INTERACTIVE EFFECTS OF GEOGRAPHY AND HOST PLANT SPECIES ON GENETIC AND PHENOTYPIC VARIATION OF COTTON FLEAHOPPER POPULATIONS

A Dissertation

by

APURBA KUMAR BARMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Entomology

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ABSTRACT

Interactive Effects of Geography and Host Plant Species on Genetic and Phenotypic Variation of Cotton Fleahopper Populations. (December 2011)

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The cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter) is a widely distributed insect across the United States. Although, it feeds on several native wild hosts, its agricultural importance lies as an economic pest of cotton in several states in the southern United States. No studies have addressed intraspecific genetic and phenotypic variation of this insect pest at a large geographic scale.

I examined genetic variation among cotton fleahopper populations associated with cotton in different geographic locations across the southern United States (Chapter II). Using dominant, neutral, nuclear molecular markers (AFLP, amplified fragment length polymorphism) and mitochondrial DNA sequences, I found that overall genetic differentiation among different geographic populations, collected from cotton in eleven cotton growing states, was low but significant. AFLP revealed the presence of three regional groups representing western (Arizona), central (Texas, Oklahoma, Arkansas, Louisiana, Mississippi and Alabama), and eastern (Florida, Georgia, South Carolina and North Carolina) populations.

I examined if there were distinct lineages of cotton fleahoppers associated with three of its host plant species: cotton (*Gossypium hirsutum*), horsemint (*Monarda punctata*) and woolly croton (*Croton capitatus*) in five different locations of Texas by using AFLP markers (Chapter III). I found two distinct host-associated lineages at three locations and local panmixia in the other two locations.

I tested if host preference of cotton fleahoppers were affected by geographic variation and prior experience. Conducting choice tests with a Y-tube olfactometer, I found that host preference in cotton fleahoppers for horsemint (one of its native host plants) is conserved and unaffected by individual's prior experience with cotton (Chapter IV).

Finally, I explored the role of host-plant species in morphological differentiation of the cotton fleahopper in two locations that differ in presence of distinct host-associated lineages. Using a geometric-morphometric approach, I detected significant effect of host plant and geography on body morphology and wing shape of cotton fleahopper populations (Chapter V). Length of antenna and rostrum were two important traits associated with morphological divergence of cotton and horsemint associated insect populations. Cotton associated individuals had relatively longer antenna and rostrum compared to individuals associated with horsemint.

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I will remember Dr. Medina's relentless effort in reading and suggesting corrections of my writings for the last several years. You have also given me enough independence to explore and execute my research questions, which of course sometime might have led me to distractions. I must say that Dr. Parajulee has trusted me enough and kept me associated with him for the past 7+ years. Without your help and inspiration, it would have been difficult for me to reach the status in which I find myself today.

The financial supports from Texas AgriLife Research and Extension, Plains Cotton Growers Inc., Cotton Incorporated, Texas State Support Committee, Texas A&M University, and Texas EcoLab made this body of research possible. I sincerely acknowledge your investments. Dr. Suh was highly instrumental in giving my research a 'jump start' with his knowledge on cotton fleahopper biology and habitat. I am grateful to Adam Jones, Department of Biology, who advised me in the area of evolutionary biology and pointed me in to the right direction. I acknowledge the collaboration with Dr. Tomas DeWitt, Dept. of Wildlife and Fishery Sciences, which expanded the scope of my study on morphological differentiation.

Here, I extend my appreciation to Dr. John Westbrook, USDA-ARS, APMRU for his cooperation and logistic help. Drs. Scott Armstrong, Roy Parker, Terry Pitts (Oklahoma) and Peter Ellsworth (Arizona) generously provided cotton fleahopper samples. I extend my thankfulness to the Cotton Entomology Program at the Lubbock AgriLife Research Center and Dr. Ram Shrestha. I am thankful to Drs. Micky Eubanks and Michel Slotman for allowing me to use their laboratory space on several occasions. I really appreciate the friendship of Kevin Deitz, who helped me in part of my research.

I visited a number of places not only in Texas but also in several other states and sampled cotton fields without knowing the farmer's name or address. In all my good intensions, two persons always watched my back: my friend, Stanley Carroll and my wife, Babi. I express my heartiest gratitude to their patience, love, and encouragement.

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CHAPTER I

INTRODUCTION

Integrated pest management (IPM) combines several pest control methods, which are economically viable, ecologically sustainable and friendly to environment (Smith 1978). Successful IPM relies on complete understanding of pests and their interactions with both biotic and abiotic factors. Considerations regarding economic viability, ecological sustainability and environmental impacts are extremely important in the design of IPM strategies. Due to the dynamic nature of IPM and the influx of novel information pertaining multi-trophic interactions, IPM approaches should be flexible enough to evolve over time. In many IPM practices, the effect of the genetic and phenotypic variation within insect pest species seems not to be taken into consideration. Several studies have described two or more cryptic or sibling species "hidden" within the pest population that were thought to be one species (Blair et al. 2005) (cryptic species examples). Similarly, several host races have been identified within the insect pest species (Bush 1969, Feder et al. 1988, Carroll and Boyd 1992, Emelianov et al. 2001, Aguin-Pombo 2002, Calcagno et al. 2007, Ohshima 2008, Peccoud et al. 2009). The realization that genetic diversity in insect population can be structured by geography, host-plant association or a combination of both has led to a series of studies addressing the mechanisms underlying genetic divergence. However, very few studies have looked at the organization of genetic diversity in economically important insect

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This dissertation follows the style of the *Environmental Entomology*.

pests of crops and even fewer have used this information to improve IPM practices. In my dissertation research, I have taken several steps to understand intra-specific phenotypic and genotypic diversity of an economically important agricultural insect pest and discussed the implications of this variation in pest management.

The present dissertation was built upon the idea that insect pests may possess intra-specific variation in their genotype and/or phenotype, which if ignored may impair the successful implementation of sound IPM practices. Here, I use the cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae), as a model species to explore intra-specific genetic and phenotypic diversity in geographic and host-associated populations. The cotton fleahopper is an economically important pest of cotton in Texas, Oklahoma and Arkansas. In 2010, cotton fleahopper infested 4.5 million acres of cotton cultivation in the United States with a total loss of 81,048 bales of cotton lint (Williams 2011). Nationwide, the yield loss was highest in Texas (81% of total loss) and the state (Texas) spent about 10 million dollars in treating cotton fleahoppers alone (Williams 2011). Although the cotton fleahopper is reported to be present in the majority of the cotton growing states in the United States, not all cotton-growing states suffer similar amount of crop losses due to this insect (Williams 2011). Thus, question arises: why is there heavy infestation of cotton fleahoppers in some regions but not in others? In Chapter II, I address this question by exploring the geographic population structure of the cotton fleahopper in the United States. Data of this kind are useful in order to have baseline information on the way in which genetic variation is structured geographically. It is likely that genetically distinct pest populations differ in traits relevant to their

control. Thus, the organization or structuring of genetic diversity in insect pests may provide insights into the variation in effectiveness of some control practices among different geographic locations.

Although the cotton fleahopper derived its name by being associated with cotton, this insect has more than 160 host plant species in 35 different families (Esquivel and Esquivel 2009). Among these host plants, two native plant species, horsemint (*Monarda punctata*) and woolly croton (*Croton capitatus*), are the most preferred host plants in Texas, both species supporting relatively high populations. Horsemint and woolly croton are considered as primary hosts of the cotton fleahopper in Texas and they are customarily presumed to be the source of cotton fleahopper populations infesting cotton. However, no studies have been conducted to demonstrate if movement of cotton fleahopper from any of these two hosts into cotton actually occurs in nature. Studies in several insect species (e.g., *Rhagoletis pomonella*, *Ostrinia nubilalis, Acyrthosiphon pisum*) have shown that insect populations associated with different host plant species occurring in sympatry may actually represent distinct host-associated lineages rather than being one panmictic population (Feder et al. 1988, Via 1999, Malausa et al. 2007). The existence of genetically distinct parasite (e.g. herbivores) lineages on different host species (e.g., plants) is referred to as host-associated differentiation (HAD). Presence of HAD in cotton fleahopper populations associated with different host plant species indicates reproductive isolation among insects associated with different host-plant species and may also suggest restricted movement of individuals between host plant species. Chapter III explores if cotton fleahopper populations associated with cotton and

two of cotton fleahopper's wild and native host-plant species (i.e., horsemint and woolly croton) are genetically distinct in several geographic locations in Texas.

Preference for particular host plant species is considered as one of the key factors explaining reproductive isolation among insect populations associated with different host plant species. The preference for specific host plants is a fundamental behavior of herbivore insect species that allows individuals to increase their fitness and advantageously exploit the resources they have available choosing the ones on which their fitness could attain its maximum potential. This preference for one host plant to another in herbivore insects could be either for feeding, oviposition or for rendezvous locations to find mating partners. Host preference in insect herbivores may present geographic variation. For example, *Helicoverpa armigera* populations from different geographic regions showed variable host preference in Australia (Firempong and Zalucki 1990). Distant geographic populations of herbivore species may experience heterogeneous host plant compositions throughout their distribution range. In such situations, insect populations would be expected to prefer the most abundant host plant at any given geographical location, either because of more frequent encounters of the insect with the most locally common plant species or just because of the adaptive evolution in insect population to a predictable resource. In Chapter IV, I explore if host preference for horsemint and cotton is modulated by geography in the cotton fleahopper and assess if prior experience with a particular plant species may alter the cotton fleahopper host plant preference.

As noted earlier, both geography and host plant composition may influence phenotypic and genotypic variation in insects. For example, behavioral differences in herbivorous insect could occur among insects associated with different host plant species at different locations. In a similar vein, it can also be hypothesized that geography and host plant species, either by itself or in combination, can create morphological variation in populations of same species. For example, insect populations from distant geographic locations may have unique evolutionary histories and exhibit different morphologies. Similarly, morphological variation in insect populations may result from their association with host plant species which are different morphologically, anatomically, and biochemically. For example, in the goldenrod aphid, *Uroleucon* sp.*,* populations associated with pubescent hosts had longer rostrum and shorter hind tarsi compared to populations associated with host plants with glabrous surfaces (Moran 1986). The native host plants of the cotton fleahopper considered in this study also vary in terms of their surface morphology and chemistry. Then the question arises: are cotton fleahopper populations associated with two different host plant species show any morphological differences? Also, how these morphological differences in cotton fleahopper populations vary geographically? Chapter V gives an account of my approach to evaluate the effect of host plant species and geography in morphological variation of different cotton fleahopper populations.

The overall conclusions from this body of work are summarized in Chapter VI. Considering that the cotton fleahopper is an economically important pest in the United States, the conclusions I provide are drawn from a pest management perspective. To my

knowledge, this is one of the few studies, which have addressed host-associated differentiation in an agricultural system and the only one that has done so by exploring HAD in a hemimetabolous, non-parthenogen, multivoltine herbivore species. This dissertation not only contributes to the overall understanding of the cotton fleahopper's evolutionary ecology in the United States, but also adds a case study involving a homopteran other than Aphididae to the scarce list of host-associated differentiation studies in agro-ecosystems.

CHAPTER II

GEOGRAPHIC STRUCTURE OF GENETIC VARIATION OF COTTON FLEAHOPPER, *Pseudatomoscelis seriatus* POPULATIONS IN THE UNITED STATES

Introduction

The cotton fleahopper, *Pseudatomoscelis seriatus* is reportedly a native insect of the southern United States and northern Mexico (Knutson et al. 2002). In the United States, the cotton fleahopper has a wide distribution, from west (California) to east (North Carolina) and north (Nebraska) to south (Texas) (Source: Plant Bug Planetary Biodiversity Inventory). Cotton fleahopper is a polyphagous insect, which has been reported from more than 160 different host plant species belonging to 35 different families (Esquivel and Esquivel 2009). However, most of the studies related to this insect have been conducted due to its association with cultivated cotton (*Gossypium hirsutum*). Cotton as a cultivated crop was introduced to the United States during the 1600's and subsequent cultivation of cotton expanded to most of the southern states (Lewis and Richmond 1966). The cotton fleahopper was first reported as a pest during the 1920's (Reinhard 1926). Currently, cotton is commercially grown in 17 states in the US (Fig. 2.1), and cotton fleahopper infestations in cotton have been recorded from 10 different states, mostly in the south-west and mid-south region (Williams 2011). However, the extent of crop losses due to cotton fleahopper infestation is not similar across all the states. Cotton in Texas, Oklahoma and Arkansas is affected by cotton fleahopper more than the other cotton producing states. Thus, a question arises: are

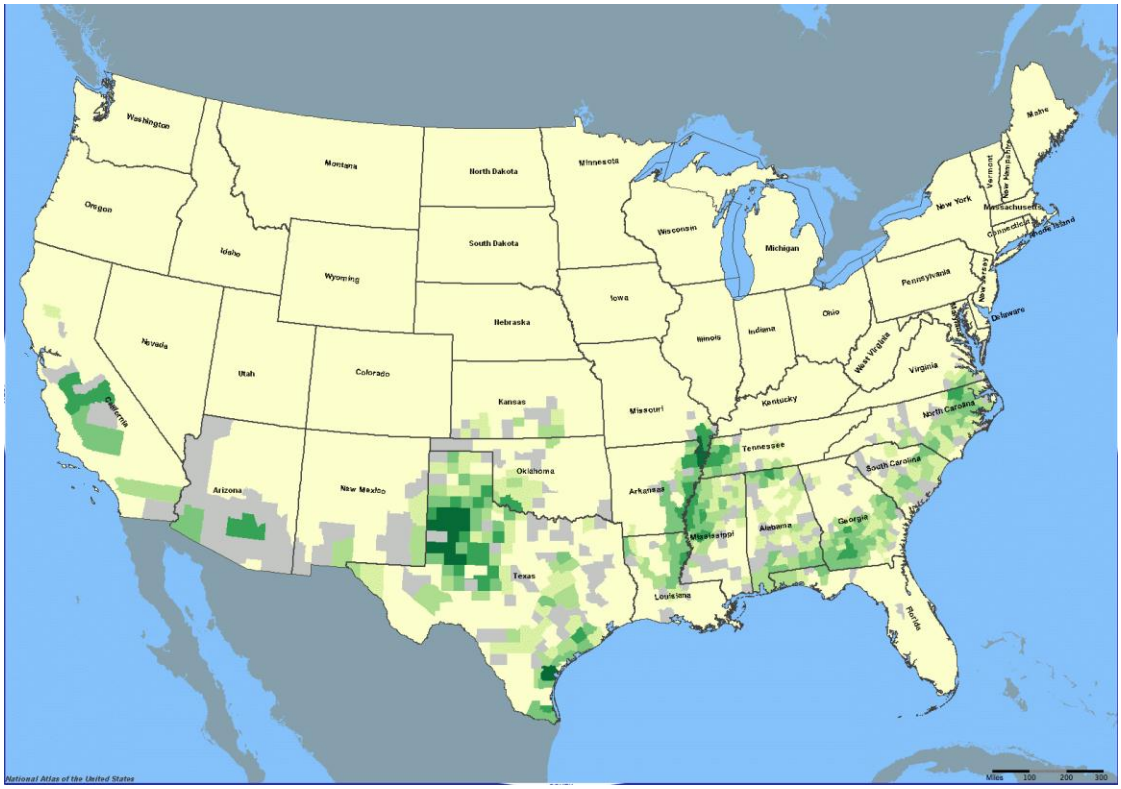

Fig. 2.1. Map illustrating areas and relative intensity of cotton production in the United States based on upland cotton harvested in 2007. Cotton production intensifies as the shading changes from light grey (lowest) through the increasing hues of green (highest) (National Atlas, 2011).

cotton fleahopper populations in different cotton growing states genetically different or are there eco-geographic factors which prevent populations from building up to an economic level at some locations?

No studies have been conducted to evaluate genetic differences among cotton fleahopper populations originating from different cotton growing states in the United States. Population genetic studies can reveal the presence or absence of genetic differentiation among populations. Genetic differences among geographic populations could be explained by historical factors (e.g., multiple introductions from distinct origins) or by the influence of micro-evolutionary processes such as natural selection, genetic drift and gene flow (Roderick 1996). One of the objectives of population genetic studies is to detect the presence of genetic structure, i.e. genetic differentiation among populations. Genetic structure in natural populations of phytophagous insects can be organized by their affiliation to different host plant species and geographic locations or sometimes by a combination of both. For example, populations of the fall armyworm, *Spodoptera frugiperda* were found to be genetically differentiated into two strains, based on their association with corn and rice (Pashley 1986). Similar examples can be found in other agricultural systems where insect populations associated with different host plant species have genetically differentiated into distinct host races (Ruiz-Montoya et al. 2003, Thomas et al. 2003, Carletto et al. 2009). In contrast to host-mediated genetic divergence, population differentiation due to geography requires physical isolation of insect populations either due to physical barriers (mountain ranges, deserts, rivers, etc.) or due to limited dispersal ability of insects. For example, Medina et al. (2010) showed

that the potato tuberworm (*Phthorimaea operculella*) populations in the western United States were genetically differentiated from eastern populations and suggested that mountain ranges between eastern and western states (Rocky and Appalachian mountains) could be the reason behind the geographically distinct patterns. In this study, I examined if the cotton fleahopper populations from different cotton producing states were genetically differentiated, I used two types of molecular markers, neutral molecular markers, AFLP (amplified fragment length polymorphisms) and mitochondrial gene sequences, specifically mtDNA COI sequences.

The two molecular markers selected in this study are widely used in population genetic studies (Grapputo et al. 2005, Ahern et al. 2009, Seyahooei et al. 2011). Gene sequences of mitochondrial genome were used to infer both taxonomic relationships and phylogeographic patterns of population differentiation (Avise 2004). Since, the mtDNA genome is maternally inherited and has lower effective populations sizes, than nuclear genomes, the mtDNA also provides unique information such as sex biased dispersal and evidence of past population bottlenecks. In addition, mtDNA genes are highly conserved and less variable than nuclear genes, which allow researchers to investigate longer evolutionary histories when compared to AFLP markers (Ahern et al. 2009). AFLP markers are highly variable and tend to reveal relatively recent population structure, which can give insight into contemporary gene flow among populations (Hewitt 2004). In this study, using two different molecular markers, I asked whether 1) geographically distant cotton fleahopper populations associated with cotton are genetically

differentiated and 2) if there is differentiation among populations, does this differentiation follow any geographic pattern?

Materials and methods

Sample collection and DNA extraction

Insect were collected from 11 cotton growing states and one location in Mexico. Insects were collected from cotton field(s) in areas where cotton is extensively cultivated within each state (Table 2.1). *P. seriatus* adults were collected during the reproductive stage of cotton using a standard sweep net and/or a motorized blower also known as a 'keep-it-simple' (KIS) sampler (Beerwinkle et al. 1997). We used a total of 282 *P. seriatus* individuals for AFLP analyses. For mtDNA analyses, we used a total of 70 individuals collected from 12 locations (Table 2.1). In the cotton producing states, where multiple sites were sampled, we pooled the insects collected from all sites within each state and considered it as a single geographic population representing that particular state. Insects were preserved in 95% ethanol at 4°C until used for DNA extractions. Genomic DNA was extracted using Qiagen® DNeasy kit (Valencia, CA) following the manufacturer's recommended protocol for animal tissue. DNA concentration and purity were measured for each specimen using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., DE). DNA was eluted in 100ul of Qiagen AE buffer.

Table 2.1. Information related to *P. seriatus* populations collected from different cotton growing states in the US

State	Population code	Latitude(North) dd min	Longitude (West) dd min	Elevation (m)	Collection date (mm-year)
Arizona	AZ	33 01.986	111 34.999	421	July-2010
Texas	TX	30 32.100	96 26.640	72	May-2010
		34 09.300	101 57.000	1066	July-2009
		31 25.320	100 08.400	358	July-2009
Oklahoma	OK	34 37.415	99 53.659	341	June-2010
Tamaulipas, Mexico	MX	26 00.057	97 44.245	17	June-2010
Arkansas	AR	36 03.035	90 22.654	77	June-2010
		36 20.537	90 13.088	87	June-2010
		35 23.609	92 23.405	208	June-2010
Mississippi	MS	33 42.902	90 59.458	39	June-2010
		33 24.454	90 54.673	71	June-2010
Louisiana	LA	32 21.057	91 30.662	33	July-2010
		32 54.643	91 44.833	32	July-2010
Alabama	AL	30 31.632	88 14.558	68	July-2010
		30 38.820	87 45.668	56	July-2010
Florida	FL	30 57.643	85 08.034	59	July-2010
Georgia	GA	31 08.327	84 48.688	49	July-2010
South Carolina	SC	33 41.586	80 41.618	86	July-2010
		33 31.659	80 44.995	63	July-2010
North Carolina	NC	36 12.165	76 26.573	4	July-2010
		35 49.683	77 36.313	36	July-2010
		35 42.379	77 49.728	40	July-2010

AFLP procedure

Amplified fragment length polymorphisms (AFLP) were generated using the protocol proposed by Vos et al.(1995) with slight modifications (see Chapter III for full description). The AFLP procedure is the same as the one described in Chapter III, except for the selective amplification step. I used three different selective primer pairs: EcoRI-ACT + MseI-CAT; EcoRI-AAC + MseI-CTC; and EcoRI-ACG + MseI-CAC. The PCR parameters were the same used for pre-selective and selective amplification in Chapter III.

Samples were analyzed using capillary electrophoresis. Each reaction was prepared by adding 0.5 µL of 400 HD-ROX-size standard (ABI 402985), 9 µL of HiDi formamide, and 1 µL of selective PCR amplification product. Samples were analyzed in an ABI 3130 genetic analyzer (Applied Biosystems, Forest City, CA). Results from capillary electrophoresis were analyzed by GeneMapper® 4.0 (Applied Biosystems, Forest City, CA). Electrophenograms in GeneMapper were evaluated using a 1 bp bin width. Only fragments between 50 and 400 bp were analyzed. The threshold for peak detection was set at 100. Thus, only fragments with relative florescent unit (RFU) of 100 or more were considered.

AFLP data analysis

Data obtained from three primer pairs were combined and analyzed as a single matrix. The percent polymorphic loci (%P) and expected heterozygosity (He) were estimated using GenAlEx 6.3 (Peakall and Smouse 2006). Principal coordinate analysis

(PCA) was performed on genetic distances among geographic populations based on Nei's genetic distance (Nei 1972). Analysis of molecular variance attributed to different hierarchical groups was calculated using ARLEQUIN version 3.1 (Excoffier et al. 2005). Pairwise F_{ST} values and their significance were calculated in ARLEQUIN version 3.1 (Excoffier et al. 2005). Bayesian clustering of individual genotypes was performed in STRUCTURE 2.3.1 (Pritchard et al. 2000, Falush et al. 2007). The STRUCTURE run followed an admixture model, with 10 replicates for each *K* assuming *K*= 1 to 10 and 50,000 burn-in followed by 50,000 MCMC replications. The best estimate of *K* was determined by the method described by Evanno *et al.* (2005) which takes into account the rate of change in the probability of data between successive *K* [Ln Pr(X|K)] values and graphically finds the uppermost hierarchical level of population structure for the tested scenario. We evaluated isolation-by-distance (IBD) by regressing genetic distance, $F_{ST}/(1- F_{ST})$ over transformed (natural log) geographic distance among populations as described by Rousset (1997) using GenAlEx 6.3. The significance of the correlation coefficient (r^2) was calculated with a Mantel test based upon 9999 random permutations.

mtDNA procedure

A fragment of ~ 600 base pairs from the mitochondrial COI gene was amplified by PCR using the primers C1-J-1718 and TL2-N-3014 (Simon et al. 1994). The PCR reaction consisted of a 25µl volume containing 2µl of template DNA, 0.5µl of 50X dNTPs, 0.5µl of each primer, 0.5µl of 50X polymerase (Clontech Laboratories, Mountain View, CA), 2.5µl of 10X buffer and 18.5µl of PCR grade water. PCR samples

were amplified in a GeneAmp 9700 thermocycler (Applied Biosystem, Valencia, CA) using the following program: 30 sec at 95°C; 45 cycles of 30 sec at 95°C, 30 sec at 54°C, 1 min at 72°C; 5 min at 72°C. Five microliters of PCR product were used for 1% agarose gel electrophoresis, to visualize the amplified products and compared them against a standard 1 kb ladder (NEB #3232). The amplified PCR products were purified following the PEG (polyethylene glycol) purification protocol (Sambrook et al. 1989). Another round of agarose gel electrophoresis of the post-purified PCR products was performed to test for quality by searching for the presence of multiple band or primer diamers of the PCR products. Purified PCR products were sequenced with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequencing reaction was carried out in a final volume of 15µl containing 1.5µl of BigDye Terminator Ready Reaction Mix, 2.25µl of Big Dye Sequencing Buffer (5×), 2.25µl of 0.25µM primer, 3.75µl of water and 5µl of purified PCR product. Cycle sequencing was performed using a GeneAmp® PCR System 9700 thermal cycler starting with 2 min at 96 °C, followed by 50 cycles of 10 sec at 96 °C, 5 sec at 55 °C and 3 min at 60 °C. Each sample was sequenced in both forward and reverse directions using the same primer combination used for initial amplification.

mtDNA sequence analysis

The mtDNA sequence for each individual was generated in both forward and reverse direction. Two sequences (forward and reverse) were then assembled and edited to obtain a consensus sequence for each individual using Sequencher 4.8 (Gene Codes

Corporation, Ann Arbor, MI). The consensus sequences for all individuals were aligned using Clustal-W in MEGA v5 (Tamura et al. 2011). We calculated nucleotide sequence diversity, and genetic differentiation among different populations using ARLEQUIN version 3.1 (Excoffier et al. 2005). A haplotype network was constructed using 95% parsimony criterion as implemented in the program TCS 1.21 (Clement et al. 2000). AMOVA was performed to partition overall genetic variation explained by population groups and individuals within a population using ARLEQUIN version 3.1 (Excoffier et al. 2005). Pairwise value of genetic significance (Φ_{ST}) was estimated based on Kimura-2-parameters using ARLEQUIN version 3.1 (Excoffier et al. 2005).

Results

Nuclear DNA analysis

The three selective primer pairs used for the AFLP analyses of 282 individuals yielded a total of 559 bands, of which 71.1% were polymorphic. The number of individuals (282) and AFLP bands (559) used in this study were adequate (SESim = 0.002) to reveal the presence of population structure according to the method described by Medina et al. (2006). Molecular diversity as indicated by percent polymorphism was highest for the Texas (TX) population and lowest for the Florida (FL) population (Table 2.2). The expected heterozygosity was similar for all the populations. Principal coordinate analysis (PCA) based on Nei's genetic distance (Nei 1972) among the 12 geographic populations sampled revealed that populations were grouped into at least four clusters (Fig. 2.2). Out of these four clusters, the cotton fleahopper population

Table 2.2. Molecular diversity for geographic populations of cotton fleahopper based on AFLP data. P%, percent polymorphic loci; He, expected heterozygosity and SE of expected heterozygosity

Populations	N	%P	He	SE of He
AZ	16	59.2	0.104	0.006
TX	21	71.0	0.110	0.006
OK	12	59.9	0.108	0.006
MX	14	57.4	0.102	0.006
AR	18	67.9	0.109	0.006
MS	16	64.9	0.108	0.006
LA	18	66.2	0.109	0.006
AL	13	55.8	0.105	0.006
FL	6	36.7	0.096	0.006
GA	20	66.9	0.107	0.006
SC	17	60.8	0.108	0.006
NC	17	60.3	0.109	0.006

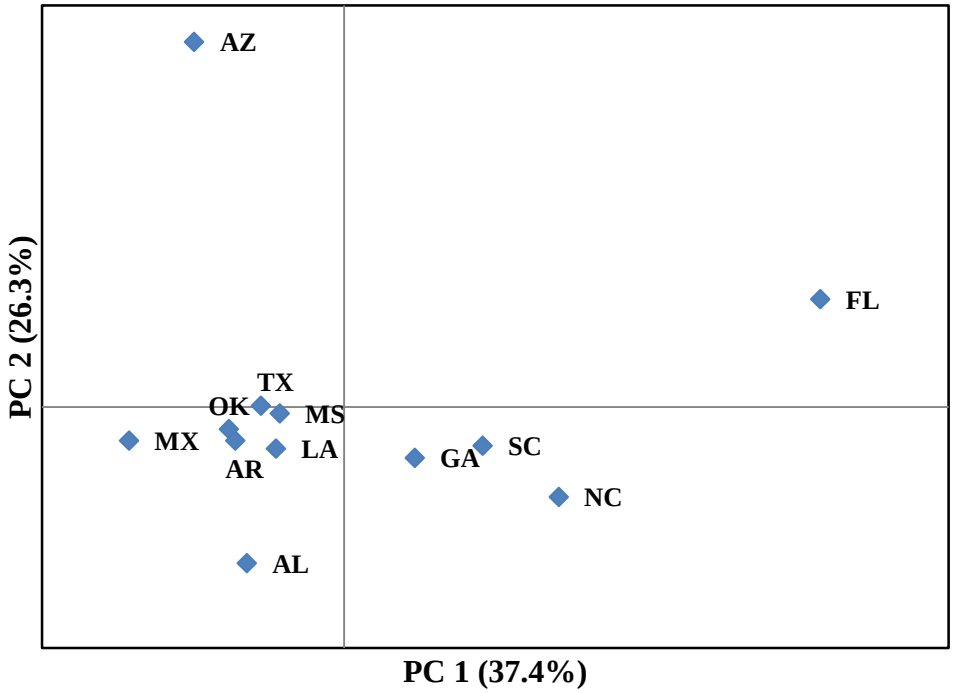

Fig. 2.2. PCA of 12 geographic populations based on their Nei genetic distances (Nei, 1972).

obtained from Florida (FL) and Arizona (AZ) formed two separate clusters. While Georgia (GA), South Carolina (SC) and North Carolina (NC) populations formed one cluster and the individuals from the remaining States (i.e., TX, OK, MX, AR, MS, LA, and AL) grouped together to form another cluster. Tests for significance of genetic differentiation indicated that overall, there was significant, yet little genetic differentiation among geographic populations ($F_{ST} = 0.02$; $P < 0.0001$). Analysis of molecular variance (AMOVA) of the12 geographic populations indicated that most of the genetic variation existed within populations (97.6%) and the remaining proportion (2.4%) of genetic variation was explained by geographic location (Table 2.3). Based on the PCA output and STRUCTURE analysis, we grouped the12 geographic populations into 3 regional groups (i.e., western, central and eastern) and performed another AMOVA. This AMOVA showed that the regional grouping was able to explain significant genetic variation (1.9%, $F_{CT} = 0.02$, $P = 0.0008$). At the next hierarchical level, within region, the geographic population(s) explained significant ($F_{ST} = 0.01$, $P < 0.0001$), but small (1.2%) genetic variation (Table 2.4). The pairwise comparisons of F_{ST} values between populations indicated that all comparisons between AZ and the reminder of the geographic populations were significantly different from zero (Table 2.5). Prior to Bonferroni correction, 52 out of 66 total pairwise comparisons showed significant F statistics. However, the total number of significant pairwise comparisons dropped to 20 after the correction (Table 2.5). The STRUCTURE analysis showed at least 5 possible genetic populations (Fig. 2.3). Although STRUCTURE suggested 5 genetic populations, based on the graphical output of STRUCTURE, it appears that three genetic populations

Table 2.3. AMOVA of 12 geographic populations without considering regional grouping

Sources of variation	df	% variation	F-statistics	P-value
Among geographic populations	11	2.4	0.023	<0.0001
Among individuals within population	176	97.6		

Table 2.4. Hierarchical AMOVA of 12 geographic populations after grouping the populations into three geographic regions

Sources of variation	df	% variation	F-statistics	P-value
Among geographic regions	2	1.9	0.019	0.0008
Among populations within region	9	1.2	0.012	<0.0001
Among individuals within population	176	96.9		

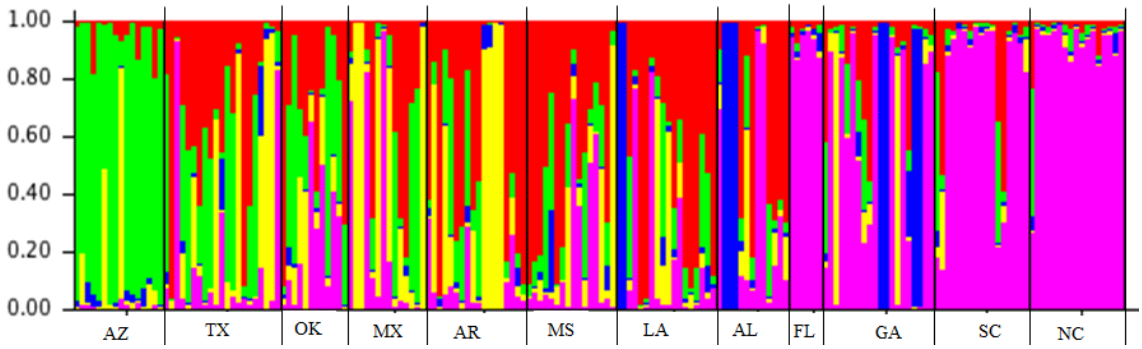

Fig. 2.3. Results of STRUCTURE analysis for 12 geographic populations based on AFLP markers at $K = 5$. Individuals are organized by their state of origin and represented by two letter state code (see Table 2.1 for population code). A vertical black line separates one population from another.

Table 2.5. Pairwise F_{ST} values (below diagonal) and geographic distance in km (above diagonal) between *P. seriatus* populations collected from 12 locations. Bold face F_{ST} values indicate significant population differentiation after Bonferroni correction at 0.05 significance level

	AZ	TX	OK	MX	AR	MS	LA	AL	FL	GA	SC	NC
AZ	-	1468	1092	1548	1982	1919	1876	2218	2497	2523	2857	3215
TX	**0.04**	-	544	556	827	576	477	763	1057	1087	1492	1923
OK	**0.04**	-0.01	-	980	896	838	817	1181	1436	1458	1766	2126
MX	**0.05**	0.01	0.01	-	1352	1054	928	1056	1348	1383	1848	2314
AR	**0.04**	0.00	0.01	0.01	-	332	459	671	761	764	915	1233
MS	**0.04**	0.00	-0.01	0.01	0.00	-	130	407	607	626	946	1355
LA	**0.05**	0.01	0.00	0.02	0.00	0.00	-	370	623	647	1018	1446
AL	**0.07**	0.02	0.02	0.03	0.02	0.02	0.02	-	301	334	793	1262
FL	**0.08**	0.05	0.05	**0.08**	0.05	0.04	0.04	0.07	-	37	516	993
GA	**0.05**	0.01	0.01	**0.03**	0.01	0.02	0.02	0.03	0.04	-	479	956
SC	**0.05**	0.02	0.01	**0.03**	0.02	0.02	0.02	0.03	0.03	0.01	-	477
NC	**0.07**	**0.03**	0.02	**0.04**	**0.03**	**0.02**	**0.03**	**0.03**	0.03	0.02	0.01	-

will make more biological sense in our data. These three genetic populations likely correspond to 1) individuals from Arizona, 2) Texas, Oklahoma, Mexico, Arkansas, Mississippi, Louisiana and Alabama and 3) Florida, Georgia, South Carolina and North Carolina. The autocorrelation analysis (Mantel test) between genetic and geographic distance among different geographic populations was significant ($r^2 = 0.26$, $P = 0.02$) (Fig. 2.4).

Mitochondrial DNA analysis

A total of 70 individuals from 12 different geographic populations were included in the mtDNA dataset. The primers (forward: CJ-1718, and reverse: NL-2-3014) amplified 609 bp of the COI gene. The consensus sequence resulted from overlapping forward and reverse sequences for individual cotton fleahoppers and comparing them for homology with other closely related published mitogenome. I found that the obtained sequence represented a segment of the COI gene corresponding to 2,260 and 2,871 bp of *Riptortus pedestris* (Hemiptera: Alydidae) obtained from GeneBank, accession no. EU427344 (Hua et al. 2008). The amplified COI segment was A-T rich (A:33.5%, C:17.1%, G:16.0%, T:33.4%) and the frequency of variable sites was 4.1%. There were 15 haplotypes (H1 to H15) in the sampled populations and 1 haplotype (H1) was present in all of the 12 geographic populations (Table 2.6). The relationships among the 15 mitochondrial haplotypes present in the 70 sampled individuals were reconstructed with a haplotype network (Fig. 2.5). AMOVA results indicated marginal support for population differentiation among geographic populations

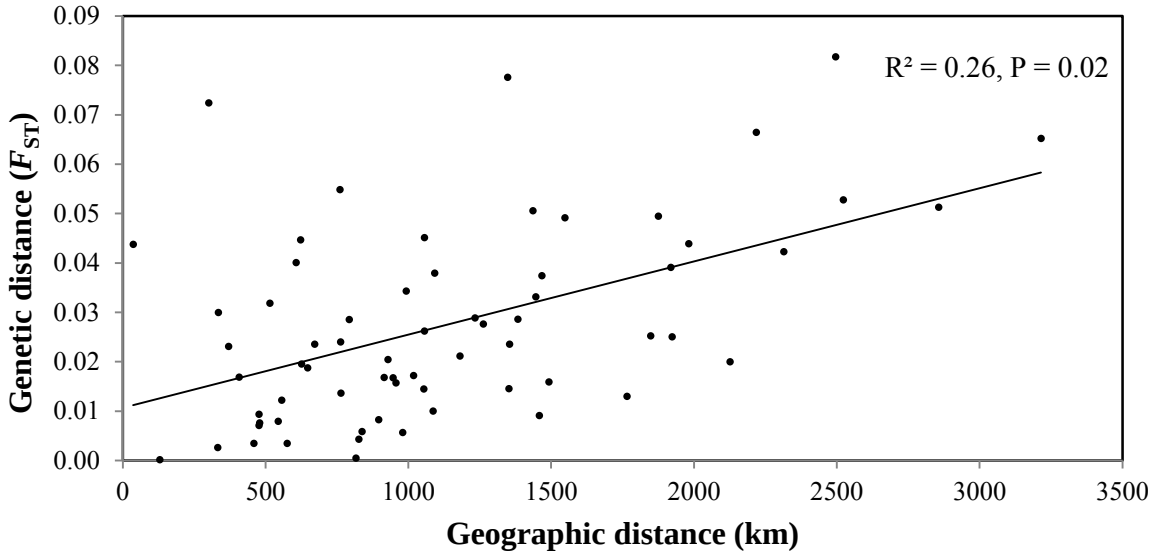

Fig. 2.4. Isolation-by-distance among 12 geographic populations based on AFLP data. Graph showing the result of autocorrelation between genetic and geographic distances among different populations.

Table 2.6. Variability in mitochondrial DNA sequence of geographic populations of *P. seriatus*. Number of analyzed individuals (N), number of individuals for each haplotype (H1-H15), nucleotide diversity (Pi) and expected heterozygosity (He) for each geographic population

Populations	N	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	Pi	He
AZ	6	2							1	1	1	1					2.5	0.0041
TX	7	5	1			1											2.8	0.0046
OK	3	3															0.0	0.0000
MX	5	4				1											2.8	0.0046
AR	7	6						1									0.9	0.0014
MS	6	4				2											3.7	0.0061
LA	5	5															0.0	0.0000
AL	7	3		1	1	1	1						1				5.2	0.0086
FL	4	2				1							1				5.3	0.0087
GA	7	5				2											3.3	0.0055
SC	5	2				2										1	4.6	0.0075
NC	8	3					1							3	1		6.3	0.0103

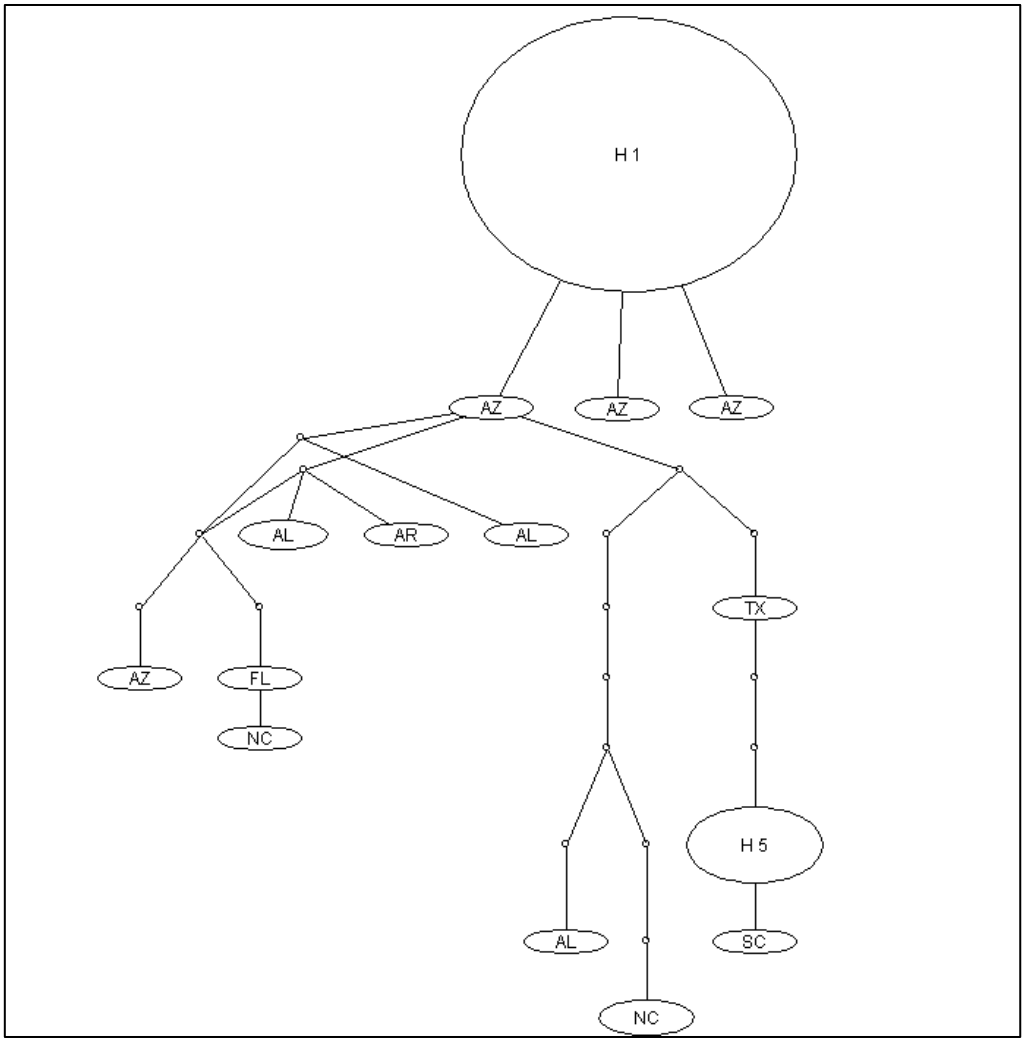

Fig. 2.5. Mitochondrial haplotype network for geographic populations of *P. seriatus*. The areas of the circles are proportional to the number of samples sharing each haplotype. Lines represent single nucleotide mutations and small back dots represent haplotype types not observed in the sample.

Table 2.7. Analysis of molecular variance (AMOVA) of *P. seriatus* populations based on mtDNA sequence data

Sources of variation	d.f.	% variation	Φ statistics	P value
Among geographic populations	11	5.7	0.06	< 0.0001
Among individuals within population	56	94.3		

Table 2.8. Pairwise Φ_{ST} between geographic populations based on Kimura 2-parameter distance between mtDNA haplotypes. No significant differences in Φ_{ST} were observed among any pairs

	AZ	TX	OK	MX	AR	LA	MS	AL	FL	GA	SC	NC
AZ	0.000											
TX	-0.051	0.000										
OK	-0.108	-0.132	0.000									
MX	-0.051	-0.250	-0.132	0.000								
AR	-0.035	-0.033	-0.167	-0.033	0.000							
LA	0.061	-0.171	0.040	-0.171	0.127	0.000						
MS	0.013	0.000	0.000	0.000	-0.055	0.161	0.000					
AL	-0.028	-0.099	-0.049	-0.099	0.042	-0.074	0.063	0.000				
FL	-0.039	-0.146	0.020	-0.146	0.118	-0.169	0.175	-0.137	0.000			
GA	0.034	-0.183	-0.010	-0.183	0.075	-0.177	0.103	-0.066	-0.143	0.000		
SC	0.319	0.102	0.363	0.102	0.444	-0.056	0.478	0.076	-0.052	0.019	0.000	
NC	0.118	0.125	0.113	0.125	0.212	0.152	0.215	0.009	0.031	0.162	0.251	0.000

(Φ_{ST} = 0.06, P < 0.0001). The majority of the molecular variation was among individuals within a population (94.3%) and only 5.7 % variation was observed among different geographic populations (Table 2.7). Different pairwise comparisons of geographic populations did not indicate any significant differentiation (Φ_{ST}) in mtDNA sequences among any of the geographic populations studied (Table 2.8).

Discussion

The current study is the first one to report population genetic information of the cotton fleahopper. Since the cotton fleahopper has gained importance due to its pestiferous association with cotton, this study was aimed at capturing the genetic diversity of this pest within the cotton growing areas in the United States. The mtDNA data indicated a lack of geographic structure of genetic variation. In contrast, AFLP data showed that the 12 different putative geographic populations studied were grouped into three broad clusters, viz. western (AZ), central (TX, MX, OK, AR, MS, LA, AL), and eastern (FL, GA, SC and NC) clusters (Figs. 2.2 and 2.3). This discrepancy between mitochondrial and nuclear DNA data suggests that the current geographic distribution of the cotton fleahopper may be relatively new and must have followed the geographic expansion of cotton cultivation.

Gene flow among geographically distant populations can have a homogenizing effect, preventing population divergence (Mallet 2001). However, genetic divergence among populations can still occur even in the face of gene flow provided there is local adaptation due to strong natural selection and/or high genetic drift due to small

population sizes. Due to its relatively wide geographic distribution, the cotton fleahopper may be experiencing divergent natural selection at different geographic locations as suggested by the geographic mosaic theory of coevolution proposed by Thompson (2005b). One of the reasons that could explain the different selection regimes experienced by the cotton fleahopper at different locations may have to do with variation in the abundance and species composition of alternative hosts in the landscape among the different geographic locations. In Chapter III, I indicate how the presence and abundance of cotton fleahopper host plants may vary even within the same state. Another possibility is that the observed differentiation among geographic populations could be caused by variation in insect population sizes, which may lead to differential rates of genetic drift at different locations. Population sizes of cotton fleahopper may vary among locations not only due to differences in the abiotic (e.g., climate) and biotic (e.g., plant health, natural enemies composition) components of the cotton fleahopper niche but also due to differences in the severity of pest control measures (e.g. insecticide applications). Except for the South-Central region of Texas and the Mississippi Delta, no other cotton growing area have been thoroughly surveyed for alternative host plants of the cotton fleahopper (Snodgrass et al. 1984, Esquivel and Esquivel 2009). Previous reports and my recent survey in several cotton growing states suggest that woolly croton (*Croton capitatus*) and evening primrose (*Oenothera speciosa*) are two common host plants widely distributed across several States.

The distribution and abundance of the cotton fleahopper's cultivated host (i.e., cotton) is well documented. Cotton was introduced in the United States during 1600's

and its cultivation started in Virginia (Lewis and Richmond 1966). Since its establishment, its cultivation range expanded westward and intensive commercial cultivation was recorded in almost all of the western states by early the 1900's. Cotton became a major crop in Arizona by 1920 and in California by 1950. Although cotton is cultivated in most of the southern belt of the United States, the overall cotton landscape is highly fragmented in terms of intensity of cotton cultivation (Fig. 2.1). For example, the Texas High Plains, the Mississippi Delta, and North Carolina's Coastal Plains are where most of the cotton cultivation takes place in the United States. These areas appear to be fragmented and isolated by distance. In this study, I collected cotton fleahopper populations from these areas. Thus, if one considers only cotton, these samples seem to represent fragmented populations of cotton fleahopper across its distribution range in the United States. Several studies have suggested that gene flow could be restricted among isolated insect populations inhabiting fragmented or geographically isolated landscapes (van Dongen et al. 1998a, Knutsen et al. 2000, Ellis et al. 2006, Exeler et al. 2010). Gene flow is depended on the dispersal potential of the organism in question and of the distance between the fragmented habitats. Currently, there is no information on the dispersal potential of cotton fleahoppers, but field observations indicate that it is a weak flier like other members in the group of plant bugs (Miridae) and do not take high altitude active flights (Almand et al. 1976). Thus, limited dispersal ability may lead to genetic differentiation among geographically isolated populations. Our AFLP data showed that the Arizona population of cotton fleahopper is genetically different from the other 11 geographic populations sampled. Similarly, the population composed by

individuals collected from Florida, Georgia, North and South Carolina, was genetically distinct from the remaining populations. In contrast, populations in the Central United States (i.e., Texas, Oklahoma, Arkansas, Mississippi, Louisiana and Alabama) were genetically similar, suggesting that individuals within this region remain connected through gene flow. Other insect species distributed across the United States have shown similar patterns of regional genetic structure. For example, the potato tuberworm, *Phthorimaea operculella* (Medina et al. 2010), the pecan nut casebearer, *Acrobasis nuxvorella* (Hartfield et al. 2011), Hessian fly, *Mayetiola destructor* (Johnson et al. 2011), sorghum plant bug, *Stenotus rubrovittatus* (Kobayashi et al. 2011), Colorado potato beetle, *Leptinotarsa decemlineata* (Grapputo et al. 2005) and the cotton boll weevil, *Anthonomus grandis* (Kim and Sappington 2006), all showed regional population structure.

The AFLP data showed that there is a positive and significant correlation between the genetic and geographic distances among cotton fleahopper populations. The isolation by distance (Wright 1943) found in the cotton fleahopper suggests that individuals may not be able to disperse beyond their respective regions (i.e., Arizona, Central United States and Eastern United States) and tend to reproduce mostly with fleahoppers that occur relatively close. A meta-analysis by Peterson and Denno (1998) indicated that phytophagous insects with high mobility show weak IBD, while insects with moderate mobility show pronounced or significant IBD. In cases of mobile insect pests of agricultural importance such as *Ostrinia nubilalis* and *Hemicoverpa zea*, there is no genetic differentiation among geographically distant populations (Krumm et al. 2008,

Groot et al. 2011). These insects are considered to have panmictic populations even though their geographic distributions comprise a relatively large geographic area. This could arise due to high dispersal abilities achieved through migratory flights (Franklin et al. 2010) or through passive dispersal using wind currents (Michel et al. 2009). Alternatively, pest movement associated with human activities may also explain the movement of some insect populations (Loxdale and Lushai 1999, Benavides et al. 2005).

In studying the population structure of agricultural insect pests, it is important to consider the diet breadth of the insect species studied. Several agricultural insect pests are polyphagous and can utilize different sets of host plant species at different geographic locations. Several insects associated with different host-plant species have been shown to present host plant associated genetic differentiation or habitat associated genetic differentiation (Brunner et al. 2004, Vialatte et al. 2005, Carletto et al. 2009, Peccoud et al. 2009, Brunner and Frey 2010). Host-associated differentiation (HAD) is the formation of genetically distinct lineages when parasites (e.g., herbivorous insects) are associated with different hosts (e.g., host-plant species) (Pashley 1986, Stireman et al. 2005, Althoff 2008). In Chapter III, I report the existence of HAD in the cotton fleahopper. Thus, the small but significant genetic differentiation observed among several geographic locations in our study could be influenced by the presence of different sets of host plant species in those locations. If one considers that the cotton fleahopper is a native pest in the study area, it could be that before cotton cultivation, fleahoppers were associated to different native plants at different locations and may have been genetically differentiated. Once cotton was introduced, fleahoppers from these

different host-plant species could have switched to cotton, making the current genetic differentiation, we found in cotton fleahoppers at different geographic locations an artifact of their prior distinct origins.

Advances in the development of molecular markers over the last few decades have led to an increased number of studies addressing the genetic population structure of several groups of organisms (Avise 2004). Currently, both multi-locus nuclear molecular markers (e.g., AFLP, microsatellite, SNP) and mtDNA sequence data are widely used in population genetic studies. Information gained from population genetic studies have the potential to improve insect pest management practices. For example, identification of pheromone races (Willett and Harrison 1999) can improve pest control practices based on mating disruption and pest monitoring using pheromone traps. Similarly, identification of host-associated races in pests and their natural enemies could enhance the success of biological control programs (Malausa et al. 2007, Lozier et al. 2008, Medina 2011), and help in the design of strategies aimed to reduce the risk of insecticide resistance (Denholm and Rowland 1992, Bourguet et al. 2000, Endersby et al. 2006). Similarly, knowing the way in which genetic variation is organized may improve the design of strategies to delay resistance to transgenic plants that express bacterial toxins (Tabashnik 1994, Carriere et al. 2010).

Nevertheless, very few studies have been able to link population genetic structure to specific traits relevant to pest control. It has become clear that individuals belonging to the same pest species are far from being genetically and phenotypically homogeneous. The next logical step is to investigate how the organization of genetic and phenotypic

variation is influenced by ecological and environmental factors and how this variation translates into vulnerability of pests to different control practices.

CHAPTER III

GEOGRAPHIC PATTERN OF HOST-ASSOCIATED DIFFERENTIATION IN THE COTTON FLEAHOPPER, *Pseudatomoscelis seriatus* (Reuter)

Introduction

Interest in ecological speciation of herbivorous insects over the past four decades has led to several proposed underlying ecological and evolutionary processes responsible for the observed diversity in this group of animals (Berlocher and Feder 2002, Dres and Mallet 2002, Bethenod et al. 2005). Host plants can exert strong natural selection on herbivorous insects, and may play a key role in the radiation of herbivore insect lineages (Ehrlich and Raven 1964, Mopper 1996, Miller et al. 2003, Funk 2010). Shifting of herbivore insects to different host plant species followed by adaptations to the newly adopted host plant, may lead to reproductive isolation among insect populations associated with different host plant species (Martel et al. 2003, Diegisser et al. 2009), resulting in host-associated lineages (Dobler and Farrell 1999, Stireman et al. 2005, Peccoud et al. 2009). Insects that use different host plant species across their geographic distribution are likely to experience divergent selection pressures by host plants at different locations (Via 1991, Thompson 1994, Sword et al. 2005). Particularly, selection pressure may vary if host plant densities and/or the communities in which the insect and its host plants are immersed (i.e., predators, competitors, alternative host plants, diseases, etc.) differ across the insect's geographic distribution.

Variation in the availability and/or abundance of host plant species within an herbivore's distribution range may generate differences in the pattern of host plant specialization or adaptation (Kuussaari et al. 2000). Herbivore populations of the same species may be specialized on different host plant species at different locations and yet be characterized as a generalist species when its entire geographic distribution is considered (Fox and Morrow 1981). Therefore, it is always more realistic to examine host-associated differentiation (HAD) throughout the entire geographic distribution of a species (Toju 2009). However, fine scale heterogeneity in vegetation composition and other ecological factors are often overlooked when studying interspecific interactions.

A growing number of studies have documented HAD of insect herbivore species (Guttman et al. 1981, Carroll and Boyd 1992, Emelianov et al. 2001, Nason et al. 2002, Brunner et al. 2004, Stireman et al. 2005, Conord et al. 2006, Ohshima 2008, Dorchin et al. 2009). Although the majority of these examples have studied unmanaged or wild systems composed of perennial plant species, there are some examples of HAD of herbivores in agro-ecosystems (Via 1991, DeBarro et al. 1995, Ruiz-Montoya et al. 2003, Vialatte et al. 2005, Alvarez et al. 2007). These examples show that HAD in herbivore insects is not uncommon in agricultural systems, which are mostly managed and prone to relatively high levels of anthropogenic disturbances.

In the present study we report HAD in the cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae). *Pseudatomoscelis seriatus* has numerous host plant species, both native wild hosts as well as introduced crop species. Previous studies indicate that several aspects of *P. seriatus* biology such as host plant preference, total

developmental time, nymphal mortality and fecundity are differentially influenced by their host plant species (Gaylor and Sterling 1976, Beerwinkle and Marshall 1999). The objectives of the present study were 1) to detect if host-associated differentiation was present in *P. seriatus* populations associated with three selected host plant species, and 2) to assess the geographic structure of host-associated differentiation at a regional scale (i.e., the state of Texas, USA). This is the first study on any genetic aspect of *P. seriatus*.

Materials and methods

The insect

Pseudatomoscelis seriatus is a native insect of North America. It is considered a generalist herbivore reported to feed on ~160 host plants species belonging to 35 different plant families (Snodgrass et al. 1984, Esquivel and Esquivel 2009). In the 1920's, heavy yield losses of cotton were attributed to *P. seriatus* in different regions of Texas (Reinhard 1926). Presently, *P. seriatus* occurs in several cotton growing regions in the United States, mostly in Texas, Oklahoma, Arkansas, Mississippi and Louisiana. *Pseudatomoscelis seriatus* is a hemimetabolous insect, which complete 8-9 generations per year and feeds externally using its sucking mouthparts on tender stems or flowering structures of its host plants. It hibernates as eggs, which hatch during March-April depending on the rainfall and temperature. After progressing through five nymphal instars, *P. seriatus* remains as an adult for about 12-15 days. A female lays about 10-15 eggs under the epidermal layer of the stem of its host plants. Adults are not active fliers but they may disperse long distances either through wind or by anthropogenic dispersal.

Host plants

For this study, we selected three host plant species of *P. seriatus viz*., *Monarda punctata* L. (Laminales: Lamiaceae) known as horsemint, cultivated cotton, *Gossypium hirsutum* L. (Malvales: Malvaceae), and *Croton capitatus* Michx. (Euphorbiales: Euphorbiaceae) known as woolly croton. These three host plant species are the most common fleahopper host plants at our study sites and they persist for a relatively long time during the spring, summer and fall, respectively, maximizing their period of interaction with *P. seriatus* (Almand et al. 1976, Holtzer and Sterling 1980b). Depending on local climatic conditions, *P. seriatus* spends between 3 to 5 generations in each of these three host plant species in our study areas. Unlike horsemint and woolly croton, cotton is an introduced plant and it is widely cultivated in the study areas. Cotton was introduced to Texas in the early 1800's and a massive expansion of cotton cultivation followed until the 1920s (Lewis and Richmond 1966). The three chosen host plant species belong to different families and carry different suits of defensive chemicals (Scora 1967, Schmidt and Wells 1990, Williams et al. 2001). These host plant species are available to *P. seriatus* at different times of the year, with some overlapping periods. For instance, in College Station, in a typical year, the native spring wild host, horsemint (*M. punctata* L.) becomes available for *P. seriatus* at the beginning of the growing season (April-June), while woolly croton (*C. capitatus* Michx.) becomes abundant from

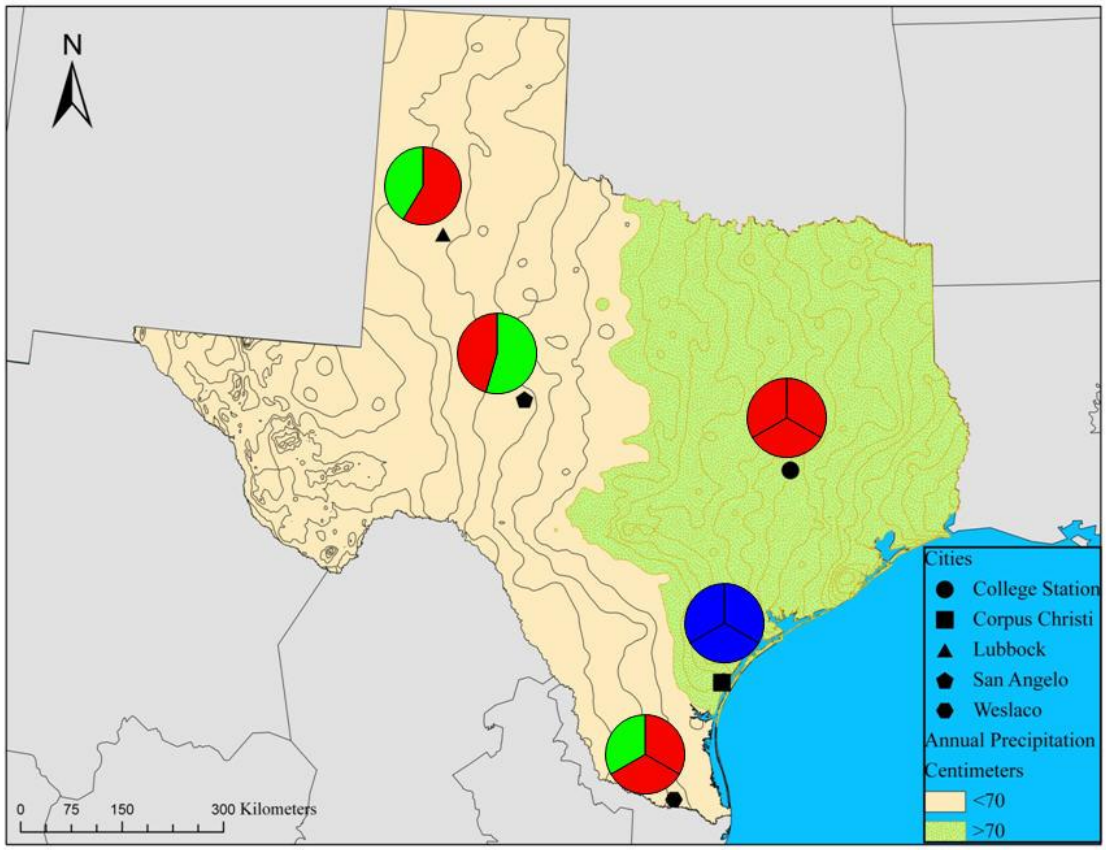

Fig. 3.1 Study locations along with the population structure of *P. seriatus* in an annual precipitation map of Texas. Presence and proportion of the three genotypes of *P. seriatus* is shown in a pie diagram for each location (within each circle, green colour represents horsemint genotype in Lubbock, San Angelo and Weslaco, red colour represents horsemint/cotton/woolly croton or all depending on the location and blue represents a locally distinct population in Corpus Christi). The rainfall map was generated in ArcGIS from available annual rainfall data for the state of Texas.

May until October. The timing of cotton cultivation varies by location within the state of Texas. For example, near College Station, cotton is normally planted during mid-April while in Lubbock and San Angelo, planting of cotton is typically optimal in mid-May.

Sample collection

Insect collections were made from five Texas locations including Lubbock, San Angelo, College Station, Weslaco and Corpus Christi (Fig. 3.1 and Table 3.1). All five locations are in areas under intensive cotton cultivation. We collected fleahoppers associated with cotton, horsemint and woolly croton from several fields (2 to 5 fields per host plant species) within each location. In two study locations, Lubbock and San Angelo, we did not find woolly croton.

Pseudatomoscelis seriatus adults were collected during the peak fleahopper activity on each host plant (which is the flowering stage of each plant) by using a standard sweep net and a motorized blower also known as a 'keep-it-simple' (KIS) sampler (Beerwinkle et al. 1997). The identity of *P. seriatus* collected from horsemint, woolly croton and cotton was confirmed by a mirid systematist **(**Dr. Joseph C. Schaffner, Texas A&M University**).** Insects were preserved in 85% ethanol at 4°C until used for DNA extractions. We used 12-20 fleahoppers per host plant species at each location for genetic analyses.

Table 3.1. Collection location, host plant, population code and geographical information (i.e., latitude, longitude, elevation) of *P. seriatus* populations used in the study

Location	Host plant	Population Code	Latitude (N)	Longitude (W)	Elevation (m)
Lubbock	Horsemint	LH	33.571	101.804	967
			33.489	101.619	874
	Cotton	LC	34.155	101.950	1066
			33.949	101.695	975
			33.981	102.078	1005
			33.641	102.079	1017
San Angelo	Horsemint	SH	31.661	100.330	618
			31.852	100.292	532
			32.064	100.305	617
			32.388	100.378	682
	Cotton	SC	31.422	100.140	358
			32.093	101.353	849
			31.328	100.161	579
			31.381	100.332	571
			31.414	100.073	555
College Station	Horsemint	CH	31.423	96.242	58
			30.842	96.617	85
			30.535	96.444	72
			30.692	96.515	70
			30.706	96.565	79
	Cotton	CC	30.535	96.444	72
			30.692	96.515	70
			30.399	96.269	62
			30.706	96.565	79
	Woolly croton	CW	30.392	90.350	58
			30.546	96.506	73
			30.844	96.622	83
Weslaco	Horsemint	WH	26.935	98.134	39
			26.799	98.412	72
	Cotton	WC	26.137	97.958	20
			26.385	98.253	44
			26.290	98.333	52
	Woolly croton	WW	26.137	97.958	20
			26.079	98.078	25
			26.119	97.968	20

Table 3.1. continued

Location	Host plant	Population Code	Latitude (N)	Longitude (W)	Elevation (m)
Corpus Christi	Horsemint	TH	29.253	96.179	30
			29.442	97.101	102
			28.950	96.208	15
	Cotton	TC	27.969	97.713	32
			29.209	96.228	30
			27.848	97.644	24
	Woolly croton	TW	27.627	97.793	21
			27.610	97.753	16
			27.952	97.684	22

Genetic methods

Genomic DNA was extracted from randomly chosen individual specimens using Qiagen ® DNeasy kit (Valencia, CA) following the manufacturer's recommended protocol for animal tissue. DNA concentration and quality were measured for each specimen using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., DE). DNA was eluted in 100ul of Qiagen AE buffer.

Amplified fragment length polymorphisms (AFLP) markers were generated using the protocol proposed by Vos et al.(1995) with slight modifications. Samples were randomly arranged in 96-well plates for AFLP analyses. Three to four samples were repeated within each plate and the same samples were repeated in all the plates used in order to check the reproducibility of our analysis. Restriction digestion and ligation steps were performed by adding 5.5 µl of genomic DNA to 5.5 µl of a master mix containing 1.1 µL of 10x T4 DNA ligase buffer, 1.1 µL of 0.5M NaCL, 0.55 µL of diluted bovine serum albumin (1 mg/mL), 0.05 µL of MseI (NEB R0525M), 0.05 µL of EcoRI (NEB R0101T), 0.03 µL of T4 DNA ligase (NEB M0202M), 1 µL of MseI and 1 µL of EcoRI adaptors (ABI 403077) and 0.61 µL of ultra-pure water (18.2 mega-ohm/cm). The entire reaction was left overnight at room temperature for adequate digestion. The next morning, each reaction was diluted to 1:18 (11 µL + 189 µL) ratio with buffer TE_{thin} (15 mM Tris of pH 8.0, 0.1 mM EDTA). Preselective PCR amplification was performed in a 20 µL reaction containing 4 µL of the diluted restricted/ligated DNA and 16 µL of a mixture containing 1 µL of EcoRI and MseI AFLP pre-selective primers mix (ABI 403078) with 15 µL of AFLP core mix (ABI 402005). The PCR protocol for the pre-

selective amplification consisted of: 95°C for 1 min followed by 20 repetitive cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 90 s with a final hold at 75°C for 5 min followed by a storing temperature of 4°C until subsequent procedure. The amplified product was diluted 20-fold by adding 190 µL of buffer TE_{thin} to each reaction.

For selective PCR amplification of restriction fragments, 4 µL of the diluted pre-selective PCR product was added with 15 µL platinum super mix (Invitrogen 11306016), 1 µL of primers EcoRI-ACT (ABI 402045) or EcoRI-AAC (ABI 4303053) and 1 µL of MseI-CAT (ABI 402018) or MseI-CTC (ABI 402016). The PCR parameters were an initial warm-up at 95°C for 30 s, 12 cycles of 95°C for 10 s, 65°C for 40 s with a lowering of 0.7°C per cycle, 72°C for 5 min, followed by 35 cycles of 95°C for 11 s, 56°C for 30 s, 72°C for 2 min and finally a hold of 75°C for 5 min before storing the samples at 4°C.

Samples were analyzed using capillary electrophoresis. Each reaction was prepared by adding 0.5 µL of 400 HD-ROX-size standard (ABI 402985), 9 µL of HiDi formamide, and 1 µL of selective PCR amplification product. Samples were analyzed in an ABI 3130 genetic analyzer (Applied Biosystems, Forest City, CA). Results from capillary electrophoresis were analyzed by GeneMapper® 4.0 (Applied Biosystems, Forest City, CA) which provides a presence (1) absence (0) matrix for each individual. Electrophenograms in GeneMapper were evaluated using a 1 bp bin width. Only fragments between 50 and 400 bp were analyzed. The threshold for peak detection was set at100. Thus, only fragments with relative florescent unit (RFU) of 100 or more were considered.

Statistical analysis

The SESim statistic (Medina et al. 2006) was used to assess the adequateness of the number of individuals and AFLP markers used to detect genetic population structure. A SESim value lower than 0.05 indicates that a given combination of markers and individuals is sufficient to detect the pattern of genetic structuring at the geographic scale considered.

Data obtained from two primer pairs (E/ACT-M/CAT and E/AAC-M/CTC) were combined and analyzed as a single matrix. Different population genetic information (percent of polymorphic loci and expected heterozygosity) were obtained after analyzing the AFLP matrix with GenAlEx 6.3 (Peakall and Smouse 2006). Principal coordinate analysis (PCA) was performed by using Nei's genetic distance matrix (Peakall and Smouse 2006) to visualize the relatedness of different populations in a two dimensional coordinate system. An unrooted neighbor joining (NJ) tree was obtained using the NEIGHBOR procedure in PHYLIP (Felsenstein 1993). The same distance matrix used to generate the NJ tree was bootstrapped 10,000 times using AFLP-SURV (Vekemans 2002). Bootstrap values were obtained using the majority rule in CONSENSE procedure under PHYLIP 3.6 (Felsenstein 1993).

Genetic differentiation was estimated by calculating F_{ST} values (Wright 1969) for host-associated populations at each location and also by calculating an overall F_{ST}. Bayesian clustering of individual genotypes was performed in STRUCTURE 2.3.1 (Pritchard et al. 2000, Falush et al. 2007). The STRUCTURE run followed an admixture model, with 20 replicates for each K assuming K= 1 to 7. 100,000 burn-in and 50,000

replications were used. The best estimate of *K* was determined by the method described by Evanno *et al.* (2005) which takes into account the rate of change in the probability of data between successive *K* [Ln Pr(X|K)] values and graphically finds the uppermost hierarchical level of population structure for the tested scenario. Analysis of molecular variance (AMOVA) was carried out using ARLEQUIN version 3.1 (Excoffier et al. 2005) to partition the genetic variation among and within populations. Individuals were grouped in three ways and analyzed with AMOVA to understand the effect that location and host plants have on genetic variation. The three groups were: 1) overall (host plant and locality combinations, i.e., 13 groups accounting for 5 locations and 3 host plants), 2) among locations (5 groups accounting for 5 locations), and 3) among host plants (3 groups accounting for 3 host plant species). We evaluated isolation by distance (IBD) by regressing genetic distance, $F_{ST}/(1- F_{ST})$ over transformed (natural log) geographic distance among populations as described by Rousset (1997) using GenAlEx 6.3. The significance of the correlation coefficient (r^2) was calculated with a Mantel test based upon 999 random permutations.

Results

We obtained DNA of adequate concentration (avg. 63 ng/µl) and quality (2.1, 260/280 ratio) from individual *P. seriatus* DNA extractions. AFLP analysis of 196 individuals with 2 primer combinations (E/ACT-M/CAT and E/AAC-M/CTC) produced 432 bands. A SESim statistic of 0.028 indicated that this number of bands and individuals were sufficient to describe *P. seriatus* genetic population structure at the

scale of this study (Medina et al. 2006). The percentage of polymorphic loci in the 13 purported *P. seriatus* populations (refer to Table 3.2 for annotated population information) varied from 39.35% (CH population) to 59.26% (TC population). The overall F_{ST} value for the *P. seriatus* populations sampled was 0.11, which was significantly different from zero (P=0.0001). Within-population variability, as indicated by the mean expected heterozygosity, was consistent over all the populations ($H_E \approx$ 0.08), with the exception of Corpus Christi in which, populations from each of the three host plant species showed a higher value ($H_E \approx 0.11$) (Table 3.2). Pairwise F_{ST} values show that the Corpus Christi population, which include individuals feeding on horsemint, cotton and woolly croton, (i.e., TH, TC and TW, respectively) was the most genetically distinct population when compared with populations from the other four locations (i.e. Lubbock, San Angelo, College Station and Weslaco) (Table 3.3).

Principal coordinate analysis (PCA) of all 13 purported populations collected from the three host plant species in the five locations considered in this study revealed that *P. seriatus* in Texas was grouped into three distinct clusters (Fig. 3.2). Similarly, three defined clusters can be observed when the populations are grouped using a neighbor-joining (NJ) tree (Fig. 3.3). Both the PCA and the NJ tree show that at three sampling locations (i.e., Lubbock, San Angelo, and Weslaco), *P. seriatus* populations associated with horsemint (i.e., LH, SH and WH) grouped together into a distinct horsemint associated cluster regardless of their geographic origin. On the other hand, *P. seriatus* populations from Corpus Christi (i.e., TH, TC and TW) were grouped together in a unique cluster regardless of their host plant association. Finally, a third cluster was

Table 3.2. Genetic diversity indices based on AFLP data among *P. seriatus* populations as coded according to location and host plant origin

Location	Host Plant	Population code	Polymorphic loci (%)	Expected heterozygosity	SE of heterozygosity
Lubbock	Horsemint	LH	48.15	0.079	0.006
	Cotton	LC	46.06	0.082	0.006
San Angelo	Horsemint	SH	52.55	0.077	0.006
	Cotton	SC	49.07	0.080	0.006
College Station	Horsemint	CH	39.35	0.075	0.006
	Cotton	CC	45.14	0.081	0.006
	Woolly croton	CW	45.83	0.079	0.006
Weslaco	Horsemint	WH	47.92	0.082	0.006
	Cotton	WC	45.83	0.076	0.006
	Woolly croton	WW	43.06	0.079	0.006
Corpus Christi	Horsemint	TH	53.94	0.111	0.007
	Cotton	TC	59.26	0.113	0.007
	Woolly croton	TW	58.33	0.115	0.007

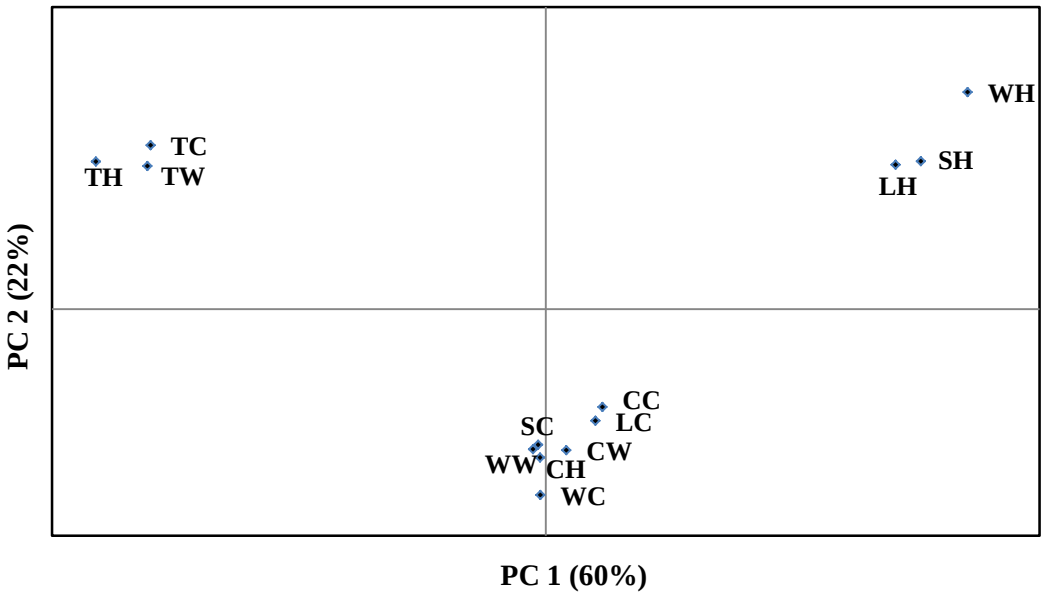

Fig. 3.2 Principal coordinate analysis of *P. seriatus* populations. The distance matrix of thirteen populations was converted to principal component scores and projected in a two dimensional space formed by principal component one (PC1, explains 60% of the variation) and principal component two (PC2, explains 22% of the variation).

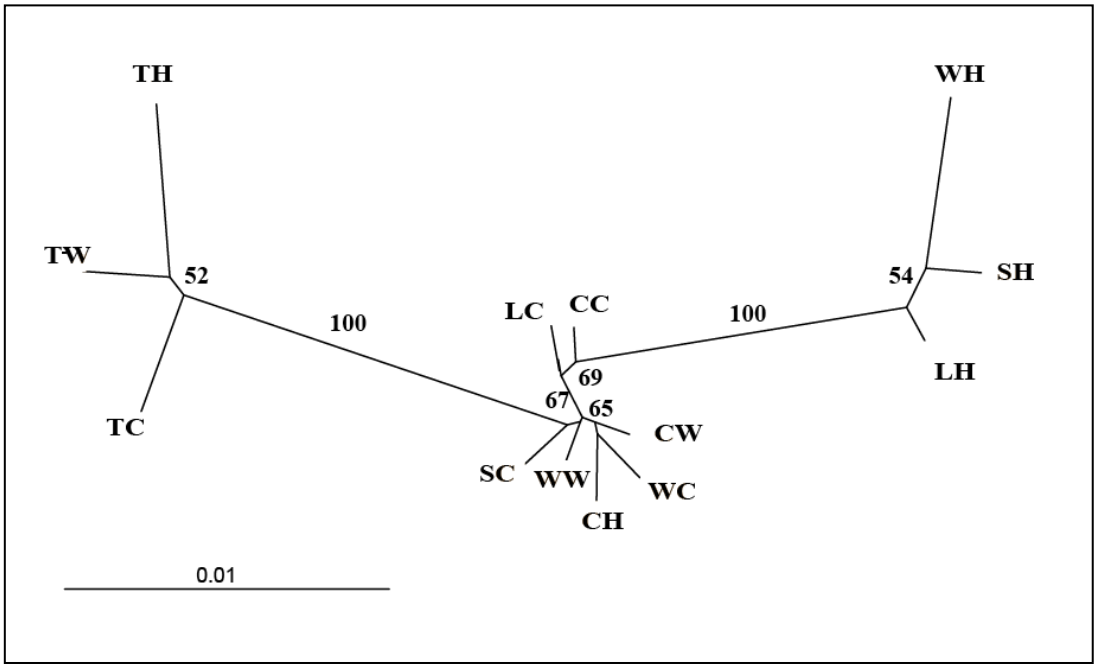

Fig. 3.3 Unrooted neighbor-joining tree of *P. seriatus* populations collected from three host plant species across Texas. Three populations (i.e., LH, SH and WH) grouped together based on their host plant association (to horsemint), while three populations (i.e., TH, TC and TW) grouped based on their geographic location (i.e., Corpus Christi). Bootstrap support (%) from 10,000 iterations is provided for each major node.

Table 3.3. Pairwise comparisons of *P. seriatus* populations. Lower diagonal values are F_{ST} value and upper diagonal values are Nei's genetic distance. Values in bold represent significant F_{ST} for the respective pair of populations compared.

	Nei's genetic distance												
	LH	LC	SH	SC	CH	CC	CW	WH	WC	WW	TH	TC	TW
LH		0.012	0.006	0.015	0.016	0.012	0.013	0.006	0.017	0.015	0.037	0.033	0.033
LC	**0.098**		0.013	0.004	0.005	0.002	0.004	0.018	0.005	0.003	0.021	0.018	0.019
SH	0.026	**0.120**		0.018	0.017	0.013	0.016	0.007	0.018	0.016	0.040	0.035	0.035
SC	**0.139**	0.000	**0.172**		0.005	0.004	0.004	0.020	0.005	0.004	0.020	0.018	0.018
CH	**0.142**	0.014	**0.158**	0.019		0.005	0.004	0.021	0.004	0.005	0.020	0.020	0.018
CC	**0.098**	0.000	**0.118**	0.009	0.022		0.004	0.017	0.005	0.004	0.021	0.018	0.019
CW	**0.111**	0.000	**0.137**	0.001	0.017	0.000		0.019	0.003	0.003	0.020	0.019	0.017
WH	0.020	**0.157**	0.037	**0.191**	**0.197**	**0.149**	**0.166**		0.022	0.022	0.043	0.040	0.039
WC	**0.132**	0.000	**0.151**	0.003	0.016	0.000	0.000	**0.180**		0.004	0.021	0.020	0.019
WW	**0.128**	0.000	**0.150**	0.009	0.028	0.000	0.000	**0.193**	0.000		0.019	0.018	0.017
TH	**0.240**	**0.138**	**0.270**	**0.141**	**0.148**	**0.145**	**0.131**	**0.277**	**0.140**	**0.131**		0.009	0.008
TC	**0.203**	**0.104**	**0.229**	**0.116**	**0.126**	**0.110**	**0.106**	**0.242**	**0.114**	**0.107**	0.019		0.008
TW	**0.215**	**0.117**	**0.238**	**0.123**	**0.131**	**0.120**	**0.103**	**0.254**	**0.120**	**0.105**	0.009	0.010	

Pairwise F_{ST} value

formed by a mixture of populations from all three host plant species (i.e. horsemint, cotton and woolly croton) from different locations (i.e., Lubbock, San Angelo, Weslaco, and College Station). Similarly, the Bayesian clustering analysis performed in STRUCTURE 2.3.1 (Pritchard et al. 2000, Falush et al. 2007) revealed that there are three (K=3) distinct genetic populations of *P. seriatus* in Texas (Fig. 3.4). The STRUCTURE output for K=3, revealed a pattern of host-associated differentiation in *P. seriatus*. In three locations (i.e., Lubbock, San Angelo and Weslaco) horsemint associated populations (i.e., LH, SH, and WH) were found. In contrast, populations in two locations (i.e., College Station and Corpus Christi) were not differentiated based on their host plant associations (Fig. 3.4). There was no host-associated differentiation in *P. seriatus* from Corpus Christi. However, *P. seriatus* from Corpus Christi represented a geographically distinct genotype, differentiated from rest of the populations at the four other locations.

AMOVA of all 13 purported *P. seriatus* populations also revealed that genetic variation was structured (Table 3.4). When the data were grouped by location or by host plant alone, there was not significant population structure. However, host plant groups within a locality or location groups within host plants showed significant population structure. A Mantel test on log transformed geographic distance and genetic differentiation among all the populations showed no correlation (r^2=0.013, P = 0.176) between these two matrices (Fig. 3.5) discarding the presence of isolation by distance.

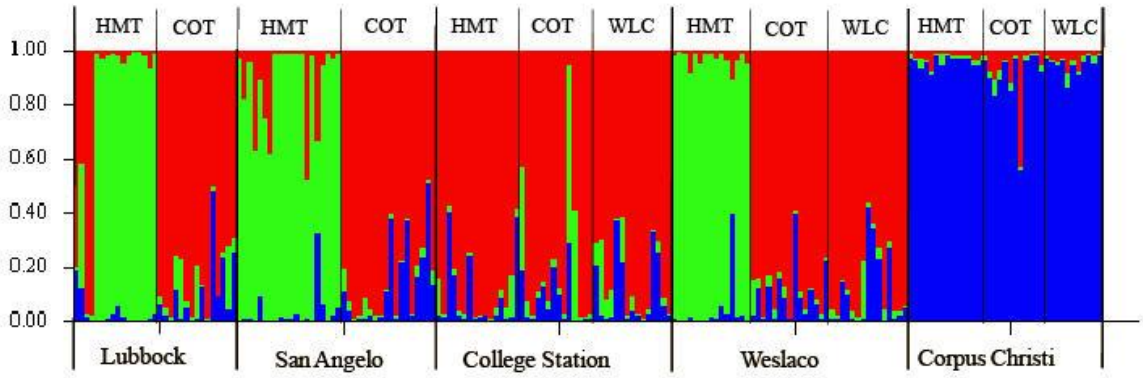

Fig. 3.4 STRUCTURE analysis of *P. seriatus* populations inferred from AFLP markers. Individuals are organized according to the location and within each location, by each host plant (horsemint = HMT; cotton = COT and wooly croton = WLC). Three inferred genetic clusters ($K = 3$) are represented by different colour (green, red and blue). Each individual is represented by a narrow vertical column, which is divided into coloured segments. Segments are in proportion to inferred membership into each of the K inferred genetic clusters.

Table 3.4. Analysis of molecular variance (AMOVA) result for *P. seriatus* populations. Populations were collected from three host plants (horsemint, cotton and woolly croton) in five locations (Lubbock, San Angelo, College Station, Corpus Christi and Weslaco). Variation was partitioned in (a) overall (host plants × location), (b) grouped by location and, (c) grouped by host plant.

	Source	d.f.	SS	Variance	Variation	*F*-statistics
(a)	Overall	12	925.85	3.34	11.07	0.111^{**}
	Individuals within	183	4912.56	26.84	88.93	
(b)	Among locations	4	435.65	1.19	3.92	0.039^{NS}
	Host plants within	8	490.20	2.313	7.62	0.079^{**}
	Individuals within	183	4912.56	26.84	88.45	
(c)	Among host plants	2	225.84	0.68	2.23	0.022^{NS}
	Locations within	10	700.01	2.87	9.44	0.097^{**}
	Individuals within	183	4912.56	26.84	88.33	

P value indicated by ** and ^{NS} represents significant and non-significant *F*-statistics, respectively.

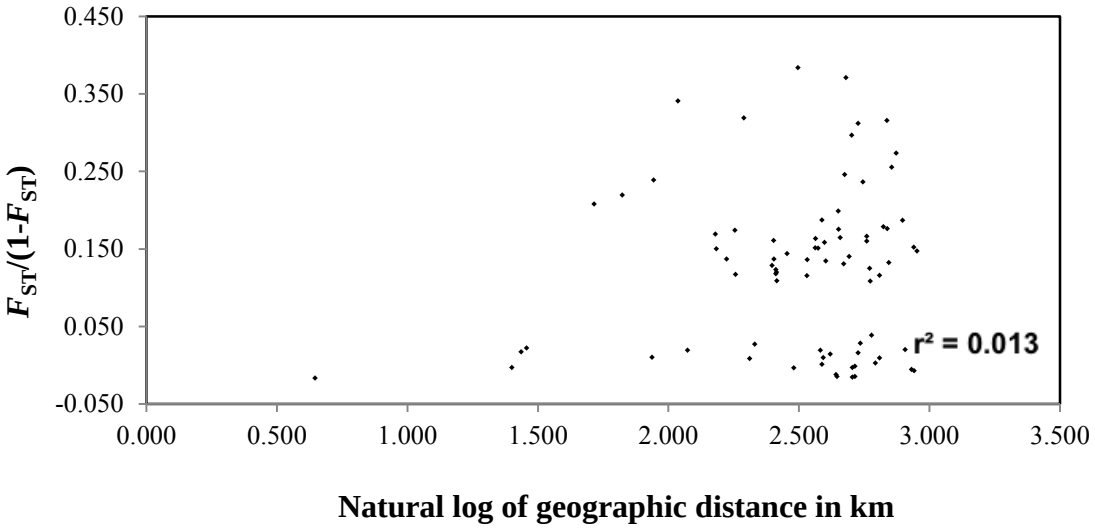

Fig. 3.5 Correlation between geographical distance and genetic distance among the *P. seriatus* populations in the study area ($P = 0.176$).

Discussion

The results of this study show host-associated differentiation (HAD) in *P. seriatus*. Interestingly, HAD is not uniformly distributed throughout the geographic distribution of this pest in Texas. In three out of five study locations (i.e., Lubbock, San Angelo, and Weslaco), *P. seriatus* shows HAD while in two locations (i.e., College Station and Corpus Christi) its populations were not genetically differentiated based on their host plant association. Further, *P. seriatus* populations in Corpus Christi were geographically differentiated from the populations in the rest of the locations we sampled. Our results indicate a geographic pattern of HAD.

Several ecological studies have reported a geographic mosaic pattern of host plant use in phytophagous insects (Thompson and Cunningham 2002, Berenbaum and Zangerl 2006, Conord et al. 2006, Rich et al. 2008, Toju 2009, Brunner and Frey 2010, Craft et al. 2010), which is the basis of the geographic mosaic theory of co-evolution (Thompson 2005a). Our results show what can be called a geographic mosaic of host-associated differentiation. In the case of *P. seriatus*, the observed geographic differences in the pattern of HAD could be due to the heterogeneity of the landscape and associated ecological factors across the geographic distribution of this insect. The five geographic locations in our study represent distinct agro-ecological zones (http://www.tpwd.state.tx.us). We observed a significantly lower abundance of horsemint near Lubbock, San Angelo, and Weslaco than in College Station and Corpus Christi. Lubbock, San Angelo, and Weslaco receive an average annual rainfall of about 52.5 cm. In contrast, College Station and Corpus Christi receive an average annual

precipitation of 87.5 cm (Fig. 3.1), which correlates with a high abundance of horsemint. Areas with abundant horsemint have relatively large populations of *P. seriatus* in neighboring cotton fields. In areas receiving low rainfall, horsemint patches are small and mainly restricted to low-lying areas or ditches where plants receive more moisture (Fletcher 1940). Therefore, *P. seriatus* populations associated with horsemint plants in low rainfall areas are small and relatively isolated from cotton. Small *P. seriatus* populations resulting from low horsemint abundance in Lubbock, San Angelo and Weslaco may lead to stronger genetic drift and could promote HAD in this locations.

The most striking difference among the geographic locations studied was the differential presence of woolly croton. Woolly croton is absent or at low numbers in the three locations at which HAD was found (Fig. 3.1). This plant species is the primary host of *P. seriatus* during the fall and serves as a hibernating substrate for eggs at the end of the cotton season (Almand et al. 1976, Gaylor and Sterling 1977). Thus, woolly croton may act as bridge connecting *P. seriatus* populations from cotton in the fall with horsemint in the spring. Therefore, the absence (e.g., in Lubbock and San Angelo) or low numbers (e.g., in Weslaco) of woolly croton is likely to keep *P. seriatus* populations in cotton and horsemint reproductively isolated, promoting their genetic differentiation. In addition, *P. seriatus* strongly prefers horsemint and woolly croton to cotton (Beerwinkle and Marshall 1999; A. K. Barman, unpublished data) strengthening the isolation of *P. seriatus* populations associated with horsemint and cotton. Host plant preference has been invoked as a mechanism keeping herbivorous insects associated with different host plant species reproductively isolated (Hokkanen 1991, Feder et al.

1994, Cunningham et al. 1999). If strong host plant preference exists, herbivorous insects may also track the phenologies of their host plants adding a temporal component to their reproductive isolation owing to phenological differences among their host plants.

Intimate association of insect herbivores with their host plants enables them to match their biology with their host plant phenologies. Differences in host plant phenology have been found to act as a pre-mating barrier among insect herbivore populations associated with different host plant species and thereby facilitate host-associated differentiation (Feder et al. 1994, Groman and Pellmyr 2000, Berlocher and Feder 2002, Dres and Mallet 2002). For example, a difference of ~25 days in fruiting phenology between apples and hawthorns translates into reproductive isolation and HAD of *Rhagoletis pomonella* (Feder et al. 1994). The three host plant species we studied (i.e., horsemint, cotton and woolly croton) show minimum to no phenological overlap at the locations in which we found HAD (i.e., Lubbock, San Angelo, and Weslaco). *Pseudatomoscelis seriatus* life spans last ~32 days (Gaylor and Sterling 1975). In Lubbock and San Angelo there is a time difference of ~30-35 days in flowering and fruiting phenology between horsemint and cotton which is long enough to reproductively isolate *P. seriatus* populations on these two host-plant species. In contrast, at the locations that did not show HAD (i.e., College Station and Corpus Christi) the three host plant species had overlapping phenologies.

Dispersal ability may play an important role in determining genetic population structure (Roderick 1996, Peterson and Denno 1998). Although the dispersal ability of *P. seriatus* has not been assessed with any mark-recapture study, personal observations

(AKB and CPCS) indicate that *P. seriatus* is a weak flier, which is consistent with reports from other insects in the same family (King 1973, Stewart and Gaylor 1994, Lu et al. 2009). Studies addressing airborne dispersal of *P. seriatus* have shown that very few individuals take high altitudinal flight and that in wind tunnels they tend to remain attached to their host plant at wind speeds as high as 48km per hour (Almand et al. 1976). Several studies have shown that habitat fragmentation may lead to increased population differentiation of insects by restricting gene flow, particularly if the insect has limited dispersal ability (Van Dongen et al. 1998b, Sato et al. 2008). In the locations at which HAD of *P. seriatus* occurs, the distribution of horsemint is fragmented.

Although both College Station and Corpus Christi *P. seriatus* populations lack HAD, the Corpus Christi population is genetically distinct from all the others (Fig. 3.3 and 3.4). The genetic differentiation between the Corpus Christi population and the rest cannot be explained by isolation-by-distance (Fig. 3.5). In addition, there are no apparent geographical barriers between Corpus Christi and remaining study locations. Without any further ecological and behavioural studies, it is difficult to speculate why the Corpus Christi population would be genetically differentiated from the rest. Perhaps a non-Texas propagule of *P. seriatus* may have colonized Corpus Christi, since marine routes connect this area. The overwintering eggs of *P seriatus* can easily be transported along with plant material from other locations. Thus human mediated movement (Kuhnle and Muller 2011) of *P. seriatus* populations cannot be ruled out. However, the high genetic diversity and higher polymorphism of the Corpus Christ population as compared with the others contradicts this hypothesis, suggesting that *P. seriatus* population in this area may be

ancestral to the fleahopper populations in our other study sites (Table 3.2). Future research using different molecular markers will be needed to further examine the above possibilities.

In summary, asynchrony in growth phenology among different host plant species, lower population abundance of horsemint, strong preference for the native host and finally the absence of woolly croton (the preferred hibernating host-plant) may have collectively contributed to the observed pattern of HAD of *P. seriatus* at the geographical scope of this study. In conclusion, our results indicate that *P. seriatus*, although considered a widespread generalist, could be a specialist at certain locations. Our results suggest that geographic variation in vegetation composition may influence population structure of herbivorous insects on different host plant species. Incorporating evolutionary ecology information in pest management practices will not only enrich our understanding of population genetic processes at a local scale but will also aid in the formulation of more efficient and sustained insect management strategies than the ones currently in place.

CHAPTER IV

HOST PREFERENCE OF COTTON FLEAHOPPER, *Pseudatomoscelis seriatus* (Reuter) IS NOT LABILE TO GEOGRAPHIC ORIGIN AND PRIOR EXPERIENCE

Introduction

Host plant selection is a fundamental behavior in herbivorous insects, which allows them to successfully feed, shelter, mate and oviposit (Bernays and Chapman 1994, Schoonhoven et al. 2005, Bruce and Pickett 2011). The process of host plant selection is broadly sub-divided into three major steps that are not always clearly delineated: searching (orientation), recognition and selection (landing and probing), and finally acceptance (feeding and oviposition) (Visser 1986, Schoonhoven et al. 2005). The orientation behavior is mainly dependent on olfactory and/or visual cues from the herbivore's host plants (Patt and Setamou 2007, Wenninger et al. 2009). Herbivorous insects are able to track specific blends of volatile chemicals (Bruce et al. 2005, Schoonhoven et al. 2005). This ability allows them to find their host plants even when they occur within diversified, complex habitats where their host plants are surrounded by non-host plant species (Bruce and Pickett 2011). Therefore, assessing the olfactory orientation of herbivorous insects is the first step towards screening their host-plant preference.

Herbivore's host-preference may be subject to geographic variation (Newby and Etges 1998, Kawecki and Mery 2003, Verdon et al. 2007, Utsumi et al. 2009). For

example, *Helicoverpa armigera* (Firempong and Zalucki 1990), *Malacosoma disstria* (Parry and Goyer 2004), *Drosophila mojavensis* (Newby and Etges 1998), *Uroleucon ambrosiae* (Funk and Bernays 2001) and *Leptopilina clavipes* (Pannebakker et al. 2008) show geographic variation in their host preference. Geographic variation in host preference in insect herbivores seems to respond to variation in the distribution and abundance of their host plant species (Kuussaari et al. 2000). Therefore, herbivore populations may specialize and prefer locally abundant host plant species (Fox and Morrow 1981, Sword and Dopman 1999). These geographic differences in host plant preference may be due to genetic variation among geographic populations (Jaenike 1990, Via 1990, Singer and Parmesan 1993) and/or due to geographic variation in the composition of host-plant species (Bernays and Funk 1999, Funk and Bernays 2001, Davis and Stamps 2004, Troncoso et al. 2005). Thus, herbivore insect's host preference cannot be generalized from observations based on just a few and/or focalized geographic locations and it needs to be investigated at multiple geographic locations across the entire distribution of the insect.

Host preference in polyphagous herbivorous insects may vary not only due to geographic differences in host plant composition and/or due to genetic variation among insect populations, but also due to insect's behavioral modification as a result of prior experience. Prior experience on a particular host plant may result into induced preference of an insect for a host plant they have experienced over another host plant they have never encountered (Jermy et al. 1968, Dethier 1982). Induced preference for experienced host plants has been shown in several lepidopteran larvae (Jermy et al.

1968, Zhang et al. 2007) and in coleopterans (Messina et al. 2009, Coyle et al. 2011). Polyphagous insects exhibiting induced preference and distributed across wide geographic areas are likely to show greater variation in host preference than insect with localized distributions, due to heterogeneity in host plant composition. However, variation in host preference is not ubiquitously present in insects. There are insect species in which host preference is unchanged (conserved) regardless of geographic differences in host composition and prior host exposure (Wehling and Thompson 1997, Davis and Stamps 2004, do Valle et al. 2011, Wellenreuther et al. 2011). Thus, host selection behavior in phytophagous insects could range from highly variable to strictly conserved.

Several insect pest management (IPM) strategies such as the use of trap crops, resistant varieties and botanicals (attractants and deterrents) are based on pest's host preference behaviors. Thus, investigation into insect's host preference will increase successes of these IPM strategies. For example, if an insect species is subjected to variation in host preference behavior due to induced preference, a trap crop would not be as useful to attract insect populations as it would be to attract pests with fixed host preferences (Hokkanen 1991, Thaler et al. 2008, Guillemaud et al. 2011, Midega et al. 2011).

The cotton fleahopper, *Pseudatomoscelis seriatus* (Hemiptera: Miridae) is an insect pest of cotton, which has a wide distribution in the United States (Henry 1991), with more than 160 host plant species belonging to 35 different families (Esquivel and Esquivel 2009). However, the host plant complex of this pest varies geographically. In

this study, we have selected two locations in Texas, USA: Lubbock and College Station (Fig. 4.1). These two locations are 620 km apart and belong to different eco-regions. These two locations differ considerably in average annual rainfall and both availability and abundance of host plants for *P. seriatus.* Horsemint (*Monarda punctata*) is very limited in Lubbock, while highly abundant in College Station. Besides, the fleahopper's primary overwintering host plant, woolly croton (*Croton capitauts*), is absent in Lubbock. Cotton is a cultivated host plant of this insect, common to both locations, but more concentrated in Lubbock compared to College Station.

The present study explored whether *P. seriatus* shows variation in host preference. We specifically asked: 1) does host plant preference of *P. seriatus* vary geographically; and 2) do prior experience result in induced preference to host plants in *P. seriatus*?

Materials and methods

Insect

The cotton fleahopper is a small (3-4 mm long) sucking insect. It completes its lifecycle (from egg to adult mortality) in 32-40 days. Adults are weak fliers and live up to 10-15 days. In this study we used adult insects of either sex. Insects were collected either using a sweep net or with an aspirator directly from the host plant. Host preference bioassays were conducted within 2-3 hours after insect collection to avoid mortality. Only active, healthy adults were used in bioassays.

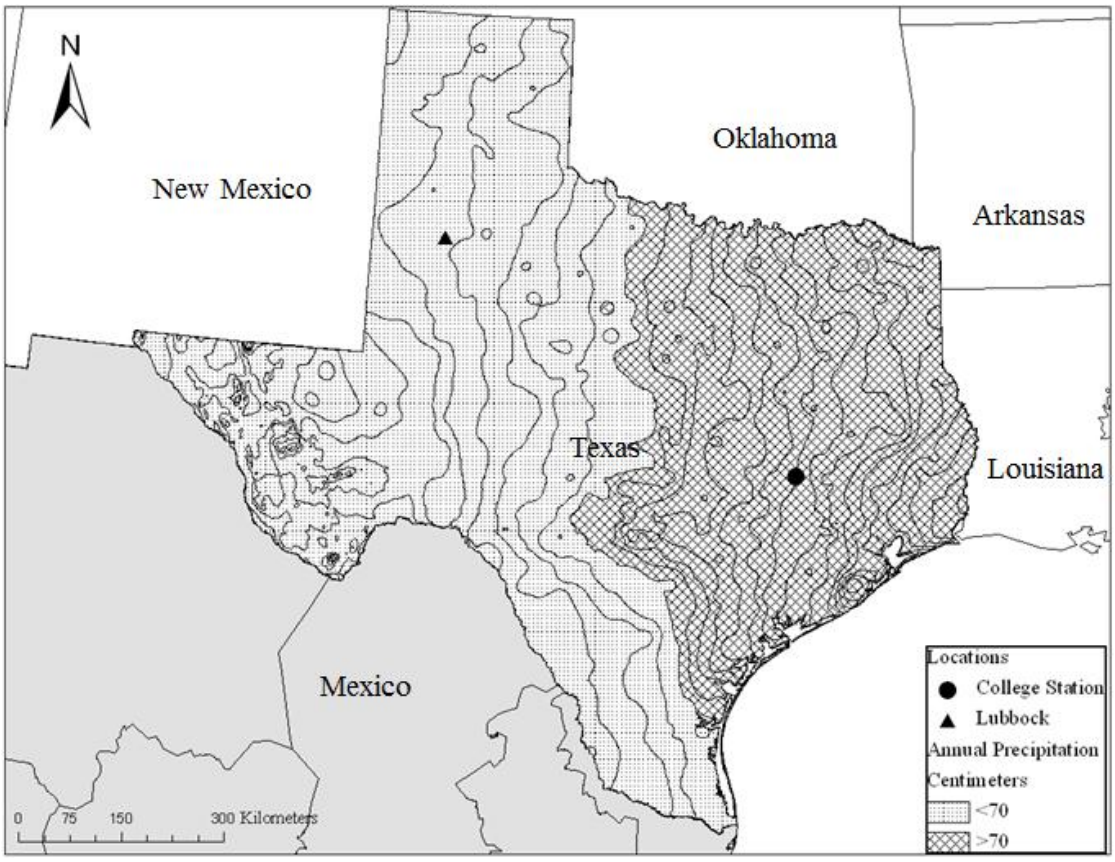

Fig. 4.1. Map indicating annual rainfall and two geographic locations for host preference study.

Host plant

We selected two host plant species, horsemint (*Monarda punctata*) and cotton (*Gossypium hirsutum*), for evaluating olfactory preference of cotton fleahopper. Horsemint is a native wild host plant species of cotton fleahopper in the study area. Although horsemint is a perennial species, fresh aboveground biomass can only be seen in our study areas from early April to late June. Horsemint plants are 0.3 to 1.5 m tall depending on the habitat. Each stem bears an apical dense whorl of flowers. We used a bouquet of 3-4 stems, wrapped with cotton wool at the bottom and soaked in water in a glass beaker as one of the odor sources for olfactometer bioassays. Cotton is a cultivated crop in the study areas and pre-flowering (squaring) stage of the plant is susceptible to cotton fleahopper attack (Reinhard 1926, Ring et al. 1993). We used 3-4 cotton branches, bearing flower buds as another odor source in olfactometer bioassays. Approximately equal amount of plant material from both plant species were put within the bioassay arena. The control treatment consisted of an empty glass beaker of the same size and with same amount of water used in the other treatments.

Olfactometer setup

A glass Y-tube olfactometer was used (12 cm common tube, 5 cm arms, and 1.3 cm internal diameter; Analytical Research System, Gainesville, FL) to conduct the bioassays. The odor sources and/or control treatment were placed in a glass chamber (15 cm internal diameter, 32 cm tall), with two openings. The glass chamber was airtight to prevent any exchange of volatiles. One opening of the glass chamber was connected to

the air-delivery system (Analytical Research System, Gainesville, FL), which allowed air flow through the glass chamber. The other opening of the glass chamber was connected to an arm of the Y-tube. Thus the two arms of the Y-tube olfactometer received odors coming out of the two glass chambers. Charcoal-filtered air was flowing through the two arms of the Y-tube at constant rate (l liter/minute, 18.5 psi) throughout the experiment.

Bioassay

Air was released for 10-15 min prior to the bioassay so that plant odors travel through the arms of the Y tube. A paper straw (equal in length to the longer arm of the Y-tube) was placed on the floor of the long arm of the Y-tube to facilitate movement of cotton fleahoppers to the point of rendezvous (where long and two short arms meet). In earlier trials without the paper straw, we found that fleahopper adults were incapable of walking on the glass surface of the Y-tube and could not make a choice. Adult cotton fleahoppers were placed individually on the rear tip of the paper straw inside the long arm of the Y-tube. Cotton fleahopper individuals took from 30 sec to 5 min to travel between the points of release and choice. Fleahoppers took more than 7 min to arrive to the rendezvous point were considered as non-respondents and were not included in the analysis. We allowed 3 additional minutes of response time for fleahoppers to make a choice (i.e., landing into either one of the two arms of Y-tube). When an individual stayed for more than 1 min in any of the arms, we consider it a ‘choice’. Orientation behavior was observed on each individual fleahopper adult only once. About 30-50

individuals were tested for each set of experiments. For every experiment, the orientation (left to right) of the Y-tube was changed by flipping it after testing a series of 5 consecutive individuals, in order to avoid any orientation biased behaviors. Prior to each bioassay, we also tested if fleahoppers show any particular orientation to either of the two arms in the absence of odor sources, and found no significant deviation from the expected ratio (0.05). After each experiment, the Y-tube, glass chambers and connecting tubes were washed with ethanol and subsequently with water, and air-dried to remove any residual odors from previous assays. Two sets of bioassays were conducted during 2009 and 2010.

Effect of geography

In this study we evaluated preference of cotton fleahopper to horsemint and cotton in two geographic locations i.e., Lubbock and College Station. At each location, cotton fleahopper adults were collected from horsemint during May (College Station) and June (Lubbock). Insect collections were made from relatively pure patches (ranging from 0.3 to 30 m^2) of horsemint at both locations. Since we observed both nymph and adults in the plants, we assumed that the collected individuals were completing their life cycle in horsemint. To test if host-preference varies at each location, three pairs of treatments were offered to the collected adult fleahoppers: 1) horsemint vs. air, 2) cotton vs. air, and 3) horsemint vs. cotton.

Effect of prior experience

This study was done during the summer of 2010 in College Station, where both cotton and horsemint are abundant. In College Station, horsemint grows naturally during April-May and cotton is planted during mid-April. Cotton fleahoppers (nymphs and adults) were collected from horsemint patches close to cotton fields. We called this the 'source population' because we used individuals from this population and reared them on cotton and horsemint in confinement. We tested the host-preference of the source population as described in the previous experiment (See Effect of Geography section above). Two similar source populations were collected from the same location and released fleahopper nymphs and adults into two field cages where cotton or horsemint plants were growing free of fleahoppers. We allowed about 50 days to pass to ensure that any fleahopper introduced in the cage as an adult was dead and that the offspring of these adults completed at least one generation on their respective host inside the cage. After 50 days, newly emerged adult fleahoppers were collected using an aspirator from their respective host plant inside the cage. Thus, adult cotton fleahoppers use in these bioassays experienced either cotton or horsemint during the entire duration of their immature stages before conducting the experiments. We refer this fleahoppers as "host-associated populations" (i.e. horsemint-associated population or cotton-associated population). An average of 50 adults from each host-associated population were tested in the Y-tube olfactometer using the same treatment combinations used for testing for geographic differences (see above). This design allowed us to test if host-preference of cotton fleahopper was influenced by prior host plant experience.

Statistical analysis

Host-preference data were analyzed as percent response using chi-square analysis ($\alpha = 0.05$) under the null hypothesis that cotton fleahopper oriented with an expected probability of 0.5 for each treatment (Proc FREQ, SAS 2006).

Results

Test for effect of geography

Cotton fleahoppers from both Lubbock and College Station preferred horsemint to cotton. In Lubbock (Fig. 4.2A) 70 per cent of tested individuals preferred horsemint to cotton ($\chi^2 = 16.0$; df = 1; $P < 0.001$). Similarly, in College Station (Fig. 4.2B) 68 per cent of tested individuals preferred horsemint to cotton ($\chi^2 = 12.96$; df =1; $P = 0.0003$). A significantly larger proportion of individuals preferred horsemint over the control (air) in both Lubbock and College Station. In contrast, preference of cotton fleahopper to cotton was non-significant when tested against the control.

Test for effect of prior experience

Source population: 80 per cent of the individuals from the source population (Fig. 4.3A) preferred horsemint to cotton ($\chi^2 = 30.5$; df = 1; $P < 0.0001$). When individuals were allowed to choose between horsemint and the control, significant preference for horsemint was observed while there was no preference to cotton when tested against the control.

Horsemint-associated population: Individuals of this population spent their all life stages on horsemint. 84 per cent of the individuals from the horsemint-associated

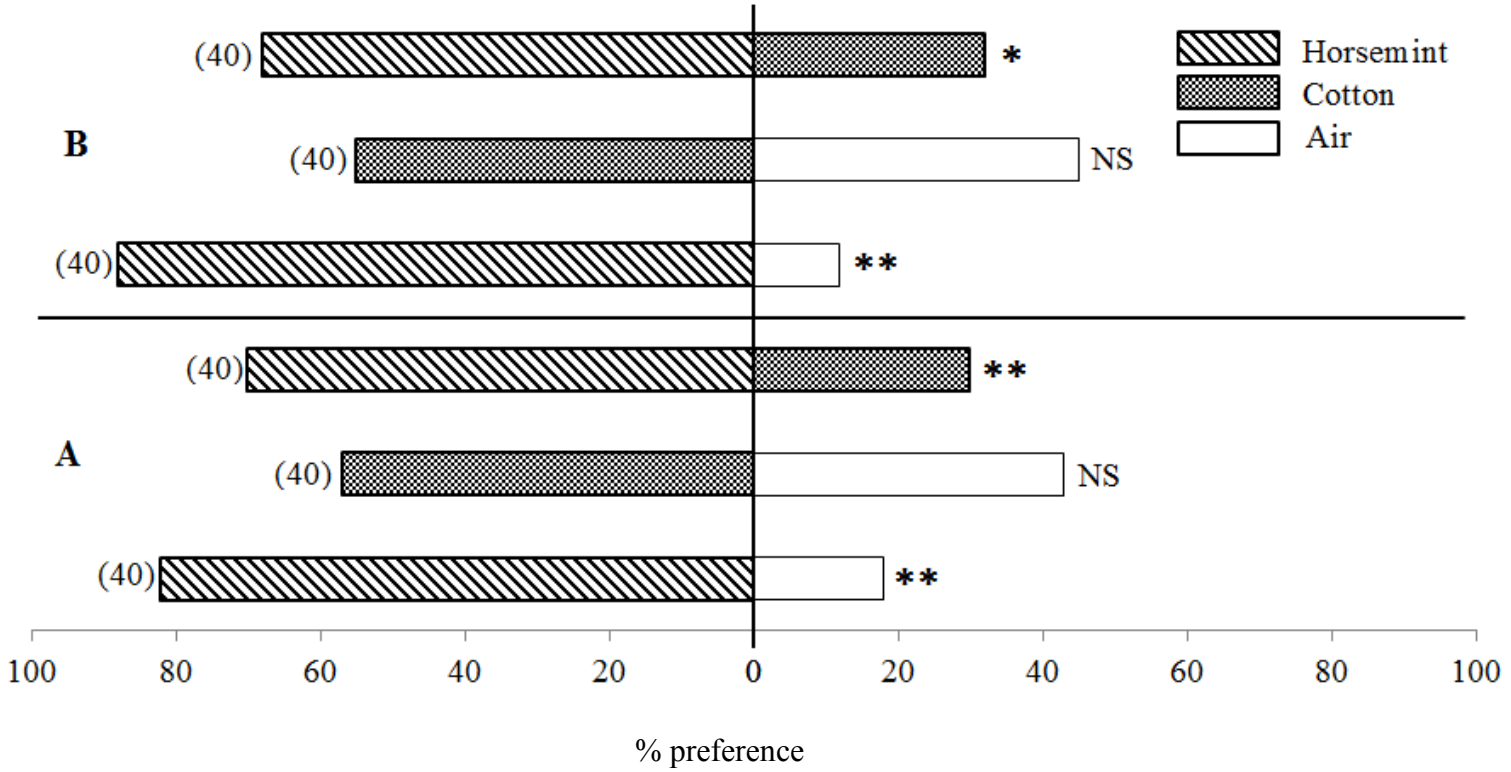

Fig. 4.2**.** Olfactory preference of two geographic populations of cotton fleahopper to cotton and horsemint. **A.** Lubbock population. **B.** College Station population. Statistically significant χ^2 test is indicated as either *=P<0.05 or **=P<0.0001. Number within parentheses on the left side of each test indicates number of individuals tested.

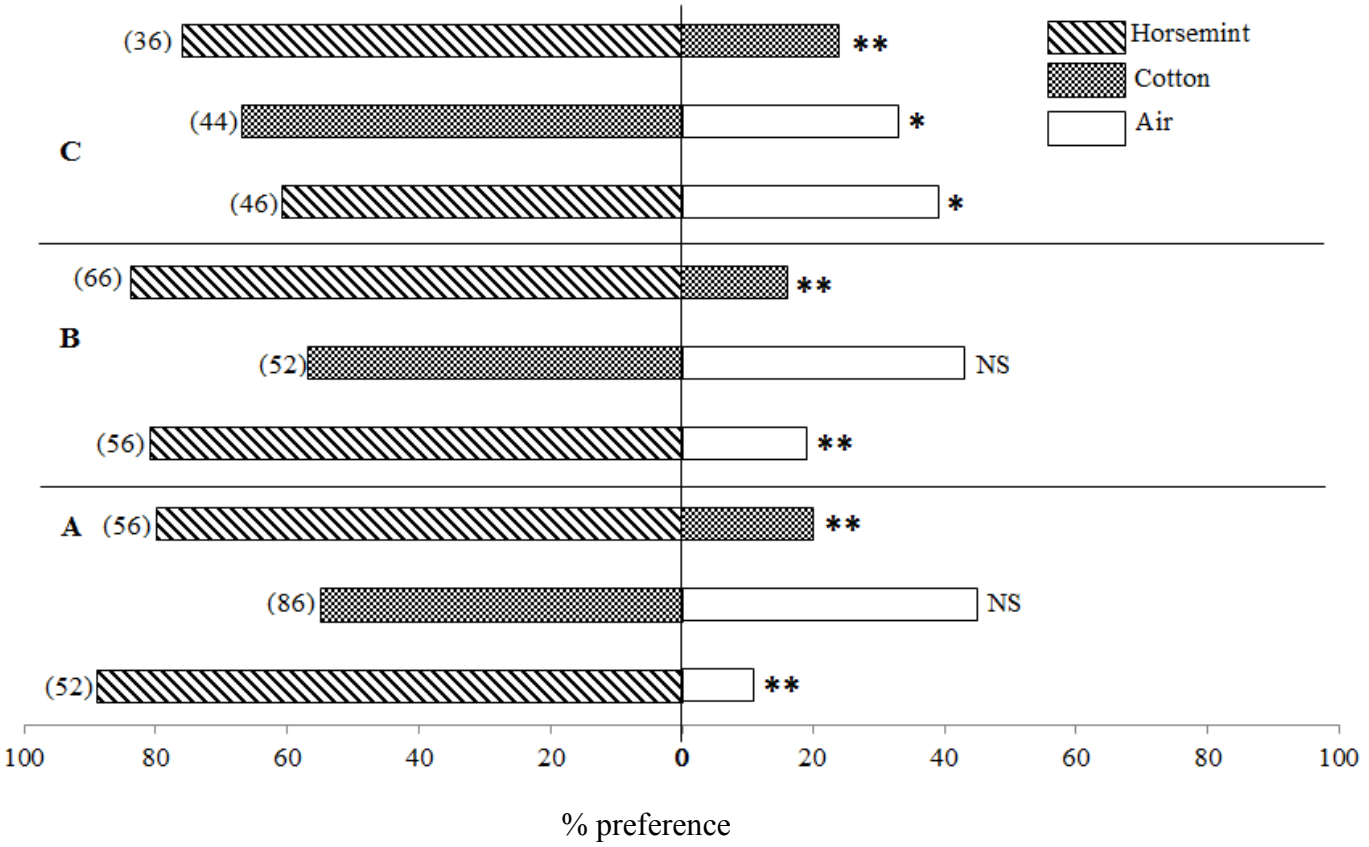

Fig. 4.3. Olfactory preference of cotton fleahopper population with and without prior experience to cotton. **A.** Source population **B.** horsemint-associated population and **C.** cotton-associated population. Statistically significant χ^2 test is indicated as either *P<0.05 or **P<0.0001. Number within parentheses on the left side of each test indicates number of individuals tested.

populations (Fig. 4.3 B) preferred horsemint to cotton ($\chi^2 = 22.2$; df = 1; $P < 0.0001$). When individuals were allowed to choose between horsemint and the control, significant preference for horsemint was observed while there was no preference to cotton when tested against the control.

Cotton-associated populations: This population consists of individuals, which completed their all life stages on cotton. 76 per cent of the individuals from the cotton-associated populations (Fig. 4.3 C) preferred horsemint to cotton ($\chi^2 = 28.4$; df = 1; $P < 0.0001$). When individuals were allowed to choose between horsemint and the control, significant preference for horsemint was observed. There were a statistically significant proportion of individuals that preferred cotton over air.

Discussion

Our results indicate that the cotton fleahopper prefers horsemint to cotton regardless of its geographic origin and prior experience. In 5 independent experiments, an average of 76 per cent of tested individuals preferred horsemint to cotton. The previous study by Beerwinkle and Marshall (1999) also found that cotton fleahopper strongly prefers horsemint over cotton and suggested its potential use as a trap crop. Unlike our study, Beerwinkle and Marshall, and others (Van Tol et al. 2002, Midega et al. 2011), focus their studies on insects collected from a single geographic location and recommended pest management options based on their host-preference results. Since, host-preference may vary among geographic populations of herbivore insect pests,

development of trap crop strategies should consider testing multiple geographic populations.

The influence of locally abundant host plants in insect host preference has been documented in several instances where insect populations are adapted to the locally most abundant host plant and prefer it over other potential hosts (Harrison 1987, Newby and Etges 1998, Funk and Bernays 2001, Kawecki and Mery 2003, Gotthard et al. 2004, Ferrari et al. 2006, Logarzo et al. 2011). Since the cotton fleahopper is a widely distributed, highly polyphagous insect and the abundance of its host-plant species varies geographically, this insect herbivore is bound to use different host-plant species at different geographic locations. If preference for a particular host is not a fixed trait then we expect to find differences in host preference among geographic populations of cotton fleahopper. However, contrary to this expectation, our results indicate that there was no difference in host-preference between geographic populations (Fig. 4.2A and B). The two locations we picked (i.e., Lubbock and College Station) belong to two distinct eco-regions and both differ in terms of annual rainfall (Fig. 4.1) and abundance of cotton and horsemint. Horsemint is limited in Lubbock, whereas abundant in College Station. Similarly, the area under cotton cultivation in Lubbock is larger than in College Station (USDA-NASS 2010). In spite of the differences between the two locations we studied, cotton fleahopper preferred horsemint to cotton in both Lubbock and College Station.

Our study also shows that preference of cotton fleahopper to horsemint is unaffected by insect's prior experience with cotton. Although both adult and nymphs spent their life on cotton, cotton fleahoppers exhibited preference to horsemint over

cotton when given a choice (Fig. 4.3 C). This indicates that host preference in cotton fleahopper is not inducible by learning; rather it seems to be a conserved behavior in this insect species. Similar results have been documented in several studies where host preference of polyphagous or oligophagous herbivorous insects does not change due to prior host experience (Janz et al. 2009, Kuhnle and Muller 2011, Midega et al. 2011). Thus, cotton fleahopper's host preference could be an evolutionarily conserved feature which is not affected by geographic differences in available host plants (Wehling and Thompson 1997) and prior experience (Kawecki and Mery 2003). This fixed host preference could be due to the evolutionary history of the cotton fleahopper and horsemint. The consistent preference for horsemint in cotton fleahopper could be the result of a longer association of this pest with horsemint than with cotton. The cotton fleahopper and horsemint, both are native to the southern United States (Henry 1991, Turner 1994, Knutson et al. 2002). In contrast, large-scale cultivation of introduced cotton in the southern United States dates back only to late 1600 (Lewis and Richmond 1966). Thus, the cotton fleahopper has been interacting with horsemint for longer time than with cotton. This long association with horsemint might have resulted in higher fitness of this insect in horsemint than in cotton. For example, cotton fleahopper took shorter to complete its life cycle and laid significantly more eggs in horsemint than in cotton (Gaylor and Sterling 1976, Holtzer and Sterling 1980a). Thus, it is not surprising that the cotton fleahopper present fixed preference for horsemint as found by our study. This as well as previous studies have shown that native host plants are preferred over cotton by the cotton fleahopper (Holtzer and Sterling 1980a, Beerwinkle and Marshall

1999). One could ask then why the fleahopper is found in cotton. The relatively high abundance of cotton compared to its native wild hosts may explain in part the presence of this insect in cotton. Although cotton may not be the host on which this insect has the highest fitness, its abundance may compensate for its relatively low resource quality. There are several examples in which herbivore insects choose plants on which their fitness is relatively low, but on which they get ecological advantages such as protection from natural enemies (Gratton and Welter 1999, Singer et al. 2004, Diamond and Kingsolver 2010), and/or a predictable food resource (González-Megías and Gómez 2001). Thus, host preference might not be strictly required for an herbivore to establish its association with a widely cultivated crop. In our study system, cotton is a sub-optimal host plant for the cotton fleahopper (Gaylor and Sterling 1976). However, its relatively high abundance makes it a predictable resource. In this scenario, the evolution of host preference for cotton might be unnecessary for the fleahopper due to this crop's extensive cultivation. In contrast, cotton fleahopper preference for wild hosts will be maintained due to its (insects) optimal fitness on these host-plants and their (wild host plants) limited abundance.

Our data suggest that wild hosts of the cotton fleahopper could be utilized as a pest management tool. For example, horsemint could be used as a trap crop, or to develop kairomone baits etc. This kind of cultural management options hold promises in the current cotton pest management scenario in Texas, where insecticide applications are unwarranted due to the introduction of transgenic Bt-cotton and successful boll weevil eradication program. Unlike the introduced polyphagous *Helicoverpa armigera*, which

may not be feasible to control through trap crops due to their induced host preferences (Cunningham et al. 1998, Cunningham et al. 1999), the conserved host preference of the cotton fleahopper suggests that trap crops could be a management option for this native pest.

CHAPTER V

MORPHOLOGICAL VARIATION IN COTTON FLEAHOPPER POPULATIONS: GEOMETRIC MORPHOMETRIC APPROACHES TO QUANTIFY THE INTERACTIVE EFFECT OF HOST PLANT AND GEOGRAPHY

Introduction

Phytophagous insects have been a subject of interest in studying host plant mediated morphological divergence (Carroll and Boyd 1992, Adams and Funk 1997, Diegisser et al. 2007, Nosil 2007). Since host plants present several layers of defense to protect themselves from herbivorous insects, insects need to overcome these barriers in order to utilize plants as a food resource. One of the defensive barriers phytophagous insects encounter is the host plant's surface morphology (Bernays et al. 1991). Host plant's surface morphology can be characterized by toughness and hairiness. Especially, hairiness of leaves or stems has shown to be an important morphological character which poses problems for an insect's attachment, movement and access to food (Bernays et al. 1991, Medeiros and Moreira 2002). Therefore, insects need to develop adaptations to some morphological structures of plants in order to increase their survival and fitness. Studies of insect species within the order Hemiptera (specifically in Aphididae) have shown morphological changes in response to plant trichomes (Moran 1986, Gorur 2003). Similarly, insect's tarsi may be selected for better attachment and locomotion on specific plant surfaces. For example, shorter hind tarsi in *Uroleucon* aphids is related to plant

surfaces with long trichomes (Moran 1986). These examples indicate that host plants can be the source of strong natural selection and induce morphological adaptations in insect populations.

Several phytophagous insects are widely distributed, likely following the geographic distribution of their host plants or due to passive dispersal through wind, water, etc. Currently, human transportation has also contributed in the expansion of some phytophagous insects' distributions. Thus, the same insect species can be found in geographically distant locations. Geographically distant or isolated insect populations may go through separate evolutionary trajectories depending on the differential strength of local micro-evolutionary forces such as genetic drift, natural selection and gene flow among populations. It is well documented that insect populations respond to spatial variation such as differences in latitude and longitude (Scharf et al. 2009), and evolve into morphologically distinct ecotypes (Sota et al. 2007, Seabra et al. 2009, Tantowijoyo and Hoffmann 2011). In geographically isolated insect populations, gene flow may be limited which can further lead to their genetic differentiation. Thus geographically isolated populations are likely to have unique evolutionary histories.

The combined effects of host plant selection and local adaptation may influence morphological traits of geographically isolated populations of phytophagous insects in different ways. An approach to tease apart the effects of local adaptation (i.e., geography) and host-plant mediated natural selection will be to consider replicated pairs of divergent host pairs in the multiple geographic locations. Documenting similar patterns of morphological divergence in populations across the same selection gradient

(i.e., different host plant species) at different geographic locations will be evidence of strong host-mediated natural selection (Schluter 2000).

Simultaneously quantifying both the effect exerted by host-plant selection and by geographic variation on the morphology of phytophagous insects can be achieved by using a generalized model of divergent selection (GMDS). This model is able to simultaneously quantify the effect of both host-plant selection and local adaptation on the observed pattern of evolutionary diversification (Langerhans and DeWitt 2004, Langerhans 2010). According to this model, one will be able to measure an organism's characters (morphological, behavioral, etc.) relevant to interacting selection gradients and to investigate shared and unique responses of organisms across different selection regimes by performing a multivariate analysis of variance (MANOVA). The broad applicability of GMDS lies on the fact that it can be used for various characters (e.g., morphological, behavioral etc.) across broad taxonomic units, which experience the same set of selection gradients (e.g., selection by different host-plant species) at multiple locations.

This study focuses on a phytophagous insect, *Pseudatomoscelis seriatus* (Hemiptera: Miridae) and two of its primary host plant species, horsemint (*Monarda punctata*) and cotton (*Gossypium hirsutum*) in two distinct eco-regions of Texas (i.e., Lubbock and College Station). The objectives of this study were 1) to evaluate if host plants exert differential natural selection on morphological traits of *P. seriatus*, and 2) to quantify the contribution of host plant species and geography in the evolution of

morphological traits in *P. seriatus*. I used traditional and geometric morphometric data to address these questions.

Materials and methods

Insect and host plants

Pseudatomoscelis seriatus, known as the cotton fleahopper, is a generalist forager reported to feed on more than 160 host plant species from 35 families (Esquivel and Esquivel 2009). Through its sucking mouthparts, *P. seriatus* mostly feeds on plant reproductive structures and occasionally on tender stems of plant terminals. It is native to the southern United States and northern Mexico and its current range extends from Baja California in the west to North Carolina in the east. *P. seriatus* completes one generation (egg to adult) within 32-40 days depending on the prevailing temperatures and feeding host. There are five immature stages (nymphs) and nymphs are restricted to using the same plant where they eclose. Thus, *P. seriatus* intimately interact with the same host plants for most of their lives, though adults are winged and can disperse across potential hosts. Available host species vary in abundance in time and space and these plant species vary chemically, nutritionally, and morphologically.

Since all recorded host plants of *P. seriatus* do not occur at any given location, the insect is found in abundance only on 3-4 dominant host plants at any given location. In my two study locations, College Station and Lubbock, I selected populations of *P. seriatus* feeding on cotton (*Gossypium hirsutum*, Malvaceae) and horsemint (*Monarda punctata*, Lamiaceae). Among these two host plants, horsemint is a native wild plant,

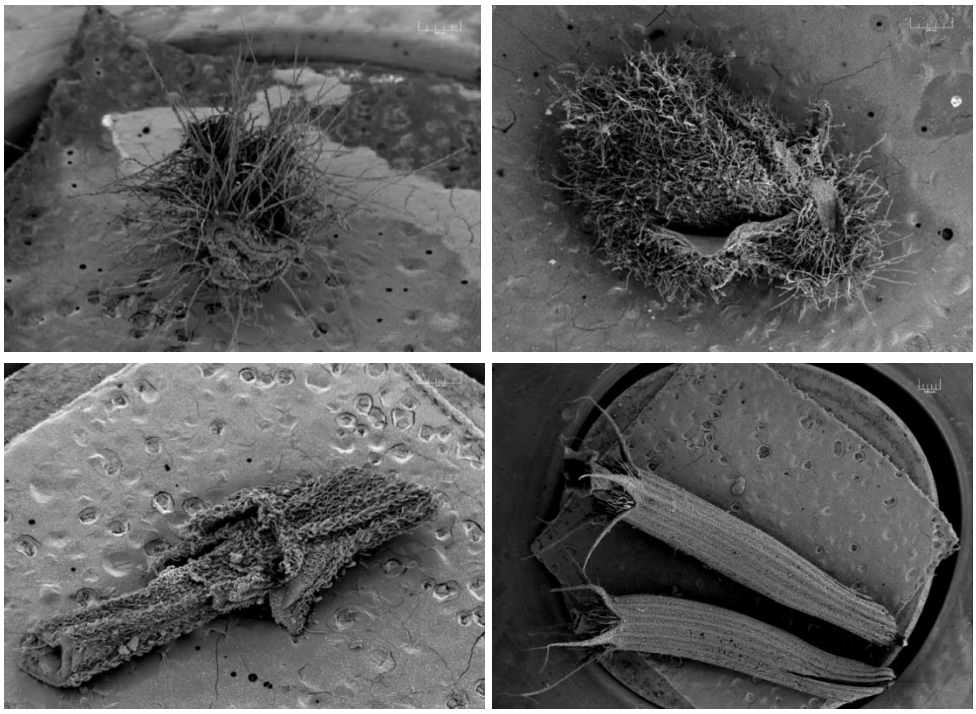

Fig. 5.1. Scanning electron microscopy (SEM) picture showing surface morphology of plant-parts utilized by *P. seriatus* in the two selected host plants. Cotton stem (upper left), cotton flower bud or square (upper right), horsemint stem (lower left), and horsemint florets (lower right).

and support a higher number of *P. seriatus* individuals than cotton, which is a cultivated crop. These two host plants potentially offer different environments to *P. seriatus* in terms of defensive chemical and surface morphology (Fig. 5.1). While horsemint is a native plant in the study areas, cotton was introduced to the east coast during the early 1600's as a commercially cultivated crop and later on expanded to the study areas (Lewis and Richmond 1966). Additionally, cotton and horsemint are good candidates to study diversification because these hosts are known to influence survival, fecundity and development of *P. seriatus* (Gaylor and Sterling 1976), and *P. seriatus* morphometry (head capsule width) is known from previous work to vary among individuals raised on cotton and horsemint (Suh 2007).

Study sites

The two locations we studied, Lubbock and College Station, are 621km apart and belong to distinct eco-regions in which cotton cultivation follows different patterns within the state of Texas, USA. College Station (N 30.5391; W 96.4491; elevation 80 m) receives an annual average rainfall of 100 cm, while Lubbock (N 33.6946; W 101.7351, elevation 985 meter) is a comparatively arid area, receiving 48 cm of rainfall annually (Fig. 5.2). The two host plant species, horsemint and cotton are spatially and temporally more apart in Lubbock compared to College Station, where phenologies of the plants and geographic distributions overlap significantly.

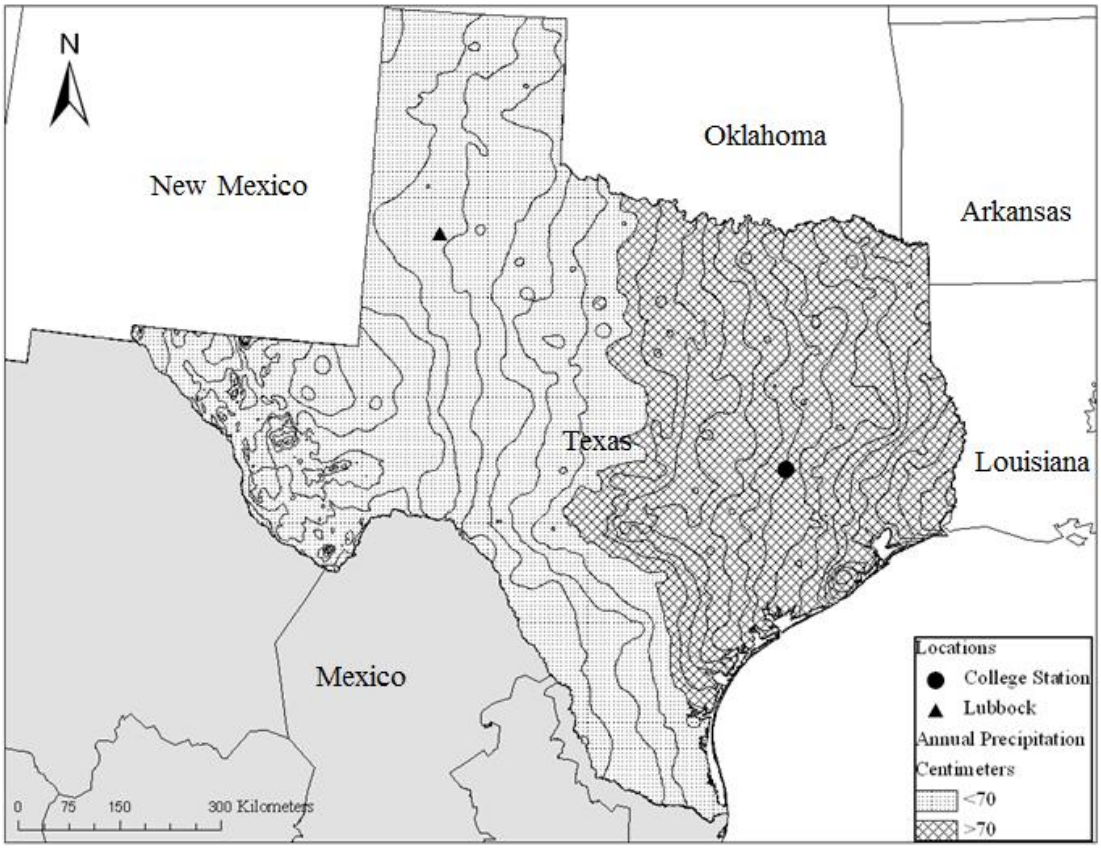

Fig. 5.2. The two study locations, Lubbock and College Station, representative of areas with low rainfall and areas with high rainfall, respectively.

Sampling and data acquisition

Insect samples were collected by sweep-net during the summer of 2010. From each host plant at each location, 30-40 individual *P. seriatus* adults were preserved in 80% ethanol until sample preparation. Only females were used for subsequent measurements, because sexual dimorphism exists in *P. seriatus.* Females tend to be bigger than male (AKB, unpublished data). I also focused on females because of their role in host plant selection for oviposition. Thus, females are ultimately responsible for the success of their offspring. To study morphological variation of *P. seriatus*, I selected several body appendages such as antenna, legs, and mouthparts (rostrum), because these appendages directly interact with host plant surfaces and are also involve in the utilization of host plants as a food source. Insects were dissected under a stereoscope to separate body appendages. Each of the dissected body appendages were temporarily slide-mounted using glycerol as a mounting medium, affixed to a cover slip, and pressed flat onto a slide to reduce measurement error due to orientation and depth of focus. Slide-mounted body appendages were photographed using an AxioCam® MRc attached to an EMS-2 stereomicroscope illuminated by CL1500 ECO lamp (Carl Zeiss, Inc., Thornwood, New York) at 50× magnification, resulting in a resolution of 488.5 pixels·mm^{-1}.

Morphometric methods

Traditional measurement: We selected 13 body parameters (individual lengths of the 1st through 4th antennal segments, summed length of 1st through 3rd rostral segments,

Table 5.1. Cotton fleahopper morphological traits considered for traditional morphometric analysis

Morphological traits	Abbreviated code
Length of 1st antennal segment	A1
Length of 2nd antennal segment	A2
Length of 3rd antennal segment	A3
Length of 4th antennal segment	A4
Total length of first 3 rostral segments	R123
Length of last rostral segment	R4
Length of fore femur	FL1
Length of fore tibia	FL2
Length of fore tarsus	FL3
Length of hind femur	HL1
Length of hind tibia	HL2
Length of hind tarsus	HL3
Width of hind femur	HL1W

length of the last segment of rostrum, length of fore femur, fore tibia, fore tarsus, length of hind femur, hind tibia, hind tarsus, and hind femur width) to be used in a traditional morphometric analysis (Table 5.1). The length and width of these body parameters were measured by digitizing points on captured images using the software tpsDIG, version 2.1 (Rohlf 2006) . Distances were calculated from point coordinates in Excel (online accessory information File 1). All linear distances measured were log transformed (Lande and Arnold 1983). Size was calculated as the geometric mean of the log data in Excel.

Geometric Shape measurement: Shape data were generated from forewings (Fig. 5.3 A and B). Shape information generated from insect forewings has been used extensively to detect variation among populations associated with host plants (Kuussaari et al. 2000, Bruce et al. 2005, Patt and Setamou 2007, Bruce and Pickett 2011). Either one of the two forewings was slide-mounted and fixed with cover slip. A picture of the dorsal side of the forewing was taken under 25× magnification as described above.

Statistical analysis

Traditional (linear distances) and geometric (landmark coordinates) data were analyzed separately, as is classically done, because each data set has an alternative, internally consistent, scaling of their covariance space (Rohlf and Corti 2000). However, multiple morphometric data sets on the same specimens are not likely to be independent. I therefore additionally explored a method to fuse data from differently scaled spaces, described below, to see if more statistical power (and analytical clarity)

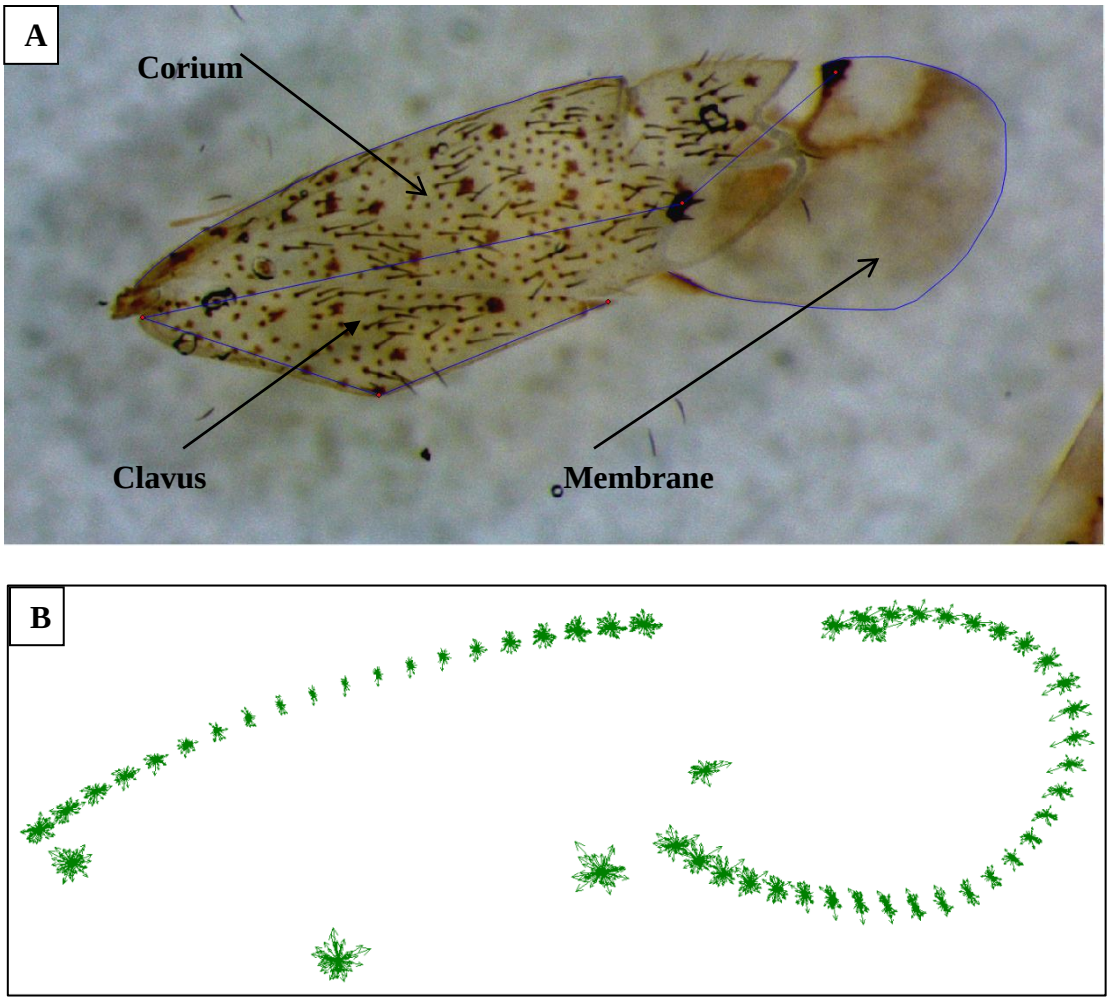

Fig. 5.3. Positions of landmarks on the forewing of the cotton fleahopper. A. Original picture with landmarks and outlines. B. Landmarks of 55 coordinates used to describe wing shape (tpsRELW analysis of 117 individuals).

could be achieved, and to see if interesting forms of trait integration were evident between body and wing morphology.

Traditional data were log transformed and the geometric mean of the log-data was calculated to serve as a measure of body size. Log-data were converted to shape data with a standard orthogonalization method (Burnaby 1966), which is equivalent to regressing size on log distances and saving residuals. A multivariate analysis of covariance (MANCOVA) was performed to estimate the nature and magnitude of shared and unique aspects of diversification across hosts (host main effect, host×region interaction), the general effect of region, and allometric covariates (e.g. host×size, region×size). Non-significant covariate terms were dropped from the final statistical models. Discriminant analysis (both linear and quadratic) was performed with canonical scores from the host effect, to provide a measure of classification success. Validation by jackknifing (one-removal) was performed to verify significance of the discriminant functions. The sample size for traditional morphometrics was 117 specimens. All statistics were conducted in JMP 9.0 (SAS Institute, Cary, NC) and verified by replication in Excel spreadsheets.

For geometric shape data, principle components of superimposed coordinates were calculated using the covariance matrix, and major components that collectively summarized 99% of the data (22 of 106) were retained for further analysis. The 22 principle components (=shape variables) were analyzed as described above for the traditional morphometric data, by the method of Langerhans and DeWitt (2004). Linear and quadratic discriminant analyses were performed above with jackknife procedure. Sample size for geometric wing shape was 113 specimens. Statistics were calculated in

JMP and Excel. Procrustes analysis was conducted. Non-shape effects of location, size, and orientation were removed from the raw coordinate data by translating, scaling and rotating each conformation to best fit with the consensus, or average conformation, rotated to its major axis. I used stereographic projection to flat shape space using tpsRelw (Rohlf 2006) and treated semilandmarks and landmarks equivalently. Deficient dimensionality (4 dimensions lost for superimposition, and one lost for each semilandmark) was corrected by omitting null vectors following principle components analysis.

The combined dataset represents a fused dataset generated from only shape data from both traditional and geometric data. The traditional and geometric data were fused by scaling each respective data block to true rank. True rank is the number of truly independent dimensions in a data space (DeWitt, in review). Higher total variance might be explained and more meaningful insights could be drawn by using a fused dataset rather than using either traditional or geometric datasets alone. For example, the true rank (number of principal components needed to summarize 99% of the variance in my dataset) of the traditional shape and geometric shape data was 11 and 22, respectively. Thus, the fused dataset originally consisted of 33 principal components.

Results

Overall, analysis of all three types of datasets (traditional measurement, geometric shape, and combined shape data) indicated that there was a significant effect of both host-plant (shared effect) and region (unique effect) on morphological variation of *P. seriatus*.

The shared effect and unique effect explained (η^2) an average of 48.4 and 53.8% of observed variation in the study organism, respectively. The MANCOVA also indicated that host-plant × region, which is also another form of unique effect, had a significant effect on *P. seriatus* morphology and explained an average of 42.1% variation across the three datasets. Detailed results from all three datasets are as follows.

Traditional morphology

The result of MANCOVA on traditional morphology data shows that effect of host-plant was significant ($F = 3.48$, df = 13, 94, $P = 0.0002$), i.e., being associated with two different host plants, correlates with *P. seriatus* individuals differing morphologically (Table 5.2). The canonical variate derived for shared effect (host-plant) also showed diversification for two host-plant associated insect populations (Fig. 5.4). Examination of the canonical loadings for effect of host-plant revealed at least three distinct morphological changes: individuals associated with cotton have longer antenna, longer rostrum and narrower femur compared to individuals associated with horsemint. The direction of morphological changes with respect to host-plant was consistent in the two studied regions. Two sources of variation, region and the interaction term (region × host-plant) can be considered as unique aspects of *P. seriatus* morphological divergence. The MANCOVA indicated that there were significant effect of both region ($F = 617$, df = 13, 94, $P < 0.0001$) and region × host-plant ($F = 3.25$, df = 13, 94, $P = 0.0004$) on morphological divergence of *P. seriatus* populations. The discriminant function analysis (DFA) based on the 13 linear measurements classified insects by their host-plant

Table 5.2. Results of MANCOVA on traditional body size measurement data of *P. seriatus*

Test for	Factors	F	df	P	(η^2) Partial variance explained (%)	DFA (LD)	DFA (QD)
Shared divergence	Host	3.48	13, 94	0.0002	32.5	72.6	83.2
Unique divergence	Region	6.17	13, 94	<0.0001	46.0	84.1	91.2
	Host × Region	3.25	13, 94	0.0004	31.0		

Table 5.3. Results of MANCOVA on wing geometric shape measurement data of *P. seriatus*

Test for	Factors	F	df	P	(η^2) Partial variance explained (%)	DFA (LD)	DFA (QD)
Shared divergence	Host	4.09	22, 89	<0.0001	50.3	80.3	96.6
Unique divergence	Region	5.23	22, 89	<0.0001	56.4	85.5	100
	Host × Region	2.86	22, 89	0.0003	41.4		

Table 5.4. Results of MANCOVA on combined data (wing shape and traditional shape data) of *P. seriatus*

Test for	Factors	F	df	P	(η^2) Partial variance explained (%)	DFA (LD)	DFA (QD)
Shared divergence	Host	3.70	30, 67	<0.0001	62.3	88.2	100.00
Unique divergence	Region	3.23	30	<0.0001	59.1	82.4	100.00
	Host × Region	2.63	30, 67	0.0005	54.1		

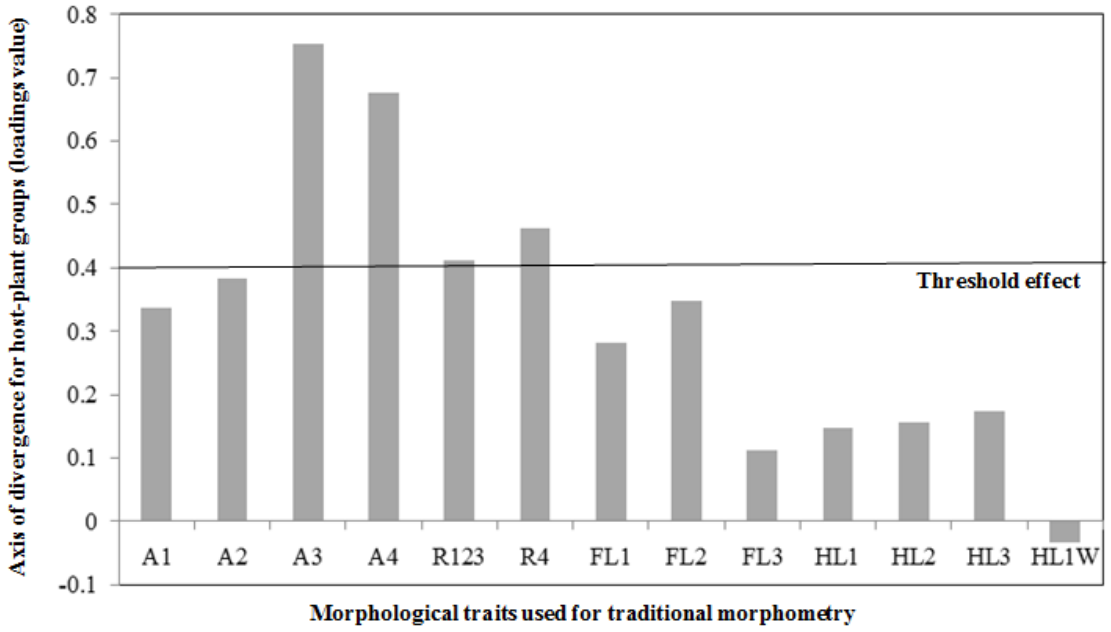

Fig. 5.4. Loadings of 13 morphological traits on the axis of divergence based on the correlation between traits measurement and canonical score. Abbreviation of morphological traits are indicated in Table 5.1 of this chapter.

association, with the null hypothesis of 50 per cent being correctly classified to either of the two host-plant groups by chance. The DFA result classified 72.6% of the individuals correctly to their respective group. Similarly, another DFA classified 84.1% of the individuals correctly to their respective region-group.

Geometric shape

The MANOVA of geometric shape data on the right forewing of ***P. seriatus*** showed that host plant has a significant effect on the observed morphological divergence ($F = 4.09$, $df = 22, 89$, $P < 0.0001$) (Table 5.3). Relative warp analysis of wing shape coordinates showed that overall; wings of individuals associated with horsemint are narrower at their proximal end than wings of cotton associated individuals (Fig. 5.5). We found a significant effect of two unique aspects on ***P. seriatus*** wing shape divergence: region ($F = 5.23$, $df = 22, 89$, $P < 0.0002$) and region × host-plant (Table 5.3). The first DFA, based on host-plant effect correctly classified 80.3% of the individuals into their respective host-plant groups. Similarly, the second DFA based on region effect correctly classified 85.5% of the individuals into their respective region groups.

Combined

The combined dataset consist of shape information obtained from both traditional morphology and wing shape data. The shape data from traditional morphology data was extracted by subtracting the size information from the original form information. PCA

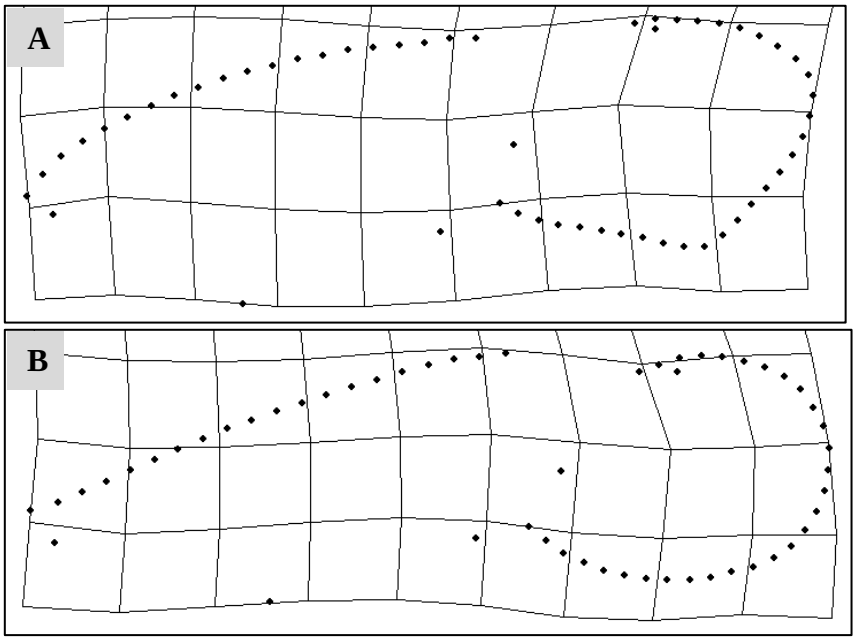

Fig. 5.5. Visualization of fore wing shape variation of two host-associated groups in thin plate spline transformation grid (10x). A. cotton associated individuals, B. horsemint associated individuals.

analysis indicated that 99% of the variation was explained by 11 and 22 PCs in traditional shape data and wing shape data, respectively. These two dataset (i.e., traditional shape and wing shape) were then combined based on their true ranking and PCA on this combined data indicated that 99% of the variation was explained by 30 PCs. Thus, at least 3 overlapping PCs were detected between the two datasets suggesting a case of phenotypic integration. Based on the 30 PCs, we conducted MANOVA, which indicated that there was a significant effect of host ($F = 3.7$, $df = 30, 67$, $P < 0.0001$), region ($F = 3.23$, $df = 30, 67$, $P < 0.0001$) and their interaction, host × region ($F = 2.63$, $df = 30, 67$, $P = 0.0005$) (Table 5.4). The DFA based on host effect, correctly classified 88.2 % of the individuals to two host groups. Similarly, 82% of the individuals were correctly classified into their respective region groups in another DFA while region was considered as an effect on morphological variation.

Discussion

Cotton fleahopper populations associated with two different host plant species showed divergence in both body size and wing shape. Variation in the magnitude of these morphological differences was also observed between two relatively distant geographic locations. The estimate of partial variance explained (η^2) by host plant species alone was almost equal to the variance explained by geographic location alone for both body size and wing shape. However, when considered together (fused data) the analysis suggested a greater role of host plant species than of geographic location in explaining morphological variance among the studied cotton fleahopper populations. In

addition, the combined dataset provided more discriminatory power (88.2% success in discriminant function analysis, DFA) than any of the individual data sets (i.e., either traditional morphometric or wing geometric-morphometric) to correctly classify cotton fleahopper individuals to their respective host plant or region. The most important result of this study was to detect significant morphological differences between cotton and horsemint associated populations in replicated locations, which supports the notion that host plant species can act as a strong agent of natural selection in phytophagous insects.

Phytophagous insects feeding on different host plant species generally encounter variable environments in terms of morphological, biochemical and anatomical host-plant features (Bernays et al. 1991). Cotton and horsemint belong to two unrelated plant families and their surface morphologies vary significantly. In general, cotton has dense, coarse, long trichomes (hirsute hairs), while horsemint appears to be glabrous (lack of hairs) (Fig. 5.1). Projecting the loadings of insect's morphological traits in a common canonical axis indicated that 3 morphological traits had high character values (threshold =0.4) (Fig. 5.4). These three morphological traits (i.e., 3rd and 4th antennal segment and the last rostrum segment) showed higher loading for cotton associated fleahoppers; whereas, width of hind-femur had relatively higher loading for horsemint associated fleahoppers. Based on the host plant's surface morphology (i.e., presence of hirsute hairs in cotton), increased rostrum length was expected for cotton associated fleahoppers. However, variation in antennal length was not expected. The longer antenna of cotton associated individuals also could be the result of allometric relationship with insect's body size.

Body size in insects has been shown to correlate with nutritional quality of their food resources. For example, insects receiving higher quality food could develop larger body size and therefore, several body appendages might grow longer. Therefore, longer antennal length or other variation in body size could be just an effect of nutrition rather than selection on morphological traits. A similar idea was suggested by Suh (2007) as an explanation for head-capsule differences in cotton fleahopper nymphs fed on different host plant species. In another example, individuals of the cabbage aphid (*Brevicorynye brassicae*) feeding on turnip (*Brassica campestris*) were found to be larger than individuals feeding on green cabbage (*B. oleracea*), and morphological measurement of different body appendages indicated a positive correlation with body size (Ruiz-Montoya et al. 2005). Thus, morphological difference among insect populations feeding on different host plant species could be the result of phenotypic plasticity.

Although, morphological divergence in phytophagous insects has been studied more in the context of insect-host plant interactions, an equally important aspect of morphological divergence in phytophagous insect is their interaction with geography. Insect populations can be locally adapted owing to variations in climatic conditions and to environmental differences such as heterogeneity in host plant composition, host-plant abundance, predator and/or parasitoid composition and abundance. Geographically isolated populations may also have different evolutionary histories such as occurrence of stochastic events generating bottlenecks, differences in the level of genetic drift, amount of gene flow with nearby populations and different degrees of local adaptation. In the case of cotton fleahopper, I found a strong influence of geography on both insect body

size and wing shape. Morphological traits such as the length of antenna and rostrum were longer in College Station than in Lubbock cotton fleahopper populations. This body size variation in College Station populations was similar to what we saw in the case of cotton associated populations, which indicate that College Station populations of cotton fleahopper are more like the "cotton type" and Lubbock populations are more like the "horsemint type". Further studies will be needed to verify if this geographic trend in morphology among fleahoppers associated with different host-plant species still persists after reciprocal transplant experiments among populations from both locations.

Geometric morphometric analysis of insect wing shape variation has been a common approach used to differentiate interspecific and intra-specific populations (Campos et al. 2011, Jorge et al. 2011). Especially, this approach has been able to detect subtle differences in wing shape, which could also be used as diagnostic tools in resolving taxonomic problems such as species/sub-species identification (Tofilski 2008). Wing shape sometimes can be more reliable to detect subtle differences than traditional measurements of body size. For example, larval food of a neotropical butterfly, *Heliconius erato* influenced wing shape more than body size and therefore wing shape was used to discriminate the host plant used by this butterfly (Jorge et al. 2011). The wing shape data (based on landmark coordinates) for cotton fleahopper used in the current study were also highly reliable to assign individuals into their respective host plant group. Thus, I was able to classify cotton fleahopper individuals to their correct host plant group with 80% success. Visualization of wing shape differences of two host-associated groups in a thin plate spline transformation grid indicated that fore wings of

cotton fleahoppers associated with cotton had a broad base, while wings of horsemint associated individuals appear to be more slender and elongated. The typical hemelytrons (fore wing of true bug) consist of three segments: corium, clavus and membrane (Fig. 5.3 A). We visualized considerable shape variation in clavus shape between the two host associated groups, where horsemint associated individuals had narrower clavus compared to cotton associated individuals. I restrain from interpreting the observed wing shape variation between the two host-associated groups I studied without knowing the flight aero dynamics involved in this insect flight. I believe these results may raise future inquiry and hypotheses regarding wing shape and its relation to dispersal in this insect.

It was suggested that insect diet can influence insect's wing shape more than its body size (Jorge et al. 2011). If such a suggestion holds to be true then it would be possible to use wing shape information in identifying the host-plant of origin of migratory pest populations. Pest individuals can be reared under laboratory/field conditions to obtain base line information on wing shape associated with their host plants and these data could be used to determine the origin of individuals from natural populations. Wing shape could also be used to understand population structure and to understand the dynamics of insect migration at local scales (Schachter-Broide et al. 2004). Identification and correct classification of insect populations is important in pest management. Geometric-morphometric studies have resolved several cases of cryptic species or sub-species, which are not only useful for identification of sub-populations of insect pest species (Shiao 2004, Kitthawee and Dujardin 2010, Lyra et al. 2010), but also helpful in differentiating biological control agents used for suppression of insect pests

(Querino et al. 2002). Another potential area for using the findings of geometric-morphometric data on insect's morphological traits would be in the breeding of crop cultivars against insect pests. For example, several studies have indicated that glabrous (hairless) cotton cultivars are more vulnerable to cotton fleahopper injury than the hirsute type (Lukefahr et al. 1970, Walker et al. 1974). If considerable variation in cotton fleahopper morphology exists in natural populations as shown in this study, breeding approaches need to consider this variation in insects to prevent failure of developed cultivars. These examples and my current work suggest that geometric-morphometric approaches can differentiate subtle morphological variation in insect populations and attest to the power to capture such variation. Incorporating information on the interactive effect of host-plant species and geography on morphology of pest and natural enemies will allow us to fine tune our pest management strategies.

CHAPTER VI

SUMMARY, SIGNIFICANCE, AND FUTURE DIRECTIONS

This dissertation research was undertaken to understand the movement of cotton fleahopper in agroecosystems. I started with the idea that wild host plants were the source of cotton fleahopper populations infesting cotton. However, the cotton fleahopper is a highly polyphagous insect (more than160 reported host plants). Thus, the number of potential sources was staggering and there were temporal asynchrony and spatial heterogeneity in the potential host plant species. These observations suggested that perhaps the importance of different host plant species for the fleahopper varied at different locations. To select the host plant species for this study, I surveyed locally abundant host plant species for presence of cotton fleahopper in several eco-regions in Texas and selected three plant species (i.e., horsemint, woolly croton and cotton) on which fleahopper populations were abundant enough to conduct the study.

Insect movement can be detected either directly by using physical marking systems (e.g., florescent dye, proteins, pollen, etc.) or indirectly by using molecular markers. While physical markers provide direct measurement of movement of dispersal potential in real time, molecular markers provide information on historical patterns of movement among insect populations. Both approaches have their utility depending on the questions being asked. Direct measurement of movement is challenging, especially with small insects. Thus, indirect measurements of movement have become the method of choice. When molecular markers are used to estimate insect movement, we are

actually measuring the gene flow among populations rather than the physical movement. I developed AFLP markers to assess gene flow among cotton fleahopper populations from cotton, horsemint and woolly croton. Lack of gene flow among individuals associated with different host-plant species is referred as host-associated differentiation (HAD). I found host-associated differentiation in the cotton fleahopper, where a distinct fleahopper genotype is associated with horsemint. However, the presence of this distinct genotype in this insect is affected by geography, suggesting that HAD is a population rather than a species trait. John N. Thompson discussed this possibility for the first time in 1994 referring to co-evolution, within the context of his geographic mosaic theory(Thompson 1994). In a similar vein, what I found can be referred to as the geographic mosaic of HAD as it shares the same theoretical underpinnings. My data validate this theoretical approach in an agricultural cropping system for the first time.

The presence of HAD in the cotton fleahopper indicates that individuals associated with one of its host-plant species (i.e., horsemint) were reproductively isolated from individuals associated with cotton. This suggests that gene flow and perhaps dispersal from horsemint to cotton was negligible. Lack of gene flow has been found in other agricultural pests associated with different host plant species (even when host plants are adjacent to each other), such as the cereal aphid, the beet armyworm and the pea aphid. However, these other studies only explored the effect of host-plant species on genetic variation without incorporating local geographic variation. The implications of HAD in agroecosystems are vast, yet largely unexplored. Several common agricultural pest management practices could be affected by the presence of HAD. For

example, genetically distinct populations among different host-plant species may exhibit differences in phenotypic traits relevant to their control. I have found that size and shape of key morphological traits of the cotton fleahopper such as, rostral length, wing shape and antennal length vary significantly among cotton fleahoppers associated with different plant species and the magnitude of this difference is modulated by geography. Host plant association could also influence other phenotypic traits of insect pest such as insecticide resistance, resistance to transgenic crops, vulnerability to natural enemies and to entomopathogens. The magnitude of the phenotypic differences in insect populations could also be modulated by geography. Having found host-associated and geographically distinct populations in cotton fleahopper it is possible that these populations may exhibit differential vulnerabilities and tolerances to several control measures.

Prior to this study, no genetic information was available for the cotton fleahopper. I have not only provided evidence of genetic variation among host associated populations in this study but I have also established baseline genetic information of this pest in cotton across most of its geographic distribution in the United States. I found that the cotton fleahopper in cotton shows a macro-geographic structure of its genetic variation. This structuring appears to be relatively new and may have occurred after the introduction of cotton, suggesting that genetically distinct populations could evolve in relatively short time (~200 years). Other insect species have been reported to differentiate genetically in comparable time scales (e.g., the apple maggot, and the soap berry bug). If such time scale is common for genetic differentiation to happen in pest

populations, this has important implications in agroecosystems since most of the agricultural crops in the new world are around 200 years old if not older.

The findings from the current research work open up several potential future studies, which could help in explaining genetic differences among insect populations and aid in cotton fleahopper management. Historically, the fleahopper population in cotton has been smaller in north-western part of Texas (Lubbock and San Angelo), while fleahopper population have been found to be significantly larger in the south-eastern part of Texas (College Station and Corpus Christi). Owing to this spatial difference in insect abundance, the current economic threshold level for fleahopper also differs in the two cotton growing regions. It would be informative to know whether the cotton fleahopper populations in these two regions differ in their potential to inflict damage to cotton. Although cotton associated fleahoppers in both regions are genetically similar, the source of these populations seem to be different. In Lubbock and San Angelo, cotton infesting fleahopper populations may disperse from host plants other than horsemint, while in College Station and Corpus Christi, the fleahopper infesting cotton may come from horsemint and/or woolly croton. Future research should explore, 1) what are the other potential host plants in Lubbock and San Angelo which can contribute to cotton infesting fleahoppers?, 2) what are the hibernating host plants of cotton fleahoppers in Lubbock and San Angelo, since woolly croton is absent in those areas?, 3) is cotton itself a hibernating host for the cotton fleahopper?, and 4) what is the state of genetic variation in cotton fleahopper populations associated with host plant species other than the three considered in this study? The results of behavioral experiments showed that preference

for horsemint in fleahopper population remain unchanged irrespective of geographic location, genotype (identified through AFLP) and prior experience. I tested two horsemint associated populations with two different AFLP genotypes, one from Lubbock and another form College Station and found that the preference for horsemint was similar in both genotypes. However, I was not able to test the fleahopper associated with cotton in Lubbock. It would be interesting and informative to explore the host preference of fleahoppers associated with cotton in Lubbock and San Angelo. Since in these locations cotton associated populations are genetically distinct from horsemint associated populations and one would be able to explore if genetically distinct, cotton associated population have lost the preference for horsemint and specializes on cotton only. Since different genotypes may have differential phenotypic expression, one could test if different genotypes of fleahoppers have differences in insecticide tolerance, differences in life history traits, and differences in dispersal potential. Presence of regional populations of fleahopper across the cotton growing areas in the United States raises the possibility that there might be differences in level of insecticide tolerance, and magnitude of polyphagy among these populations. Future studies should consider these possibilities by formulating appropriate experiments in contexts of cotton fleahopper's importance as a pest of cotton.

As I have discussed, presence of genetic differentiation among insect populations may influence several pest management practices. Integrated pest management (IPM) programs that aim to be realistic and sustainable should therefore consider this phenomenon more explicitly. In the past few decades, ecology has found an important

place in pest control practices and theory. The time has come for evolution to find its place in our pest management practices and paradigms.

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