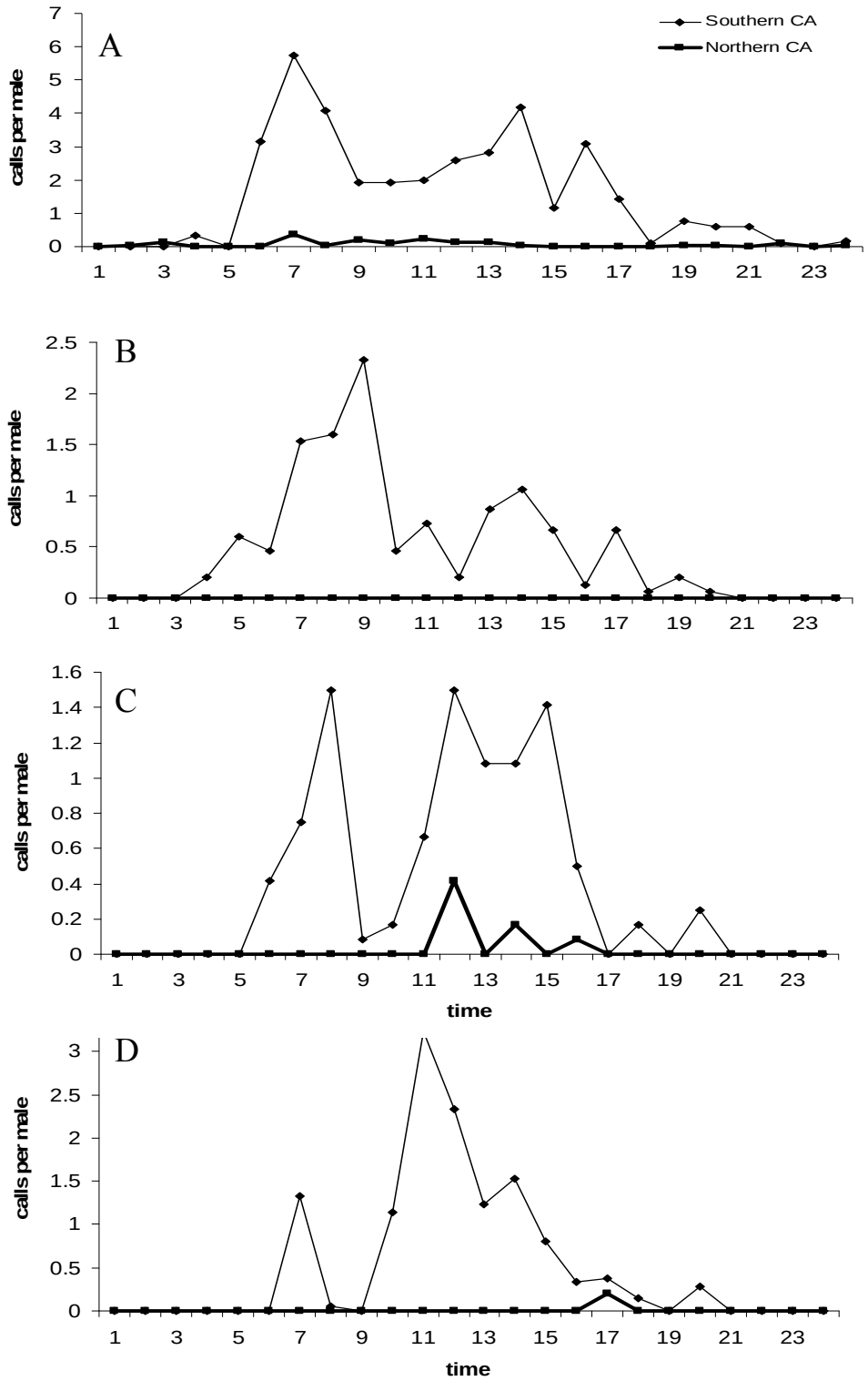


**Figure II.6.** Periodicity and frequency of *Graphocephala atropunctata* male chirping calls over four consecutive two-wk periods. A. July 8-30 B. Aug 8-Aug 15 C. Aug 17-Aug 27 D. Aug 31-Sept 2008.

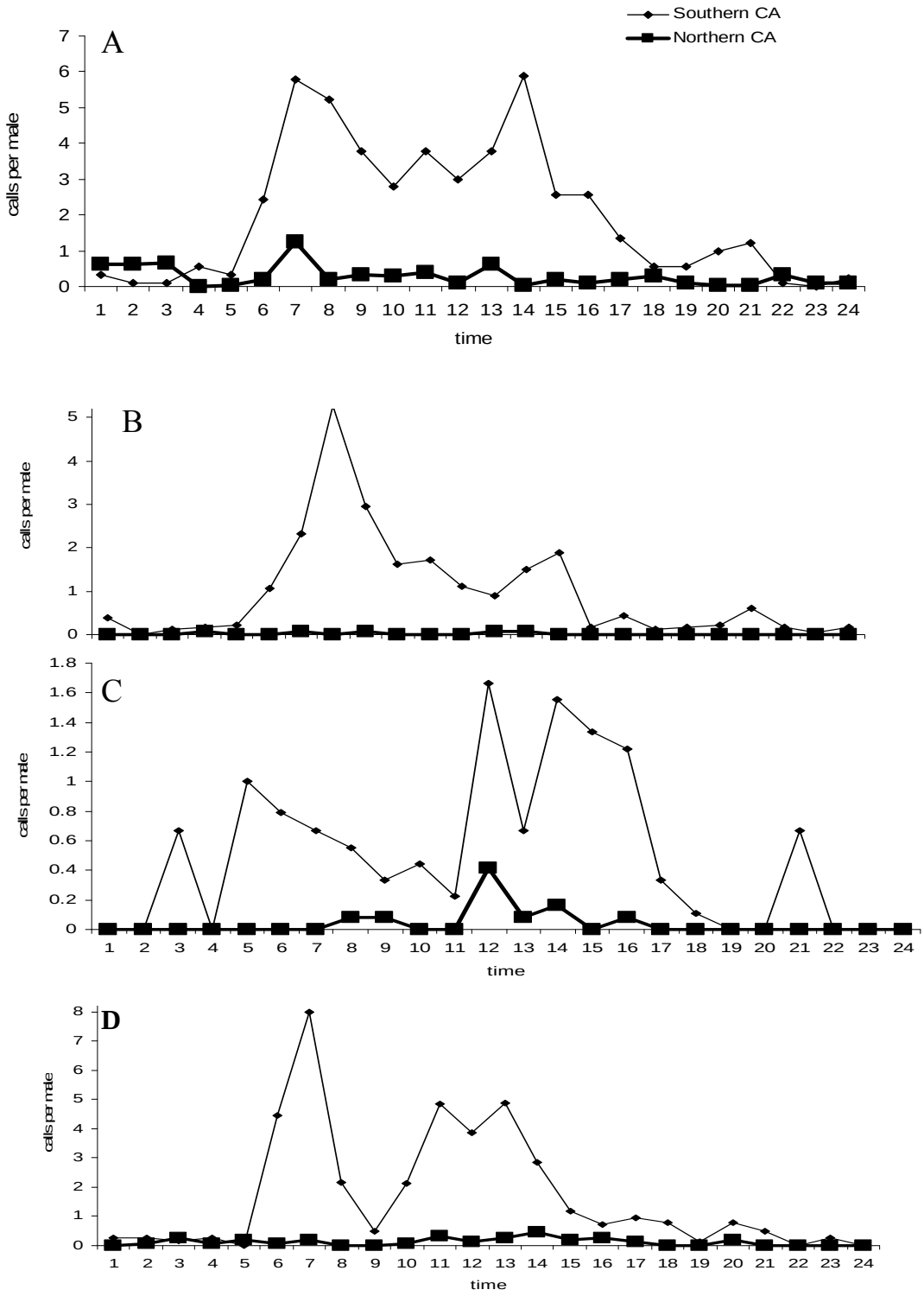


**Figure II.7** Periodicity of male mating calls, gulping calls, and chirping calls across entire study of *Graphocephala atropunctata* from southern California.

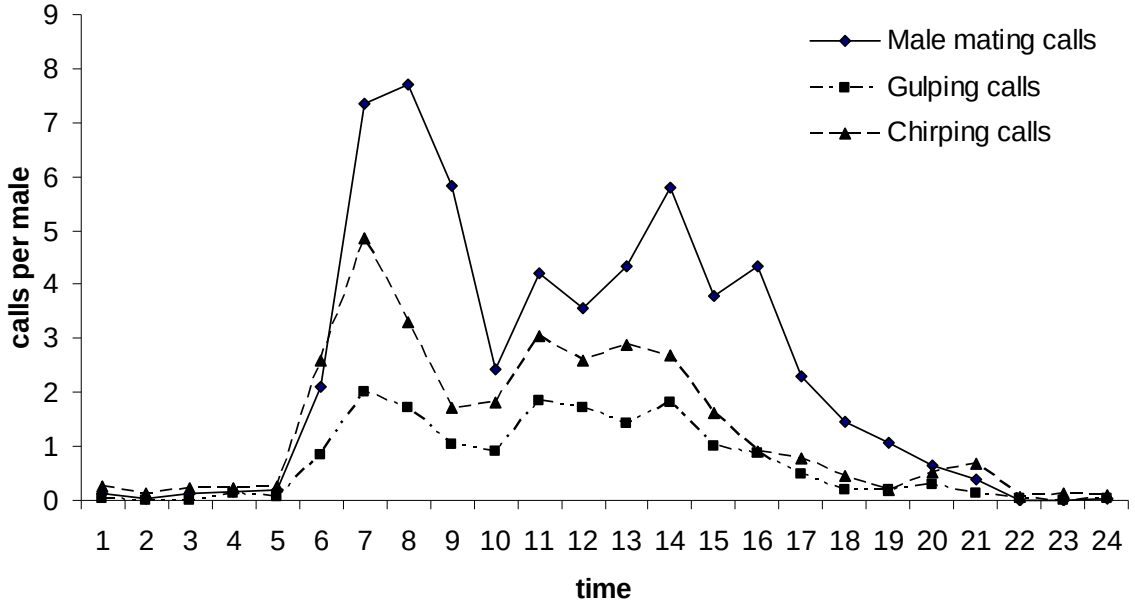



**Figure II.8** Periodicity of male mating calls, gulping calls, and chirping calls across entire study of *Graphocephala atropunctata* from northern California.

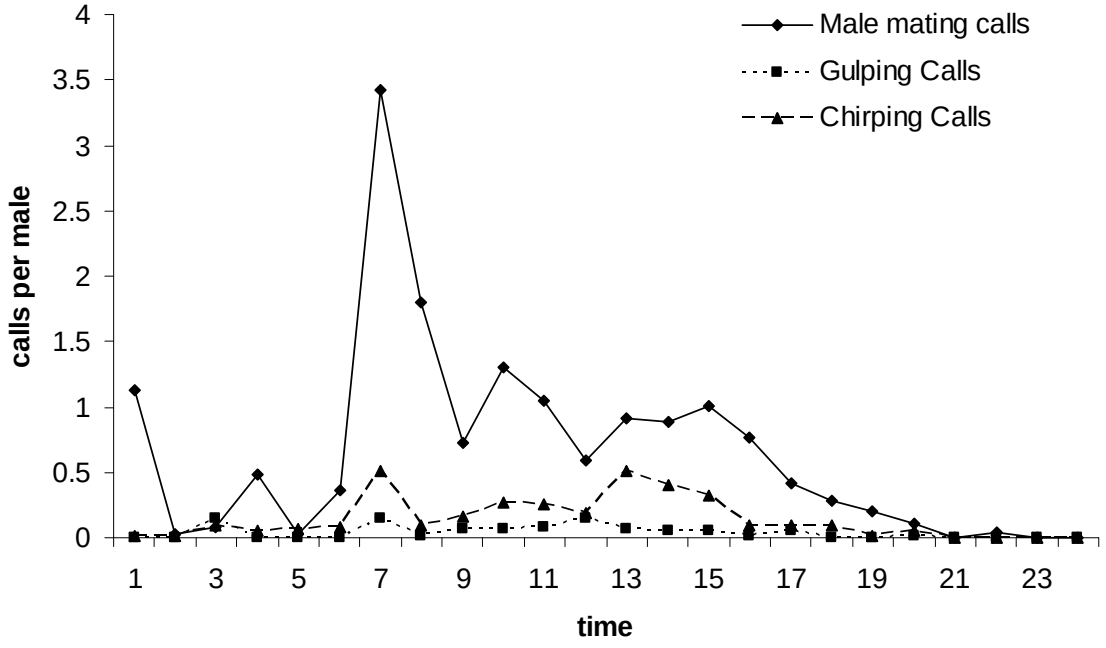
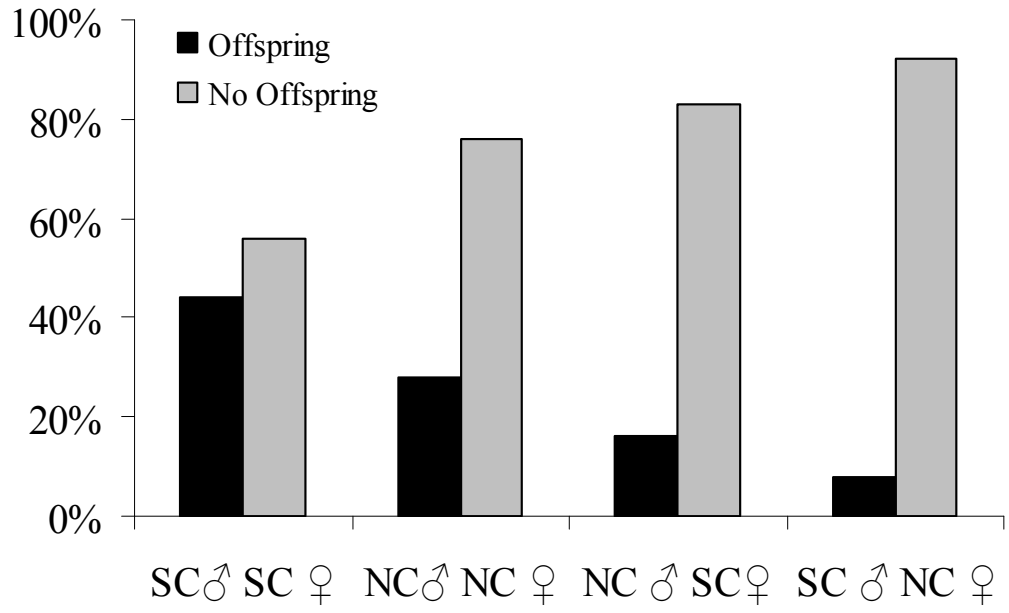


**Table II.4** Results of cross-breeding trials between populations of *Graphocephala atropunctata* from northern California (NC) and southern California (SC).

| | Offspring | No Offspring | % Offspring | % No Offspring |
|---|---|---|---|---|
| NC♂ NC ♀ | 8 | 20 | 29% | 71% |
| SC♂ SC ♀ | 16 | 20 | 44% | 56% |
| NC ♂ SC♀ | 6 | 32 | 16% | 84% |
| SC ♂ NC ♀ | 3 | 39 | 7% | 93% |

**Figure II.9** Cross-breeding results of *Graphocephala atropunctata*. Black bars represent percentage of trials with offspring, and grey bars indicate percentage of trials without offspring.



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