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Genetic Variability of Two Leaffooted Bugs, *Leptoglossus clypealis* and *Leptoglossus zonatus* (Hemiptera: Coreidae) in the Central Valley of California

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Abstract

Leaffooted plant bugs (LFPBs) (*Leptoglossus* spp., Guérin-Méneville) (Hemiptera: Coreidae) are large seed-feeding bugs native to the Western Hemisphere. In California, several *Leptoglossus* spp. feed on almonds, pistachios, and pomegranate and are occasional pests. The objective of this study was to survey the different species of *Leptoglossus* present in almond, pistachio, and pomegranate orchards in the Central Valley of California. We used two molecular markers, amplified fragment length polymorphisms (AFLPs) and mitochondrial DNA COI, to determine the number of species or strains of each species, and to infer whether individuals of each species move and possibly interbreed with populations from the other host plants. Two species of leaffooted bugs were abundant, *Leptoglossus clypealis* Heidemann, and *Leptoglossus zonatus* (Dallas). *L. clypealis* was collected in almond and pistachio, while *L. zonatus* was found on all three host plants, but was the dominant species in pomegranate. The AFLP results indicated that *L. clypealis* consisted of one species, which suggests it moves between almonds and pistachios during the growing season. Mitochondrial DNA COI for *L. clypealis* found 1–2% divergence between sequences, and a high haplotype diversity of 0.979 with 17 haplotypes. The AFLP results for *L. zonatus* found two genetically divergent populations which were morphologically similar. The mtDNA COI sequences for *L. zonatus* were used for haplotype analysis; three haplotypes were found in California, with one haplotype shared with collections from Brazil. The importance of genetic variability and cryptic species for pest management are discussed.

Key words: Heteroptera, cryptic species, host plant strains, integrated pest management, haplotype

The genus of insects, *Leptoglossus* Guérin-Méneville (Hemiptera: Coreidae) commonly known as leaffooted plant bugs (LFPBs) is widely distributed in the Western Hemisphere from southern Canada into South America and the Caribbean (Allen 1969, Brailovsky and Barrera 1998, Brailovsky and Barrera 2004), with at least 61 species currently documented in the Americas. LFPBs feed on seeds, nuts, and fruits and several species are considered agricultural or forest pests (Allen 1969, Brailovsky 2014). In the Central Valley of California, three species *Leptoglossus zonatus* (Dallas), *Leptoglossus clypealis* Heidemann, and *Leptoglossus occidentalis* Heidemann are recorded on almonds, pistachios, and pomegranate and are occasional pests (Essig 1958, Daane et al. 2005, Zalom et al. 2012). However, in recent years, *L. zonatus* and *L. clypealis* have become more apparent on these host plants (Haviland 2007, Joyce et al. 2013), perhaps due to their increased cultivation.

L. zonatus is polyphagous and occurs widely throughout much of the Western Hemisphere (Allen 1969, Brailovsky and Barrera 1998, Gonzaga-Segura et al. 2013) (Table 1). It presents a highly variable life history, with developmental time ranging from 54 to 83 d depending on the host crop (Matrangolo and Waquil 1994, Grimm 1999, Grimm and Somarriba 1999, Tepole- García 2011). A broad range of host plants is recorded for *L. zonatus*, which is reported to migrate from one crop to another for feeding or to complete its development (Grim and Guharay 1998, Grimm and Somarriba 1999) (Table 1). Detection of fruits or seeds is often by olfaction of the host-plant associated volatiles (Xiao and Fadamiro 2009). Aldrich et al. (1979) and Soares et al. (1994) suggest that chemical cues produced and detected are specific for each leaffooted bug species but also vary among the life stages and sexes within the same species (Gonzaga-Segura et al. 2013).

The second leaffooted bug species, *L. clypealis*, is common in California yet has a more restricted distribution range, spanning from the south of the United States into northern México and the southwest United States (Heidenmann 1910, Allen 1969). The

Table 1. Native and exotic plant species consumed by *L. zonatus* and *L. clypealis*

L. zonatus					
Number	Family	Species	Common Name	Origin	Reference
1	Acanthaceae	—	—	—	Fernandes and Grazia 1992
2	Anacardiaceae	*Pistacia vera*	Pistachio	Exotic	Bolkan et al. 1984
3	Anacardiaceae	*Mangifera indica*	Mango	Exotic	Pires et al. 2012
4	Anacardiaceae	*Anacardium occidentale*	Cashew	Native	Grimm and Somarriba 1999
5	Arecacea	*Phoenix dactylifera*	Date palm	Exotic	Solomon and Froeschner 1981
6	Asteraceae	*Cirsium horridulum*	Spiny thistle	Exotic	Henne et al. 2003
7	Asteraceae	*Sonchus oleraceous*	Sowthistle	Exotic	Henne et al. 2003
8	Asteraceae	*Helianthus spp.*	Sunflower	Native	Mitchell 2000; Gonzaga-Segura et al. 2013
9	Asteraceae	*Cynara cardunculus*	Artichoke thistle	Exotic	Tepole-García 2011
10	Bignoniaceae	*Spathodea campanulata*	African tuliptree	Exotic	Souza et al. 1999
11	Bignoniaceae	*Chilopsis linearis*	Desert willow	Native	Jones 1993
12	Bixaceae	*Bixa orellana*	Lipstick tree	Native	Tepole-García 2011
13	Cactacea	*Hylocereus costaricensis*	Pythaya	Native	Grimm and Somarriba 1999
14	Cucurbitaceae	*Citrullus lanatus*	Watermelon	Exotic	Allen 1969; Solomon and Froeschner 1981
15	Cucurbitaceae	*Cucumis sp*	—	Exotic	Duarte-Sánchez 2008
16	Cucurbitaceae	*Momordica charantia*	Bitter melon	Exotic	Duarte-Sánchez 2008
17	Cucurbitaceae	*Cucumis melo*	Sweet melon	Exotic	Mitchell 2000
18	Cucurbitaceae	*Luffa cylindrica*	Luffa	Exotic	Tepole-García 2011
19	Euphorbiacea	*Triadica sebiferum*	Chinese tallow	Exotic	Henne et al. 2003
20	Euphorbiacea	*Jatropha curcas*	Physic nut	Native	Grimm 1999; Yepes-Rodríguez et al. 2012; Morales-Morales et al 2011
21	Fabaceae	*Glycine max*	Soybean	Exotic	Panizzi 1989
22	Fabaceae	*Phaseolus vulgaris*	Beans	Native	Panizzi 1989
23	Graminae	*Sorghum bicolor*	Sorghum	Exotic	Solomon and Froeschner 1981; Metranlogo and Waquil 1994
24	Graminae	*Zea mays*	Maize	Native	Solomon and Froeschner 1981; Panizzi 1989; Fernandes and Grazia 1992; Matrangolo and Waquil 1994; Pazzini 2004
25	Juglandaceae	*Carya illinoinensis*	Pecan	Native	Tarango et al. 2007; Tarango and González 2009; Tepole-García 2011
26	Lauraceae	*Persea americana*	Avocado	Native	Gonzaga-Segura et al. 2013
27	Lythraceae	*Punica granatum*	Pomegranate	Exotic	Solomon and Froeschner 1981; Raga et al. 1995
28	Malpighiacea	*Malpighia emarginata*	Barbados cherry	Native	Pires et al. 2012
29	Malvaceae	*Gossypium hirsutum*	Cotton	Native	Jackson et al. 1995
30	Malvaceae	*Triumfetta sp*	Burbark	Native	Silva et al. 1968
31	Moracea	*Morus nigra*	Blackberry	Exotic	Pires et al. 2012
32	Myrtaceae	*Psidium guajava*	Guajava	Native	Pires et al. 2013
33	Oxalidaceae	*Averrhoa carambola*	Star fruit	Exotic	Pires et al. 2011
34	Passifloraceae	*Passiflora edulis*	Passion fruit	Native	Rodrigues Netto and Guilhem 1996; Grimm and Somarriba 1999
35	Pedaliaceae	*Sesamum indicum*	Sesame	Exotic	Mitchell 2000; Gonzaga-Segura et al. 2013
36	Rosaceae	*Prunus persica*	Peach	Exotic	Solomon and Froeschner 1981; Xiao and Fadamiro 2009
37	Rosaceae	*Prunus dulcis*	Almond	Exotic	Joyce et al. 2013
38	Rutaceae	*Citrus* spp.	Citrus	Exotic	Kubo and Batista 1992; Grimm and Somarriba 1999; Henne et al. 2003
39	Rutaceae	*Citrus sinensis*	Orange	Exotic	Henne et al. 2003
40	Rutaceae	*Fortunella spp*	Kumquat	Exotic	Henne et al. 2003; Tepole-García 2011
41	Rutaceae	*Citrus tangelo*	Tangelo	Exotic	Duarte-Sanchez et al. 2008
42	Rutaceae	*Citrus limon*	Lemon	Exotic	Tepole-García 2011
43	Rutaceae	*Citrus unshiu*	Mandarin	Exotic	Henne et al. 2003; Xiao and Fadamiro 2009; Xiao and Fadamiro 2010;
44	Solanaceae	*Sonalum melogena*	Eggplant	Exotic	Mitchell 2000
45	Solanaceae	*Lycopersicon esculentum*	Tomato	Native	Grimm and Somarriba 1999; Xiao and Fadamiro 2009
46	Solanaceae	*Sida sp,*	Wireweed	Native	Silva et al. 1968
47	Solanaceae	*Solanum americanum*	American black nightshade	—	Duarte-Sanchez et al. 2008

Table 1. Continued

L. zonatus					
Number	Family	Species	Common Name	Origin	Reference
48	Solanaceae	*Solanum tuberosum*	Potato	Native	Mitchell 2000; Gonzaga-Segura et al. 2013
L. clypealis					
1	Acanthaceae	*Juniperus ashei*	Juniper	Native	Mitchell 2000
2	Anacardiaceae	*Pistacia vera*	Pistachio	Exotic	Allen 1969; Bolkan et al. 1984
3	Anacardiaceae	*Rhus aromatica*	Sumac	Native	Mitchell 2000
4	Rosaceae	*Prunus dulcis*	Almond	Exotic	Heidemann 1910
5	Rosaceae	*Prunus spp*	Plums	Native, exotic	Allen 1969; Mitchell 2000
6	Cupressaceae	*Thuja spp*	Arborvitae	Native	Allen 1969; Mitchell 2000
7	Ericaceae	*Arctostaphylos pungens*	Manzanita	Native	Mitchell 2000
8	Agavaceae	*Dasylirion wheeleri*	Desert spoon	Native	Mitchell 2000
9	Asteraceae	*Helianthus spp.*	Sunflower	Native	Mitchell 2000
10	Fabaceae	*Phaseolus vulgaris*	Beans	Native	Mitchell 2000

Dashed lines indicate that a species or a center of origin is unknown.

developmental time from nymph to adult is 31–34 d (Mitchell 2000). *L. clypealis* has been noted to aggregate during winter under the bark of the trees and in leaf litter (McPherson et al. 1990), is recorded to feed on a much smaller number of host plants than *L. zonatus* (Table 1), and can migrate from one crop to another. Generally, it consumes fruits and seeds but also attacks the stem and the leaves of trees (Mitchell 2000). Chemical cues such as alarm pheromones and sex pheromones are also documented for *L. clypealis* (Aldrich et al. 1979, Wang and Millar 2000).

L. zonatus is recorded on at least 48 plant species in 24 families while *L. clypealis* is noted on 10 plant species in eight families, respectively, consisting of both native and exotic species (Table 1). Host plants include numerous economically important crops, and can result in crop damage and decreased yields (Bolkan et al. 1984, Rice et al. 1985, Marchiori 2002, Henne et al. 2003, Xiao and Fadamiro 2009, Xiao and Fadamiro 2010, García et al. 2012) (Table 1). For example, feeding by *L. zonatus* reduces the yield on the satsuma mandarin, *Citrus unshiu* (Xiao and Fadamiro 2009, Xiao and Fadamiro 2010), and can reduce yield in corn by 15% (*Zea mays*) (Marchiori 2002). *L. clypealis* is attributed to reducing the yield of pistachios by 30% (Bolkan et al. 1984, Rice et al. 1985, Michailides et al. 1987; Michailides 1989). In addition to yield losses in crops, both species have been recorded as vectors of plant pathogens. *L. zonatus* can transmit a yeast disease (*Nematocera coryli*) to fruit (Henne et al. 2003, Xiao and Fadamiro 2010) as well as transmitting *Trypanosomatids* to corn (*Zea mays*) (Jankevicius et al. 1993), and *Eremothecium (=Stigmatomycosis)* to pomegranate (Michailides and Morgan 1990) and pistachio. *L. clypealis* can transmit the fungal pathogens, *Botryosphaeria dothidea* (Rice et al. 1985) and *Eremothecium coryli* (Michailides and Morgan 1990, 1991). Finally, the damage caused by *L. zonatus* and *L. clypealis* generates wilted fruits and predisposes the fruit to colonization by other insects and pathogens (Pires et al. 2011).

Pheromones and biological control using parasitoids or predators could be included as components of an integrated pest management (IPM) program (Blatt and Borden 1996) for these species. Using pheromones and biological control effectively requires knowledge of the pest species and whether or not there are host-plant-associated strains or geographically divergent populations of each pest in order to best use these pest control tools. For example, parasitoid wasps are often host specific (Hoffman et al. 1991). Management of these two leaffooted bugs through biological control and the potential to use pheromones has been investigated (Grimm and Guharay 1998, Souza and Amaral Filho 1999, Marchiori 2002). For instance, the eggs of *L. zonatus* can be parasitized by *Trissolcus* spp. (Marchiori 2002), *Anastus* spp. and *Gryon* sp. (Souza and Amaral Filho 1999) while adults of *L. zonatus* can be parasitized by *Trichopoda pennipes* (Souza and Amaral Filho 1999) and *Trichopoda* spp. (Duarte-Sanchez et al. 2008). Entomopathogens such as the fungi *Beauveria bassiana* and *Metharhizium anisopliae* have been effective generating mortality rates that range from 88 to 99% and 91% for *L. zonatus* attritubed to each pathogen, respectively (Grimm and Guharay 1998).

The presence of cryptic species or genetically divergent strains of either *L. zonatus* or *L. clypealis* is suggested by the variation in biological traits such as dietary plasticity and variability in developmental time, such as that observed for *L. zonatus* raised on *Jatropha curcas* (Grimm and Somarriba 1999) and *Zea mays* (Fernandes and Grazia 1992). The two taxonomic revisions of the genus *Leptoglossus* by Allen (1969) and Brailovsky (2014) are based on anatomical and morphological characters, and currently molecular tools have not been used to investigate variability within the genus or these species. In some insect systems, molecular markers have uncovered that insects with large native distribution ranges can consist of genetically distinct strains or cryptic species, which are morphologically similar but genetically and behaviourally distinct (Herbert et al. 2004, Joyce et al. 2014). For example, the moth *Diatraea saccharalis* (Lepidoptera: Crambidae) has a range throughout much of the Western Hemisphere and has been considered one species based on morphology. Using molecular markers, evidence for three potential species was uncovered (Joyce et al 2014). Hebert et al. (2004) working with the moth *Astraptes fulgerator* (Lepidoptera: Hesperiidae) found through use of molecular markers that this one species of moth actually consisted of 10 divergent host-plant-associated lineages. For moths, genetic divergence of populations in the range of 2–3% suggests the presence of cryptic species. For the Heteroptera, it has been suggested that a larger genetic divergence among populations in the range of 5% could suggest the presence of cryptic species (Park et al. 2011). The large distribution range of *L. zonatus* through the Western Hemisphere along with the wide host plant use and variation in developmental time suggest that this species may consist of genetically variable populations or possibly a cryptic species complex. In contrast, *L. clypealis* has a more restricted range of host plants and a more limited geographic distribution (Table 1). No previous molecular studies of genetic variability of either of these economically important species have been conducted.

The goal of this study was to examine the genetic variability of *L. zonatus* and *L. clypealis* in the Central Valley of California, to determine if there were any genetically divergent populations or cryptic species of either species present in California. The population genetic structure of each species was examined using two molecular markers. Knowledge of genetic variability, cryptic species or strains could improve IPM programs for these two species.

Materials and Methods

Collecting Adult Leaffooted Bugs

Adult leaffooted bugs are large insects (1–2 cm) yet are difficult to detect when sampling, as bugs sense motion and move into treetops or hide behind plant parts. The presence of leaffooted bug feeding in almond orchards is commonly detected when the characteristic defensive response or sap is observed on almonds. In addition, these insects are sometimes observed at harvest when almonds or pistachios are shaken from trees. Adult leaffooted bugs in this study were collected opportunistically in almonds, pistachios, and pomegranate between May 2013 and October 2014 when abundant and when obtained from collaborators throughout the Central Valley of California (Fig. 1; Table 2). Adult leaffooted bugs were identified to species using the key from McPherson et al. (1990) and Brailovsky (2014). Insects were stored in 80% ethanol or frozen and host plant of collection was noted, along with GPS coordinates of collection sites. When nymphs were collected along with adults, photographs of leaffooted bug life stages were taken, as insects were reared into the adult stage (Fig. 2). DNA was subsequently extracted to examine whether there were cryptic species or strains (Vos et al. 1995, Joyce et al. 2014, Park et al. 2011). Within Hemiptera, up to 3% intraspecific divergence is often observed; genetic divergence of 5% or more among populations of Hemiptera is sufficient to consider the presence of cryptic species (Park et al. 2011).

Molecular Identification of Species and Strains

DNA was extracted from the thorax of male adult LFPBs using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA), using standard tissue protocols and a 1 h incubation at 55°C (Qiagen 2006). The DNA quantity was measured using the Qubit dsDNA HS Assay kit (ThermoFisher, Waltham, MA). Amplified fragment length polymorphism (AFLP) markers were developed to investigate population structure, search for cryptic species, and investigate whether there were potential host-plant-associated strains or biotypes (Vos et al.1995, Joyce et al. 2014). Samples were randomized on two 96-well plates. Two primer combinations were used (M-CAT, E-ACT; M-CAC, E-ACG) to produce fragments for comparison. Details of AFLP reactions are elaborated in Joyce et al. (2014). Prior to capillary electrophoresis, 0.4 µl of GeneScan Liz 500 size standard and 0.9 µl of HiDi formamide (ThermoFisher, Waltham, MA) were added to 1 µl of the final product of each sample. Samples were run on a 3730 Genetic Analyzer. Genemapper 5.0 software was used to determine the presence or absence of each allele. The peak detection threshold was set for each primer combination and was typically 100 luminescent units. Phylip 3.65 was used to calculate Nei's pairwise genetic distance and to generate a neighbor-joining tree used to visualize genetic similarity of individuals. Structure 2.3.4 software (Pritchard et al. 2007) was run using the following parameters: no a priori assignment of individuals to a known population, analysis for diploid individuals, a length of burn-in of 50,000, followed by 50,000 iterations, an admixture model, and independent loci. The number of potential populations for K was estimated as the number of geographic sampling locations plus 4 (K = 3 sites + 4 = 7 for *L. clypealis*; K = 8 sites + 4 = 12 for *L. zonatus*) as suggested by Pritchard et al. (2000), and each iteration was run 20 times. Subsequently, Structure output was used to run Structure

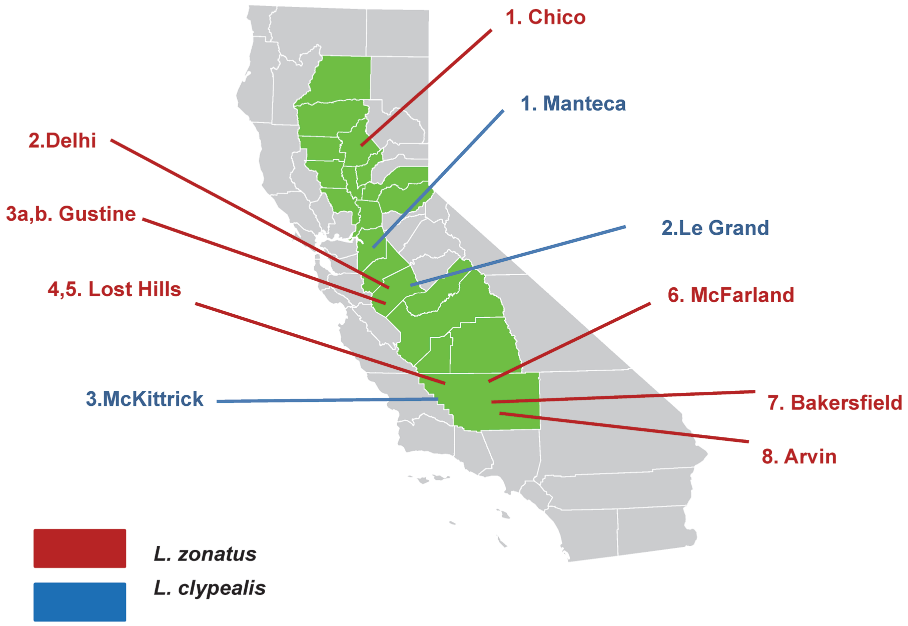

Fig. 1. Map of collections sites for *L. clypealis* and *L. zonatus* from the Central Valley of California (see Table 2).

Table 2. Leaffooted bug species, host plants, collections dates and geographic coordinates associated with collections used in this study (see Fig. 1)

L. zonatus			
Site collected	Host plant	Date	Latitude, Longitude
1.Chico	Pomegranate	Oct., Nov. 2013	39.754909, –121.802367
2. Delhi	Almond	Aug. 2013	37.403583, –120.798897
3a. Gustine	Pomegranate	Sept. 2014	37.1983313, –121.626997
3b. Gustine	Almond	Aug. 2014	37.1297699, –121.024452
4. Lost Hills	Pomegranate	Aug. 2014	35.7347774, –119.7970986
5. Lost Hills	Pomegranate	June 2013	35.62103301, –119.9289746
6. McFarland	Pomegranate	Aug. 2014	35.676542, –119.234569
7. Bakersfield	Pomegranate	Aug. 2014	35.402925, –118.918141
8. Arvin	Pomegranate	Oct. 2013	35° 12′07.39″ N, 118° 49′58.76″ W
L. clypealis			
1. Manteca	Almond	May 2013	37.8579571, –121.2501047
2. Le Grand	Pistachio	Sept. 2013	37.1928503, –120.2696643
3. McKittrick	Pistachio	May 2013	35.51284524, –119.7043273

Harvester to determine K based on the method by Evanno et al. (2005), the mostly likely number of population clusters for each species (Evanno et al. 2005, Earl and VonHoldt 2012). CLUMPAK software was used to run Distruct and visualize permutated results (Kopelman et al. 2015). Analysis of molecular variance (AMOVA) was run using the AFLP data to examine the genetic variation at two levels, among populations and by geographic region, using GenAlEx 6.0 (Peakall and Smouse 2006). For *L. clypealis*, there were three sampling locations, one with almonds (Manteca) and two locations with collections from pistachios (LeGrand, McKittrick). There was an unbalanced design with respect to host plant for both species, so AMOVA was used to run a comparison of molecular variation among the eight collections and by geography (northern and southern Central Valley). For *L. zonatus*, there were eight sites sampled (Table 2). AMOVA was also run on two factors, among populations and by geography (north–south). Pairwise comparisons of populations were subsequently made among populations of F_{ST} values with Bonferroni corrections, in order to determine which populations were significantly different.

For each insect, DNA was also used to sequence a ~650 bp region of mitochondrial DNA cytochrome oxidase 1 (CO1) (known as the 'bar code') using a universal forward primer LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse primer HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), or forward primer LepF2_t1 (5′-TGTAAAACGACGGCCAGTAATCATAARGATATYGG-3′) and reverse primer LepR1 (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) (Park et al. 2011). A polymerase chain reaction (PCR) mix for six samples consisted of the following: 195.6 μl sterile ultra pure water; 2.4 μl Taq polymerase (Clonetech, Mountainview,CA); 30 μl Taq 10× buffer; 24 μl dNTPs; 6 μl forward primer; and 6 μl reverse primer. For each reaction, 6 μl template DNA was added to each vial and the contents were vortexed and spun down. The PCR program was the following: an initial 1 min warm-up at 95°C; then 40 cycles of a touchdown program consisting of 92°C for 30 s, 43–52°C for 30 s (with a 0.3 °C temperature increase each s), and 72°C for 60 s; after 40 cycles, a 68°C final extension for 10 min and then a hold at 4°C.

PCR products were run on a 1.5% agarose gel to visualize the amplification of products of ~650 bp. Samples were cleaned-up using the Exo-sap-it (Affymetrix, Inc, Santa Clara,CA) cleanup kit and run on a 3730 Genetic Analyzer. Resulting sequences were analyzed using Geneious 7 (Biomatters, Aukland, New Zealand) software to produce consensus sequences (Kearse et al. 2012). Additionally, 22 sequences of mtDNA CO1 from *L. zonatus* were selected from Genbank for comparison with the sequences produced for *L. zonatus* in this study; these sequences represented genetic diversity of *L. zonatus* sequences available in GenBank. The additional mtDNA COI sequences in Genbank were all *L. zontatus* collected in Brazil; only samples from Brazil were available in GenBank for comparison. For *L. clypealis*, no existing mtDNA COI sequences were found in Genbank for comparison. Sequences were aligned in Geneious 7.0 using the Clustal W alignment function and used to produce an unrooted neighbor-joining tree (Kearse et al. 2012). Bootstrap support values were obtained by 1000 pseudoreplicates of the aligned data set, and those above 80% are shown below supported nodes (Joyce et al. 2014).

For both *L. clypealis* and *L. zonatus*, mitochondrial DNA COI sequences were used to determine the number of haplotypes, haplotype diversity, nucleotide diversity, and Tajima's D using DNAsp 5.10 (Librado and Rozas 2009). Results were used to construct a haplotype network using Popart 1.7 and a TCS network (Leigh and Bryant 2015).

Results

Field Collections of Leaffooted Bugs

Leaffooted bugs were obtained from sites through the Central Valley of California from almonds, pistachios, and pomegranates (Fig. 1; Table 2). All collections consisted of *L. clypealis* and *L. zonatus*. Both species were obtained primarily from the mid to southern Central Valley, with the exception of *L. zonatus* samples collected in Chico, Butte County (Fig. 1). Prior to this study, *L. zonatus* was not reported as a pest in almonds or pistachios. Photographs of the first instars show newly emerged first instars of *L. zonatus* and *L. clypealis* are distinct in appearance, with *L. zonatus* first instars being orange in color while first instars of *L. clypealis* are green (Fig. 2a and d). Adults of these two species can be distinguished as well; *L. clypealis* has a pointed clypeus, a spine-like projection at the front of its head, while *L. zonatus* adults have two prominent yellow-orange spots on the prothorax behind the head (Fig. 2c and f). Collections in this

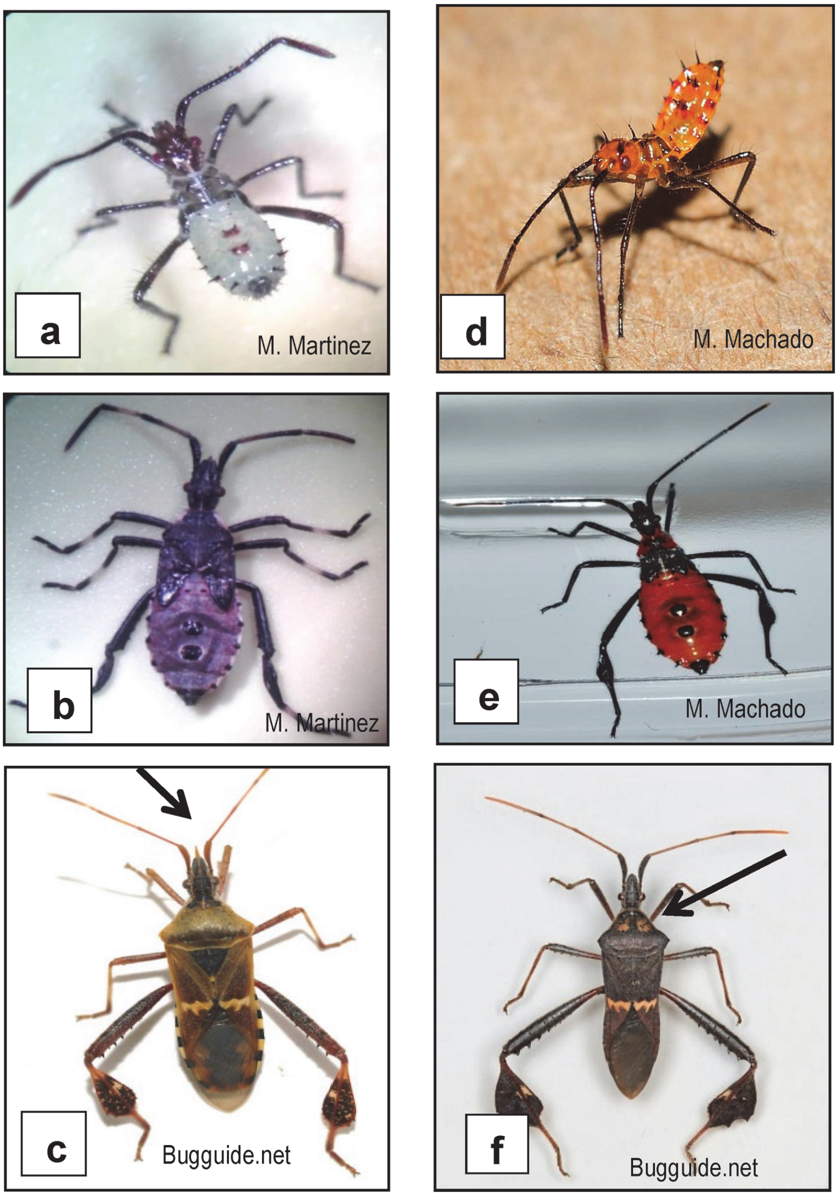

Fig. 2. (a) *L. clypealis* first instar nymph, (b) *L. clypealis* third instar, (c) *L. clypealis* adult with a spine-like tylus on the head, (d) *L. zonatus* first instar nymph, (e) *L. zonatus* second or third instar, (f) *L. zonatus* adult with two distinct spots on the anterior pronotum.

study from 2013 found *L. clypealis* on almonds and pistachios, and *L. zonatus* on pomegranate, almonds, and pistachio (Table 2). In 2013, *L. clypealis* was detected early in the almond growing season (May) when almonds were still forming, and *L. zonatus* was more abundant near almond harvest time (August, September). In 2014, *L. zonatus* and *L. clypealis* were both collected in almonds and pistachios; additionally, *L. zonatus* was obtained from pomegranate. In 2014, leaffooted bugs were more notable at almond and pistachio harvest time in August and September, and less abundant early in the growing season.

Molecular Identification of Species and Strains

AFLPs for *L. clypealis* were obtained for 46 male adults using two primer combinations, producing 209 AFLP markers, of which 204 markers were polymorphic. There were 14 *L. clypealis* adults from almonds in Manteca, 20 from pistachios in LeGrand, and 12 from pistachios in McKittrick, which were used for AFLP work. Structure Harvester found K = 2 using the method by Evanno et al. (2005). However, a visual inspection of the structure output suggests that there is one interbreeding group of *L. clypealis* on almonds and pistachios (Fig. 3). The AMOVA analysis among the three populations and by geography found 2% of variation among populations; however, the difference was marginally significant ($F = 0.02$, $P = 0.055$) and 0% of variation was attributed to geography. Pairwise comparisons of F_{ST} values found the population from almonds in Manteca and the population from pistachios in Le Grand were significantly different ($P < 0.01$), but the other population comparisons were not.

Mitochondrial DNA COI sequences were generated for 20 *L. clypealis*, seven individuals from almonds, and 13 from pistachios using the same individuals used to produce AFLP markers. The mtDNA COI sequences had 1–2% genetic divergence between them. A haplotype analysis found 17 haplotypes, with a haplotype diversity of 0.979, and a nucleotide diversity of Pi = 0.01381. Tajima's

D value was -1.20 and was not significant ($P < 0.10$) (Fig. 5). No previous *L. clypealis* sequences were available in GenBank for comparison. A blast search of nucleotide sequences in GenBank found the closest match to *L. clypealis* mtDNA COI was a sequence from *L. occidentalis* collected in Nova Scotia with 94.9% similarity (Park et al. 2011).

The second species, *L. zonatus* had 146 males used to produce AFLP markers. This species was more abundant on pomegranate; 122 individuals were collected from pomegranate, 16 from almond, and eight from pistachio. Collections yielded the following number of insects used for DNA work from each location; Chico 16, Gustine 25, Delhi 11, McFarland 9, Arvin 22, Lost Hills(1) 33, Lost Hills(2) 20, and Bakersfield 9. A total of 164 AFLP markers were obtained using two primer combinations; of these, 159 markers were polymorphic. Structure Harvester found K = 2 using the Evanno et al. (2005) method, indicating the presence of two genetically divergent groups within the *L. zonatus* collections (Fig. 4). Most populations belonged to one cluster (red), but at the Lost Hills site there were many individuals of two genetically distinct types of *L. zonatus*, collected from Lost Hills in 2013 and 2014 (green and red bars) (Fig. 4). Nei's pairwise genetic distance was determined among the eight populations. The larger genetic distances were between the Lost Hills(1) population (green in structure) and all other populations (0.048–0.06), with the largest genetic difference between Lost Hills(1) and McFarland (0.08) (Table 3). Other large genetic distances were between Lost Hills(1) and Lost Hills(2) (0.05), and between McFarland and Bakersfield (0.06). Populations with the smallest genetic distances were Gustine, Chico, Delhi, Arvin, and Lost Hills(2) which were all in the range of 0.02–0.40. The AMOVA analysis of the eight populations found 7% variation among the eight collection sites which was significant ($P = 0.001$), with 0% variation attributed to geography (Table 4). The F_{ST} values were significantly different among most populations (Table 5). The largest F_{ST} values were between Lost Hills(1) and McFarland at 0.184, followed by Lost Hills(1) and Arvin (0.144), and Bakersfield and McFarland (0.140). Finally, Lost Hills(1) had an F_{ST} of 0.125

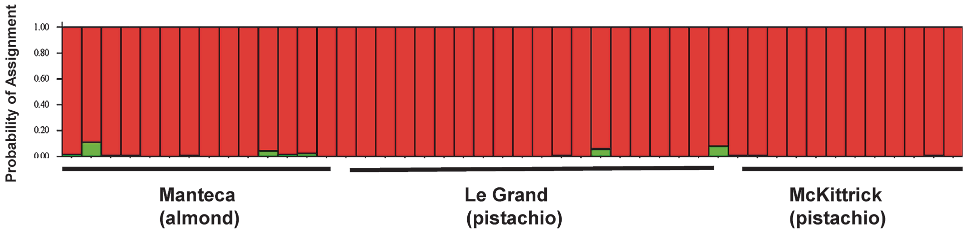

Fig. 3. Structure analysis of AFLPs from *L. clypealis* collected in the Central Valley of California on almonds and pistachios from May 2013 to September 2014. Structure 2.3.4 was run using the following parameters: diploid individuals; 50,000 iterations; admixed data; and independent loci. The collection site and host plant is listed below the bar (see also Table 2, Fig. 1).

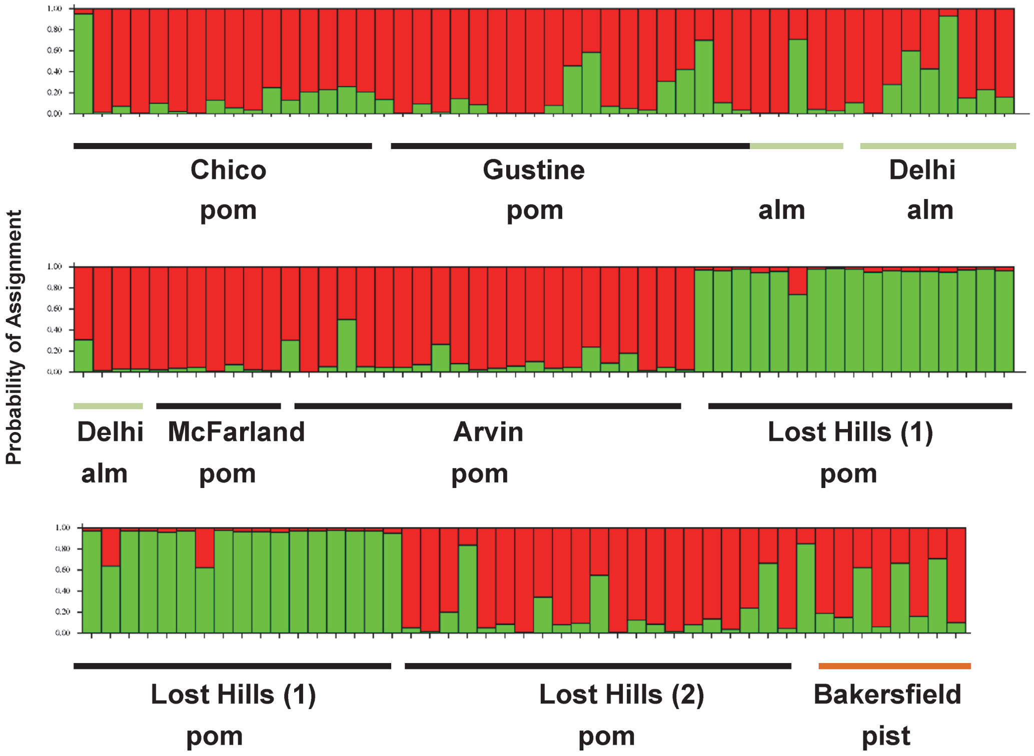

Fig. 4. Structure analysis of AFLPs from *L. zonatus* collected in the Central Valley of California from May 2013 to September 2014. Structure 2.3.4 was run using the following parameters: diploid individuals; 50,000 iterations; admixed data; and independent loci. Structure Harvester found that K = 2, and individuals could be assigned to two genetically distinct populations. The collection sites are listed below the bars on the figure. Host plants are abbreviated below collections site, with pomegranate = pom, almond = alm, and pistachio = pist (see also Table 2, Fig. 1).

Table 3. Nei's genetic distance among eight populations based on AFLPs for *L. zonatus* from collections in the Central Valley of California

	Chico	Gustine	Delhi	McFarland	Arvin	LH-1	LH-2	Bakersfield
Chico	0.000	0.022	0.033	0.042	0.028	0.053	0.023	0.039
Gustine		0.000	0.023	0.035	0.026	0.052	0.014	0.037
Delhi			0.000	0.05	0.039	0.056	0.029	0.049
McFarland				0.000	0.048	0.085	0.037	0.06
Arvin					0.000	0.061	0.027	0.047
Lost Hills-1						0.000	0.053	0.060
Lost Hills-2							0.000	0.039
Bakersfield								0.000

Table 4. AMOVA for *L. zonatus* for two factors, by region and among populations

	Factor	df	Sum of squares	Variation (%)
L. zonatus	By region	1	45.266	0
	Among collections	5	196.366	7
	Individuals within group	134	2333.758	91

Table 5. Pairwise comparisons of genetic divergence estimates (F_{ST}) between *L. zonatus* populations from eight collections in the Central Valley of California

	Collection Site	1	2	3	4	5	6	7	8
L. zonatus									
	1. Chico	0	P = 0.001*	P = 0.001*	P = 0.001*	P = 0.001*	P = 0.001*	P = 0.003*	P = 0.001
	2. Gustine	0.042	0	P = 0.025	P = 0.001*	P = 0.001*	P = 0.001*	P = 0.048	P = 0.001*
	3. Delhi	0.059	0.024	0	P = 0.001*	P = 0.001*	P = 0.001*	P = 0.003	P = 0.001*
	4. McFarland	0.106	0.073	0.104	0	P = 0.001*	P = 0.001*	P = 0.001*	P = 0.001*
	5. Arvin	0.062	0.058	0.076	0.117	0	P = 0.001*	P = 0.001*	P = 0.001*
	6. Lost Hills (1)	0.125	0.125	0.107	0.184	0.144	0	P = 0.001*	P = 0.001*
	7. Lost Hills (2)	0.040	0.012	0.036	0.079	0.056	0.124	0	P = 0.001*
	8. Bakersfield	0.075	0.062	0.072	0.140	0.097	0.109	0.064	0

F_{ST} values are listed below the diagonal. P values are above the diagonal. Comparisons made at $P < 0.05$, after Bonferroni corrections were made.
*Comparison between populations is significant.

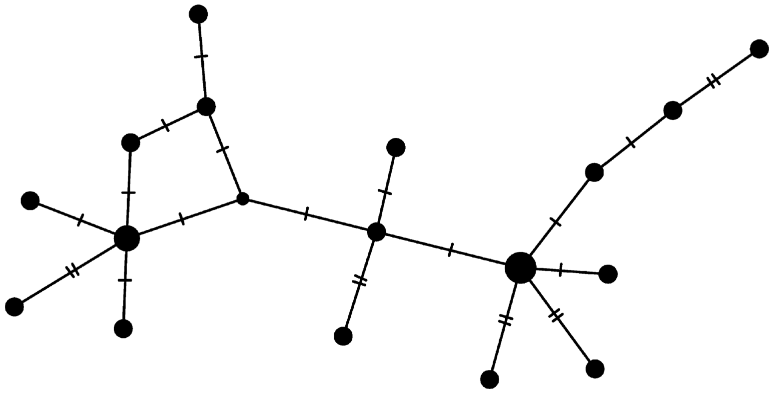

Fig. 5. Haplotype network based on 20 mitochondrial DNA COI sequences of *L. clypealis* collected in the central valley of California. Seventeen haplotypes were found, with a haplotype diversity of 0.979.

from both Chico and Gustine. The lowest F_{ST} values which were not significantly different were between Gustine and Delhi (0.024), and between Lost Hills(2) and Chico (0.04), Lost Hills(2) and Gustine (0.12), and Lost Hills(2) and Delhi (0.032) (Table 5).

For *L. zonatus*, we generated mtDNA COI sequences for 41 individuals from the central valley of California, and combined them with 22 mtDNA COI sequences available in GenBank to produce a neighbor-joining tree (Fig. 6). The main portion of the tree consisted of two primary regions. The first main cluster consisted of 24 *L. zonatus* from California collections from Chico, Delhi, Gustine, Lost Hills(1), Lost Hills(2), Bakersfield, and McFarland; the second region of the tree consisted of 17 California individuals from three sites (Lost Hills(1), Lost Hills(2), and McFarland) along with 13 individuals from Brazil Genbank accessions, and a smaller sub-branch

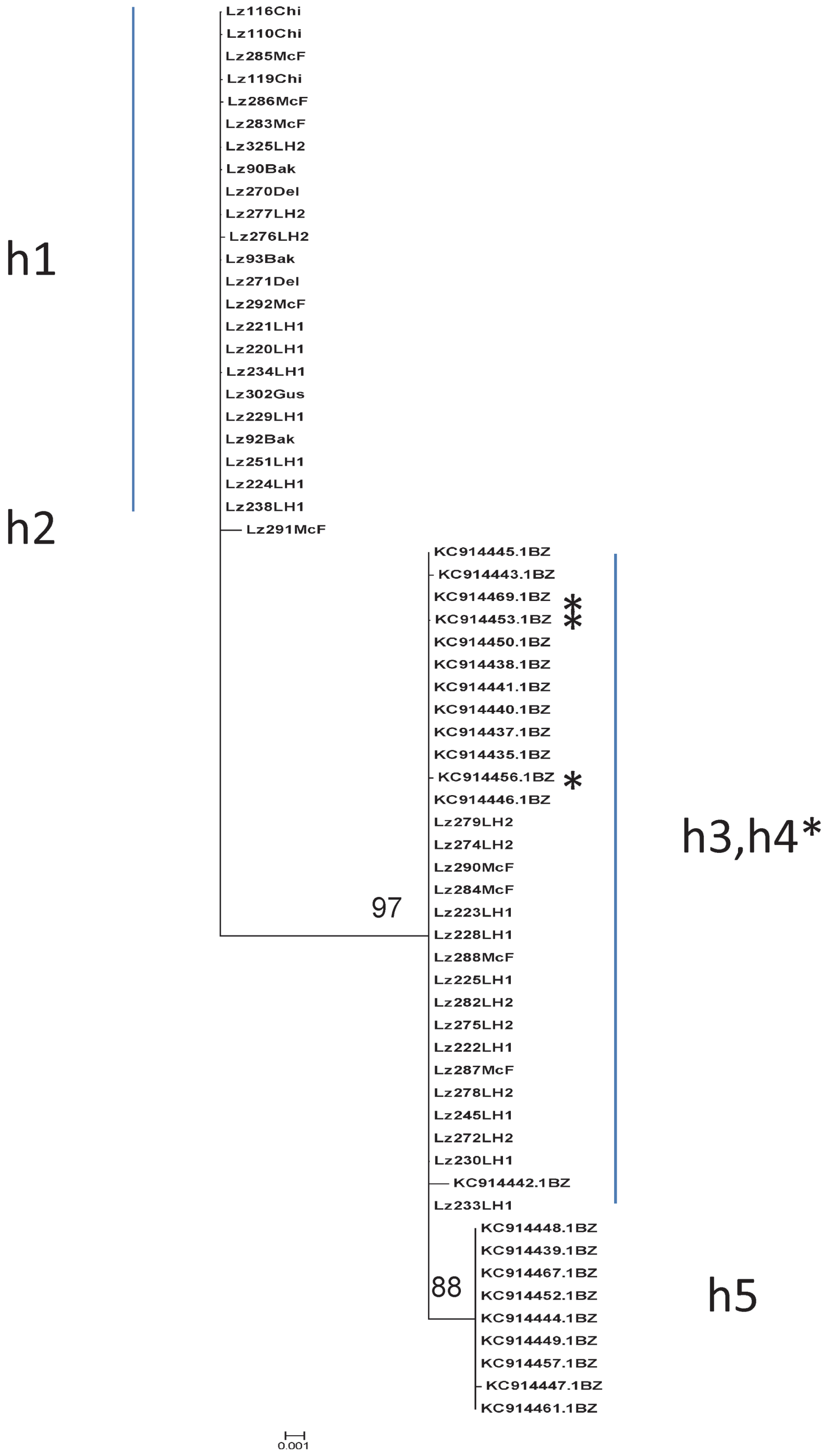

Fig. 6. Mitochondrial DNA COI sequences from 41 *L. zonatus* collected in California combined with 22 *L. zonatus* previously sequenced from GenBank, collected from Brazil. California sites are abbreviated as follows: Lost Hills 2 = LH2; McFarland = McF; Gustine = Gus; Lost Hills 1 = LH 1; Delhi = Del; Bakersfield = Bak; Chico = Chi (see also Table 2 for host plants). GenBank accessions begin with KC and end with BZ (i.e., KC914469.1BZ). Neighbor-joining tree, 1,000 psuedoreplicates were run and nodes with support above 80% are indicated (see also Fig. 7 for haplotypes). Haplotype 1 (h1) = California collections only, h2 = individual from McFarland, h3 = California collections from LH1, LH2, and McFarland, and GenBank accessions from Brazil, h4 = 3 GenBank accessions from Brazil, KC 914469.1, KC914453.1, KC914456, h5 = GenBank accessions from Brazil.

with nine more individuals from GenBank from Brazil (Fig. 6). The genetic diversity between individuals from the two main regions was typically in the range of 2% and ranged up to 2.3% (Supp Table 1 [online only]). For example, individuals from Chico were ~2% divergent from samples from the second main region of the tree, including accessions from Brazil. Interestingly, there were individuals from Lost Hills(1) and Lost Hills(2) collections on both of the main branches of the tree, which were about 2% divergent as well.

A haplotype analysis of the mtDNA COI sequences from *L. zonatus* collections in California found three haplotypes, with a haplotype diversity of 0.526, and a nucleotide diversity of 0.008. For the California collections, the Tajima's *D* value was 2.70, and was highly significant ($P < 0.01$). For accessions from Brazil, there were four haplotypes, with a haplotype diversity of 0.645, and a nucleotide diversity (Pi) of 0.002; Tajima's *D* was 0.64 ($P > 0.10$). Combining California and Brazil samples resulted in a total of five haplotypes, a haplotype diversity of 0.658, and a nucleotide diversity (Pi) of 0.009 (Fig. 7).

Finally, the pattern of population divergence found with the AFLP markers was compared with that found with mtDNA COI sequences for *L. zonatus*. The AFLP analysis assigned most individuals in the study to either the population illustrated in red, or the second population shown in green (Fig. 4). Most individuals were represented by red bars, suggesting gene flow among those individuals, while the Lost Hills(1) population was the second group in green. Lost Hills(1) contains individuals with two lineages of mtDNA COI about 2% divergent, and there appears to be gene flow among those individuals as they are assigned to one group with the AFLP markers. The Lost Hills(1) group shown in green in the AFLP analysis may represent a group of more recently introduced individuals, or more closely related individuals that those in the other collections. For *L. zonatus*, the 2% divergence in the mtDNA COI is not high enough to suggest the presence of cryptic species; however, there were two distinct genetically divergent groups present in California (Figs. 6 and 7).

Discussion

Both *L. clypealis* and *L. zonatus* were collected in the Central Valley of California, with *L. clypealis* found on almond and pistachio and *L. zonatus* collected from almonds, pistachios, and pomegranate. Prior to this study, *L. zonatus* was not noted as a pest on these host plants; however, it appears to have become more abundant and expanded its range from southern California northward into Butte County in northern California (Joyce et al. 2013). In 2014, *L. zonatus* was observed more frequently in the Central Valley than *L. clypealis*. The apparent increased abundance in *L. zonatus* could be due to increased plantings of almonds, pistachios, and pomegranate, or possibly an introduction of an exotic population.

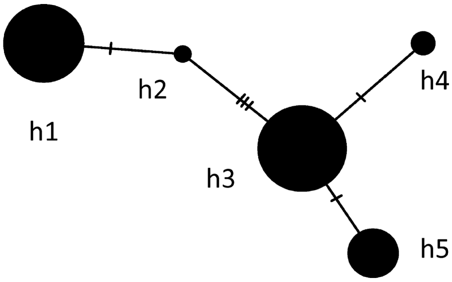

Fig. 7. Haplotype network based on 63 *L. zonatus* sequences, including 41 *L. zonatus* sequences collected in the central valley of California, and 22 GenBank accessions from Brazil. The analysis found three haplotypes in California, and five haplotypes in the combined data set. h1 = California collections only, h2 = Lz291McF, h3 = California and Brazil GenBank accessions, h4 = only GenBank KC914442.1BZ from Brazil, h5 = GenBank accessions from Brazil. One haplotype (h3) is shared for some collections from California and some existing GenBank accessions from Brazil (see Fig. 6).

For *L. clypealis,* there was no apparent host-plant-related genetic structure or biotypes detected from the AFLP genetic analyses. Individuals collected in the mid-Central Valley were collected both on almond and pistachio trees, while those collected in the southern Central Valley were more abundant on pistachio than almonds. Overall, *L. clypealis* individuals on the two host plants throughout the state were found to consist of one interbreeding population (Joyce et al. 2013). It is likely that once *L. clypealis* can no longer feed on almonds due to the hardening of the almond shell these insects move into pistachios which remain susceptible a bit later into the growing season. It is helpful for management to know that *L. clypealis* appears to be moving between the two host plants. There was a modest amount of genetic divergence of up to 2% detected in the mtDNA COI sequences. The AMOVA analysis found a significant difference between the almond population of *L. clypealis* from Manteca and the pistachio population of *L. clypealis* from LeGrand. However, no host-plant strains or cryptic species were apparent from the AFLP markers for *L. clypealis*. The 17 haplotypes found in the *L. clypealis* sequenced from the Central Valley of California represented a relatively high haplotype diversity, suggesting the insect is in its native range.

L. zonatus populations exhibited significant genetic structure. The AFLP results suggested at least two genetically divergent populations, as did the mitochondrial DNA COI sequences. The AMOVA analysis among *L. zonatus* populations found 7% variation. Some possibilities for the genetic structure observed among the populations of *L. zonatus* include: 1) host-plant associated populations; 2) variation in geographical distribution between divergent populations; and/or 3) cryptic species. On pomegranates, *L. zonatus* was common in the fall (after September) throughout the Central Valley of California; individuals were also occasionally abundant and collected on almonds and pistachios during the growing season and at harvest. Leaffooted bugs such as *L. zonatus* are elusive and are difficult to collect unless they are abundant. Most *L. zonatus* used to examine genetic diversity in this study were collected from pomegranate, due to the larger populations of insects more easily detected on this host plant. The study was limited in that most collections of *L. zonatus* were from pomegranate, and additional collections from almonds and pistachios would be required to test the hypothesis of host-plant-adapted populations. However, there was significantly genetic diversity among the populations sampled, which was not due to geographic distribution or region (north–south). For *L. zonatus*, the AFLP data indicated a genetically distinct population collected from the southern portion of the Central Valley near Lost Hills. *L. zonatus* were collected from the same geographic region (Lost Hills) in 2013 and 2014, yet the two collections are genetically divergent. The presence of two genetically distinct groups from the AFLP results suggests that there are two or more types or strains in the *L. zonatus* populations present in California at the time of this study. This is further supported by the mitochondrial DNA COI sequences.

Over 40 mtDNA COI sequences from *L. zonatus* were produced from California collections and combined with 22 sequences from GenBank collections from Brazil; no GenBank *L. zonatus* sequences were available from other countries for comparison. The *L. zonatus* in this study exhibited a moderate degree of genetic diversity, suggesting *L. zonatus* may consist of subspecies. One of the main regions of the neighbor-joining tree (Fig. 6) consisted of *L. zonatus* from California only, with ~2% divergence between them and the other main region of the tree. The other principal region of the tree contained additional *L. zonatus* from California along with the 22 GenBank accessions of *L. zonatus* from Brazil. The haplotype analysis for *L. zonatus* from California consisted of 41 sequences, and found that three haplotypes occurred in the California collections. Haplotype 1 was found at seven collection sites, haplotype 2 consisted of one individual from McFarland, while haplotype 3 had individuals from Lost Hills and McFarland; haplotype 3 was also shared with a number of *L. zonatus* GenBank accessions from Brazil. The Tajima's *D* value (2.70) for California *L. zonatus* collections was highly significant, suggesting a recent contraction of the population. Based on personal observations and communications with farmers and entomologists, *L. zonatus* seems to be increasing in abundance in the Central Valley of California. Haplotype 3 which is found in the Central Valley and also occurs in Brazil could have been introduced into California, or it could be a haplotype found throughout the distribution range of this insect. Due to the potential of this insect to cause economic damage to nut crops such as almonds and pistachios in California, a larger study of the genotypes of *L. zonatus* from throughout the range of this insect could be beneficial. Future studies of the genetic variability of this species would benefit by including more populations from outside of California, perhaps insects from Mexico and the southern United States, to help understand patterns of genetic diversity and to help pinpoint the center of origin of this species.

Documenting the presence of different strains, biotypes or haplotypes of *L. zonatus* in California and through the range of this insect is important, as genetically divergent populations differ in their susceptibility to biological control agents, vary in their host plant preferences, and may use different pheromone blends to communicate, all of which could impact the effective management of this insect. In this study of *L. zonatus* and *L. clypealis*, the 2–3% divergence between populations based on mtDNA COI did not meet the ~5% divergence criteria suggested by Park et al. (2011) to suggest the presence of cryptic species. To the knowledge of the authors, this is the first study that applies DNA analyses to *Leptoglossus* spp., and this study reveals that *L. zonatus* encompasses at least two genetically divergent groups. *L. zonatus* is currently considered a single generalist polyphagous species with a wide distribution in the Western Hemisphere. The possible subspecies or strains of *L. zonatus* might be separated by host plant or distribution. Other insects presumed to be dietary generalists and now are considered well established species complexes, including for beetles (Blair et al. 2005), butterflies (Herbert et al. 2004), guilds of herbivorous insects (Stireman et al. 2005), dipterans (Smith et al. 2006), and hymenopteran parasitoids (Molbo et al. 2003, Kankare et al. 2005). The application of molecular analysis to other populations of the widely distributed *L. zonatus*, especially those from different regions or different host plants, may reveal the existence of genetic variability within the species.

Factors that generate population genetic divergence include habitat selection or host-plant preference (Henry 1994). Host-plant preference is a critical barrier to gene flow for *Rhagoletis pomonella* (Feder and Bush 1989, Feder et al. 1994, Feder 1998). Differential use of host-plants causes prezygotic isolation among host races of *Rhagoletis* (Feder et al. 1994). The same factor can affect other guilds of herbivorous insects (Stireman et al. 2005). *L. zonatus* could consist of multiple genetically diverse populations encompassed under this taxon, with each group preferring a particular group of crop plants. Previous evidence has shown the treehopper species *Enchenopa binotata* consists of a complex of sympatric species whose life cycles are strongly related with the phenology of its host plant (Cocroft et al. 2008). It has been recognized that the development time of *L. zonatus* differs among some of its host plants such as *Jatropha curcas* (Grimm and Somarriba 1999) and *Zea mays* (Fernandes and Grazia 1992). Host plants could potentially contribute to population divergence, but the extent of this mechanism would need to be investigated.

Taxonomic studies may overlook less conspicuous characters such as variation in biochemical or behavioral traits among populations (Shaw and Mullen 2011). Frequently these phenotypes evolve rapidly yet they are not often included in species characterizations or taxonomy (e.g., Mullen et al. 2007, 2008; Bjaerke et al. 2010). However, cryptic species behavioural characters are the most prominent aspects in their differentiation (Mullen and Shaw 2014). For example, the males of the Hawaiian swordtail cricket of the genus *Laupala* produce courtship songs that are very distinct and previously considered variants within a single species; however, it is now accepted that this group includes 38 morphologically similar cryptic species recognized based on their courtship songs and many of them occur sympatrically (Mendelson and Shaw 2002). *L. zonatus* populations might vary in acoustic, behavioural, or ecological traits which could contribute to population genetic divergence within this group. It is of interest to understand how morphologically similar species coexist, and whether mechanisms like partitioning of resources, microhabitat preferences, biochemical or behavioral traits contribute to their persistence (Stuart et al. 2006). Bickford et al. (2006) suggest why morphological change might not correlate with species boundaries. For example, cryptic species can rely primarily on non-visual mating signals or behaviors like sex pheromones or mating calls, but the morphological structures needed to produce different acoustic or olfactory signals need not differ appreciably. Information on behavioral mechanisms such as courtship vibrational signals and sex pheromones is limited for *L. clyplealis* and *L. zonatus*. However, some research has been conducted on alarm pheromones and other chemical components generated by leaffooted bugs (Aldrich et al. 1979, Soares et al. 1994, Wang and Millar 2000, Gonzaga-Segura et al. 2013). Thus, further research on acoustic or chemical ecology could provide evidence of behavioural mechanisms that could contribute to the identification of cryptic species or genetically divergent populations in the genus *Leptoglossus*.

The identification of genetic variability within a species is important for pest management strategies, as the proper identification of a species helps to maximize the success of outcomes of biological control and the identification of invasive pest species. Development of control measures for crop pests and invasive species often exploit species-specific interactions between parasites or pathogens and their hosts (Souza and Amaral Filho 1999; Marchiori 2002; Joyce et al. 2014). Therefore, failing to recognize genetic variability limits the effectiveness of these programs and could cause rejection of potentially valuable species as control agents. Consequently, the detection and identification of the leaffooted bugs and genetically divergent strains or cryptic species is crucial to promote the appropriate management and pest-control strategies especially on the crops of pistachio, almonds, and pomegranate that are economically important in the Central Valley of California.

This is the first study that describes the genetic diversity of the leaffooted bugs *L. clypealis* and *L. zonatus* in the crops of almonds, pistachios, and pomegranates, and also to report the extensive distribution of the leaffooted bug *L. zonatus* into the northern portion of California's Central Valley. This study suggests that further genetic study of *L. zonatus* throughout its range could contribute to the recognition of additional genetically divergent populations, which could contribute to pest management strategies for *L. zonatus*.

Data Availability

Lc248_COI MF669742, Lc151_COIpi MF669743, Lc148_COIpi MF669744, Lc147_COIpi MF669745, Lc146_COIpi MF669746, Lc143_COIpi MF669747, Lc138_COIpi MF669748, Lc128_COIpi MF669749, Lc82_COIpi MF669750, Lc78_COIpi MF669751, Lc76_COIpi MF669752, Lc75_COIpi MF669753, Lc53_COIpi MF66975, Lc31_COIalm MF669755, Lc30_COIalm MF669756, Lc29_COIalm MF669757, Lc28_COIalm MF669758, Lc27_COIalm MF669759, Lc18_COIalm MF669760, Lc17_COIalm MF669761, Lz325_COILH2 MF669762, Lz302_COIGus MF669763, Lz292_COIMcF MF669764, Lz291_COIMcF MF669765, Lz290_COIMcF MF669766, Lz288_COIMcF MF669767, Lz287_COIMcF MF669768, Lz286_COIMcF MF669769, Lz285_COIMcF MF669770, Lz284_COIMcF MF669771, Lz283_COIMcF MF669772, Lz282_COILH2 MF669773, Lz279_COILH2 MF669774, Lz278_COILH2 MF669775, Lz277_COILH2 MF669776, Lz276_COILH2 MF669777, Lz275_COILH2 MF669778, Lz274_COILH2 MF669779, Lz272_COILH2 MF669780, Lz271_COIDel MF669781, Lz270_COIDel MF669782, Lz251_COILH1 MF669783, Lz245_COILH1 MF669784, Lz238_COILH1 MF669785, Lz234_COILH1 MF669786, Lz233_COILH1 MF669787, Lz230_COILH1 MF669788, Lz229_COILH1 MF669789, Lz228_COILH1 MF669790, Lz225_COILH1 MF669791, Lz224_COILH1 MF669792, Lz223_COILH1 MF669793, Lz222_COILH1 MF669794, Lz221_COILH1 MF669795, Lz220_COILH1 MF669796, Lz119_COIChi MF669797, Lz116_COIChi MF669798, Lz110_COIChi MF669799, Lz93_COIBak MF669800, Lz92_COIBak MF669801, Lz90_COIBak MF669802.

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Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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