HOST-ASSOCIATED DIFFERENTIATION IN AN INSECT COMMUNITY

A Dissertation

by

AARON MICHAEL DICKEY

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 2010

Major Subject: Entomology

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Approved by:

Chair of Committee,	Raul F. Medina
Committee Members,	Marvin K. Harris
	Micky D. Eubanks
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ABSTRACT

Host-Associated Differentiation in an Insect Community. (December 2010)

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Host-Associated Differentiation (HAD) is the formation of genetically divergent host-associated lineages maintained by ecological isolation. HAD is potentially an important route to ecological speciation in parasites including many insects. While HAD case studies are accumulating, there is a dearth of negative results in the literature making it difficult to know how common the phenomenon really is or whether there are specific traits of parasites which promote HAD. To address these two problems, studies are needed which both publish negative results (i.e., parasites not showing HAD) and test for HAD in multiple parasite species on the same pair of host species (i.e., control for host plant effects).

In this study, HAD was tested in three species of herbivorous insects and one parasitoid species on the same two host tree species: pecan and water hickory. The insects were selected based on the presence or absence of two traits, parthenogenesis and endophagy. A test for HAD was considered "positive" when population substructure was explained by host-association. To test for the presence of HAD, insects were sampled

sympatrically to eliminate geographical isolation as a confounding factor, sampling was replicated spatially to assure that HAD persisted, and multiple loci were sampled from each individual. Genetic data was analyzed using cluster analyses. HAD was found in both pecan leaf phylloxera and yellow pecan aphid but not in pecan bud moth or in the parasitoid of the yellow pecan aphid, *Aphelinus perpallidus*. Interestingly, both taxa showing HAD are parthenogenetic and both taxa not showing HAD reproduce sexually.

Species showing HAD were tested for the presence of a pre-mating reproductive isolating mechanism (RIM) which could be maintaining HAD despite the potential for gene flow. Selection against migrants to the alternative host was tested in yellow pecan aphid using a no-choice fitness experiment. The overall contribution of this RIM to total isolation was positive and ranged from 0.614 to 0.850. The RIM of "habitat preference" was tested in pecan leaf phylloxera using a dual-choice preference experiment. In this species, preference was only detected for phylloxera originating from water hickory suggesting that host discrimination ability may be a less important factor promoting differentiation in phylloxera.

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I also thank the landowners and managers who provided access to field sites: USDA Pecan Genetics, College Station Parks and Recreation, Texas Parks and Wildlife, Pat Caraway, Bobby Murphy, and Wendel Legally. I am grateful to the Office of Graduate Studies, the College of Agriculture, and the Department of Entomology for providing funding support throughout this research.

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NOMENCLATURE

AMOVA	Analysis of Molecular Variance
PCA	Principal Coordinates Analysis
HAD	Host-Associated Differentiation

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CHAPTER I

INTRODUCTION

In the forward to Warren Abrahamson and Arthur Weis's 1997 book, *Evolutionary Ecology Across Three Trophic Levels*, May Berenbaum posits the following: "*Solidago*, *Eurosta*, and *Eurosta's* natural enemies may well come to be regarded as a model interaction, as it were, for the fields of ecology and evolutionary biology" (Abrahamson & Weis, 1997). The "interaction" of which Berenbaum speaks, begins with a pair of unassuming North American wildflower species in the genus, *Solidago*. These two species possess demography and species associations which make them well suited for research in the field of Evolutionary Ecology; first, they occur sympatrically, and second, they share a "community" of insect species (Stireman et al., 2005). These insect species feed directly on the plants themselves, or they feed on insects and fungi which in turn feed on the plants. This pattern of geography and herbivory is, in fact, not unique to these wildflowers but is replicated many times over in plant genera throughout the world. I wanted to do a parallel study to that of Stireman et al. (2005) and to that end I introduce a new pair of host plant species exhibiting this pattern, both in the genus, *Carya*.

This dissertation follows the style of the *Journal of Evolutionary Biology*.

The first insect to gain fame on *Solidago* was the herbivorous fly, *Eurosta*. *Eurosta*, as it turned out, was not a generalist species feeding on both species of *Solidago* in sympatry but rather showed a pattern of Host-Associated Differentiation (HAD). That is, populations of the fly were genetically distinct (Waring et al., 1990) and showed a high degree of reproductive isolation when feeding on the two sympatrically occurring plant species (Itami et al., 1998). HAD is the presence of these genetically divergent, host associated populations (Pashley, 1986, Hernandez-Vera et al., 2010). These genetically divergent populations could be considered 'host races' i.e. incipient sympatric species *sensu stricto* Berlocher and Feder (2002) or they could be cryptic species which evolved in allopatry along with their hosts and which now occur in sympatry. Alternatively, genetically divergent populations could be local, host associated demes which may be ecologically isolated at small geographic scales but which periodically exchange genes preventing race formation at larger geographic scales (Baer et al., 2004).

Eurosta was neither the first, nor the last, insect to show HAD. But until our recent publication (Dickey & Medina, 2010), *Solidago* was the only host-plant pair for which HAD had been explicitly tested in a community of herbivores. In *Solidago*, at least four insect herbivores, including *Eurosta*, consist of host-associated lineages. These insects are members of three different insect orders and all of them are associated with galls (Waring et al., 1990, Stireman et al., 2005, Blair et al. 2006). Thus, the present research program is only the second such program to test HAD in a community of herbivores feeding on the same two host plant species.

As alluded to, multiple herbivores show HAD on related species of host-plants (Dres & Mallet, 2002, Funk et al., 2002) but such case studies come from a wide range of host plants and therefore do not control for host plant effects. Thus their utility in testing the ubiquity of HAD or the factors that might promote it is limited. Stireman et al. (2005) argued that by testing HAD in an herbivore community one can test the ubiquity of HAD provided that all negative results (herbivores not showing HAD) are reported. If negative results are not reported, the ubiquity of HAD will be overestimated. Stireman et al. (2005) also argued that testing HAD in an herbivore community can be used to test whether specific herbivore traits promote HAD. Two such proposed traits are endophagy (Mopper, 2005) and parthenogenesis (Dixon, 1998). Because these traits have been proposed to promote HAD, three herbivores were selected for HAD testing based on the presence of one or both of these traits. In addition I report negative results.

Once HAD is demonstrated, several questions can be asked. First, is there a reproductive isolating mechanism (RIM) operating to limit gene flow in a particular species maintaining HAD in sympatry? Second, has HAD in herbivores cascaded up to promote sequential radiation at the third trophic level? I addressed the first question in two separate herbivores and I addressed the second question in an aphid parasitoid.

In summary, HAD was tested in an insect community on a second host-plant system intended to be a parallel host-plant system to *Solidago*. Members of the genus *Carya* have two characteristics thought to promote HAD; like *Solidago* they are native to the

study area and they are long-lived perennials. Insects were selected from this system to test HAD based on two traits which are thought to promote HAD, endophagy and parthenogenesis (Chapters II and IV). Sequential radiation, or “cascading HAD”, was also tested (Chapter V). By testing HAD in an insect community, the effect of host-plant species is controlled for facilitating comparisons between insects. In addition, by reporting negative results (Chapter II), I eliminate a possible bias caused by underreporting. Lastly, for those species of insects showing HAD, the presence of a pre-mating reproductive isolating mechanism (RIM) was tested to investigate its role in maintaining HAD in sympatry (Chapters III and IV).

Unfortunately, neither the present work nor that of Stireman et al. (2005) tested HAD in enough herbivores so as to test statistically either the ubiquity of HAD or whether specific herbivore traits could promote it. As such, these two systems both await further HAD tests in additional herbivores in order to obtain sufficient statistical power to fully address these questions. These and other suggestions for future study are discussed in Chapter VI.

CHAPTER II

TESTING HOST-ASSOCIATED DIFFERENTIATION IN A QUASI-ENDOPHAGE AND A PARTHENOGEN ON NATIVE TREES*

Synopsis

Host Associated Differentiation (HAD) is the formation of genetically divergent host associated sub-populations. Evidence of HAD has been reported for multiple insect herbivores to date, but published studies testing more than one herbivore for any given host-plant species pair is limited to herbivores on goldenrods. This limits the number of pair-wise comparisons that can be made about insect life-history traits that might facilitate or inhibit host-race development in general. Two traits previously proposed to facilitate HAD include endophagy and parthenogenesis. We tested for HAD in two herbivores, a quasi-endophagous caterpillar and a parthenogenetic aphid, feeding on two closely related species of hickories. We found that the quasi-endophage is panmictic while the parthenogen exhibits HAD on their sympatric host-plants, pecan and water hickory, at a geographic mesoscale. This is an important first step in the characterization of HAD in multiple insect herbivores using North American hickories, a host-plant system with many shared parthenogens.

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*Reprinted with permission from Dickey, A.M. and Medina, R.F. 2010. "Testing host-associated differentiation in a quasi-endophage and a parthenogen on native trees." *Journal of Evolutionary Biology*, **23** 945-956.

Introduction

Flowering plants, herbivorous insects, and insect parasitoids together comprise more than 50% of the world's species (Godfray, 1994, Price, 1980, Schoonhoven et al., 1998). Because insects often have narrow host breadths (Mitter et al., 1988, Godfray, 1994) and tight associations with their hosts they are prime candidates for host associated differentiation (HAD). HAD is the formation of genetically divergent host associated sub-populations (Bush, 1969, Abrahamson et al., 2003). HAD has been proposed as a mechanism promoting adaptive radiation of host-associated lineages resulting over time in increased species diversity (Mitter et al., 1988, Stireman et al., 2006, Funk et al., 2002). HAD can be detected when genotypes cluster by host plant species despite sampling from geographically separated populations (Berlocher & Feder, 2002, Stireman et al., 2006, Scheffer & Hawthorne, 2007).

HAD is a special case of ecological speciation (Schlutter 2001; Rundle & Nosil, 2005) wherein the disruptively selective environments experienced by diverging populations of phytophagous insects are different host plant species (Dres & Mallet, 2002). The ecological literature contains a growing body of HAD case studies (Dres & Mallet, 2002, Stireman et al., 2005, Vialatte et al., 2005, Sword et al., 2005, Dorchin et al., 2009, Lozier et al., 2007, Magalhaes et al., 2007, Peccoud et al., 2009) but these, with a single exception (Stireman et al., 2005), involve no more than one herbivore tested per host-plant pair. Many reports provide a plausible set of herbivore traits hypothesized to facilitate HAD but testing the relative importance of those traits will depend on studies

which 1) Test HAD for multiple herbivores on the same host-plant species pair and 2) Publish negative as well as positive results. While there are several traits that could promote HAD, two herbivore traits proposed to facilitate HAD are addressed in this study; endophagy (Dreger-Jauffret & Shorthouse, 1992, Stireman et al., 2005) and parthenogenesis (Sunnucks et al., 1997, Loxdale, 2008, Dixon, 1998, Vialatte et al., 2005).

Endophagous insects are thought to be more prone to exhibit HAD because they are less exposed to selective regimes imposed by generalist predators and environmental conditions and because they are more likely to be selected by host plant traits than exposed feeders (Stilling & Rossi, 1998, Stireman et al., 2005, Cornell et al., 1998). The only case of HAD tested in multiple herbivores on the same host-plant pair involves insects on sibling species of native perennial goldenrods (Abrahamson & Weis, 1997). In this system HAD was found in two thirds of the endophages tested but was not found in either of two tested exophages. HAD was found in six of eleven species tested and in insects belonging to different insect orders and different trophic levels (Waring et al., 1990, Eubanks et al., 2003, Stireman et al., 2006, Stireman et al., 2005).

Cyclic parthenogenic insects, such as aphids, may also develop host associated lineages faster than sexually reproducing insects because favorable mutations can become fixed more quickly when sex is limited (Hartl, 1972, Lynch, 1984, Neiman & Linksvayer, 2006). Most aphids are holocyclic apomictic parthenogens (Blackman & Eastop, 1994,

Loxdale, 2008) restricting sex to a single season. Thus, prior to sex, rapid succession of asexual generations can amplify and accelerate the response to selection (Lynch & Gabriel, 1983, King, 1993, King & Murtaugh, 1997, Vialatte et al., 2005, Loxdale, 2008). If parthenogens occupy different host-plant species and sex is initiated in response to host-plant mediated cues, then a difference in the timing of sexual reproduction may be a consequence of ecological differences between host plant species (Guldemond & Mackenzie, 1994, Serra et al., 1998). To date, HAD has been reported in several aphid species (Akimoto, 1990, Guldemond et al., 1994, Vanlerberghe-Masutti & Chavigny, 1998, Via & Hawthorne, 2002, Simon et al., 2003, Brunner et al., 2004, Vialatte et al., 2005, Lozier et al., 2007, Peccoud et al., 2009).

Testing for HAD in an insect community feeding on the same two host plant species is an optimal way to examine the role of endophagy and parthenogenesis in promoting HAD because multiple herbivores can be selected for study within a system based on the presence/absence of these traits without the confounding factors involved when comparing insects from different host-plant study systems (Stireman et al., 2005).

We have selected a host-plant study system that we think maximizes the possibility of finding HAD. First, the system is native (i.e., native tree and insect species). While rapid evolution of host races has been documented in native insects feeding on introduced plants (Bush, 1969, Feder et al., 1999, Strauss et al., 2006), the increased evolutionary time of insect community and host plant interaction afforded by native systems should

increase the probability of finding HAD. Second, the host plant species chosen are trees. HAD, to our knowledge, has been documented in arthropods feeding on annual plants in only a rare number of cases; *Spodoptera frugiperda, Sitobion avenae, Nilaparvata lugens*, and *Ostrinia nubilalis* feeding on cultivated grasses (Dres & Mallet, 2002, Martel et al., 2003, Vialatte et al., 2005). In contrast, trees offer relatively more stable, long-lived genotypes to which many generations of arthropods can adapt (Edmunds & Alstad, 1981, Mopper, 2005, Magalhaes et al., 2007) and most cases of HAD are from such perennial systems (Magalhaes et al., 2007, Dres & Mallet, 2002). The native trees pecan *Carya illinoinensis* Koch and water hickory *C. aquatica* Michx (Fagales: Juglandaceae) have been selected as host plants for this study. These deciduous trees share a large and diverse native insect fauna (Table 1).

This study is just the first step in testing HAD in a community of phytophagous insects. Insects from table 1 were selected for this study based on the presence of two traits of interest, endophagy and parthenogenesis in addition to their relative abundance at multiple study sites, the relative ease of collecting them in the field, and the relative ease of rearing them to adulthood in the laboratory.

Table 1: A list of insects shared by pecan and water hickory. This list is not comprehensive.

	Insect species	Endophagous	Parthenogenetic
Lepidoptera	*Actius luna*		
	Amorpha juglandis		
	Cameraria caryaefoliella	X	
	Catocala agrippina		
	Catocala maestosa		
	Citheronia regalis		
	Cydia caryana	X	
	Datana integerrima		
	Gretchena bolliana	X	
	Hyphantria cunea		
	Malacosoma disstria		
	Megalopyge opercularis		
	Satyrium calanus		
	Stigmella juglandifoliella	X	
Hymenoptera	*Aphelinus perpallidus*		
	Periclista marginicollis		
	Pteromalus sp.	X	
Hemiptera	*Clavaspis crypta*		
	Empoasca fabae		
	Goes pulcher	X	
	Gypona octolineata		
	Metcalfa pruinosa		
	Phylloxera devastatrix	X	X
	Phylloxera notabilis	X	X
	Phylloxera texana	X	X
	Velataspis mimosarum		X
	Melanocallis caryaefoliae		X
	Monellia caryella		X
	Monelliopsis pecanis		X
	Curculio caryae	X	

The X's denote those species exhibiting parthenogenesis and endophagy (B. Ree, Texas A&M University, College Station, Pers. Comm.; A. Dickey Pers. Obs.)

HAD was tested using cluster analyses of AFLPs (Vos et al., 1995). AFLPs are anonymous dominant molecular markers which are advantageous for HAD studies because they are often neutral, can be generated quickly, are cost effective, and can be used to determine both the structure of populations, and the assignment of individuals to populations (Sword et al., 2005, Falush et al., 2007, Meudt & Clarke, 2007).

Methods

Study system

The genus *Carya* contains thirteen North American species and five Asian species (Manning, 1978) of large deciduous trees. The genus has been present in North America for at least 34 million years based on evidence from fossilized fruits with extant species dating to the Pleistocene (Manchester, 1987). Pecan and water hickory are common to the river and creek bottoms in the hardwood forests of eastern North America (Fralish & Franklin, 2002) (Figure 1). Pecan is the most economically important indigenous nut crop in the US (Grauke et al., 2003). Water hickory is a species closely related to pecan but unlike pecan has a flat, wrinkled and bitter nut (Stone et al., 1965). The bitterness is likely due to increased phenolics content in water hickory nuts relative to pecan; a pattern which does not extend to the foliage of the two species (APPENDIX A). A detailed phylogenetic hypothesis does not exist for the genus *Carya* but within the genus, *C. aquatica* and *C. illinoinensis* have been grouped together in the section Apocarya along with three other species of North American hickories and 5 other species of Asian hickories (Thompson & Grauke, 1991). Grauke *et al.* (1987)

documented phenological differences among sympatric *Carya* species in Louisiana and found no temporal overlap in pollen shed and pistil receptivity between pecan and water hickory with water hickory budding and flowering approximately three weeks later in the spring than pecan. Phenological differences between host plants have been shown to be important sources of ecological isolation of insect populations in sympatry (Komatsu & Akimoto, 1995, Feder & Filchak, 1999, Mopper, 2005), which could drive HAD. Despite phenological differences, pecan and water hickory hybridize in the wild (Stone et al., 1965, Grauke et al., 1987) but hybrids can be recognized by nut and bud phenotypes intermediate to parent species (Grauke et al., 1987, Thompson & Grauke, 1991). The genus *Carya* is very promiscuous and other hybrids including either pecan or water hickory have been documented (Thompson & Grauke, 1991).

The yellow pecan aphid *Monelliopsis pecanis* Bissel (Hemiptera: Aphididae) is a holocyclic parthenogenetic exophage feeding on the lower surfaces of leaflet tertiary veins (Tedders, 1978). The pecan bud moth *Gretchena boliana* Granovsky (Lepidoptera: Tortricidae) is a sexually reproducing foliage feeder but early instars are inquilines, feeding inside of *Phylloxera sp.* galls (Mitchell et al., 1984) leading us to designate it as a quasi-endophage. Later instars fold leaves (Mizell & Schiffhauer, 1986). We have found and reared both herbivores commonly on both tree species and confirmed both our tree and insect species identity with systematists (G. Miller, USDA-SEL, L.J. Grauke, USDA-ARS, J. Brown, USDA-SEL). To the best of our knowledge, neither herbivore is recorded from another species of *Carya*.

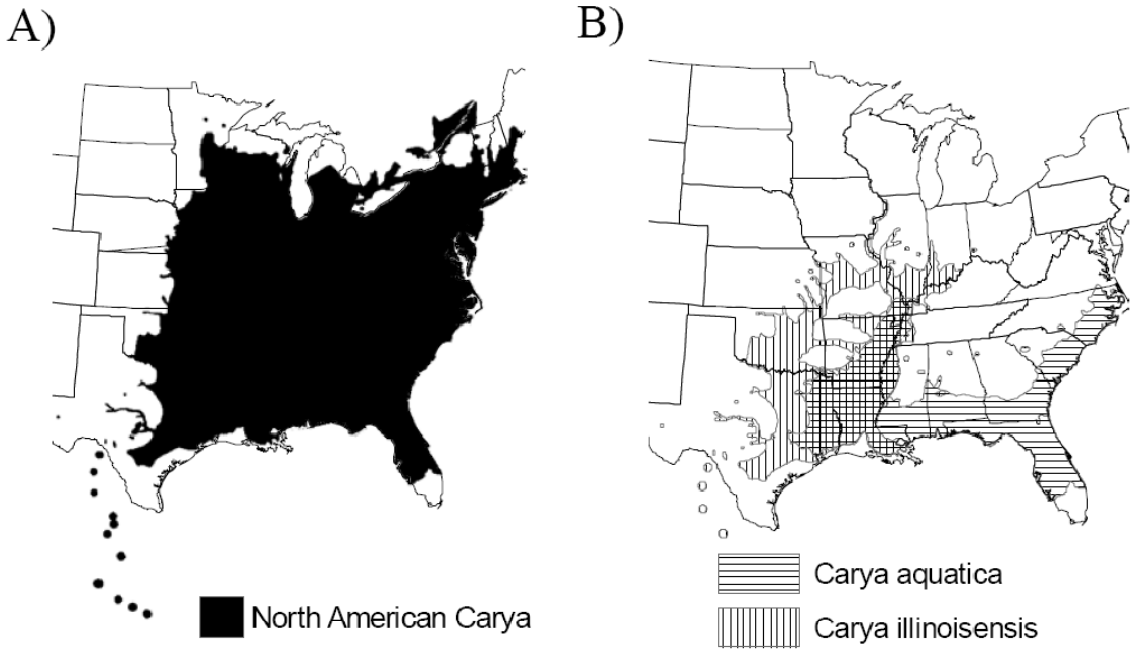


Figure 1: Distribution maps for A) the genus *Carya* in North America and B) pecan and water hickory; after Little (1971, 1977).

Insect sampling

The study area is a four county area in central Texas (Figure 2). Within this area both pecan and water hickory grow wild and pecan is also planted both as an ornamental and crop tree. Populations of each tree species were sampled for target insects throughout the growing season. For each insect, a minimum of three populations were sampled for each tree species (Table 2) with the two sites furthest apart for each tree species separated from one another by at least 80 Km. Our aim was to test the role of host plant species in promoting reproductive isolation of insects while accounting for geography. As a starting point, we sought to capture between fifteen and twenty individuals of each herbivore species from each tree species. After genotyping was completed, the data set was evaluated with the SESim method (Medina *et al.*, 2006) to determine if individual and marker sampling was adequate. If it was not, more molecular markers and/or more individuals could have been added to the project. Within this framework, we characterized at least three sites per host-plant species and sought to get herbivores from as many trees as possible within a site. We characterized sites and identified trees within each site which we sampled regularly throughout the summers of 2007 and 2008. As new sites and trees were discovered, we incorporated them into our sampling effort. Maximizing the number of trees represented within a site was also the goal when sub-sampling individuals for AFLP work. Where possible, each individual genotyped is from a different tree (Table 2).

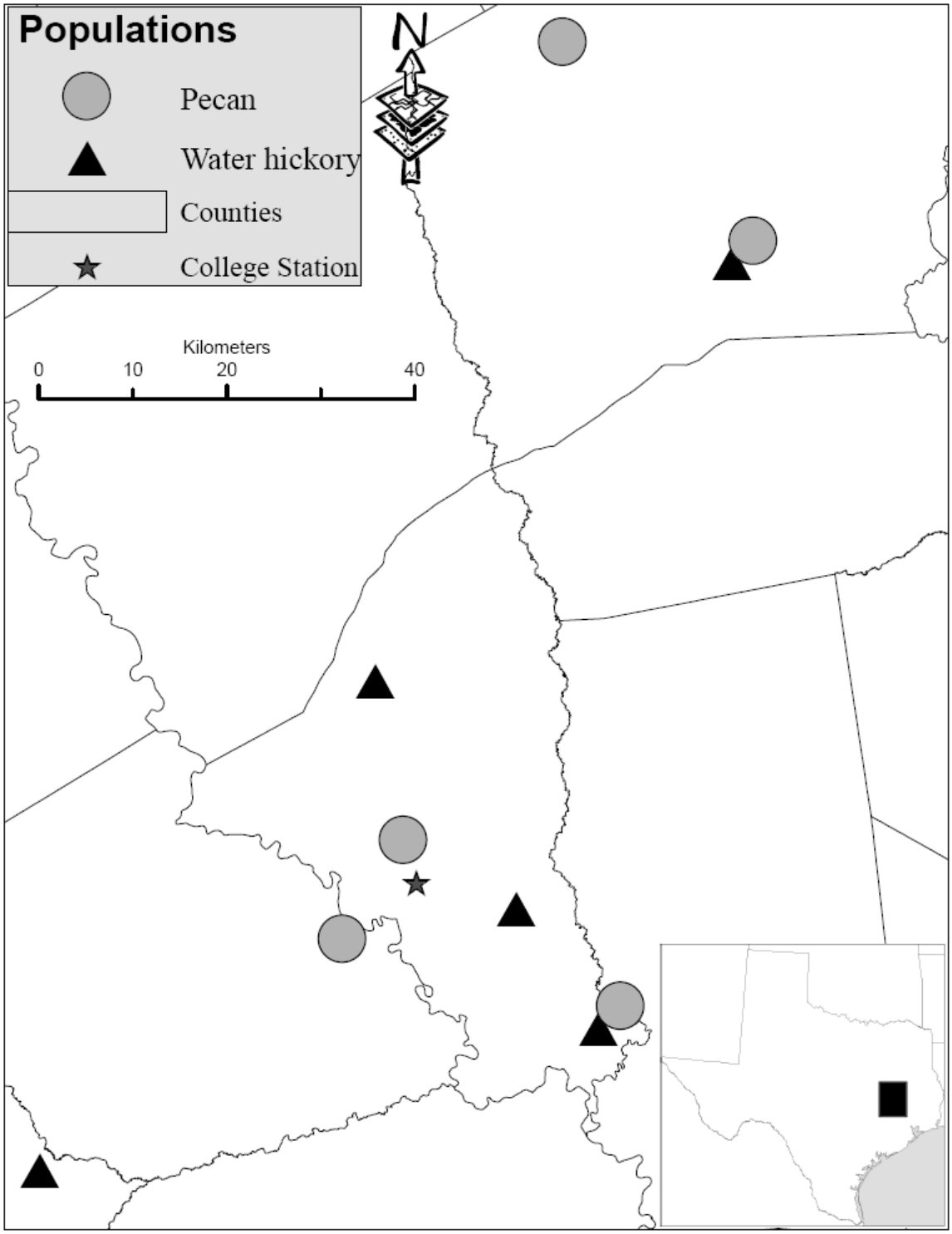


Figure 2: Location of pecan and water hickory populations sampled for pecan bud moth and yellow pecan aphid within the central Texas study area.

Table 2: Site names, locations, and number of insects genotyped per site.

Site	Location (degrees decimal)	Tree Species	Bud moths Genotyped	Yellow Aphids Genotyped
Somerville Wildlife Management Area	96.742 W 30.318 N	water hickory	2 (1 tree)	0
USDA-Pecan Genetics	96.434 W 30.517 N	pecan	10 (4 trees)	5 (5 trees)
North College Station	96.328 W 30.616 N	pecan		5 (3 trees)
Tabor	96.365 W 30.789 N	water hickory		4 (4 trees)
Lick Creek Park	96.222 W 30.561 N	water hickory		4 (4 trees)
Navasota	96.127 W 30.441 N	water hickory pecan	10 (5 trees) 7 (1 tree)	0 0
Jewett	96.147 W 31.346 N	pecan		5 (3 trees)
Fort Boggy State Park	95.979 W 31.189 N	water hickory pecan	7 (2 trees) 1 (1 tree)	5 (3 trees) 0

Insects were reared to adulthood in the laboratory to make sure the genotyping was not complicated by parasitoids and to make sure the insects genotyped had developed completely on the host plant species they were collected from. Pecan bud moths were collected as caterpillars during 2007 and 2008 from foliage and from inside *Phylloxera sp.* leaf galls and reared to adulthood in 2 ounce Cometware™ glasses (WNA, Covington, KY). Fresh leaflets from the caterpillar's host trees of origin were added weekly to the rearing glasses until pupation. Because we did not know if galls collected contained caterpillars until they emerged, survival data is not available for the period preceding emergence from galls. Following emergence from galls, survival of pecan bud moth was 100%. Yellow aphid nymphs were collected in 2008 from the underside of infested leaflets and reared to adulthood in 16 ounce Newspring DELItainer® (Pactiv Corp., Lake Forest, IL) containers with fresh leaflets from their host tree of origin provided weekly. Nymphs were commonly collected from large, mixed species infestations of aphids and were not counted or identified to species until maturation. Thus, laboratory survival rates on the two hosts were unknown. When individuals of both species matured, they were frozen at -80^{0}C for genetic analysis or saved for vouchers.

DNA isolation and AFLP reactions

Comparable numbers of each insect species were genotyped from each tree species (Table 2). Whole genomic DNA was extracted from individual insects using a DNeasy blood and tissue kit (Qiagen Corp., Valencia CA) following the manufacturer's

instructions. Amplified fragment length polymorphism (AFLP) profiles were generated from ~60ng of DNA from each sample (Vos et al., 1995, Saunders et al., 2001, Gompert et al., 2006) using the following selective primer pairs: *Mse*1-CTC/*Eco*R1-AAC, and *Mse*1-CAT/*Eco*R1-ACT. Polymerase Chain Reactions (PCR's) were run in GeneAMP® 9700 thermocyclers and diluted amplified selective products were submitted to fragment analysis on an ABI 3130 capillary sequencer with a co-loaded fluorescent (GeneScan™ 400HD [ROX™Dye]) size standard ladder (Applied Biosystems, Forest City, CA) according to manufacturer's instructions. Thermocycling conditions were as follows: the samples undergoing preselective amplification were held at 95^0C for 1 min followed by 20 cycles of 95^0C for 10 s, 56^0C for 30 s, and 72^0C for 90 s followed by a hold at 75^0C for 5 min. For the selective amplification, samples were held at 95^0C for 30 s followed by 47 cycles of 95^0C for 10 s, 65^0C-56^0C for 40 s, and 72^0C for 90 s, followed by a hold at 75^0C for 5 min. The second temperature in the selective amplification cycle started at 65^0C and was lowered by 0.7^0C for the first 12 cycles until it reached 56^0C. Absence of contamination was assured by negative controls and accuracy and repeatability of DNA fingerprints within species was verified by repeating all PCR steps for one individual from each species. For each insect and selective primer combination, resulting electrophenograms were examined and analyzed using GeneMapper® 4.0 (Applied Biosystems, Forest City, CA) with the default allele calling threshold of 100 reflectance units. Selective primer combinations were then consolidated into a single 1/0 matrix for each insect species.

The SESim statistic (Medina et al., 2006) was calculated for each insect species to determine if individual and molecular marker sampling was adequate for the host associated differentiation study. The two selective primer combinations produced 235 and 79 polymorphic loci for pecan bud moth and yellow pecan aphid respectively which gave SESim values of 0.034 and 0.038 for pecan bud moth and yellow pecan aphid respectively. Since Medina et al. (2006) showed that population structure began to break up due to inadequate sampling when SESim values were greater than 0.05, we determined that marker and individual sampling was adequate for our study.

Data analysis

The AFLP phenotype data matrix for each insect species was analyzed independently. Bayesian cluster analyses were executed in STRUCTURE 2.2 (Pritchard et al., 2007) using the recessive alleles model for dominant marker data assuming admixture and correlated alleles (Falush et al., 2007). Admixture is a general attribute of most species occurring in sympatry and the "alleles correlated" model deviant has been shown to be the most sensitive to the presence of population structure in simulated data (Falush *et al.*, 2003). STRUCTURE assumes that within a population, loci are in Hardy-Weinberg equilibrium and linkage equilibrium and assigns individuals to separate populations so as to eliminate violations of these assumptions. The output of STRUCTURE is the log probability of the data (X) given the number of clusters (K) assumed or [Ln Pr(X|K)]. Where parameter estimates indicated K>1, the *ad hoc* ΔK statistic (Evanno et al., 2005) was used to predict the most likely number of clusters (K) in the data. This involves

calculating the second-order rate of change of [Ln Pr(X|K)]. Evanno *et al.* (2005) showed that the K corresponding to a spike in this value accurately predicts the number of populations represented by the data. Additionally, the most probable number (K) of clusters present in the data was determined using Bayes' law to calculate the probability of the number of clusters (K) given the data (X) or Pr(K|X); equation 4 in Pritchard *et al.* (2007). The model was run for 100,000 generations with a burn-in period of 10,000 generations for 20 iterations each from K=1 to K= 7. Because pecan bud moth had a high number of alleles at low frequency, the parameter lambda was first inferred (Pritchard *et al.*, 2007) and found to be 0.43. The default lambda of 1.0 was used for yellow pecan aphid.

AFLPsurv 1.0 (Vekemans *et al.*, 2002) was used to estimate genetic diversity, percent polymorphic loci, and Fst among host plant species and among collecting sites using Bayesian analyses with a non-uniform prior distribution of loci (Zhivotovsky, 1999) and the estimation procedures of Lynch & Milligan (1994). The estimated Fst values were tested against the null hypothesis Fst = 0 using 9,999 random permutations of the data. AMOVA (Excoffier *et al.*, 1992) and principal coordinates analyses (PCO) were conducted among host plants and among collecting sites within host plants using the software GenAlEx 6.2 (Peakall & Smouse, 2006). For pecan bud moth, the Somerville and Fort Boggy pecan samples were removed prior to the AMOVA due to low sample sizes but they were retained in separate AMOVA analyses testing host plant and site separately.

Results

Pecan bud moth

Runs of STRUCTURE 2.2 indicated that the model parameter, alpha, fluctuated greatly over the course of a STRUCTURE run, an indication of a lack of population structure (Pritchard et al., 2007). Additionally, all individuals were assigned with relatively equal probability to both hypothetical populations when K was set to 2 (Figure 3), further indication of a lack of population structure. The most probable number of populations was K=1; Pr(K|X) = 1. Triangle plots (not shown) also produced single clusters, even when running STRUCTURE for K>1.

Expected heterozygosity is similar among sites and among host plants (Table 3) and Fst is not significantly different from zero by site (Fst=0.0188, P=0.152) or by host plant (Fst=0.0017, P=0.1425). 99% of molecular variation occurred within collecting sites (Table 4) and there was no significant effect of host plant species or collecting sites. Principal coordinates 1 and 2 explain 44.97% of the molecular variation and show no pattern by either host plant or collecting site (Figure 4A).

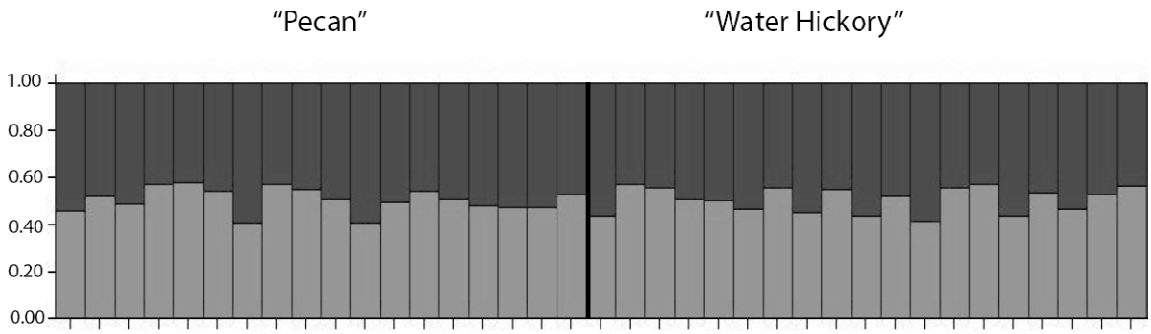


Figure 3: Bayesian population assignment probabilities (y-axis) for pecan bud moth individuals (x-axis) collected from pecan and water hickory using the recessive alleles model for dominant marker data in STRUCTURE 2.2. All individuals are assigned with relatively equal probability to both hypothetical populations when two populations are assumed indicating a lack of population structure.

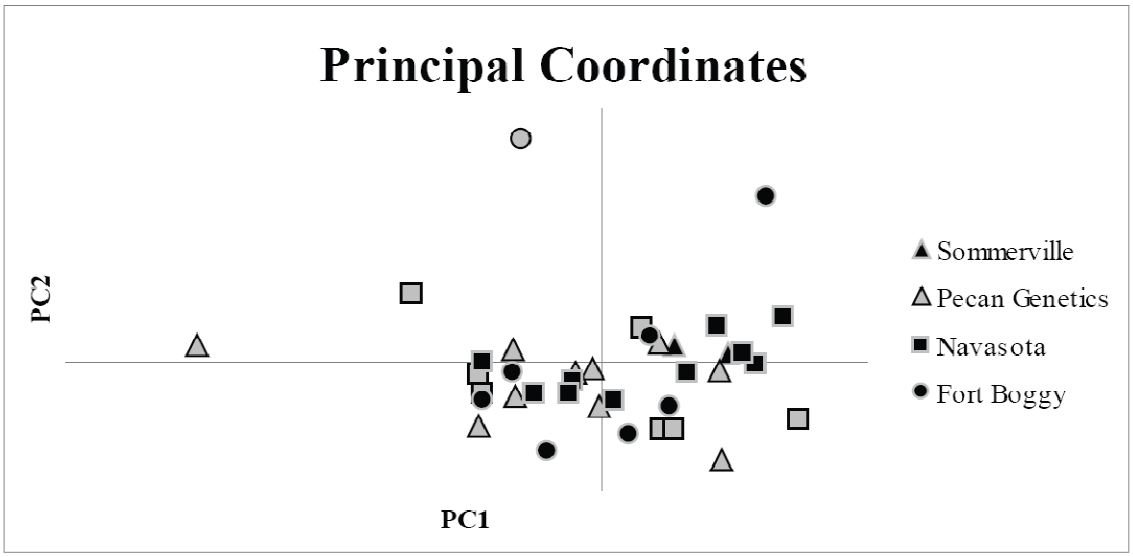
Table 3: Diversity statistics for pecan bud moth and yellow pecan aphid. N=sample size, #Loci=number of polymorphic AFLP loci genotyped for the species, #PL=number of polymorphic loci for a given collecting site or host plant, %PL=percent polymorphic loci, and Hj=expected heterozygosity (Nei's gene diversity) for a given collecting site or host plant.

	N	#Loci	#PL	%PL	Hj
Pecan Bud moth					
Collecting Site					
Somerville	2	235	73	31.1	0.18897
Pecan Genetics	10	235	171	72.8	0.17494
Navasota	17	235	140	59.6	0.17626
Fort Boggy	8	235	151	64.3	0.18357
Host Plant					
Pecan	18	235	144	61.3	0.17302
Water Hickory	19	235	159	67.7	0.17237
Yellow Pecan Aphid					
Collecting Site					
Pecan Genetics	5	79	56	70.9	0.24107
College Station	5	79	46	58.2	0.1869
Tabor	4	79	52	65.8	0.24301
Lick Creek Park	4	79	49	62	0.20309
Jewett	5	79	55	69.6	0.20609
Fort Boggy	5	79	49	62	0.20047
Host Plant					
Pecan	15	79	56	70.9	0.19195
Water Hickory	13	79	52	65.8	0.2006

Table 4: Variance components (VC) and percent molecular variation due to host plant and collecting sites within host plant for pecan bud moth and yellow pecan aphid from AMOVA. Significance testing was done using 9999 permutations of the binary distance parameter φPT, an analog of Fst in GenAlEx 6.2.

AMOVA	Pecan bud moth			Yellow pecan aphid		
Source of variation	VC	% variation	P-value	VC	% variation	P-value
Among host plants	0.270	1%	0.160	16.665	71%	<0.001
Within host among sites	0.000	0%	0.670	0.215	1%	0.314
Within collecting sites	26.406	99%		6.520	28%	

A) Pecan bud moth from pecan (grey) and water hickory (black)



B) Yellow pecan aphid from pecan (grey) and water hickory (black)

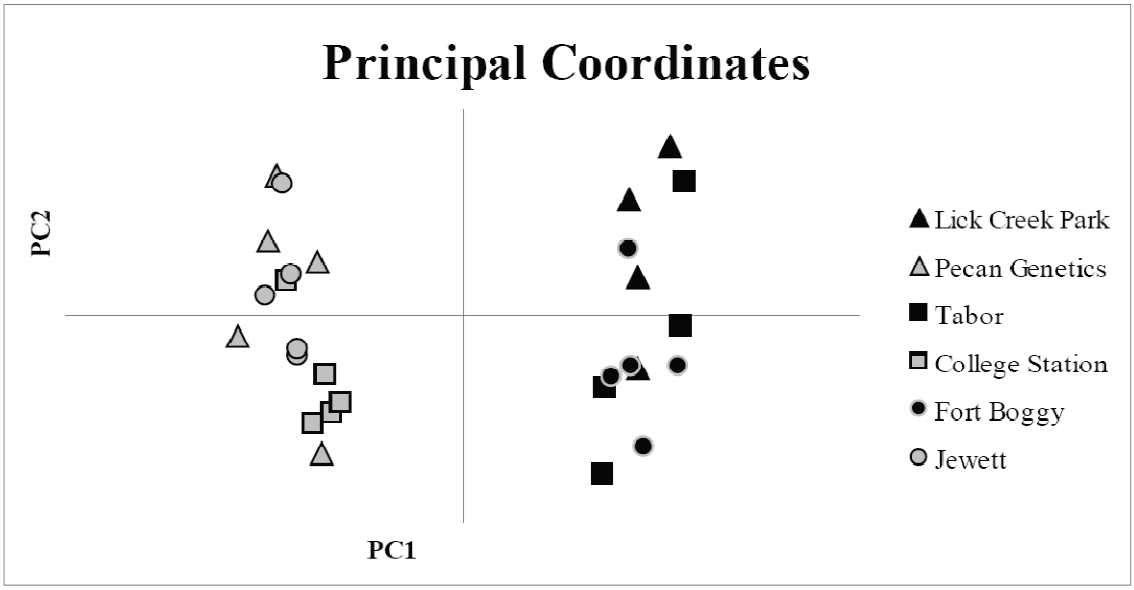


Figure 4: Eigenvectors of principal coordinates 1 (x-axis) and 2 (y-axis) for A) pecan bud moth and B) yellow pecan aphid. Symbol shapes denote collecting sites and symbol colors denote host plant species. PC 1 separates the water hickory (black) and pecan (grey) populations of yellow pecan aphid.

Yellow pecan aphid

At K=2, all individuals were assigned with high (>99.7%) probability to one of the two populations (Figure 5). Furthermore, the two populations detected by STRUCTURE corresponded exactly to host-plant species of origin. Ten loci were diagnostic for the pecan host race and seven loci were diagnostic for the water hickory host race (Table 5). An additional six loci were strongly host-plant associated, being present in >90% of individuals from the associated host, and in <10% of individuals from the host's congener (Table 5). The second order rate of change in K, (Evanno's ΔK) peaked for K=2 populations (data not shown) further indicating that two populations best explained the yellow pecan aphid data. The model produced gradually increasing values of LnPr(X|K) with K>2 but in these cases, all individuals were assigned with high (>86%) probability to one of the two host plant associated populations.

Expected heterozygosity is similar among sites and among host plants (Table 3). The Fst among host plants is significantly different from zero (Fst=0.5752, P<0.0001) but among sites within host plant species Fst is not significantly different from zero (pecan by site Fst=-0.0029, P=0.6550; water hickory by site Fst=0.0154, P=0.3143). 71% of molecular variation occurred among host plants (Table 4) but only 1% of molecular variation occurred among sites within host plant species (Table 4). Principal coordinates 1 and 2 explain 88.87% of the molecular variation with principal coordinate 1 strongly segregating aphids by host plant species (Figure 4B).

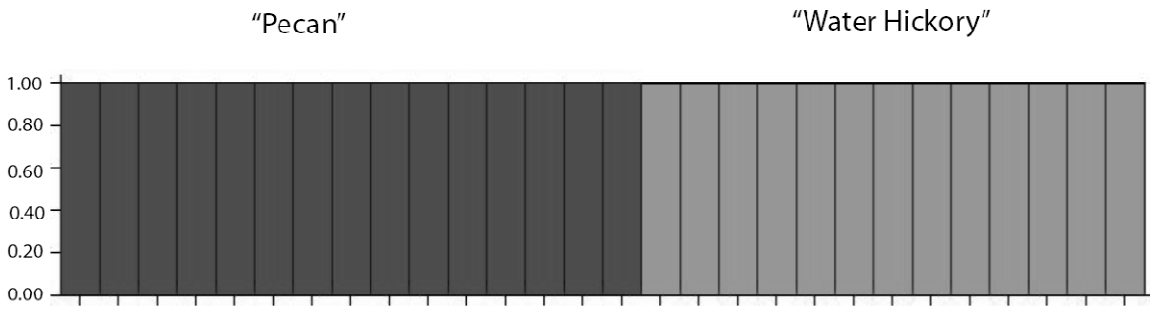


Figure 5: Bayesian population assignment probabilities (y-axis) for K=2 populations of yellow pecan aphid individuals (x-axis) collected from pecan and water hickory using the recessive alleles model for dominant marker data in STRUCTURE 2.2. Two host associated populations (light grey and dark grey) are indicated.

Table 5: Frequency of the twenty three most strongly host associated AFLP loci in the pecan and water hickory host races of yellow pecan aphid. The selective primer combination used and the size of the DNA fragment in base pairs comprise the locus name.

Locus	Pecan	Water hickory
Mse1-CAT/EcoR1-ACT _90	100%	0%
Mse1-CAT/EcoR1-ACT _103	100%	0%
Mse1-CAT/EcoR1-ACT _136	100%	0%
Mse1-CAT/EcoR1-ACT _193	100%	0%
Mse1-CAT/EcoR1-ACT _263	100%	0%
Mse1-CTC/EcoR1-AAC _63	100%	0%
Mse1-CTC/EcoR1-AAC _78	100%	0%
Mse1-CTC/EcoR1-AAC _86	100%	0%
Mse1-CTC/EcoR1-AAC _232	100%	0%
Mse1-CTC/EcoR1-AAC _268	100%	0%
Mse1-CAT/EcoR1-ACT _57	93%	0%
Mse1-CAT/EcoR1-ACT _236	93%	0%
Mse1-CTC/EcoR1-AAC _59	7%	92%
Mse1-CAT/EcoR1-ACT _176	0%	92%
Mse1-CTC/EcoR1-AAC _83	0%	92%
Mse1-CTC/EcoR1-AAC _116	0%	92%
Mse1-CAT/EcoR1-ACT _62	0%	100%
Mse1-CAT/EcoR1-ACT _141	0%	100%
Mse1-CAT/EcoR1-ACT _147	0%	100%
Mse1-CAT/EcoR1-ACT _214	0%	100%
Mse1-CTC/EcoR1-AAC _88	0%	100%
Mse1-CTC/EcoR1-AAC _113	0%	100%
Mse1-CTC/EcoR1-AAC _195	0%	100%

Discussion

The parthenogen and the quasi-endophage

We conclude panmixia for pecan bud moth among populations sampled from pecan and water hickory. We also conclude that yellow pecan aphid exhibit host associated differentiation and consists of at least two distinct host races, one feeding on pecan, and one feeding on water hickory. Host races are diagnostic at seventeen out of seventy nine (>20%) of polymorphic loci indicating strong barriers to gene flow between them (Table 5). A possible reason for the presence of HAD in yellow pecan aphid but not in pecan bud moth could be parthenogenesis. If a mutant aphid colonizes a novel host, that mutation can be amplified greatly over the course of a growing season. When males are produced, inbreeding is likely and will occur on the novel host (Dixon, 1998). This scenario may be particularly prevalent in monecious aphids (aphids which do not migrate to a second host plant for reproduction) and yellow pecan aphid is monecious. Sexual females are apterous and cannot leave the natal host. In contrast, males, while winged, may suffer a fitness penalty for leaving the natal host if they have the same mutation for host use as their mother. Male aphids inherit the entire genome minus one X chromosome and so are likely to inherit such mutations intact (Ward, 1991, Guldemond & Mackenzie, 1994). Unlike the parthenogenetic yellow pecan aphid, pecan bud moth reproduces sexually. Compared to parthenogens, sexually reproducing organisms experience more gene flow, which counteracts differentiation (Slatkin, 1973, Hendry et al., 2001, Nosil, 2009).

Endophagy has also been implicated in the propensity to show HAD and pecan bud moth, a quasi-endophage, was expected to show HAD due to its intimate larval association with *Phylloxera* galls. Despite this, the pecan bud moth population was found to be panmictic and did not consist of host associated populations. Furthermore, yellow pecan aphid is not an endophage and yet does show strong evidence for pecan and water hickory host associated populations.

Several authors have suggested univoltinism as one factor facilitating HAD because univoltine insects must be linked very tightly to the phenology of their hosts (Nyman, 2002, Mopper, 2005). For example, the apple and hawthorn host races of the univoltine apple maggot break diapause to coincide with the fruiting of their respective hosts as does their parasitoid (Feder & Filchak, 1999, Forbes et al., 2009). In contrast, neither of the insects under study is strictly univoltine; both are present and feeding on foliage throughout the growing season. However, yellow pecan aphid males are univoltine, only produced late in the season. Tedders (1978) found males from October 14th to November 22nd on pecan in Georgia. This type of sexual univoltinism is a general feature of the biology of aphids and may contribute to aphid susceptibility to HAD. Thus, if the timing of sex is coupled to differential host-plant phenologies, reduced gene flow between host races should be favored over time (Stam, 1983, Butlin, 1990, Emelianov et al., 2003).

Some authors argue that enemy free space can be an important driver of diversification (Feder, 1995, Stireman et al., 2008). Unfortunately, the dominant predators of pecan bud

moth are not known and no parasitoids have been documented nor were any reared during this study. Thus, we can say very little at this time regarding the possibilities of third trophic level effects on pecan bud moth on either host plant species. We reared two parasitoids from yellow pecan aphid, *Aphelinus perpallidus* (Gahan) and an unidentified braconid. *A. perpallidus* was the most common parasitoid accounting for >99% of parasitized aphids. Documenting parasitism rates of yellow pecan aphid by this parasitoid is ongoing but preliminary data suggests that parasitism rates are similar between the two host races (likelihood ratio test χ^2 =0.016, P=0.9, df=1; 13 parasitoids from n=109 aphids on pecan, eight parasitoids from n=71 aphids on water hickory). Thus, preliminary data suggests that neither host race of pecan aphid enjoys reduced attack, at least from *A. perpallidus*.

Pecan bud moth and yellow pecan aphid HAD tests represent an informative comparison because they are herbivorous insects on the same host-plant species pair in the same geographic location. However, this comparison is not without caveats, first, it is but a single data point and more species should be tested within this host-plant system to determine the extent to which generalities can be made. Second, this was a mesoscale geographic study and did not encompass the entire geographic range of the insects in question or their host plants. Therefore, our findings may not translate to larger geographic scales, or to other localities. Also, pecan bud moth feeds on foliage and inside buds in addition to *Phylloxera* gall tissue (Mizell & Schiffhauer, 1986). Thus, while it may be common to find pecan bud moths in *Phylloxera* gall tissue, perhaps it

should not be considered an obligate endophage for the sake of comparison in the way that the inquiline beetle, *Mordellistena*, is on *Eurosta* goldenrod galls (Eubanks et al., 2003).

Aphids in general exhibit very strong host specificity with 99% of species considered specialists (Eastop, 1973). We found literature reports of host races or host associated aphid genotypes in 18 aphid species (Table 6). These reports extend back over 150 years when Francis Walker recorded a host switch of *Aphis persicae* from sloe to peach following the introduction of the latter to England (Walker, 1850). Most cases of HAD in aphids involve food crops but recently, Peccoud (2009) found 11 host races of the pea aphid *Acyrthosiphon pisum* throughout Europe, eight of which were specific to wild hosts. This suggests that genetic diversification of aphids on wild host plants is probably much greater than previously thought. Our results add further evidence that surveys of wild host plants are likely to result in the discovery and genetic resolution of increased numbers of host differentiated aphids.

Table 6: Aphids showing host-associated differentiation.

Aphid Species	Hosts	Author
Acyrthosiphon malvae	at least 4 host races	(Dixon, 1998)
Acyrthosiphon pisum	at least 11 host races	(Peccoud et al., 2009)
Acyrthosiphon solani	multiple hosts	(Dixon, 1998)
Amphorophora sp.	*rubus sp.*	(Blackman et al., 1977)
Aphis fabae	*Vicia sp., Tropaeolum sp.*	(Dixon, 1998)
Aphis frangulae	multiple hosts	(Dixon, 1998)
Aphis gossypii	at least 5 host races	(Carletto et al., 2009)
Aphis spirea	*Citrus sp., Spirea sp.*	(Dres & Mallet, 2002)
Cryptomyzus galeopsidis	*Ribes sp.*	(Dres & Mallet, 2002)
Daktulosphaira vitifoliae	*Vitis sp.*	(Downie et al., 2001)
Dysaphis crataegi	*Crataegus sp., Daucus sp.*	(Dixon, 1998)
Eriosoma yangi	*Ulmus sp.*	(Akimoto, 1988)
Hyalopterus amygdale	*Prunus sp.*	(Lozier et al., 2007)
Monelliopsis pecanis	*Carya sp.*	(Dickey & Medina, 2010)
Myzus cerasi	*Prunus sp.*	(Guldemond & Mackenzie, 1994)
Myzus persicae	*Nicotania sp.*, multiple hosts	(Margaritopoulos et al., 2007)
Pemphigus bursarius	*Lactuca sp.* and *Matricaria sp.*	(Miller et al., 2005)
Schizaphis graminum	at least 3 host lineages	(Anstead et al., 2002)
Sitobion avenae	at least 3 host lineages	(Vialatte et al., 2005)
Tetraneura yezoensis	*Ulmus sp.*	(Akimoto, 1990)
Uroleucon sp.	*Centaurea sp., Cirsium sp.*	(Guldemond & Mackenzie, 1994)

Testing the importance to HAD of parthenogenesis *per se* will be challenging because there are other aphid traits proposed to facilitate HAD; host alternation, host specificity, and the relative commonness of apterous sexuals (Dixon, 1998), in addition to univoltinism of sex (mentioned previously). Furthermore, apomictic parthenogenesis is likely the ancestral state of the Aphidomorpha (Dixon, 1998) and it is not common to other herbivorous taxa making it difficult to test for HAD in taxonomically diverse parthenogens as one can with taxonomically diverse endophages. Two possible exceptions to this could be thrips (Order Thysanoptera), and sawflies (Family Tenthredinidae), some of which have been shown to be parthenogenetic (Arakaki et al., 2001, Muller et al., 2004, Nault et al., 2006). There have been five species of thrips and three species of sawfly documented on pecan (Smith et al., 1996, Ree, Pers. Comm.). Pecan and water hickory share at least one sawfly species, *Periclista marginicollis* (Norton) but it is not parthenogenetic. In addition to aphids, future impetus should be given to uncovering taxonomically diverse parthenogens in this system if possible. If none are found, shared haplo-diploid herbivores such as mites (Order Acari), sawflies (Family Tenthredinidae), and thrips should be tested for HAD since haplo-diploidy is predicted to promote a level of recombination intermediate to that of parthenogenesis and sexual reproduction (Hartl, 1972).

Carya *hickories: an ideal system for HAD investigation*

The North American hickories selected for this study offer many advantages for HAD study; 1) They are native, 2) They are long lived trees, 3) They are sympatric over a

fairly large part of their native range, 4) They are ecologically similar – both bottomland species and so can be found in mixed stands, 5) They are genetically similar – both are diploid as opposed to tetraploid *Carya* species, and 6) They host many herbivores (over 400 species documented on pecan alone). The results from our first two shared species tested provide impetus to test additional parthenogens in this system. Pecan and water hickory share at least seven species of aphids, three of them endophagous phylloxerans (B. Ree, Pers. Com.; A. Dickey, Pers. Obs.). It will be interesting to see if the abundance of shared parthenogens will make this system a good counterpoint to the *Solidago* system which has an abundance of shared endophages.

CHAPTER III

SELECTION AGAINST MIGRANTS TO THE ALTERNATIVE HOST MAINTAINS HOST-ASSOCIATED DIFFERENTIATION IN A TREE DWELLING APHID

Synopsis

Host Associated Differentiation (HAD) is the formation of genetically divergent host associated populations and may be an important driver of parasite biodiversity. A feature of studies testing HAD is the study of sympatric populations associated with different hosts, however, simply documenting the presence of host-related genetic structure in a species neither confirms nor denies that the populations arose in sympatry. Despite this, it raises the question of which mechanism or mechanisms maintain such structure despite the proximity of populations in space and time. We tested for selection against migrants to the alternative host plant in yellow pecan aphid *Monelliopsis pecanis* using a laboratory reciprocal transplant experiment. Aphids fed leaflets from the alternative host species enjoyed ~22% of the lifetime fecundity and their nymphs had ~11% of the viability of aphids who were fed leaflets from the natal host species. This selection regime, repeated over multiple clonal generations prior to sexual reproduction in the fall, would provide a strong pre-mating isolation barrier to yellow pecan aphid host races. Although selection against migrants has been tested in the model pea aphid, this is the first such explicit test for an exclusively tree dwelling aphid.

Introduction

Host-associated differentiation (HAD) or the formation of genetically divergent host associated sub-populations (Bush, 1969, Abrahamson et al., 2003) has been postulated as a process that could explain insect diversity (Funk et al., 2002, Mitter et al., 1988, Stireman et al., 2006). HAD is a special case of ecological speciation (Schluter, 1996, Schluter, 2001, Rundle & Nosil, 2005) where the disruptively selective habitats are defined largely by different host plant species (Dres & Mallet, 2002). Although several examples of this phenomenon have been reported, the mechanisms by which reproductively isolated populations are maintained in the face of gene flow have been studied in relatively few study systems. Sources of reproductive isolation between host associated populations are subdivided into premating, prezygotic and postzygotic barriers based on when in the life-history of the organism they operate (Coyne & Orr, 2004, Nosil et al., 2005). Selection against migrants to an alternative habitat is one of the first reproductive isolating mechanisms to evolve during ecological speciation (Nosil et al., 2005, Hendry et al., 2007) but it is often a pleiotropic effect of adaptation to those divergent habitats. These habitats could be phenologically divergent (Feder & Filchak, 1999, Wood & Keese, 1990, Bearhop et al., 2005), chemically divergent (Nolte et al., 2006, Jain & Bradshaw, 1966, Silvertown et al., 2005), and/or structurally divergent (Lode, 2001, McNett & Cocroft, 2008). In the well known case of *Rhagoletis pomonella*, 'apple' and 'hawthorn' host races are adapted to phenologically divergent habitats since fruiting in the two host plants is separated by around three weeks (Feder & Filchak, 1999). These flies are also adapted to chemically and structurally divergent habitats

since the two host fruits produce different volatile blends and are of different sizes (Forbes & Feder, 2006).

Although within a species, some aphid lineages reproduce entirely asexually, most species have a brief, late season sexual phase and are thus considered cyclical parthenogens (Dixon, 1998). Selection against migrants to an alternative host plant habitat is more easily quantified in cyclically parthenogenetic taxa such as aphids than in obligate sexual taxa because mating is not a prerequisite for measuring fecundity of mothers or viability of their offspring. In the well known pea aphid *Acyrthosiphon pisum*, selection against migrants (Via et al., 2000) is one of several ecologically based reproductive isolating mechanisms quantified on two of its hosts in North America, alfalfa and clover (Caillaud & Via, 2000, Del Campo et al., 2003, Ferrari et al., 2006, Via, 1999, Via & Hawthorne, 2002). Selection against migrants has also been demonstrated for several host races of cotton aphid *Aphis gossypii* (Guldemond et al., 1994, Carletto et al., 2009) although not by quantifying fitness of individuals. These two aphid species are generally associated with herbaceous plants or are host-alternating (reproducing and overwintering in trees but spending most of the warm season feeding on herbaceous plants). In contrast, selection against migrants has not been tested in an exclusively tree dwelling aphid.

Recently, we showed that yellow pecan aphid *Monelliopsis pecanis* Bissel (Hemiptera: Aphididae) exhibits host associated differentiation on two different host plant species,

pecan (*Carya illinoinensis* Koch) and water hickory (*C. aquatica* Michx) (both Fagales: Juglandaceae) at a geographic mesoscale (Dickey & Medina, 2010). Host associated populations of the yellow aphid are divergent at more than 22 AFLP loci indicating strong barriers to gene flow between them. Cyclically parthenogenic insects, such as aphids, may develop host dependent lineages faster than sexually reproducing insects because favorable mutations can become fixed more quickly when sex is limited (Hartl, 1972, Lynch, 1984, Neiman & Linksvayer, 2006). Since yellow pecan aphid is a holocyclic aphid species, males and sexual females are only produced in the fall restricting sex to a single season. Thus, prior to sex, rapid succession of asexual generations can amplify and accelerate their response to selection (King, 1993, King & Murtaugh, 1997, Loxdale, 2008, Lynch & Gabriel, 1983, Vialatte et al., 2005). These arguments may also apply for maintaining host association in the face of potential gene flow provided by the sympatric occurrence of host-associated populations.

Yellow pecan aphids are restricted to North American *Carya sp.* (Blackman & Eastop, 1994, Quednau, 2003). Apterous sexual females and winged sexual males are only produced during the last month prior to leaf loss in the fall (Tedders, 1978). Prior to the opportunity for sexual reproduction, yellow pecan aphid will have between 22 and 32 overlapping generations of viviparous parthenogens (Tedders, 1978). All of these viviparae are winged and thus, have the potential to migrate from their natal host plant species to a novel host plant species providing the potential for selection to act directly on the fitness of the migrant and the viability of her nymphs if the ‘wrong’ choice is

made. That all viviparae are winged is a common feature of tree dwelling aphids unlike herb feeding species which are often unwinged until prompted to produce alates due to crowding (Moran, 1988, Hales et al., 1997, Dixon, 1998). Pecan and water hickory are not only sympatric over a portion of their geographic range (Figure 1), but also syntopic, co-occurring occasionally in bottomland hardwood forests (Dale et al., 2007) further increasing the potential for between tree species migrations of yellow pecan aphid.

To test for selection-against-migrants in yellow pecan aphid, we conducted a reciprocal transplant feeding experiment. We measured fecundity and longevity of wild collected mothers (fecundity selection), and measured development time and proportion survival of their nymphs (viability selection) on both their natal tree species and its congener. This design allowed us to implement "forced migrations" (Via et al., 2000) of winged aphids to two potential host plant habitats, one with leaflets of the natal species and one with leaflets of the alternate species, and quantify their fitness. Selection against migrants could operate on yellow pecan aphid because all parthenogenetic females are winged and thus potentially migratory (Tedders, 1978). Selection against migrants could prevent colonization of the alternative host and favor those individuals which are able to discriminate between host plant species. This selection, if present, could act both on the fecundity of the parthenogenetic mother, and subsequently on the viability of her daughters born on the chosen plant.

Methods

Fecundity and longevity measurements

Wild, yellow pecan aphid nymphs were collected from three central Texas sites 8-Jul-2009 through 11-Jul-2009. From Site 1: Lick Creek Park, Brazos County (96.222^0 W 30.561^0 N), nymphs were collected from water hickory only. From Site 2: Fort Boggy State Park, Leon County (95.979^0 W 31.189^0 N), nymphs were collected from both pecan and water hickory. And from Site 3: USDA Pecan Genetics, Burleson County (96.434^0 W 30.517^0 N), nymphs were collected from pecan only. Because nymphs generally feed singly on leaflets (Tedders, 1978), and multiple trees were sampled at each site, we assume that the nymphs collected represent multiple clonal lineages within each site. Nymphs were brought back to the laboratory and reared to adulthood on excised leaflets from their natal host tree. Upon alation, aphids were 'migrated' to individual 473 ml Newspring DELItainer® (Pactiv Corp., Lake Forest, IL) containers in a reciprocal transplant design. Experimentally caged adults were set up for the first 100 hours following nymph collection except for those from Lick Creek Park which were set up for the first 120 hours following nymph collection to achieve more similar sample sizes from each site. For sample sizes see Table 7. When set up, aphids were fed 1.0g of freshly excised leaflets of either pecan or water hickory (migration treatments). 0.5g of fresh leaflets was added to each container every other day and old leaflets were removed as they browned. The leaflets for experimental rearing came from nine adult pecan trees and nine adult water hickory trees present in a common garden at Texas A&M University's Eli Whitely Medal of Honor Park (96.349^0 W30.615^0 N). Each day

throughout the experiment, leaflets were harvested from three randomly selected trees of each species. Aphid containers were haphazardly arranged and maintained in a room with a 12H:12H Light: Dark cycle. The room was heated and lit by a 1000W metal halide bulb so temperature fluctuated between 26^0C (immediately prior to the light phase) and 35^0C (average 30^0C) which closely approximated natural temperature fluctuations during the study. Nymphs were counted and removed daily and both adult longevity and total fecundity was summed for each aphid. The experiment ran until the last aphid died on 7-Aug-2009.

Viability and development time measurements

Firstborn nymphs from each aphid adult (those produced in the first 24-48 hours) were set up for rearing under the same husbandry conditions as the adults. Nymphs from the same mother were kept together so the number of nymphs per cup ranged from one to eleven. The proportion surviving to adulthood (viability) was recorded for each cohort, and development time was measured for each surviving nymph (sample sizes in Table 7).

Table 7: Sample sizes from each site for the yellow pecan aphid reciprocal transplant experiment. The numbers in each box represent respectively; the number mothers "migrated", the number of nymph cohorts used to compare viability, and the number of individual nymphs used to measure development time.

	Transplanted Host	
Original Host	pecan	water hickory
water hickory (site 1)	**10 / 7 / 0**	**11 / 9 / 4**
water hickory (site 2)	**10 / 11 / 2**	**11 / 11 / 17**
pecan (site 2)	**13 / 9 / 3**	**12 / 5 / 0**
pecan (site 3)	**18 / 15 / 9**	**18 / 13 / 2**

Data analysis

All statistical tests were conducted using SPSS 14.0 for Windows (SPSS Inc). Fecundity, longevity, offspring viability, and offspring development time were analyzed using restricted maximum likelihood (REML) mixed linear models. Host plant species of origin (original host), host plant species of transplant (transplanted host), and the interaction of the two were treated as fixed effects in the models. Collecting site and the number of days between nymph collection and alation were treated as random effects. For the offspring viability and development models, the size of the initial cohort was treated as an additional random effect. SPSS also models the residual (data value minus predicted value) as a covariance parameter. Random effects were removed from a model if non-significant. In SPSS, the significance of variance among levels of fixed effects in each model are assessed using an F-test and the significance of variance among levels of random effects are assessed using a Wald statistic. For the fecundity and viability models, estimated marginal means were used from the mixed models to calculate selection against migrants relative to residents (Via et al., 2000). Post-hoc significant tests between treatment means were conducted with Mann-Whitney U tests. Estimated Marginal Means (EMM) of fecundity and viability were used to calculate relative fecundity and viability with respect to: 1) Natal host; e.g., to calculate relative fecundity for aphids originating from water hickory, we divided the fecundity of aphids migrating to an alternative host (reared on pecan) by the relative fecundity of aphids migrating to a natal host (reared on water hickory) (Via et al., 2000); and 2) Alternative host; e.g., to calculate the relative viability for migrants to pecan, we divided the offspring viability of

aphids originating from water hickory (migrants) by the offspring viability of aphids originating from pecan (residents) (Via et al., 2000). We also calculated fecundity selection against migrants, which is 1 minus the relative fecundity with respect to the alternative host, and viability selection against migrants' daughters, which is 1 minus the relative viability with respect to the alternative host.

Results

REML models of fitness variables and post hoc comparisons among treatments

Yellow pecan aphids had significantly lower fecundity, longevity and offspring viability on the alternative host relative to the natal host (original host * transplanted host interaction, $p<0.001$ for each variable, Table 8, Figure 6).This did not hold true for nymph development time ($p=0.150$, Table 8), however, only two nymphs from each alternate host transplant treatment survived to maturity. There was also a significant effect of original host and transplanted host on longevity (original host, $p=0.017$; transplanted host, $p= 0.048$) and fecundity (original host, $p=0.002$; transplanted host, $p=0.003$) (Table 8). The residual was the only significant random effect in all REML models (Wald Test, $p<0.001$ for fecundity, longevity, viability, and development time). The effects of collecting site and the number of days between collection and assay were insignificant and so these random effects were removed from the model. There was a significant effect of transplanted host on development time in the model ($p<0.001$, Table 8). This was because nymphs took longer to develop on pecan (Mann-Whitney U test, $p<0.03$; Figure 6). The remainder of comparisons between pairs of transplant treatments

was as expected from the results of the REML models for fecundity, viability, and longevity (different letters in Figures 6a, 6b, 6c respectively; $p<0.03$).

Relative fecundity, relative viability, and selection against migrants

The estimated marginal means for fecundity (in number of nymphs) and viability (in percent survival to maturity) respectively for each transplant treatment are as follows: water hickory to water hickory (16.682, 25.8%), water hickory to pecan (2.750, 2.3%), pecan to pecan (7.129, 18.8%), and pecan to water hickory (2.500, 2.2%). Thus, by natal host, water hickory aphids migrating to an alternative host (reared on pecan) achieved 2.750/16.682 = 0.165 or 16.5% of the fecundity of water hickory aphids migrating to a natal host (reared on water hickory). Additionally, their nymphs on pecan achieved 8.9% of the viability of their counterparts reared on water hickory. Likewise, pecan aphids migrating to an alternative host (reared on water hickory) achieved 35.1% of the fecundity of pecan aphids migrating to a natal host (reared on pecan) and their nymphs achieved 11.7% of the viability of pecan aphids migrating to a natal host (Table 9). By alternative host, migrants reared on pecan were 38.6% as fecund and produced offspring who were 12.2% as viable as residents. Migrants on water hickory were 15.0% as fecund and produced offspring who were 8.5% as viable as residents. Fecundity selection against migrants on pecan is 1-0.386 (38.6%) = 0.614. Viability selection against migrant's daughters on pecan is 0.878. The strength of fecundity and viability selection against migrants on water hickory is 0.850 and 0.915 respectively.

Table 8: Restricted maximum likelihood mixed linear model results for four yellow pecan aphid fitness variables; fecundity, longevity, offspring viability, and offspring development time. Fixed effects are original host, transplanted host, and the interaction of original host and transplanted host. A significant interaction effect is the signal of selection against migrants. Fixed effects contributing significantly ($\alpha<0.05$) to the model according to F-tests are noted with bold typeface.

Dependent Variable	Fixed Effects	F	Sig.
Fecundity	Intercept	88.390	**<.001**
	Original Host * Transplanted Host	36.057	**<.001**
	Original Host	10.057	**0.002**
	Transplanted Host	9.058	**0.003**
Longevity	Intercept	139.244	**<.001**
	Original Host * Transplanted Host	38.470	**<.001**
	Original Host	5.858	**0.017**
	Transplanted Host	4.023	**0.048**
Viability	Intercept	22.347	**<.001**
	Original Host * Transplanted Host	14.822	**<.001**
	Original Host	0.463	0.498
	Transplanted Host	0.435	0.512
Development Time	Intercept	1077.163	**<.001**
	Original Host * Transplanted Host	2.171	0.150
	Original Host	0.018	0.894
	Transplanted Host	17.245	**<.001**

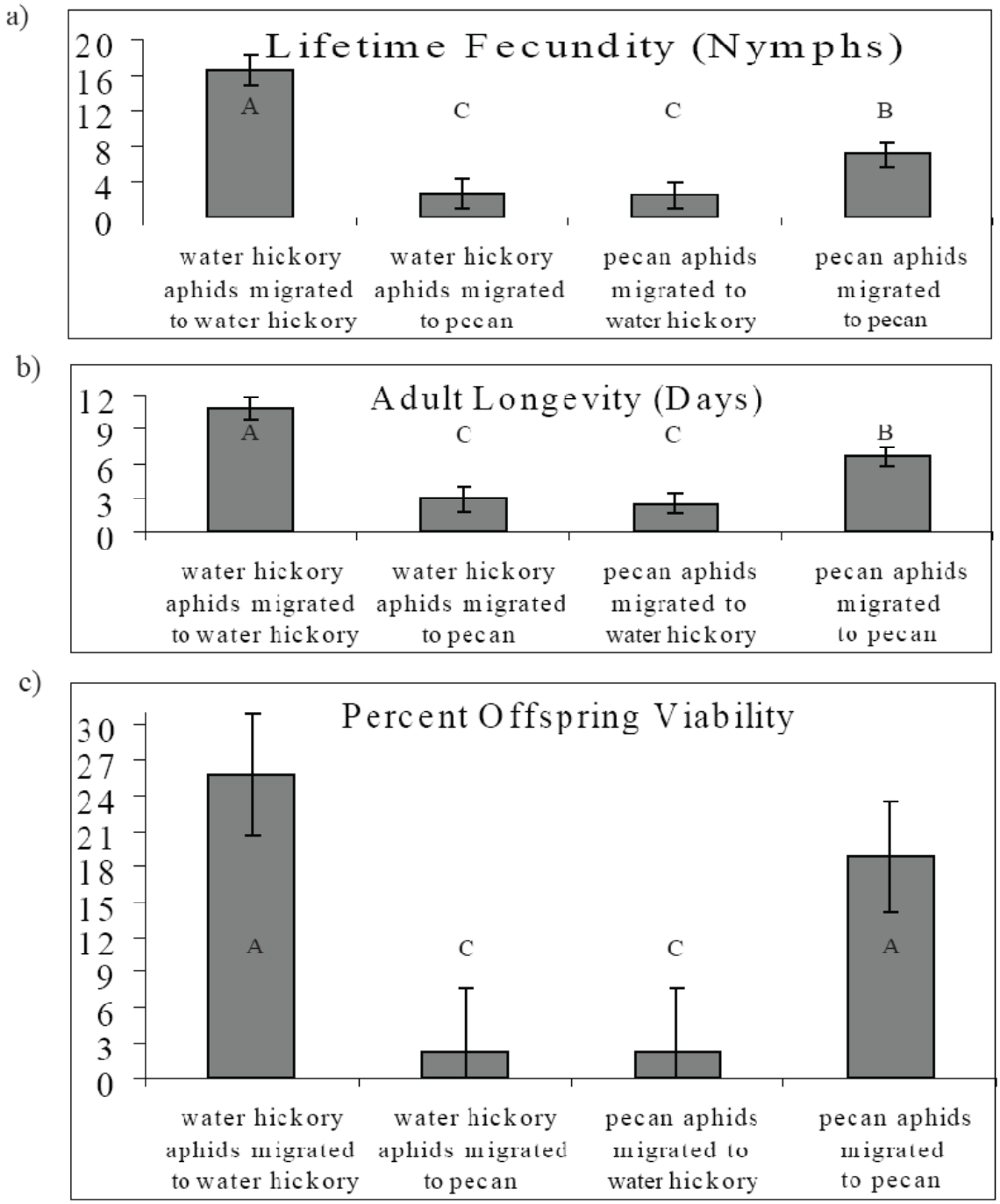


Figure 6: Mean lifetime fecundity (a), mean adult longevity (b) and mean percent viability (c) of yellow pecan aphid migrants in four treatments (Y axes). Aphids had lower fitness ($p<0.03$, Mann-Whitney U test) when force migrated to an alternative host. Error bars are mean standard errors. From left to right, sample sizes for Figure 2a and 2b are 22, 20, 30, and 31 adult aphids. Sample sizes for Figure 2c are 20, 18, 18, and 24 nymph cohorts.

Table 9: The relative fecundity and offspring viability of yellow pecan aphids calculated with respect to a) The natal host and b) The alternative host. Calculations are based on the estimated marginal means of fecundity and viability from restricted maximum likelihood mixed models. In both calculations the host listed in the first column is the natal host. c) The contribution to reproductive isolation of selection against migrants and their offspring is calculated as 1 minus fecundity/viability with respect to the alternative host.

	Relative fecundity on the alternative host	*Relative offspring viability on the alternative host*
a) Natal Host		
Water hickory	2.750/16.682 = **0.165**	0.023/0.258 = **0.089**
Pecan	2.500/7.129 = **0.351**	0.022/0.188 = **0.117**
b) Alternative Host	*Relative fecundity of migrants to the alternative host*	*Relative offspring viability of migrants to the alternative host*
Water hickory	2.500/16.682 = **0.150**	0.022/0.258 = **0.085**
Pecan	2.750/7.129 = **0.386**	0.023/0.188 = **0.122**
c) Alternative Host	*Contribution to reproductive isolation of selection against migrants to the alternative host*	
Water hickory	1-0.150 = **0.850**	1-0.085 = **0.915**
Pecan	1-0.386 = **0.614**	1-0.122 = **0.878**

Discussion

It is clear from these results that host plant species identity strongly affects yellow pecan aphid fitness and does so in a manner consistent with the conclusion of separate pecan and water hickory host races. Aphids had significantly higher fitness (fecundity and viability) when fed leaflets from the natal host species than when fed leaflets from its congener (Figure 7). Yellow pecan aphids will incur a direct cost of migrating to the alternative host plant and thus should possess the ability to discriminate among hosts in a heterogeneous landscape. Showing preference for the natal host would allow yellow pecan aphid migrants to maximize fitness and minimize cost.

Only four aphids were able to mature on the alternative host plant. For these aphids, development time was explained by the transplanted host (Figure 7, Table 9). For example, pecan nymphs on water hickory had similar development times to water hickory nymphs on water hickory. We were able to rear a few pecan aphids on water hickory leaflets for an additional generation following termination of the experiment, but were unable to do so with water hickory aphids on pecan despite several attempts.

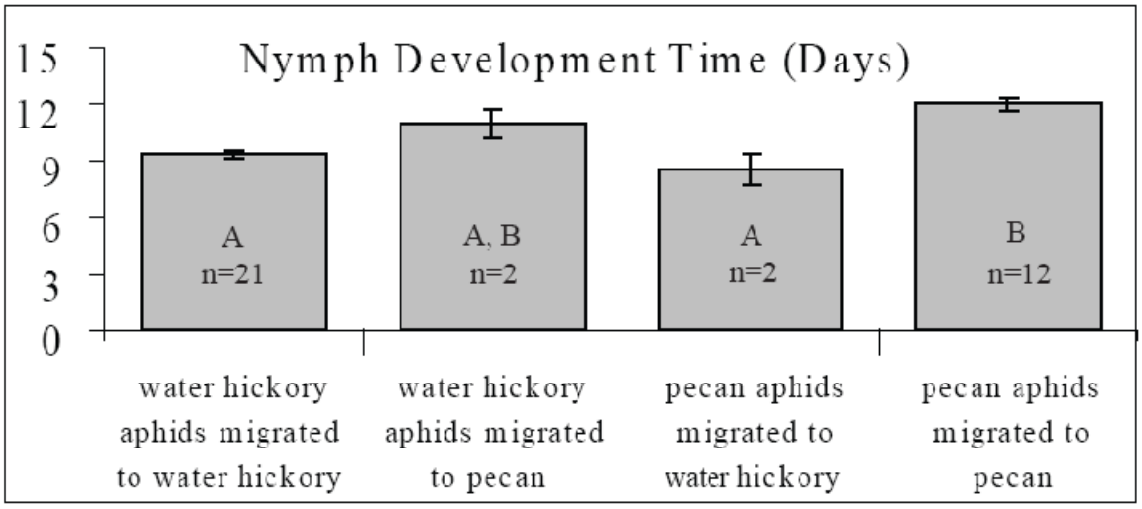


Figure 7: Mean offspring development time of yellow pecan aphid migrants to divergent habitats. Aphid nymphs took longer to develop on pecan than water hickory (outer bars; $p<0.03$, Mann-Whitney U test). Only two nymphs successfully developed in each alternative host treatment (inner bars). Error bars are mean standard errors.

Not only did aphid mothers produce fewer progeny when reared on the alternative host (Figure 6a), but those progeny had lower survival (Figure 6c). In fact, the strength of viability selection (average ~0.9) was higher than the strength of fecundity selection (average ~0.73) imposed by host plant species. Development time, another measure of viability selection, did not show the same trend though too few nymphs survived on the alternate host to make much of a comparison (Figure 7). Our results suggest that viability selection against migrants' offspring could be an important component of total selection against migrants for aphids.

Fecundity and viability selection against migrants are multiplicative in their effect as pre-mating contributors to reproductive isolation in cyclic parthenogens such as aphids. In fact, aphids will face multiple generations of fecundity and viability selection as clones prior to sex in the fall. Consider that the relative fecundity of the water hickory aphid migrant to pecan is 0.386 and the relative viability of the water hickory migrant to pecan is 0.122 (Table 9: transplanted host). This means that if a pecan resident produces 100 daughters, a migrant from water hickory to pecan will produce only 38.6 daughters. Since the survival of water hickory aphids that migrate to pecan is 0.122, the total number of mature daughters produced on pecan by a migrant from water hickory will be 38.6*0.122 = 4.709 mature daughters. Thus, while fecundity selection against migrants to pecan is 1-0.386=0.614, the selection against migrants for one and one half generation including viability selection is 1 – 0.386*0.122=0.952. The higher values indicate increasing selection against aphids migrating to the alternative host. Under this

multiplicative selection regime it will only take 3 clonal generations of fecundity selection and viability selection for the selection coefficient to exceed 0.999. Given a mean development time of 10.2 days and a 244 day activity period from leaf flush to first frost, yellow pecan aphid will have approximately 24 generations. This estimate is within the 22-32 generations estimated by other studies of the yellow pecan aphid (Tedders, 1978, Kaakeh & Dutcher, 1992).

In an innovative study involving alfalfa and clover host races of pea aphid, Via et al. (2000) demonstrated the genetic effects of selection against migrants in action. They showed that the frequency of the 'alfalfa' host associated allozyme allele at the Pep-GL locus increased in frequency between early season and late season in newly planted alfalfa fields while the frequency of the 'clover' host associated Pep-GL allele decreased. The converse was found in newly planted clover fields. Our estimates of selection against migrants in pecan and water hickory races of yellow pecan aphid (0.614 and 0.850) are lower than those found for alfalfa and clover races of pea aphids (0.992 and 0.940) (Via et al., 2000). The lower values for yellow pecan aphid could be due to within tree variability in host plant quality (Whitham & Slobodchikoff, 1981).

The results of this study suggest that yellow pecan aphid clones should have the ability to discriminate between species of hickory. Such habitat discrimination by parthenogens, if present, could be the predominant absolute contributor to reproductive isolation between yellow pecan aphid host races because it acts as a barrier earlier in life history

than selection against migrants (Nosil et al., 2005). Because yellow pecan aphids are cyclically parthenogenetic, both habitat discrimination and selection against migrants will precede any pre-mating or post-mating barriers associated with the sexual generation in the fall.

Aphids are ideal model organisms for testing the fitness trade-offs i.e., reduced performance on an alternative host, sensu Antolin et al. (2006), which are expected to accompany HAD (Mackenzie, 1996, Via et al., 2000, Najar-Rodriguez et al., 2009). These fitness trade-offs, if present in adults capable of migration, demonstrate selection against migrants to the alternative host (Via et al., 2000). Selection against migrants is a pre-mating reproductive isolating mechanism in both sexual and parthenogenetic organisms (Nosil et al., 2005). However, in cyclic parthenogens, inviability of clonal offspring is also a pre-mating reproductive isolating mechanism. In contrast, for obligate sexually reproducing organisms, offspring inviability is a post-mating reproductive isolating mechanism (Nosil et al., 2005). This means that in cyclic parthenogens two pre-mating barriers: selection against migrants and offspring inviability, will be compounded. In our experiment, these two pre-mating barriers compound to exceed 0.999 in between two and three clonal yellow pecan aphid generations. The compounding of these two pre-mating reproductive isolating mechanisms in cyclic parthenogens may be an additional factor promoting the relative abundance of HAD case studies in both tree dwelling and herb feeding aphids.

CHAPTER IV

HOST-ASSOCIATED GENETIC DIFFERENTIATION IN PECAN LEAF PHYLLOXERA

Synopsis

Host-Associated Differentiation (HAD) is the formation of genetically distinct host-associated populations. One of the genotypic signatures of HAD is that populations exhibit stronger differentiation by host plant species than by geographic isolation. HAD, as a mechanism promoting ecological speciation, has been invoked to explain phytophagous insect diversity. Two traits proposed to promote HAD are endophagy and parthenogenesis. Using AFLPs, we tested for the presence of HAD in pecan leaf phylloxera *Phylloxera notabilis* (Hemiptera: Phylloxeridae), an endophagous, gall inducing, and cyclically parthenogenetic insect on sympatric pecan and water hickory at a geographic mesoscale. This species shows strong host associated differentiation. While the effect of collecting locality was significant, accounting for 12% of molecular variation, host plant species identity accounted for 48% of molecular variation. In addition, a choice test indicated that pecan leaf phylloxera originating from water hickory showed weak but significant host discrimination ability for leaflets of the natal host while pecan leaf phylloxera originating from pecan did not. This is the first such study of a species of arboreal Phylloxeridae, a poorly known insect group. This is also the first endophage and the second parthenogen in our hickory host plant system to show evidence of HAD. This hickory system could be a good parthenogen rich counterpoint to

the goldenrod system in the study of host-associated differentiation in an insect community.

Introduction

Insect herbivores have long been considered model organisms for the study of reproductive isolation and speciation (Funk & Nosil, 2008, Walsh, 1864, Thorpe, 1930, Funk et al., 2002, Brues, 1924, Bush, 1975). As parasites, they tend to be highly specialized, feeding on one or few host plant taxa (Price, 1980, Bernays & Chapman, 1994). To insects, host plants are ephemeral sources of nutrition and rendezvous sites. They attract natural enemies (Rasmann et al., 2005, Kessler & Baldwin, 2001, De Moraes et al., 1998) and in some instances provide herbivores with chemical or physical defenses against those enemies (Heinz & Parrella, 1994, Karban & Agrawal, 2002, Aliabadi et al., 2002, Muller et al., 2001). In each of these roles, host plants can mediate disruptive natural selection and promote insect population divergence (Rice & Hostert, 1993, Maynard Smith, 1962, Bush, 1994, Nosil & Crespi, 2006). Host-associated differentiation (HAD) is the formation of the genetically distinct subpopulations that result from this divergence (Abrahamson & Blair, 2008, Dres & Mallet, 2002) and that can ultimately lead to ecological speciation (Rundle & Nosil, 2005, Schluter, 2001).

There are a number of case studies documenting HAD in herbivorous insects (Hernandez-Vera et al., 2010, Dickey & Medina, 2010, Dres & Mallet, 2002, Peccoud et al., 2009, Stireman et al., 2005, Sword et al., 2005, Hendry et al., 2007, Althoff et al.,

2006) but only Stireman et al. (2005) and Dickey & Medina (2010) have specifically tested for the presence of HAD in more than one herbivore per host-plant pair. Additionally, these two reports published negative results; that is cases where HAD was absent. Stireman (2005) argued that studies which 1) Test HAD for multiple herbivores on the same host-plant species pair and 2) Publish negative as well as positive results were both needed in order to determine the relative frequency of HAD in insect herbivores. We further argue that such studies are also needed to elucidate which herbivore traits promote HAD. Two herbivore traits proposed to promote HAD are endophagy (Dreger-Jauffret & Shorthouse, 1992, Stireman et al., 2005) and parthenogenesis (Sunnucks et al., 1997, Loxdale, 2008, Dixon, 1998, Vialatte et al., 2005). Stireman et al. (2005), adding to a large body of previous work testing HAD in herbivores on two species of goldenrods, provided some support for the endophagy hypothesis. In the goldenrod system, HAD was found in four of six endophagous herbivores and zero of two exophagous herbivores (Abrahamson & Weis, 1997, Blair et al., 2005, Waring et al., 1990, Stireman et al., 2005). Dickey and Medina (2010) have introduced another host plant system for testing HAD in an herbivore community. This system involves two hickory species: Pecan *Carya illinoinensis* Koch and water hickory *C. aquatica* Michx (both Fagales: Juglandaceae). On these two hickories Dickey and Medina found that HAD was present in an aphid but not in a caterpillar. The aphid was tested because it is cyclically parthenogenetic and the caterpillar was tested because early instars are inquilines of *Phylloxera* galls (Mitchell et al., 1984). In the present study we tested for HAD in an endophagous parthenogen, the gall inducer.

Pecan and water hickory are both bottomland species common to the oak-hickory woodlands of eastern North America. These trees are thought to be closely related and are sympatric in our study area. Pecan leaf phylloxera *Phylloxera notabilis* Pergande (Hemiptera: Phylloxeridae) is an early season (April-May) secondary pest of pecan (Harris, 1983) which also feeds on water hickory in our study area. *P. notabilis* is also recorded from pignut hickory in Virginia (Payne & Schwartz, 1971), a host plant species not present in our study area. Stem mothers of *P. notabilis* induce bladder-shaped galls on leaflets which are morphologically distinct from two other species of leaf feeding phylloxera, *P. russellae,* and *P. texana* (Stoetzel, 1985, Stoetzel, 1981). *P. notabilis* is also easily distinguished from *P. devastatrix* which is normally a stem feeder but occasionally induces galls from the woody tissue in the leaflet midrib (Stoetzel, 1985). Species in the genus *Phylloxera* feed on trees in the Fagaceae and Juglandaceae and more than half of the nominal *Phylloxera* species are recorded from North American *Carya* hickories (Blackman & Eastop, 1994). With ~60 species, tree feeding *Phylloxera* are more representitive of the Phylloxeridae as a whole, but are poorly known relative to the monogeneric grape phylloxera *Daktulosphaira vitifoliae*.

P. notabilis is cyclically parthenogenetic and induces up to five within-year gall cohorts each year in our area of study (Figure 8). Sexually reproducing males and females are produced by each cohort in addition to parthenogenetically reproducing alate migrants. The first generation is reported to be the most damaging to pecan and each subsequent generation produces fewer and smaller galls than the previous one (Stoetzel, 1985). This

suggests that while 12 consecutive parthenogenetic generations are possible each year prior to sexual reproduction (five gall cohorts * 2 parthenogenetic generations per cohort = 10 within year + 2 between year parthenogenetic generations), the majority of stem mothers hatching in the spring are likely to be the product of sexual reproduction occurring after only 2 parthenogenetic generations (Figure 8).

Given that pecan leaf phylloxera is both cyclically parthenogenetic and endophagous, we predicted finding HAD and alate preference to their respective natal host plant species. We used cluster analyses of amplified fragment length polymorphisms, AFLPs (Vos et al., 1995), and a choice test with alate phylloxera to test our predictions. We found 1. Pecan leaf phylloxera in our study area consists of genetically distinct host plant associated populations, and 2. Significant alate preference for leaflets of the natal host plant species was found for those from water hickory but not for those from pecan.

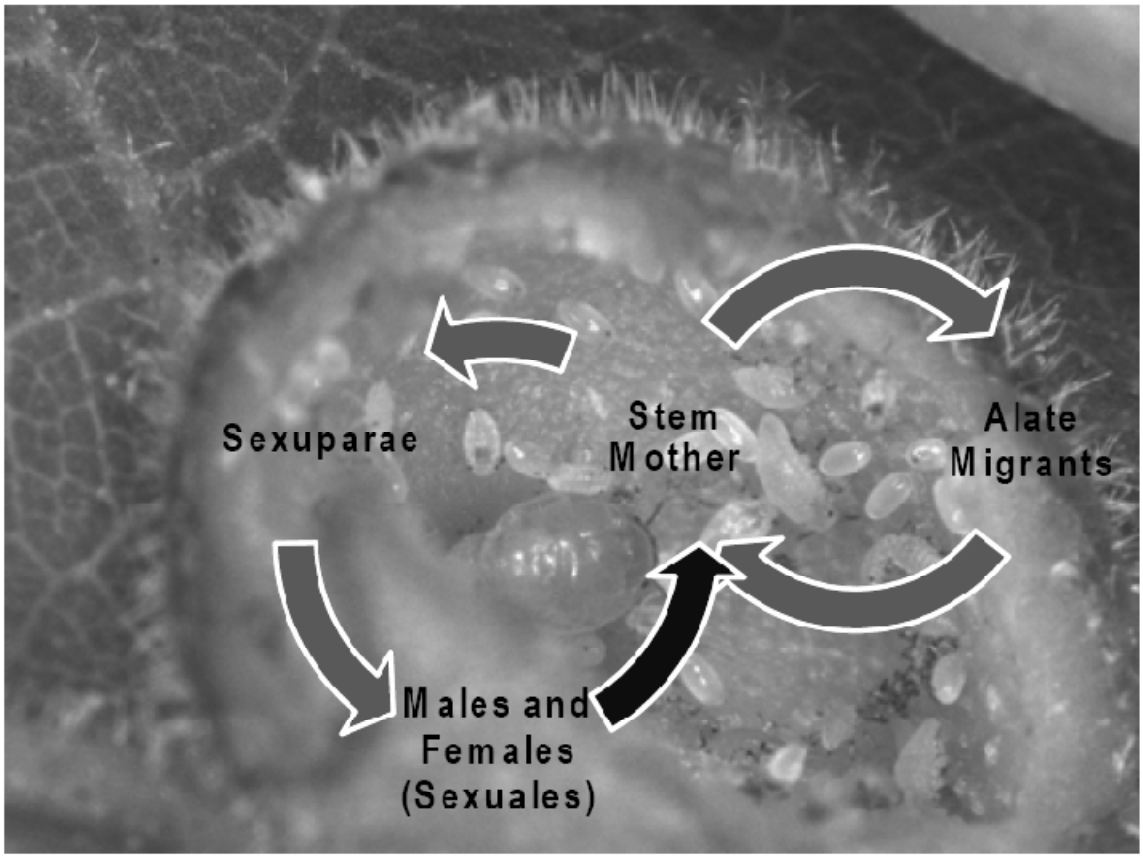


Figure 8: Between (left) and within (right) year life cycles of pecan leaf phylloxera. The grey arrows indicate parthenogenetic reproduction and the black arrow indicates sexual reproduction resulting in overwintering eggs. 5 consecutive within year life cycles are possible prior to sexual reproduction but sex can also occur after as few as two parthenogenetic generations. After Stoetzel (1985). The Stem Mother can be seen in the photograph and is the large, round individual in the center of the gall.

Methods

Insect sampling

Our study area consisted of four counties in the East Texas (Figure 9). Within this area both pecan and water hickory grow wild and pecan is also planted as an ornamental and orchard crop tree. Our aim was to test the role of host plant species in promoting reproductive isolation while preventing confounding geographic effects. For genetic analyses, four to five pecan leaf phylloxera populations were sampled for each tree species from June 2008 to May 2009 with the two sites furthest apart for each tree species separated from one another by at least 80 Km (Table 10).

Pecan leaf phylloxera were stored at -80°C within three days of being collected. Because all the adults in the gall were clones, more than one individual from the same gall was occasionally stored in the same storage tube for DNA extraction. Pecan leaf phylloxera not used for genetic analyses were stored in 95% ethanol as vouchers. Initially, AFLP fingerprints were obtained for 15-20 pecan leaf phylloxera clones from each tree species. The SESim statistic (Medina et al., 2006) was used to determine if individual and marker sampling was adequate for a host-associated differentiation study. If not adequate, more molecular markers and/or samples could be added.

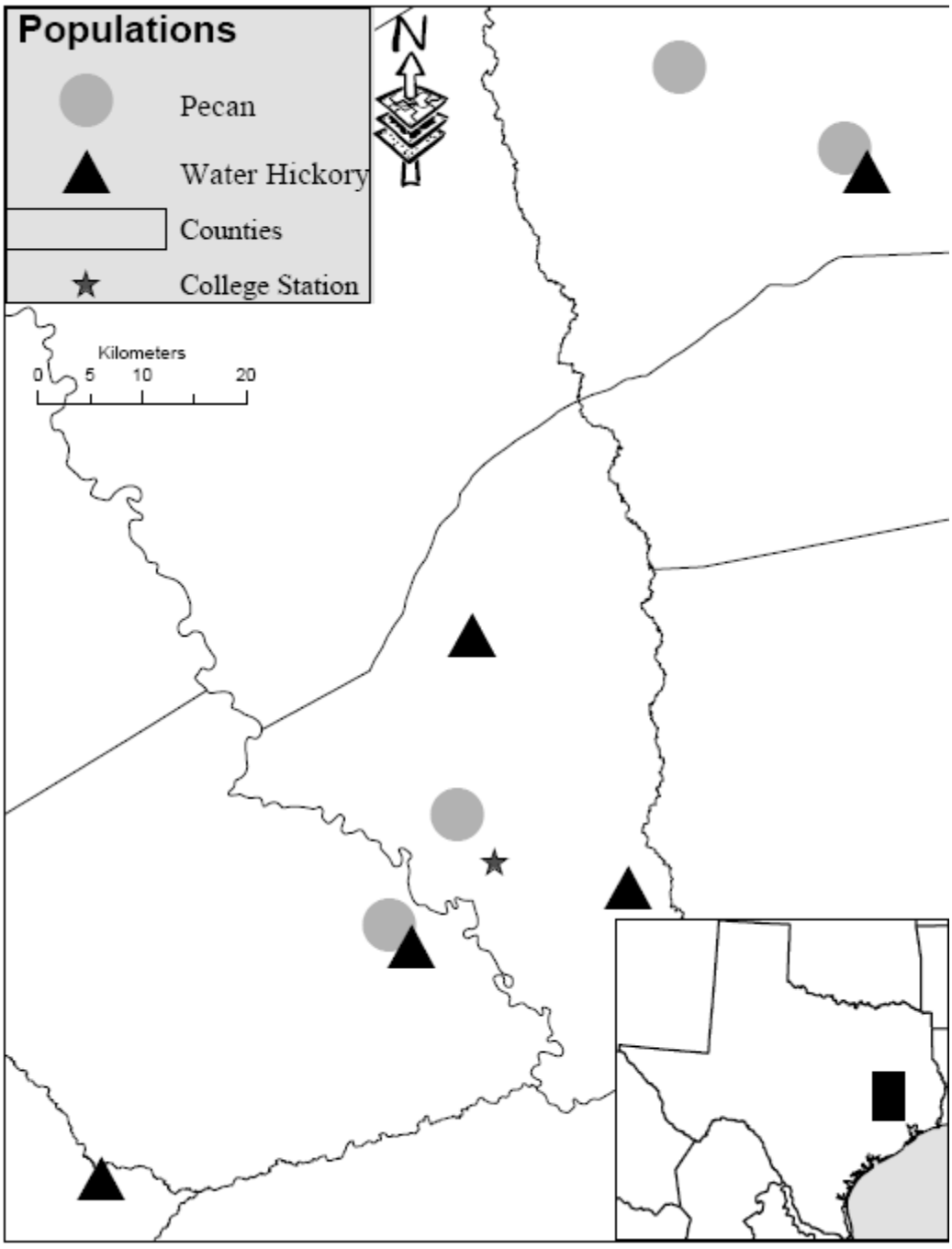


Figure 9: Locations of pecan leaf phylloxera collecting sites containing pecan and water hickory trees in our East Texas study area.

Table 10: Site names, locations, and number of pecan leaf phylloxera genotyped per site.

Site	Location (degrees decimal)	Tree Species	Clones Genotyped
Somerville Wildlife Management Area	96.742 W 30.318 N	water hickory	5 (2 trees)
USDA-Pecan Genetics	96.434 W 30.517 N	pecan	3 (3 trees)
		water hickory	2 (2 trees)
North College Station	96.328 W 30.616 N	pecan	4 (2 trees)
Tabor	96.365 W 30.789 N	water hickory	2 (2 trees)
Lick Creek Park	96.222 W 30.561 N	water hickory	3 (3 trees)
Centerville	96.104 W 31.252 N	pecan	4 (1 tree)
Fort Boggy State Park	95.979 W 31.189 N	water hickory	6 (3 trees)
		pecan	3 (1 tree)

For the alate preference assay, galls were collected opportunistically from pecan at four sites and water hickory at two sites from 8 May through 19 May 2010. From Fort Boggy State Park, Leon County (95.979^0 W 31.189^0 N), galls were collected from both pecan and water hickory. From Centerville, Leon County (96.104^0 W 31.252^0 N), galls were collected from pecan only. From Lick Creek Park, Brazos County (96.222^0 W 30.561^0 N), galls were collected from water hickory only. From USDA Pecan Genetics, Burleson County (96.434^0 W 30.517^0 N), galls were collected from pecan only. And from North College Station, Brazos County ($96. 328^0$ W 30.616^0 N), galls were collected from pecan only.

DNA isolation and AFLP reactions

Whole genomic DNA was extracted from phylloxera clones using the DNeasy blood and tissue kit (Qiagen Corp., Valencia CA) following the manufacturer's instructions. AFLP (Vos et al., 1995, Gompert et al., 2006, Saunders et al., 2001) profiles were generated from ~60ng of DNA from each sample using the following selective primer pairs: *Mse*1-CTC/*Eco*R1-AAC, and *Mse*1-CAT/*Eco*R1-ACT. Polymerase Chain Reactions (PCR's) were run in GeneAMP® 9700 thermocyclers and diluted amplified selective products were analyzed with an ABI 3130 capillary sequencer with co-loaded fluorescent (MapMarker® 1000XL X-Rhodamine) size standard ladder (BioVentures, Inc., Murfreesboro, TN) according to manufacturer's instructions. Thermocycling conditions were as follows: samples undergoing preselective amplification were held at 95^0C for 1 min followed by 20 cycles of 95^0C for 10 s, 56^0C for 30 s, and 72^0C for 90 s followed by

a hold at 75^0C for 5 min. For the selective amplification, samples were held at 95^0C for 30 s followed by 47 cycles of 95^0C for 10 s, and 12 cycles starting at 65^0C for 40s (decreasing 0.7^0C per cycle until reaching 56^0C) and 72^0C for 90 s, followed by a hold at 75^0C for 5 min. Absence of contamination was assured by negative controls and accuracy and repeatability of DNA fingerprints within species was verified by repeating all PCR steps for one clone. For each insect and selective primer combination, resulting electrophenograms were examined and analyzed using GeneMapper® 4.0 (Applied Biosystems, Forest City, CA). An allele calling threshold of 50 reflectance units was selected since this was more than 1.5 times the baseline noise of all electrophenograms. Private alleles were removed from all samples. Five alleles were removed from the *Mse*1-CTC/*Eco*R1-AAC primer data set due to their presence in the negative control.

The SESim statistic (Medina et al., 2006) was calculated to determine if individual and molecular marker sampling was adequate for the host-associated differentiation study. 10,000 iterations of the SESim algorithm were employed. The two selective primer combinations produced 111 loci, which gave a SESim value of 0.041. Since Medina et al. (2006) showed that population structure began to break up due to inadequate sampling when SESim values were greater than 0.05, we determined that marker and individual sampling was adequate for our study.

Molecular data analysis

Bayesian cluster analyses were executed in STRUCTURE 2.2 (Pritchard et al., 2007) using the recessive alleles model for dominant marker data assuming admixture and correlated alleles (Falush et al., 2007). Admixture is a general attribute of most species occurring in sympatry and the "alleles correlated" model deviant has been shown to be the most sensitive to the presence of population structure in simulated data (Falush et al., 2003). STRUCTURE assumes that within a population, loci are in Hardy-Weinberg equilibrium and linkage equilibrium and assigns individuals to separate populations so as to eliminate violations of these assumptions. The output of STRUCTURE is the log probability of the data (X) given the number of clusters (K) assumed or [Ln Pr(X|K)]. Where parameter estimates indicated K>1, the *ad hoc* ΔK statistic (Evanno et al., 2005) was used to predict the most likely number of clusters (K) in the data. This involves calculating the second-order rate of change of [Ln Pr(X|K)]. Evanno *et al.* (2005) showed that the K corresponding to a spike in this value accurately predicts the number of populations represented by the data. The model was run for 40,000 generations with a burn-in period of 2,000 generations for 20 iterations each from K=1 to K= 6.

Nested AMOVA (Excoffier et al., 1992) and principal coordinates analyses (PCO) were conducted among host plants and among collecting sites within host plants using the software GenAlEx 6.2 (Peakall & Smouse, 2006). The Tabor and Pecan Genetics water hickory samples were removed prior to the nested AMOVA due to low sample sizes but they were retained in separate AMOVA analyses testing host plant and site separately. In

a second nested AMOVA analysis, North College Station pecan clones were also removed as they contributed heavily to both site and host-plant effects. AFLPsurv 1.0 (Vekemans, 2002) was used to estimate genetic diversity, percent polymorphic loci, and Fst among host plant species, and among collecting sites within host plant species using Bayesian analyses with a non-uniform prior distribution of loci (Zhivotovsky, 1999) and the estimation procedures of Lynch and Milligan (Lynch & Milligan, 1994). To determine if the estimated Fst values were significantly different from 0, permutation tests were conducted with 9,999 random permutations of the data.

Alate preference assay

Choice tests were run from 8 May through 24 May 2010. For each choice test, five alates from a gall were placed into clear, 50mL centrifuge tubes with 0.1g of excised leaflet from each host plant species and allowed one hour to feed and oviposit. The trees providing leaflets for each assay were randomly selected from five adult pecan trees and five adult water hickory trees present in a common garden at Texas A&M University's Eli Whitely Medal of Honor Park (96.349^0 W30.615^0 N). After one hour, alates and eggs present on each leaflet were counted under 12x magnification. If no eggs were present after one hour, the same alates and leaflets could be used in subsequent assays so long as all five alates remained alive. Assays resulting in no alates present on either host and assays resulting in no eggs present on either host were not retained for analysis. For phylloxera originating from pecan, 48 assays produced at least one egg and 47 assays produced at least one alate "choice". For phylloxera originating from water hickory, 28

assays produced at least one egg and 32 assays produced at least on alate "choice". Egg count data was then converted to a preference index using the following formula:

$$\frac{(\text{\# of eggs on pecan} - \text{\# of eggs on water hickory})}{\text{Total \# of eggs}} \times 100$$

Alate count data was converted to a preference index in the same manner. Preference indices ranged from +100 indicating all eggs or alates on pecan for a given assay to -100 indicating all eggs or alates on water hickory for a given assay.

Preference indices were averaged within each natal host plant species and then tested against the null hypothesis of no preference (index value = 0) using a monte carlo test in Microsoft Excel with 1000 random permutations of the raw data where the sign (+ or -) of the preference index was allowed to vary at random. This is justified because if for a given assay, 1 egg was laid on pecan and 2 on water hickory, this would result in an index value of (1-2)/3*100=-33.3. The randomization test would allow the egg counts to switch hosts within an assay resulting in (2-1)/3*100=33.3. Thus, only the sign of the index value needed to be randomized in the monte carlo test.

Results

Host-associated differentiation

At K=2, all individuals were assigned with high (>94.2%) probability to one of two populations (Figure 10). These two populations corresponded to host-plant species of origin with the exception of 2 clones. The 2 clones were collected from a single pecan tree but clustered with the clones collected from water hickory. Six loci were diagnostic

for the pecan associated population and seven loci were diagnostic for the water hickory associated population. An additional nine loci were strongly host-plant associated, being present in $\geq$90% of individuals from the associated host, and in $\leq$10% of individuals from the host's congener. The second order rate of change in K, (Evanno's ΔK) peaked for K=2 populations further indicating that two populations best explained the pecan leaf phylloxera data.

Principal coordinates 1 and 2 explain 84.4% of the molecular variation with principal coordinate 1 strongly segregating clones by host plant species (Figure 11). As with the structure results, 2 clones collected from a pecan tree cluster with the water hickory associated population. The Fst between host plants, STRUCTURE delimited AFLP phenotypes, and the overall Fst among sites within water hickory were all significantly different from zero (Table 11). After removing North College Station clones (asterisks in Figure 10) from the nested AMOVA, significant molecular variation occurred among host plants (63%), among sites within host plant species (8%), and within sites (29%) (Table 12).

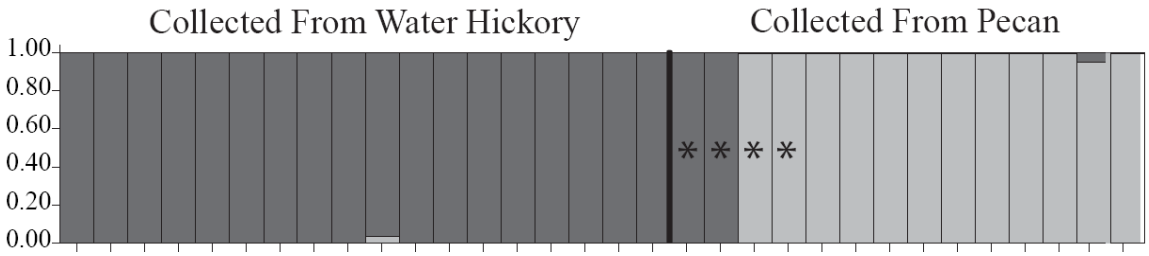


Figure 10: Bayesian population assignment probabilities (y-axis) for pecan leaf phylloxera clones (x-axis) collected from pecan and water hickory using the recessive alleles model for dominant marker data in STRUCTURE 2.2. Two host-associated populations (light grey and dark grey) are indicated. Four clones collected from pecan trees in North College Station are indicated with asterisks, 2 of these cluster with the clones collected from water hickory.

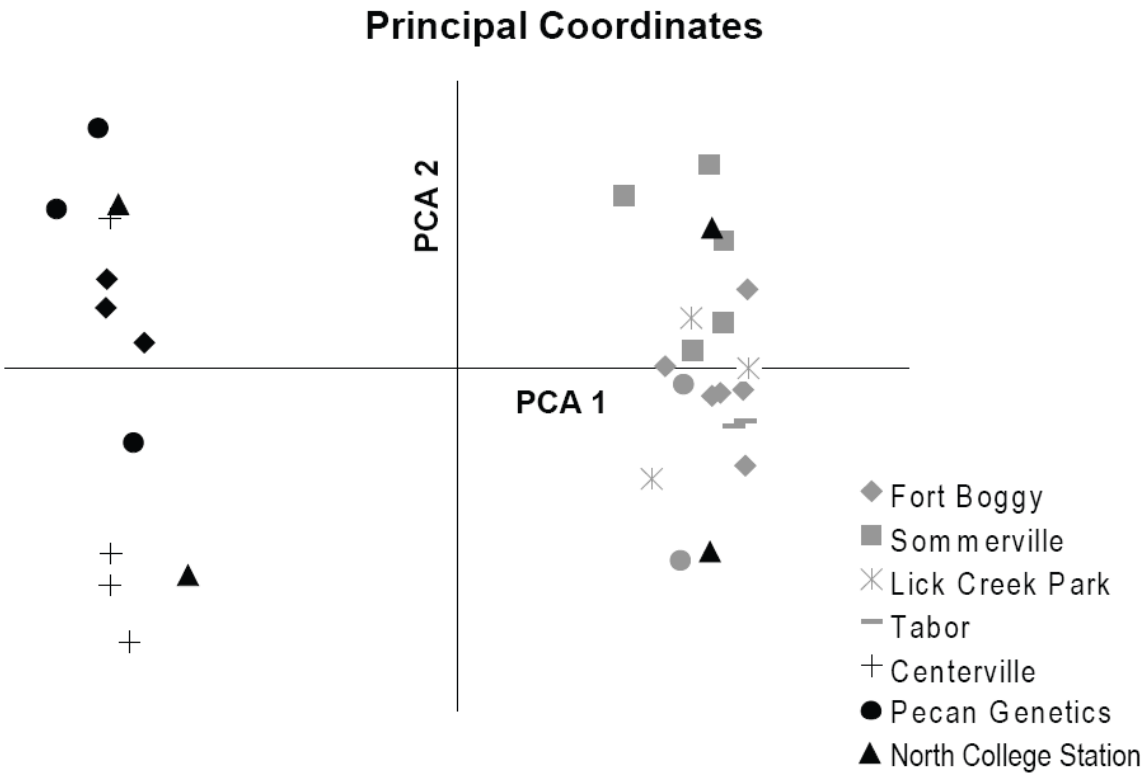


Figure 11: Eigenvectors of principal coordinates 1 (x-axis) and 2 (y-axis) for pecan leaf phylloxera. Symbol shapes denote collecting sites and symbol colors denote host plant species. PC 1 separates the water hickory (grey) and pecan (black) populations with the exception of two clones from a pecan tree in North College Station which cluster with water hickory.

Table 11: Genetic differentiation in pecan leaf phylloxera by host plant, STRUCTURE delimited AFLP phenotype, and the overall Fst by site within each host plant associated AFLP phenotype.

Source of differentiation	Fst	p-value
Host Plant	0.3629	<0.0001
AFLP Phenotype	0.4864	<0.0001
Site within Water Hickory	0.1275	0.0016
Site within Pecan	0.1574	0.0618

Table 12: Variance components (VC) and percent molecular variation due to host plant and collecting sites within host plant for pecan leaf phylloxera from AMOVA (a). Since clones collected from North College Station confounded host plant and site effects (see Figure 4), they were removed in a second AMOVA (b). Significance testing was done using 9999 permutations of the binary distance parameter φPT, an analog of Fst in GenAlEx 6.2.

	Source of variation	VC	% variation	P
(a)	Among host plants	10.268	48%	<0.001
	Within host among sites	2.623	12%	0.009
	Within collecting sites	8.543	40%	<0.001
(b)	Among host plants	14.696	63%	<0.001
	Within host among sites	1.940	8%	0.025
	Within collecting sites	6.814	29%	<0.001

Alate preference

A statistically significant preference for the natal host over the alternative host is evident for phylloxera collected from water hickory (alate choice: p=0.046, oviposition: p=0.042) but not for those collected from pecan (alate choice: p=0.328, oviposition: p=0.054) (Figure 12).

Discussion

The endophagous parthenogen

The pecan leaf phylloxera shows a genetic pattern indicating host-associated differentiation and consists of at least two distinct host races, one feeding on pecan, and one feeding largely on water hickory. Host races are diagnostic at thirteen out of 99 (13%) polymorphic loci. Pecan leaf phylloxera was tested in our system because it shows two traits proposed to facilitate HAD, endophagy and parthenogenesis. Compared with obligate sexually reproducing organisms, cyclical parthenogens such as phylloxera experience less gene flow which would counteract differentiation. Endophagy could also promote HAD because of the intimate association between the plant and herbivore. While we did not find HAD in a phylloxera inquiline caterpillar (Dickey & Medina, 2010), we have now found it in the gall inducer. Unlike inquilines, gall inducers must be able to manipulate the plant into producing tissue which it would not normally produce requiring a tight association between the insect and the plant (Weis et al., 1988, Granett et al., 2001, Tooker & De Moraes, 2009).

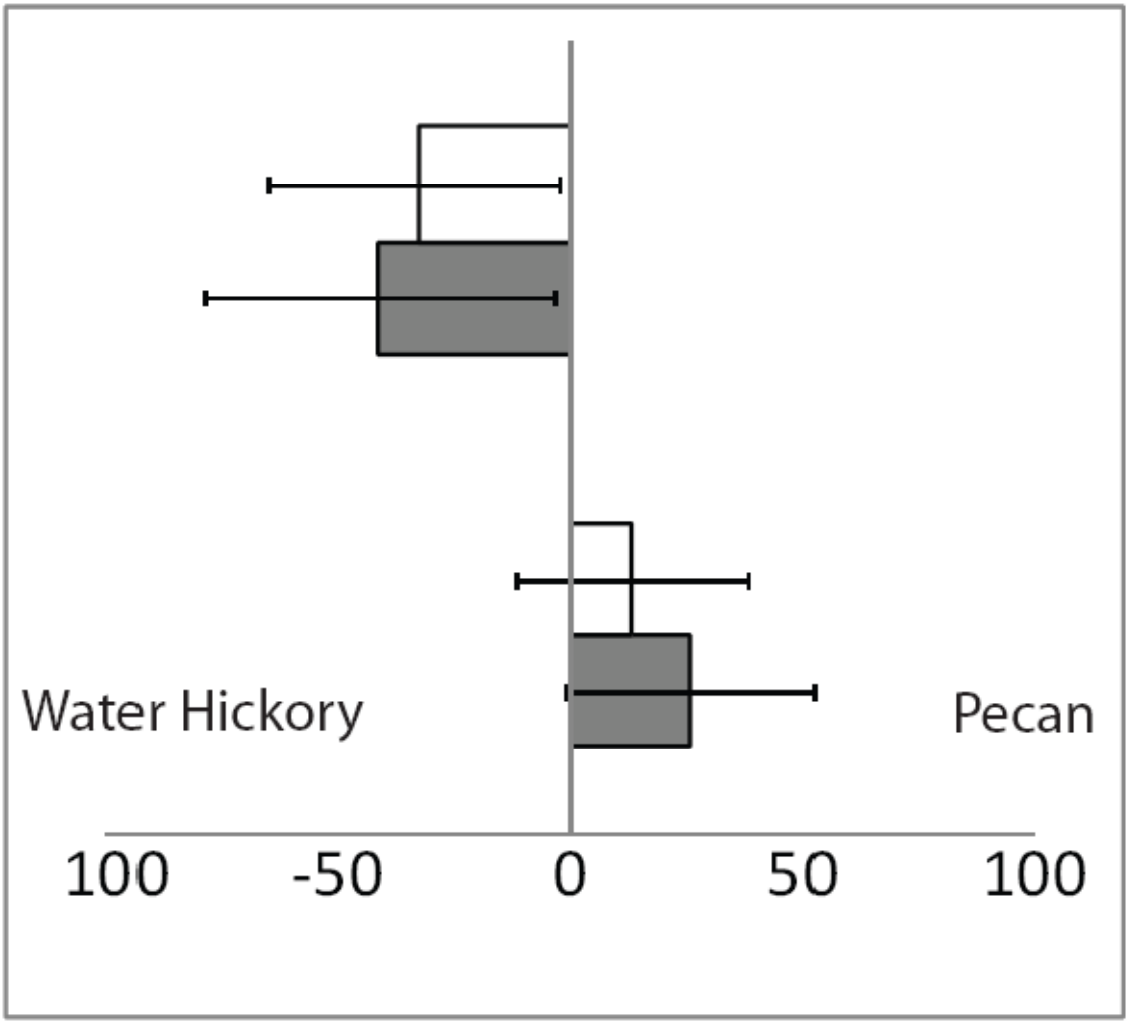


Figure 12: Two host preference indices: oviposition preference (grey) and alate "choice" (white) for *Phylloxera notabilis* collected from water hickory (top) and pecan (bottom) galls. Negative values indicate preference for water hickory and positive values indicate preference for pecan. Error bars are 95% confidence intervals based on 1000 Monte Carlo permutations.

Insect univoltinism could promote HAD because univoltine insects must be linked tightly to the phenology of their hosts (Yukawa, 2000, Nyman, 2002, Mopper, 2005, Komatsu & Akimoto, 1995). For example, the apple and hawthorn host races of both the apple maggot and its parasitoid break diapause to coincide with the fruiting of their respective host plants (Feder & Filchak, 1999, Forbes et al., 2009). While pecan leaf phylloxera is not univoltine, the first cohort of galls coincides with leaflet formation in the spring and water hickory tends to be about 3 weeks behind pecan in its phenology. Being able to track their host-plant phenology must be critical for the establishment of the first gall cohort since hatching of overwintering eggs occurs at the time of bud break (Stoetzel, 1985, Mitchell et al., 1984). If gall formation is offset between pecan and water hickory, this should then offset the release of alates, males, and females from the galls between host species reducing the likelihood of gene flow between host races. During the spring of 2010, we found that gall formation was indeed about 2 to 3 weeks behind in water hickory relative to pecan. There also seemed to be relatively little overlap in the production of alates from galls. Alates from pecan were seen from 28 April to 15 May and alates from water hickory were seen from 12 May to 24 May.

Isolation by distance

Because of the relatively small but significant site effect in our AMOVA analyses (Table 3), Nei's unbiased genetic distances after Lynch and Milligan (1994) were calculated in AFLPsurv (Vekemans, 2002) and geographic straight-line distances were obtained using the measure function in ArcMap (ESRI, Redlands, CA). Isolation-by-distance was tested

using regression analysis of genetic and geographic distances for each pair of same-host collecting sites. The two North College Station pecan clones clustering with water hickory were removed as they contributed to both site and host-plant effects. The correlation between genetic distance and geographic distance for same-host species site pairs was weakly significant ($F_{1,14}$=5.13; p=0.04) (Figure 13). This appeared to be largely due to the effect of the six pairs of pecan sites.

Furthermore, we visualized this trend by creating a neighbor joining phenogram in PHYLIP using the genetic distances calculated between each pair of locations. Bootstrap support came from 9,999 pseudoreplicates generated in AFLPsurv (Figure 14). The phenogram shows the pattern of isolation by distance between a northeast collecting site, central sites, and the southwest collecting site for water hickory associated phylloxera. The phenogram also shows the same pattern between the northeast collecting sites and a central site for phylloxera clones collected from pecan.

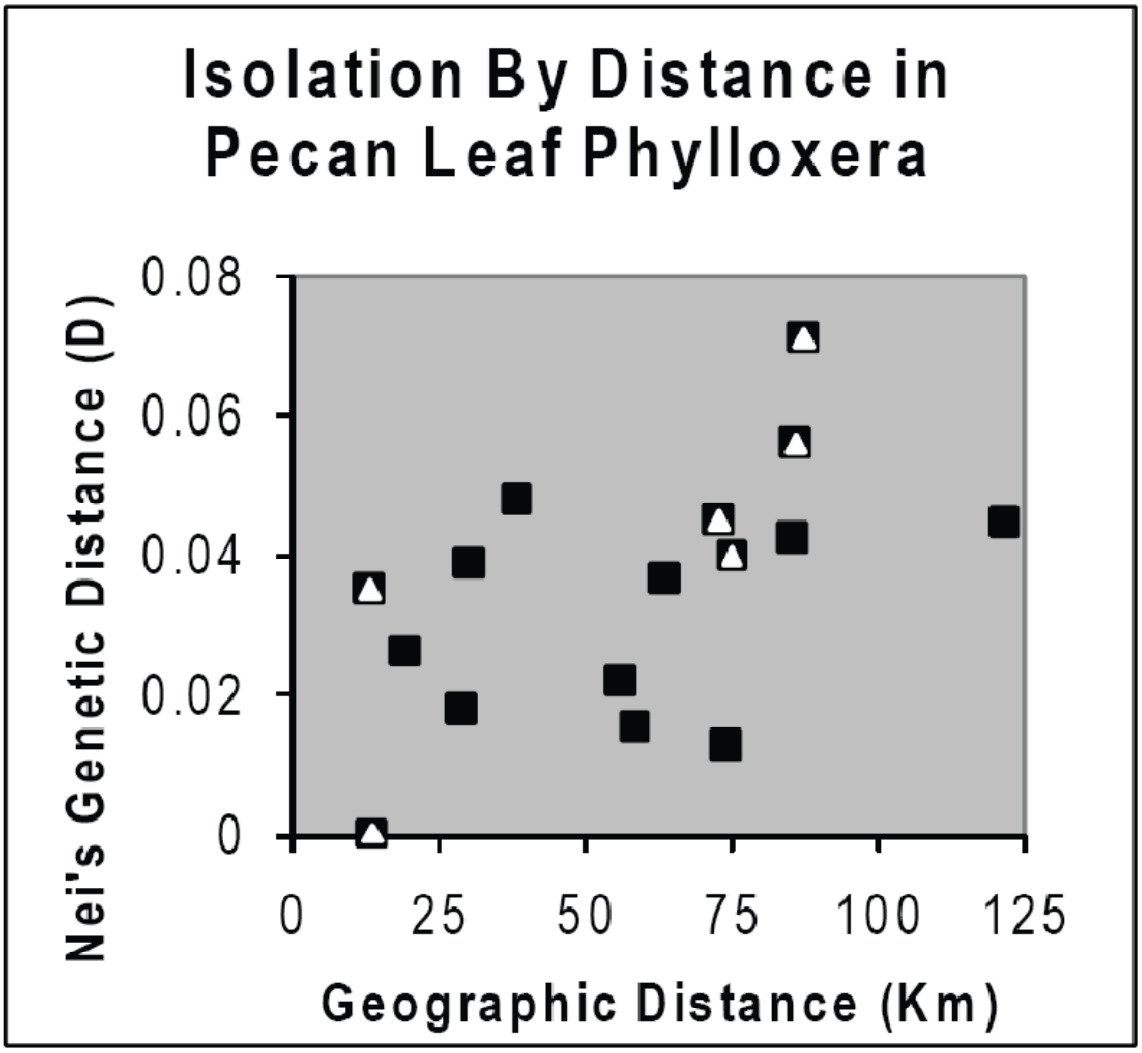


Figure 13: Overall pattern of genetic isolation by distance in pecan leaf phylloxera (r^2=0.27, p=0.040). Points are same-host site pairs. The pattern is largely due to the effect of the six pecan site pairs (white triangles; r^2=0.67, p=0.046) as the water hickory site pairs are not significantly correlated (black squares; r^2=0.066, p=0.47).

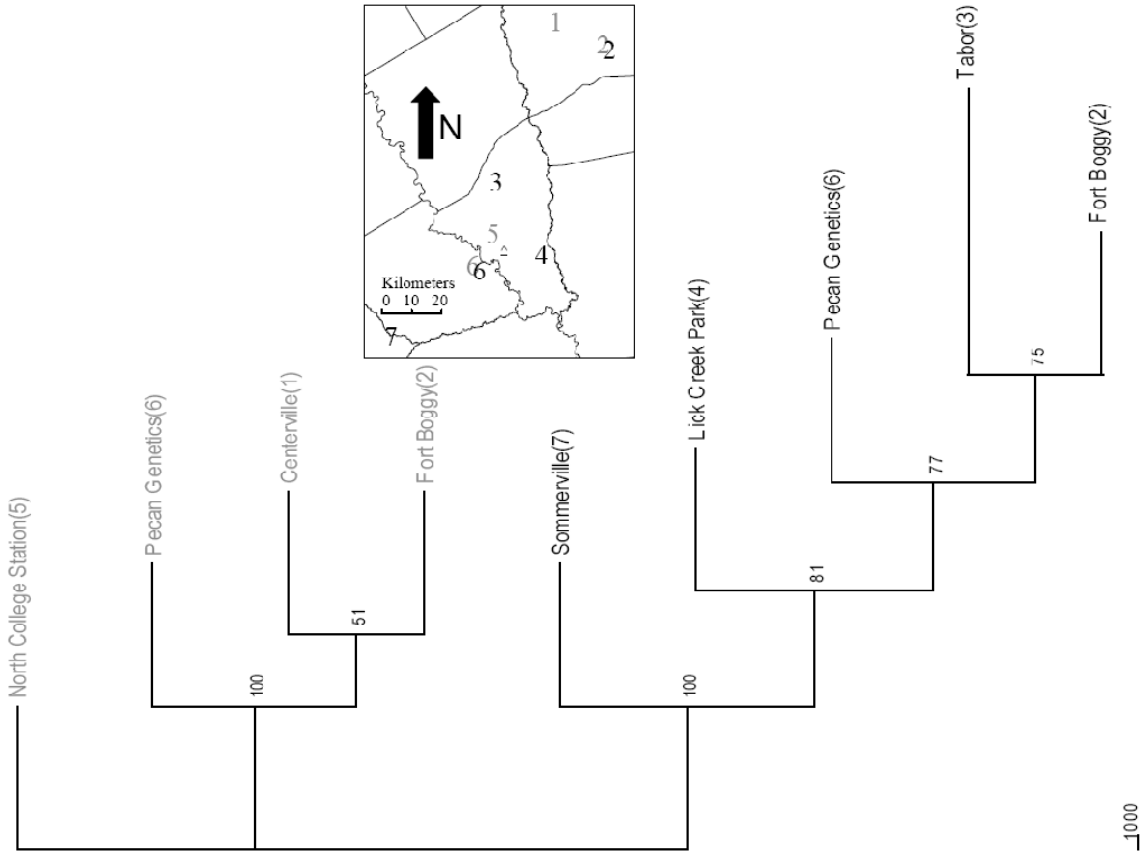


Figure 14: Phenogram showing a pattern of isolation by distance between northeast collecting sites (1 and 2), central sites (3-6) and the southwest collecting site (7) for pecan leaf phylloxera clones from pecan (grey) and water hickory (black). The neighbor-joining phenogram was constructed using PHYLIP using Nei's unbiased genetic distances (D) between locations calculated in AFLPsurv along with 9,999 bootstrap pseudoreplicates. The support at each node is a bootstrap percentage. The North College Station location was used to root the phenogram since this pecan site contained clones which clustered with both host-associated AFLP phenotypes.

It should be noted that we sampled phylloxera clones so as to account for the effect of geography rather than to test the effect of geography outright. Nonetheless, we can state that the between site molecular variance is significant. This could be due to limited between site migrations of phylloxera which has been inferred in the better characterized grape phylloxera (Vorwerk & Forneck, 2006, Forneck & Huber, 2009, Lin et al., 1999). Future studies should investigate the causes of geographic isolation in greater depth including the possibility of obligate asexuality as the predominant reproductive mode as was found in European grape phylloxera (Vorwerk & Forneck, 2006). Alates may be weak fliers and prefer to oviposit near the natal gall which could limit migration and males and females may have limited dispersal which could promote inbreeding.

Because our insect sampling did not encompass the entire geographic range of the insects in question or their host plants, our findings may not translate to other pecan leaf phylloxera populations elsewhere and the genetic clusters identified may not represent monophylys. For example, grape phylloxera shows a pattern of reduced gene flow among host species at local (Corrie et al., 2003) and regional (Downie et al., 2001) scales but host-associated genotypes, associated with graybark grape are polyphyletic (Downie et al., 2001).

Host plant discrimination in pecan leaf phylloxera

Pecan leaf phylloxera alates originating from water hickory showed a weak but statistically significant oviposition and “choice” preference for leaflets from water

hickory. Those originating from pecan did not show a similar preference for their natal host. For the choice tests in 2010, we did not use galls from the single pecan tree that yielded the "water hickory-genotype" phylloxera in 2009. Even so, there is a possibility that our pecan-sourced phylloxera contained the water hickory genotype. Another possibility is that pecan could be the more recently colonized host and as a consequence, phylloxera from pecan show reduced discrimination ability. A similar argument was made by Dorchin et al. (2009) for host-associated populations of the gall midge *Dasineura folliculi* on two sympatric goldenrods (Dorchin et al., 2009) in infering the directionality of a putative host switch. We used alates as these are the most likely to immigrate to a new tree upon leaving the opened gall. Our preference data, taken together, suggest that host plant species discrimination may be a less important reproductive isolating mechanism in pecan phylloxera than has been shown in other insects (Craig & Itami, 2009). Indeed, Granett et al. (2001) suggested that grape phylloxera might be limited in its colonization success by its ability to distinguish hosts. We predict then a differential ability to induce galls on the two hosts between the two host races. We know that the "water hickory" race can induce galls on pecan because of our genetic data but perhaps gall formation ability is reduced in the alternative host.

Preference for individual trees

Phylloxera alates showed weak to no preference for their natal host plant species. In contrast, they showed strongly differential preference for specific trees used in the dual-choice assay (Table 13). One tree of each species was universally preferred and one tree

of each species was universally avoided, regardless of the host plant species from which the phylloxera were collected. For the two universally preferred trees, the number of eggs laid was more than 50% higher than expected by chance and for the two universally avoided trees, the number of eggs laid was more than 50% lower than that expected by chance. Interestingly, one water hickory tree was strongly preferred by phylloxera originating from pecan and strongly avoided by phylloxera originating from water hickory.

Phylloxera and the understudied Aphidomorpha

Phylloxeridae are relatively understudied compared to their better characterized Aphididae relatives. A June 4, 2010 web of science search for "phylloxera" yielded 321 peer reviewed articles while a search for "aphid" yielded 12,270 peer reviewed articles. Of the 321 phylloxera articles, only 26 dealt with phylloxera species other than grape phylloxera. For example, while several endosymbionts have been described and studied in Aphididae (Baumann, 2005, Koga et al., 2003, Moran et al., 2005, Scarborough et al., 2005, Douglas, 1998), it is presently unknown if Phylloxerids, other than the grape phylloxera (Vorwerk et al., 2007), possess endosymbionts or how those endosymbionts might have evolved with their hosts. It is predicted however, that if endosymbionts are found, they will show a pattern of co-speciation with their hosts as has been shown with Aphididae species (Clark et al., 2000, Baumann et al., 1995).

Table 13: Differential oviposition preference of *Phylloxera notabilis* alates for specific trees in dual-choice preference assays. Positive numbers indicate more eggs were laid than expected by chance and negative numbers indicate fewer eggs were laid than expected by chance.

			% Deviation from Expected Egg Number		
Tree ID	Tree Species	Response	Phylloxera from water hickory	Phylloxera from pecan	All Phylloxera
IL6	Pecan	Strongly Preferred	147%	79%	98%
AQ1	Water hickory	Strongly Preferred	55%	68%	72%
AQ3	Water hickory	Mixed Response	146%	-60%	32%
IL10	Pecan	Mixed Response	-9%	24%	30%
AQ5	Water hickory	Mixed Response	-81%	71%	4%
IL9	Pecan	Avoided	-43%	-35%	-35%
AQ4	Water hickory	Avoided	-10%	-70%	-43%
IL7	Pecan	Avoided	-16%	-60%	-47%
AQ2	Water hickory	Strongly Avoided	-61%	-63%	-61%
IL8	Pecan	Strongly Avoided	-81%	-79%	-81%

HAD in an herbivore community

Pecan leaf phylloxera is the third herbivore species for which HAD has been tested on the present host plant species pair. The first two were yellow pecan aphid *Monelliopsis pecanis* Bissel (Hemiptera: Aphididae), a cyclic parthenogen for which HAD was found and pecan bud moth *Gretchena boliana* Granovsky (Lepidoptera: Tortricidae) a sexually reproducing phylloxera gall inquiline which did not show a genetic pattern of HAD (Dickey & Medina, 2010). These three species represent an informative comparison because they are all herbivorous insects on the same host-plant species pair sampled at the same geographic mesoscale. While the current report provides a second example of HAD in a parthenogen and the first example of HAD in an endophage in this system, more species should be tested within this host-plant system. Pecan and water hickory host many herbivores (over 400 species documented on pecan alone). To date, 28 herbivores have been documented to be shared by the two host plants (Dickey & Medina, 2010) and more are likely to be reported. Evidence is still scant with two out of two parthenogens tested showing HAD however if more host-associated aphid biodiversity is found in this system, it will help make the *Carya* system a good counterpoint to the *Solidago* system in which HAD has been tested and found in multiple shared endophages but in which no parthenogens have been compared. Pecan and water hickory share at least seven species of aphids, three of them endophagous phylloxerans (Dickey & Medina, 2010).

CHAPTER V

LACK OF SEQUENTIAL RADIATION IN A PARASITOID OF A HOST-ASSOCIATED APHID

Synopsis

Sequential radiation occurs when novel biodiversity at low trophic levels 'cascades' up to high trophic levels generating further biodiversity. When putatively generalist herbivore species consist of host plant specialized populations, sequential radiation can be tested for their parasites and predators. We tested for sequential radiation in *Aphelinus perpallidus* Gahan (Hymenoptera: Aphelinidae), a parasitoid wasp of the yellow pecan aphid. The yellow pecan aphid has been shown to consist of at least two genetically distinct host-tree associated populations, one feeding on pecan and one feeding on water hickory. We found that this wasp consists of three genetically distinct populations with unique molecular phenotypes but these populations did not correspond to host plant species of origin. Collecting site accounted for 20% of the molecular variance found. We suggest future research towards the elucidation of the biological basis of the three populations detected in this study. We also discuss the implications of our findings in the context of 1) habitat location in aphid parasitoids, and 2) previous studies of genetic differentiation in parasitoids, including those documenting sequential radiation. Sequential radiation may be less common in aphid parasitoids than in other parasitoid groups due to specific aspects of their biology: namely multivoltinism and learned habitat preference.

Introduction

Host-plant-associated genetic differentiation in arthropod herbivores is well documented (Dickey & Medina, 2010, Dres & Mallet, 2002, Funk et al., 2002, Hendry et al., 2007, Magalhaes et al., 2007). Several of these herbivorous insects are ideal models for the study of ecological (Funk & Nosil, 2008, Rundle & Nosil, 2005) and sympatric (Bush, 1975, Via, 2001, Schwarz et al., 2005) speciation. One unanswered question is how often host-associated differentiation in herbivores triggers an evolutionary 'cascade' of sequential radiation in their predators and parasitoids (Forbes et al., 2009, Abrahamson & Blair, 2008). The answer to this question may have implications for the ongoing selection and release of biological control agents (Lozier et al., 2009). More broadly, the answer to this question has implications for the study of speciation at the third trophic level as a whole (Tauber & Tauber, 1989, Stireman et al., 2006, Feder & Forbes, 2010). Tauber and Tauber (1989) suggested that not only herbivores, but any host or habitat specialist insect, including predators and parasitoids, could be susceptible to habitat based disruptive selection which could promote genetic differentiation and eventual speciation.

Parasitoids are insects which are free-living as adults and parasitic as larvae, killing a single host (Eggleton & Belshaw, 1992). As a group, parasitoids are quite speciose, accounting for about 10% of all described insect species (Eggleton & Belshaw, 1992). The parasitic hymenoptera account for roughly 70,000 described species with many more waiting to be described (Sharkey, 2007). Hymenopteran parasitoids possess several

traits that may predispose them to sequential radiation. First, the endoparasitic lifestyle found in many parasitic hymenoptera gives them essentially a "concealed" mode of feeding which could necessitate extensive physiological adaptations to the immune system of their insect hosts (Pennacchio & Strand, 2006, Strand & Pech, 1995, Lawrence, 1986). Second, their haplo-diploid genetic system is predicted to promote a relatively rapid rate of evolution; intermediate between that of cyclically parthenogenetic organisms and diploid sexual organisms (Hartl, 1972). Third, many parasitoids use substrate-borne vibrations during courtship and these signals may transmit differently on different plant species which could promote pre-mating isolation mediated by the different host plants (Joyce et al., 2010).

Aphelinus perpallidus Gahan (Hymenoptera: Aphelinidae) is a parasitic wasp attacking foliar feeding aphids in the family Drepanosiphidae on North American shade trees (Tedders, 1978, Zuparko, 1997). It is the primary parasitoid of yellow pecan aphid, *Monelliopsis pecanis* Bissel (Hemiptera: Drepanosiphidae) (Tedders, 1978). The yellow pecan aphid shows a strong pattern of host-associated genetic differentiation when occurring on two different host plant species: pecan, *Carya illinoinensis*, and water hickory, *C. aquatica,* with more than 20% of AFLP loci fixed within host associated population, suggesting very little gene flow between them (Dickey & Medina, 2010).

The genus *Aphelinus* are all aphid parasitoids in the hyperdiverse superfamily Chalcidoidea. These parasitoids are quite small (1-2mm) and are often very difficult to

distinguish morphologically. We suspected this genus might be particularly subject to sequential radiation following a host-plant shift by their aphid hosts for two reasons. First, two different species in this genus, *A. varipes* (Heraty et al., 2007, Hopper et al., 2007, Woolley et al., 2007) and *A. asychus* (Kazmer et al., 1996, Chen et al., 2002), have recently been shown to consist of morphologically cryptic species complexes. Several species within the *A. varipes* complex specialize on a single host aphid species and all sympatric species in the complex are reproductively isolated from each other because females reject courting heterospecific males (Heraty et al., 2007, Hopper et al., 2007). Members of the *A. varipes* complex also show some of the lowest divergence time estimates among sister species of any animal with one being as low as 72,000 years (Heraty et al., 2007). Second, *Aphelinus* are poor dispersers who generally forage on foot (Heraty et al., 2007, Rao et al., 1999). This behavior is likely to decrease migration rates and increase inbreeding rates in species belonging to this genus (Heraty et al., 2007).

We used cluster analyses of AFLP molecular markers to test for sequential radiation of *A. perpallidus* at the same geographic scale used to test for host-plant associated genetic differentiation in its host aphid.

Methods

Insect sampling

Our study area consisted of three counties in East Texas (Figure15). Within this area both pecan and water hickory grow wild and pecan is also planted as an ornamental and

orchard crop tree. Our aim was to test the role of host plant species in promoting reproductive isolation while preventing confounding geographic effects. For genetic analyses, three to four *A. perpallidus* populations were sampled for each tree species from June 2007 to October 2009 with the two sites furthest apart for each tree species separated from one another by at least 70 Km (Table14).

A. perpallidus were reared in the lab from wild collected yellow pecan aphid nymphs and stored in 95% ethanol or at -80°C after emergence from their mummified host. *A. perpallidus* not used for genetic analyses were stored in 95% ethanol as vouchers or submitted to J. Woolley (Texas A&M University) to confirm species identification. AFLP fingerprints were obtained for ~15 *A. perpallidus* from each tree species. The SESim statistic (Medina et al., 2006) was used to determine if individual and marker sampling was adequate for a host-associated differentiation study.

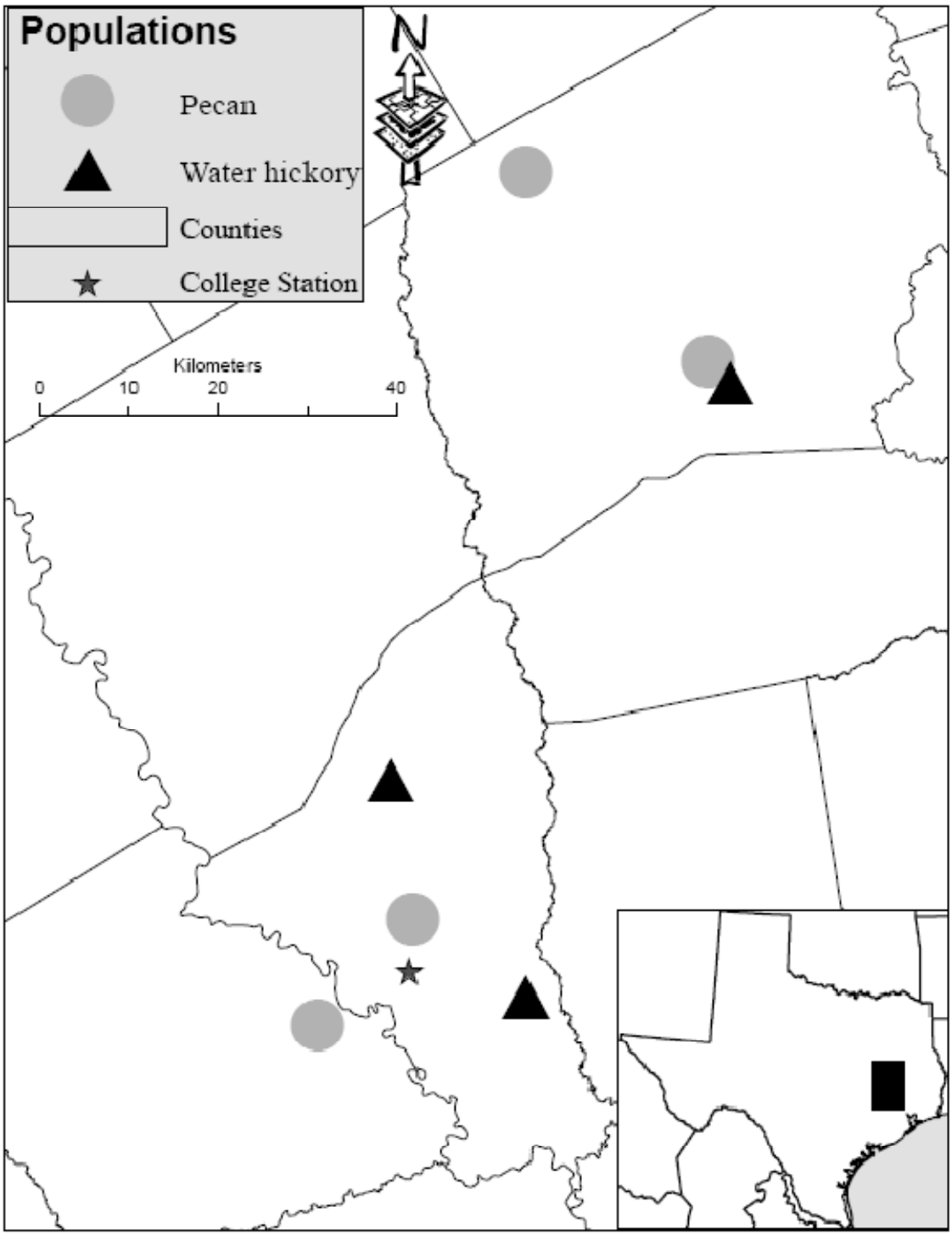


Figure 15: Locations of *Aphelinus perpallidus* collecting sites in the East Texas study area.

Table 14: Site names, locations, and number of *Aphelinus perpallidus* genotyped per site.

Site	Location (degrees decimal)	Tree Species	Wasps Genotyped
Fort Boggy State Park	95.979 W 31.189 N	water hickory	6
		pecan	2
Jewett	96.147 W 31.346 N	pecan	2
Tabor	96.365 W 30.789 N	water hickory	3
Wolf Pen Creek	96.301 W 30.621 N	pecan	5
Lick Creek Park	96.222 W 30.561 N	water hickory	5
USDA-Pecan Genetics	96.434 W 30.517 N	pecan	6

DNA isolation and AFLP reactions

Whole genomic DNA was extracted from *A. perpallidus* using the DNeasy blood and tissue kit (Qiagen Corp., Valencia CA) following the manufacturer's instructions. AFLP (Vos et al., 1995, Gompert et al., 2006, Saunders et al., 2001) profiles were generated from ~90ng of DNA from each sample using the following selective primer pairs: *Mse*1-CAA/*Eco*R1-ACG, and *Mse*1-CAT/*Eco*R1-AC. Polymerase Chain Reactions (PCR's) were run in GeneAMP® 9700 thermocyclers and diluted amplified selective products were analyzed with an ABI 3130 capillary sequencer with co-loaded fluorescent (MapMarker® 1000XL X-Rhodamine) size standard ladder (BioVentures, Inc., Murfreesboro, TN) according to manufacturer's instructions. Thermocycling conditions were as follows: samples undergoing preselective amplification were held at 95^0C for 1 min followed by 20 cycles of 95^0C for 10 sec, 56^0C for 30 sec, and 72^0C for 90 sec followed by a hold at 75^0C for 5 min. For the selective amplification, samples were held at 95^0C for 30 sec followed by 47 cycles of 95^0C for 10 sec, and 12 cycles starting at 65^0C for 40 sec (decreasing 0.7^0C per cycle until reaching 56^0C) and 72^0C for 90 sec, followed by a hold at 75^0C for 5 min. Absence of contamination was assured by negative controls and accuracy and repeatability of DNA fingerprints within species was verified by repeating all PCR steps for one sample. For each insect and selective primer combination, resulting electrophenograms were examined and analyzed using GeneMapper® 4.0 (Applied Biosystems, Forest City, CA). An allele calling threshold of 50 reflectance units was selected since this was more than 1.5 times the baseline noise of all electrophenograms. Private alleles were removed from all samples. Six alleles were

removed from the *Mse*1-CAA/*Eco*R1-ACG primer data set due to their presence in the negative control.

The SESim statistic (Medina et al., 2006) was calculated to determine if individual and molecular marker sampling was adequate for the host-associated differentiation study. 10,000 iterations of the SESim algorithm were employed. The two selective primer combinations produced 298 loci which gave a SESim value of 0.028. Since Medina et al. (2006) showed that population structure began to break up due to inadequate sampling when SESim values were greater than 0.05, we determined that marker and individual sampling was adequate for our study.

Molecular data analysis

STRUCTURE 2.2 (Pritchard et al., 2007) recessive alleles model for dominant marker data assuming admixture and correlated alleles (Falush et al., 2007) was used to determine the number of populations represented by the data. Admixture is a general attribute of most species occurring in sympatry and the "alleles correlated" model deviant has been shown to be the most sensitive to the presence of population structure in simulated data (Falush et al., 2003). STRUCTURE assumes that within a population, loci are in Hardy-Weinberg equilibrium and linkage equilibrium and assigns individuals to separate populations so as to eliminate violations of these assumptions. The output of STRUCTURE is the log probability of the data (X) given the number of clusters (K) assumed or [Ln Pr(X|K)]. Where parameter estimates indicated K>1, the *ad hoc* ΔK

statistic (Evanno et al., 2005) was used to predict the most likely number of clusters (K) in the data. This involves calculating the second-order rate of change of [Ln Pr(X|K)]. Evanno *et al.* (2005) showed that the K corresponding to a spike in this value accurately predicts the number of populations represented by the data. The model was run for 100,000 generations with a burn-in period of 10,000 generations for 20 iterations each from K=1 to K= 8 by compute clusters at Cornell University's Computational Biology Service Unit via the BioHPC web interface (http://cbsuapps.tc.cornell.edu/structure.aspx). Because *A. perpallidus* had a high number of alleles at low frequency, the parameter lambda was first inferred for K=1 (Pritchard et al., 2007) and then fixed at 0.66 for all STRUCTURE analyses.

Nested AMOVA (Excoffier et al., 1992) and principal coordinates analyses (PCO) were conducted among host plants, among collecting sites, and among STRUCTURE delimited populations using the software GenAlEx 6.2 (Peakall & Smouse, 2006). AFLPsurv 1.0 (Vekemans, 2002) was used to estimate Nei's gene diversity, percent polymorphic loci, and Fst among host plant species, collecting sites, and STRUCTURE delimited populations using Bayesian analyses with a non-uniform prior distribution of loci (Zhivotovsky, 1999) and the estimation procedures of Lynch and Milligan (Lynch & Milligan, 1994). To determine if the estimated Fst values were significantly different from 0, permutation tests were conducted with 9,999 random permutations of the data. To determine if estimates of Nei's gene diversity differed among host plants or STRUCTURE delimited phenotypes, 95% confidence intervals were constructed around

the estimates by multiplying the standard error by the critical value from the Student's distribution with degrees of freedom equal to the number of AFLP loci minus one.

Results

The second order rate of change in K, (Evanno's ΔK) peaked for K=3 populations indicating that three populations best explained the *A. perpallidus* AFLP data (Figure 16) however these populations did not correspond to host-plant species of origin or collecting site. At K=3, all individuals were assigned with high (>78%) probability to one of the three populations.

Each STRUCTURE delimited population had its own diagnostic AFLP multilocus phenotype. One locus was diagnostic for population 1, six loci were diagnostic for population 2, and 4 loci were diagnostic for population 3. Additionally, one locus was universally present in both populations 1 and 2 but absent from population 3 (Table 15).

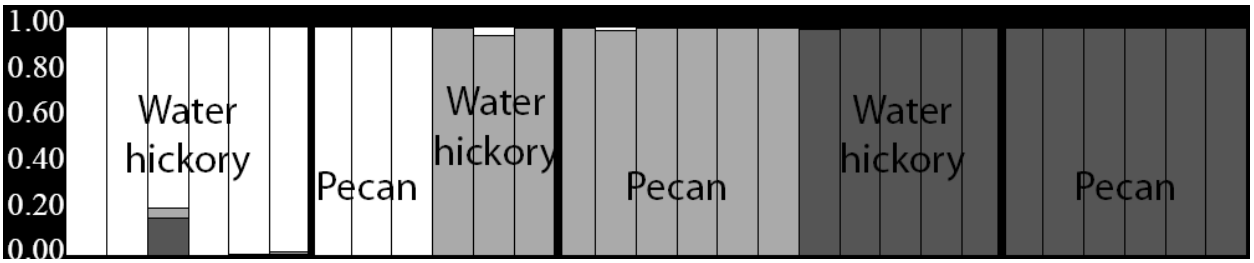


Figure 16: Bayesian population assignment probabilities (y-axis) for *Aphelinus perpallidus* individuals (x-axis) collected from pecan and water hickory using the recessive alleles model for dominant marker data in STRUCTURE 2.2. Three populations (white, light gray, and dark gray) are indicated. STRUCTURE delimited populations do not correspond to host plant of origin or collecting site.

Table 15: Diagnostic AFLP loci for each of the three STRUCTURE delimited populations of *Aphelinus perpallidus*. Each AFLP Locus name contains the selective primer combination used and the size of the DNA fragment in base pairs.

AFLP Locus	**Population 1**	**Population 2**	**Population 3**
Mse1-CAA/EcoR1-ACG _55	**X**	**X**	
Mse1-CAA/EcoR1-ACG _60		**X**	
Mse1-CAA/EcoR1-ACG _96			**X**
Mse1-CAA/EcoR1-ACG _118		**X**	
Mse1-CAA/EcoR1-ACG _151	**X**		
Mse1-CAA/EcoR1-ACG _169		**X**	
Mse1-CAA/EcoR1-ACG _293			**X**
Mse1-CAA/EcoR1-ACG _336			**X**
Mse1-CAA/EcoR1-ACG _348		**X**	
Mse1-CAT/EcoR1-AC _96		**X**	
Mse1-CAT/EcoR1-AC _195		**X**	
Mse1-CAT/EcoR1-AC _213			**X**

The overall Fst among STRUCTURE delimited populations was significantly different from zero (Fst = 0.3339, p <0.0001). The overall Fst among sites was also significantly different from zero (Fst = 0.2055, p = 0.0007). Significant molecular variation occurred among sites (20%), and within sites (80%), but not among host plants. Principal coordinates 1 and 2 explain 72.8% of the molecular variation. As with the structure results, the wasps were grouped into 3 clusters in the principal coordinates analysis (Figure 17). The significant among site variation found is likely due to the fact that at most sites, only 1 or 2 STRUCTURE delimited populations were present (Figure 18).

Discussion

Why is K>1?

Pritchard et al. (2001) warned against reading too much into STRUCTURE values of K>1 populations in the absence of a biological/ecological explanation. Among parasitoids multiple sympatric populations have been found using AFLP data for *Nasonia giraulti, Leptopilina clavipes* and *Baryscapus servadeii* (van Opijnen et al., 2005, Pannebakker et al., 2004, Simonato et al., Unpublished Data). In the case of *L. clavipes*, 2 of 3 populations were unique to wasps known to be exclusively parthenogenetic and the other was diagnostic for sexually reproducing wasps. In the case of *B. servadeii*, the populations may also correspond to a sexually reproducing population and to a parthenogenetic population.

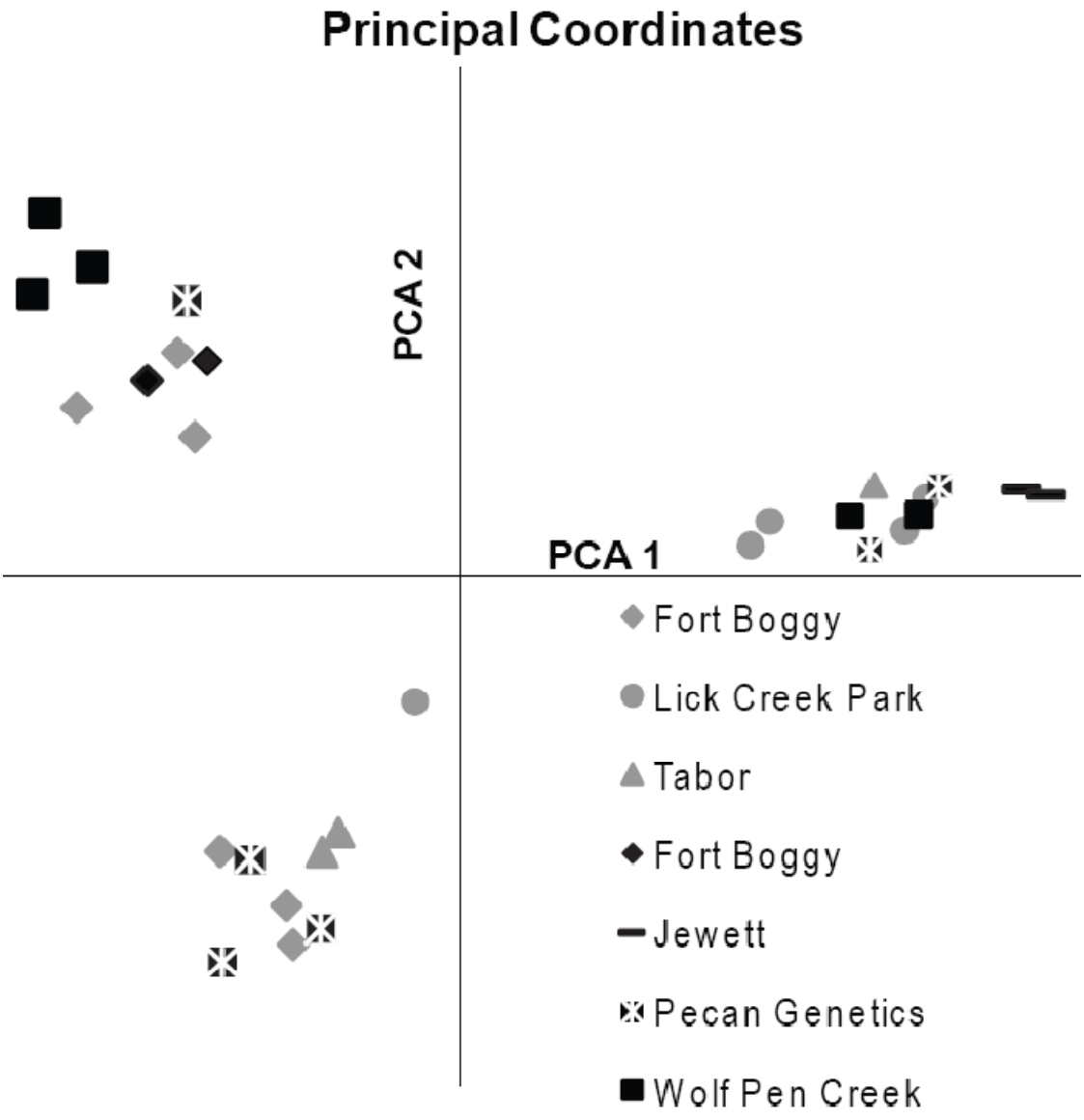


Figure 17: Eigenvectors of principal coordinates 1 (x-axis) and 2 (y-axis) for *Aphelinus perpallidus*. Symbol shapes denote collecting sites and symbol colors denote host plant species. Grey = water hickory, Black = pecan

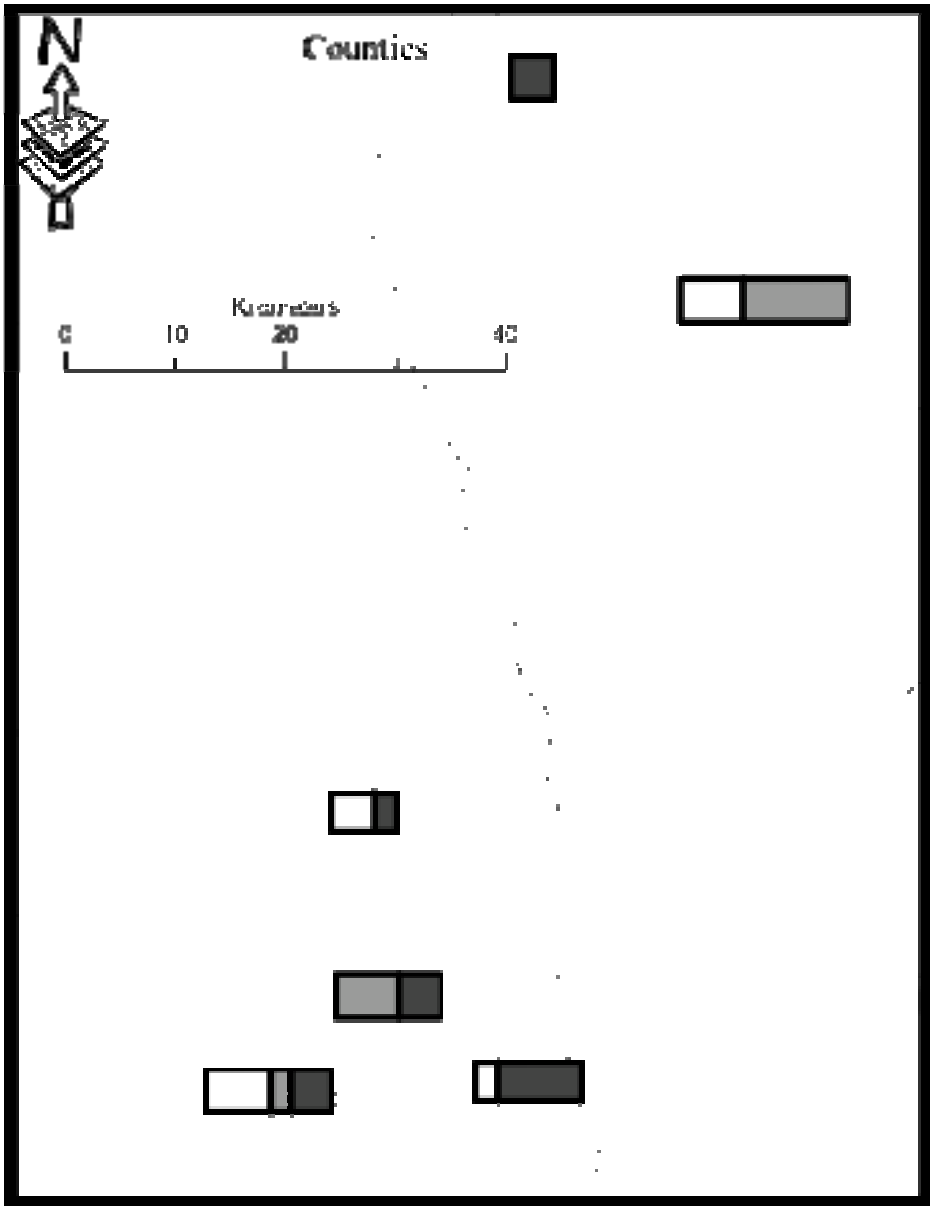


Figure 18: Frequencies of three genetically different *Aphelinus perpallidus* populations at each site. The total length of the bar corresponds to the number of female wasps genotyped.

The result of STRUCTURE finding more than one population for *A. perpallidus* is interesting. That these distinct molecular phenotypes do not correspond to geographic locality or host plant species indicates that this apparent genetic structuring is, however, without an explanation at present. The significant molecular variation due to collection site is likely due to the absence of at least 1 STRUCTURE delimited population from most sites (Figure 18). Neither the identity nor the proportions of the particular genetically distinct populations present at each site appear to conform to a pattern of isolation by distance. None-the-less, we could have exclusively parthenogenetic lineages in our data set as has been found in other parasitoid species (Schneider et al., 2002, Pannebakker et al., 2004). Other authors have noted that *A. perpallidus* is male biased (Bueno & VanCleave, 1997) however our collections were slightly female biased. Populations 2 and 3 have a lower percentage of polymorphic loci, lower estimates of genetic diversity (Table 16) and overall higher genetic similarity (Figure 19) than population 1. These are all genetic characteristics of parthenogenetically reproducing populations. Future studies should investigate the possibility of obligate parthenogenetic populations and/or reproductive incompatibility induced by endosymbionts in *A. perpallidus*.

Table 16: Diversity statistics for *Aphelinus perpallidus.* n=sample size, %PL=percent polymorphic loci, Hj=expected heterozygosity (Nei's gene diversity), and SE(Hj) is the standard error of Hj for each STRUCTURE delimited population. For gene diversity estimates, different letters denote non-overlapping 95% confidence intervals between phenotypes.

Population	N	%PL	Hj	SE(Hj)
1	9	68.1	0.22365_{a}	0.00978
2	9	57	$0.18759_{a,b}$	0.01044
3	11	54.7	0.17435_{b}	0.01041

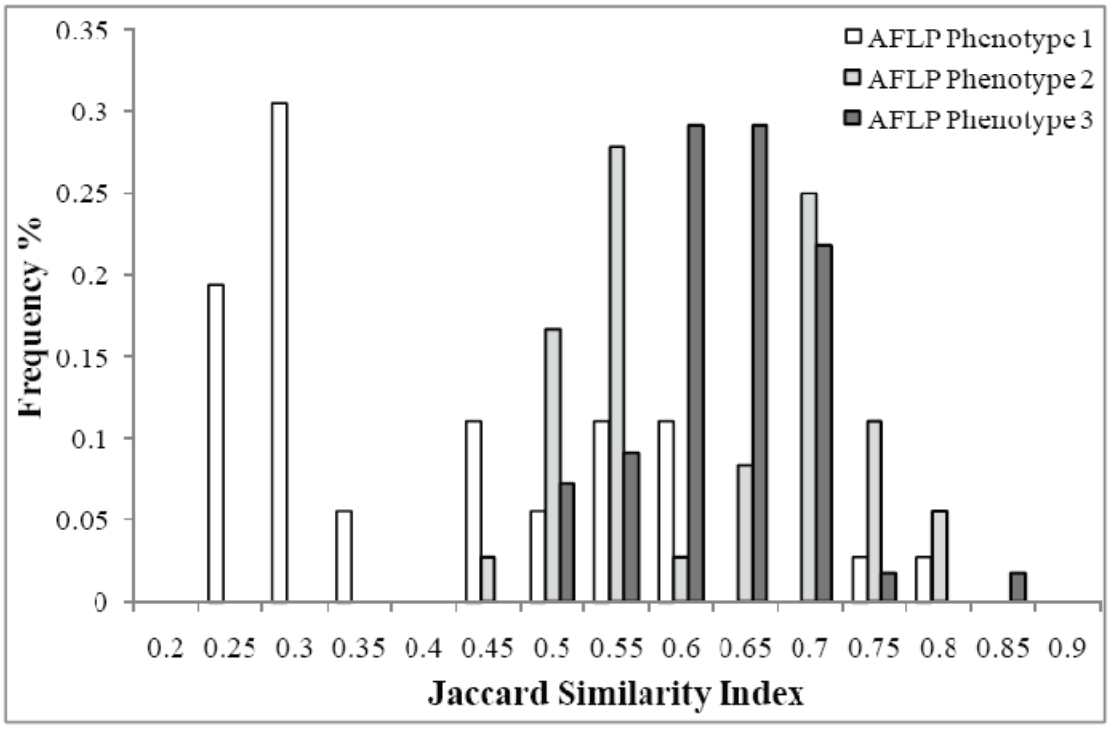


Figure 19: Distribution of pairwise genetic similarities (Jaccard's index) calculated between *Aphelinus perpallidus* individuals inside three STRUCTURE delimited AFLP phenotypes.

Host location in parasitoids

In order to locate their insect hosts, parasitic wasps must first locate the host's habitat (Vinson, 1998, Vinson, 1976, Vet & Dicke, 1992). In the case of parasitoids of herbivorous insects, the insect host's habitat is defined in large part by their host plants. To find an adequate host-plant, a parasitoid may rely on genetically determined chemical preferences (Reed et al., 1995, Vaughn et al., 1996) and/or on chemical information gathered from the host remains upon emergence (Villagra et al., 2007, van Emden et al., 2008, Gandolfi et al., 2003, Storeck et al., 2000). The naive wasp then orients toward specific chemicals emitted by the host-plants on which their insect hosts feed (Wickremasinghe & Vanemden, 1992, Read et al., 1970, Vaughn et al., 1996), their insect hosts (DeFarias & Hopper, 1997), and/or the host-plant complex (Reed et al., 1995, Vet & Dicke, 1992, Wickremasinghe & Vanemden, 1992). The degree to which the initial habitat or plant preferences of specific parasitoids is genetically determined or learned is not known but it is likely to be primarily learned (Bogahawatte & vanEmden, 1996), at least in aphid parasitoids (van Emden et al., 2008, Morgan & Hare, 1998, Takemoto et al., 2009).

Our molecular data indicate that *A. perpallidus* is not a host-plant specialist. Likewise it has also not specialized on the yellow pecan aphid host-associated populations found. Two lines of reason argue against finding sequential radiation in aphid parasitoids. First, in studies to date, parasitoids have generally shown stronger preference for the aphid host alone or for the host-plant complex while weaker or no preference has been found

for the plant alone (Bertschy et al., 2001, DeFarias & Hopper, 1997, Kalule & Wright, 2004, Meiners & Hilker, 1997, Reed et al., 1995, Souissi et al., 1998, Wickremasinghe & Vanemden, 1992). Second, while learned chemical cues have been shown to be specific to taxonomic levels below plant species, including specific wheat cultivars (Kalule & Wright, 2004), and even to the broader multi-plant species habitat, for example wheat grown near tomato plants (van Emden et al., 2008), genetically determined responses to plant cues probably derive from taxonomic levels higher than species (Reed et al., 1995). For example Brassicaceae that produce isothyanates (Blande et al., 2007, Bradburne & Mithen, 2000).

Sequential radiation in parasitoids: how common is it?

Aphidius ervi collected from pea aphid on alfalfa was not genetically differentiated from *A. ervi* collected from grain aphid on wheat (Daza-Bustamante et al., 2002). This pattern was consistent with *A. ervi's* plastic behavior whereby individuals preferred the host plant complex on which they had most recently been reared rather than the complex from which their populations had originally been collected. That *A. ervi* does not show heritability of host/habitat preference genes has been validated by behavioral results from additional authors (Villagra et al., 2007, van Emden et al., 2008). Rather, *A. ervi* relies exclusively on imprinting on the chemical signals present on host remains or larval waste products present therein. This imprinting may be further behaviorally reinforced by conditioning during oviposition (van Emden et al., 2008).

The combination of behavior and genetic data reinforces the designation of *A. ervi* as a generalist, at least at the level of aphid genus and plant sub-class. In contrast, host plant preference in *Diaeretiella rapae* has a partial genetic basis; wasps strongly prefer cruciferous plants, regardless of plant source population, imprinting, or conditioning (Vaughn et al., 1996). This has led to the designation of *D. rapae* as a habitat specialist and the degree of specialization seems to be at the level of plant genus. Despite this genetically controlled bias, *D. rapae* parasitizes 60 different aphid species and though it has formed genetically differentiated populations on cruciferous-cabbage aphid and wheat-Russian wheat aphid host-plant complexes at a local scale, (Vaughn & Antolin, 1998) this pattern does not extend throughout the entire geographic range (Baer et al., 2004). This pattern of local adaptation has led Antolin et al. to designate *D. rapae* a serial habitat specialist (Antolin et al., 2006).

Daza-Bustamante et al. (2002), Vaughn and Antolin (1998), and Baer et al. (2003) tested for genetic differentiation in parasitoids feeding on different host-plant complexes (neither the aphids nor the plants making up the host-plant complexes were closely related). In contrast, both our study and (Lozier et al., 2009) tested for sequential radiation in aphid parasitoids (the host aphids representing sister taxa or strongly differentiated host-races and the host plants in the same genus). Only Vaughn and Antolin (1998) found evidence of genetic differentiation at the local scale in *D. rapae* and this was later found not to persist at larger geographical scales. Taken together, these 5 studies suggest that host-plant habitat explained divergence and sequential radiation

may not be particularly common in parasitoids of exophytic aphids. Interestingly, all the cases where sequential radiation has been found involve insects associated with endophagous herbivores inducing galls or feeding in fruits. These include the parasitoids *Diachasma alloeum*, on apple maggot differentiated on hawthorn and apple (Forbes et al., 2009); *Platygaster variabilis* on *Rhopalomyia sp.* differentiated on two species of goldenrods (Stireman et al., 2006); and *Copidosoma gelechiae* on *Gnorimoschema gallaesolidaginis* on two species of goldenrods (Stireman et al., 2006, Kolaczan et al., 2009).

The notion of sequential radiation has only recently started to gather steam and has been highlighted by recent empirical studies (Lozier et al., 2009, Kolaczan et al., 2009, Forbes et al., 2009, Stireman et al., 2006) and reviews (Abrahamson & Blair, 2008, Feder & Forbes, 2010). Abrahamson and Blair (2008) reviewed 5 examples of sequential radiation out of 6 tested and suggested that the conditions often leading to host race formation of herbivores were likely to be the same conditions leading to sequential radiation. These are 1) A host switch occurs, 2) There is a genetic basis of habitat selection and host fidelity, 3) There is a host-based oviposition preference, 4) The insects phenology mirrors that of the host, and 5) Differential insect fitness is host-associated. We cannot speak about conditions 3 and 5 regarding *A. perpallidus*, however we can speak to some degree about conditions 1, 2, and 4. For condition 1 (a host shift occurs) to be met, condition 2 (genetic control of host preference) must also be met. Otherwise the insect would only increase its diet breadth, not switch hosts. Based on the present

study, we infer that in *A. perpallidus*, diet breadth extends at least to multiple host-races of yellow pecan aphid and based on other host records, possibly includes other derepaosiphid aphids feeding on pecan leaves (Tedders, 1978, Bueno & VanCleave, 1997). However, the possibility of sequential radiation based on host aphid species and genera deserves further attention in *A. perpallidus*. With regard to condition 4, the phenology of *A. perpallidus* is unlikely to be intimately linked to that of its host since its host is not univoltine. All the cases of sequential radiation documented to date involve univoltine insects on univoltine hosts (Forbes et al., 2009, Stireman et al., 2006, Abrahamson & Blair, 2008). This condition will probably not be met in parasitoids of exophytic aphids as they are multivoltine with overlapping generations (Dixon, 1998). Exophytic aphids themselves are probably prone to undergo host-associated genetic differentiation for reasons other than univoltinism per se (Dixon, 1998, Dickey & Medina, 2010).

CHAPTER VI

SUMMARY AND EPILOGUE

Carya: A Parallel System to *Solidago*

As mentioned in Chapter I, neither the present work on *Carya* herbivores, nor the parallel work on *Solidago* herbivores tested HAD in enough herbivores so as to assess statistically either the ubiquity of HAD or whether specific herbivore traits could promote it. Thus, impetus should be given to testing HAD in additional shared herbivores in these two systems. Such future studies must report negative results otherwise the ubiquity of HAD will be overestimated. The Medina laboratory is in the process of accumulating such studies and unpublished results suggest that HAD is present in two additional parthenogenetic species but absent in two other parthenogenetic species . This brings the total number of herbivorous parthenogens tested in this system to 6 and HAD has been found in 4 of the 6 parthenogenetic species. 7 herbivorous endophages have been tested for HAD on *Solidago* and HAD is present in 4 of the 7 endophagous species (Stireman et al., 2005, Waring et al., 1990, Blair et al., 2005) (Table 17).

Table 17: A comparison of herbivores tested for HAD from the *Solidago* and *Carya* host plant systems. X denotes the presence of endophagy, parthenogenesis, and HAD.

Solidago altissima and *Solidago gigantea* ~126 shared insect species documented			
Insect Order	**Herbivore Species**	**Endophagous**	**HAD**
Diptera	*Asteromyia carbonifera*	**X**	
Lepidoptera	*Epiblema scudderiana*	**X**	
Diptera	*Eurosta solidaginis*	**X**	**X**
Lepidoptera	*Gnorimoschema gallaesolidaginis*	**X**	**X**
Coleoptera	*Mordellistena convicta*	**X**	**X**
Diptera	*Procecidochares atra*	**X**	
Diptera	*Rhopalomyia solidaginis/capitata*	**X**	**X**
Coleoptera	*Trirhabda convergens*		
Coleoptera	*Trirhabda virgata*		

Carya aquatica and Carya illinoinensis ~30 shared insect species documented			
Insect Order	**Herbivore Species**	**Parthenogenetic**	**HAD**
Lepidoptera	*Gretchena bolliana*		
Hemiptera	*Phylloxera devastatrix*	**X**	**X**
Hemiptera	*Phylloxera notabilis*	**X**	**X**
Hemiptera	*Phylloxera texana*	**X**	
Hemiptera	*Melanocallis caryaefoliae*	**X**	
Hemiptera	*Monellia caryella*	**X**	**X**
Hemiptera	*Monelliopsis pecanis*	**X**	**X**

Are the Categories (Endophagy and Parthenogenesis) Too Broad?

As I mentioned in Chapter II, all of the parthenogens studied so far in the *Carya* system are aphids. This is problematic because HAD is not being tested in phylogenetically diverse parthenogens and there are other aphid traits thought to promote HAD. Thus, can we really test the role of parthenogenesis in promoting HAD *per se*? Or can we merely test the role of "aphidness" more broadly? A similar problem exists with the endophages on *Solidago*. While it is true that they are phylogenetically diverse, representing the orders Coleoptera, Lepidoptera, and Diptera; they are all gall makers with the exception of one gall inquiline. How common is HAD in seed feeders, leaf miners, root feeders, and other guilds of endophages? Parthenogenesis and endophagy may be overly broad categories. Thus, once sufficient numbers of species with vs. without these traits have been tested for HAD in a single host-plant system, the next step might be to think about these traits as continua.

For example, parthenogenesis in the animal kingdom exists in several forms. Furthermore, while all forms of parthenogenesis reduce recombination rates compared to obligate sexual reproduction, not all do so equally. Because recombination can break up host associated linkage groups (Hartl, 1972, Hawthorne & Via, 2001), the likelihood of HAD should increase as recombination rates decrease. In automictic parthenogenesis ova undergo meiosis, in apomictic parthenogenesis ova undergo mitosis, in arrhenotokous parthenogenesis males develop from unfertilized ova, and in theletokous

parthenogenesis females develop from unfertilized ova (Suomalainen, 1962). Table 18 shows some taxa with these kinds of parthenogenesis.

While recombination is eliminated in very few of these taxa, recombination is highest in Arrhenotokous species (Hartl, 1972) because all females are produced sexually. Automictic thelytoky will have a higher recombination rate than apomictic thelytoky because chromosomes in gametes are crossing over during meiosis (Suomalainen, 1962). Recombination rate should be lowest in apomictic thelytoky because meiosis is absent and thus, there is no crossing over. However, species exhibiting apomictic thelytoky vary in the number of parthenogenetic generations between sexual generations and so they vary in the amount of recombination. For example, a holocyclic aphid species might undergo 30 generations of apomictic thelytoky prior to sexual reproduction and recombination (Chapter III), but univoltine phylloxera or cynipid wasps might only undergo 1 generation of apomictic thelytoky prior to recombination (Chapter IV). Thus recombination rate might vary by an order of magnitude even among apomictic thelytokous parththenogens because of the number of parthenogenetic generations prior to sex. I predict that the propensity for HAD should increase along such a recombination continuum (Figure 20).

Table 18: Some selected parthenogenetic insect taxa. Not all taxa in a clade may possess parthenogenesis but taxa are listed if parthenogenesis has been documented.

	Thelytokous	**Arrhenotokous**
Apomictic	Odonata: Coenagrionidae, Diptera: Chironomiidae, Hemiptera: Coccidoidea*, Hemiptera: Aphidoidea, Hymenoptera: Cynipoidea, Hymenoptera: Vespoidea, Hymenoptera: Tenthredinoidea, Ephemeroptera	
Automictic	Hymenoptera: Formicidae, Symbiont incuded Hymenoptera, Hymenoptera: Chalcidoidea, Hymenoptera: Apoidea, Hymenoptera: Vespoidea, Hymenoptera: Ichneumonoidea, Hymenoptera: Tenthredinoidea	Hymenoptera, Acari, Thysanoptera, Colepotera: Scolytidae, Hemiptera: Coccidoidea

*Some coccids are technically automictic but with the same genetic outcome as apomixis)

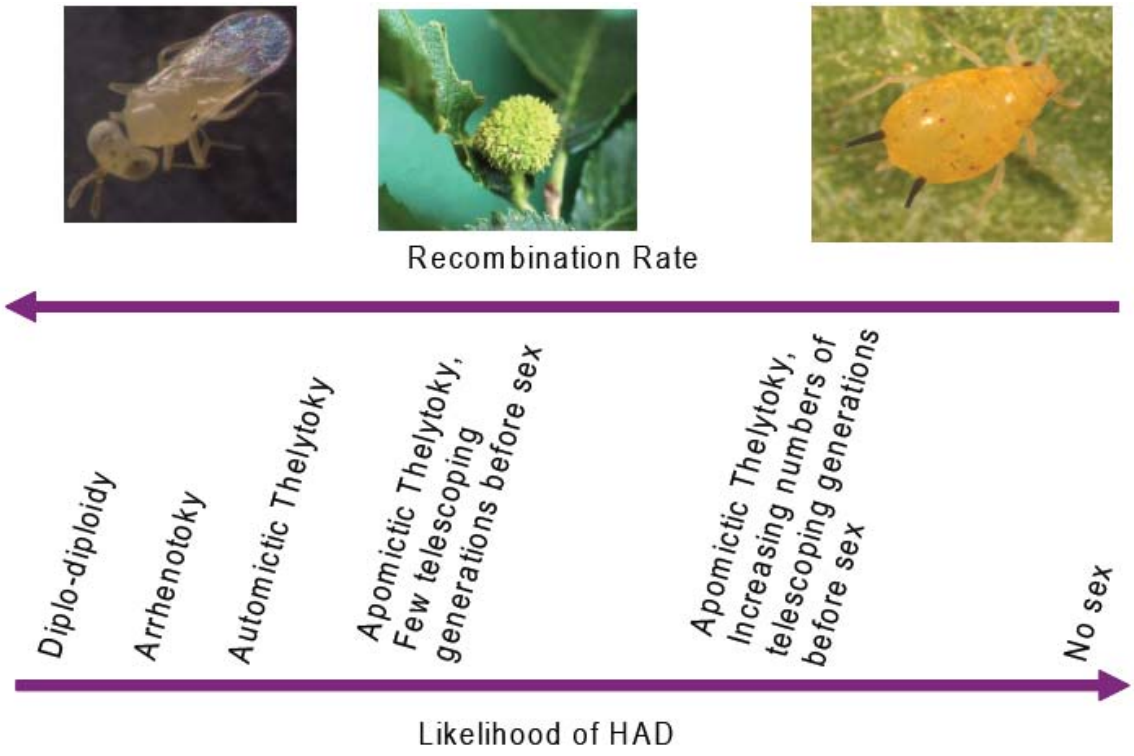


Figure 20: A hypothesized relationship between HAD and recombination rate. The propensity for HAD may increase along a hypothesized parthenogenesis continuum due to decreasing relative recombination rate.

A similar continuum might be considered with endophagy as well. The propensity for HAD should increase with the degree of intimacy with the host. For example, gall inducing taxa must be able to directly manipulate the host's immune system requiring increased genetic intimacy with the host (i.e., a gene-for-gene interaction). Gall makers should thus have the most intimate associations with their host. A less intimate association for an endophage might be a physical dependence on the size of a plant part. For example the growth of seed feeding endophages can be constrained by the volume of the seed, a trait which is likely very labile within a plant species. Some endophages (e.g. tree trunk borers) are not constrained in either of these ways (genetic or physical) and their constraints are likely to be more similar to those faced by exophages such as plant chemistry or plant toughness. An "intimacy" continuum for endophages and a recombination continuum for parthenogens, may be useful concepts to consider in the future of HAD studies addressing these traits.

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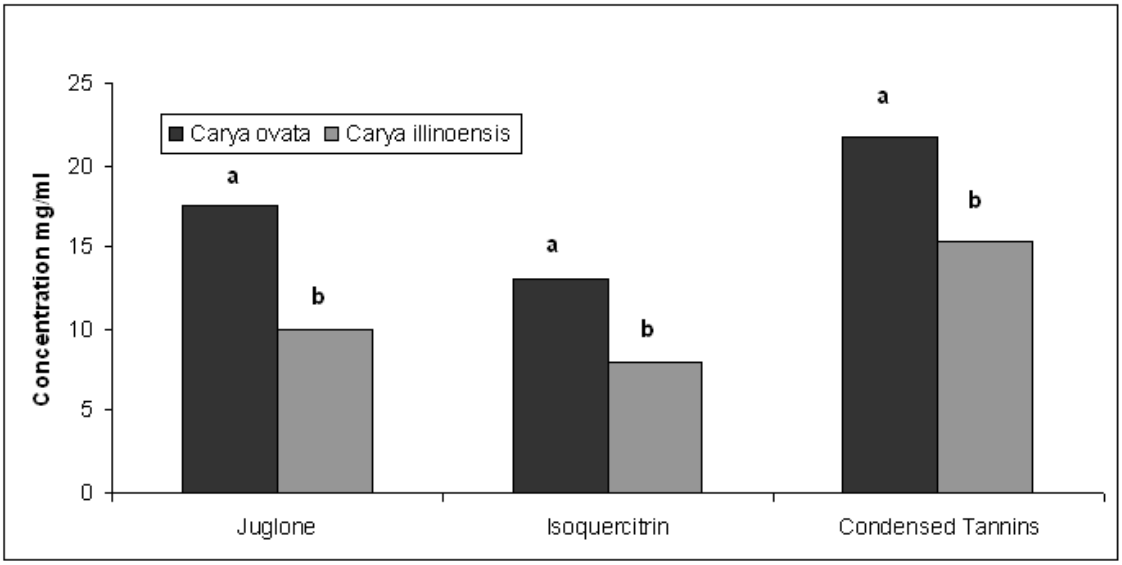
APPENDIX A

TOTAL PHENOLICS ANALYSIS OF PECAN AND WATER HICKORY FOLIAGE

Introduction

Pecan is the state tree of Texas and the most economically important indigenous nut crop in the US, valued at over 200 million dollars annually (Grauke et al., 2003). Water hickory is a closely related species but unlike pecan has a flat, wrinkled and bitter nut relative to pecan (Stone et al., 1965). Differences in nut oils are documented between these two *Carya* species (Stone et al., 1965). While differences in the phenolic compounds and condensed tannins between leaves of pecan and water hickory have not been measured, they have been documented between pecan and another species of North American hickory, *Carya ovata* (Appendix A, Figure 1). Thus, it is likely that similar differences occur between pecan and water hickories, particularly considering the differences in the bitterness of their nuts. A Folin-Denis assay was used to measure total phenolics of pecan and water hickory foliage.



Appendix A, Figure 1: Mean juglone, isoquercitrin, and condensed tannin concentrations in leaves of shagbark hickory *Carya ovata* and pecan *C. illinoinensis*. For each compound, different letters above bars designate significant differences (P=0.05) between species according to Duncan's multiple range test. Data from Diehl et al. 1992.

Methods

Sample collection

Leaf discs were collected in the field from both tree species from May 28 to June 1, 2008 using an 8m sectional pole saw with a pruner head (Jameson, Clover, SC) and a single hole-punch. Samples were put in 1.5mL microfuge tubes on ice in 0.5mL 70% acetone solution with 1mM ascorbic acid. A second leaf disc was collected from each leaf and stored in individual labeled coin envelopes in order to express total phenolics as a proportion, adjusted by dry mass. The two leaf discs collected from each leaf were symmetrical and equidistant from the leaflet midrib and all samples were taken from one of the three terminal leaflets of the compound leaf. 10 leaf samples were collected per tree, up to three trees per species were sampled at each collecting site (Appendix A, Table 1), and samples were collected from as many different branches as possible throughout the tree. Samples for dry mass were dried for 48 hours in a 70^0C drying oven and weighed in mg with a MX/UMX balance (Mettler Toledo, Columbus, OH).

Sample extraction

Sample extraction was conducted from 3 June to 6 June, 2008. Leaf discs were macerated with scissors, crushed with a pestle, and vortexed in an analog vortex mixer (VWR, West Chester, PA). Samples were centrifuged at 14,000 RPM for 4 minutes and the effluent was removed. The last three steps were repeated four times with an additional 0.1mL of acetone solution added each time for a final volume of 0.8mL. The acetone was then evaporated at 45^0C for a final volume of 0.24mL.

Appendix A, Table 1: Collecting sites for pecan and water hickory leaf samples. 10 leaf samples were taken per tree.

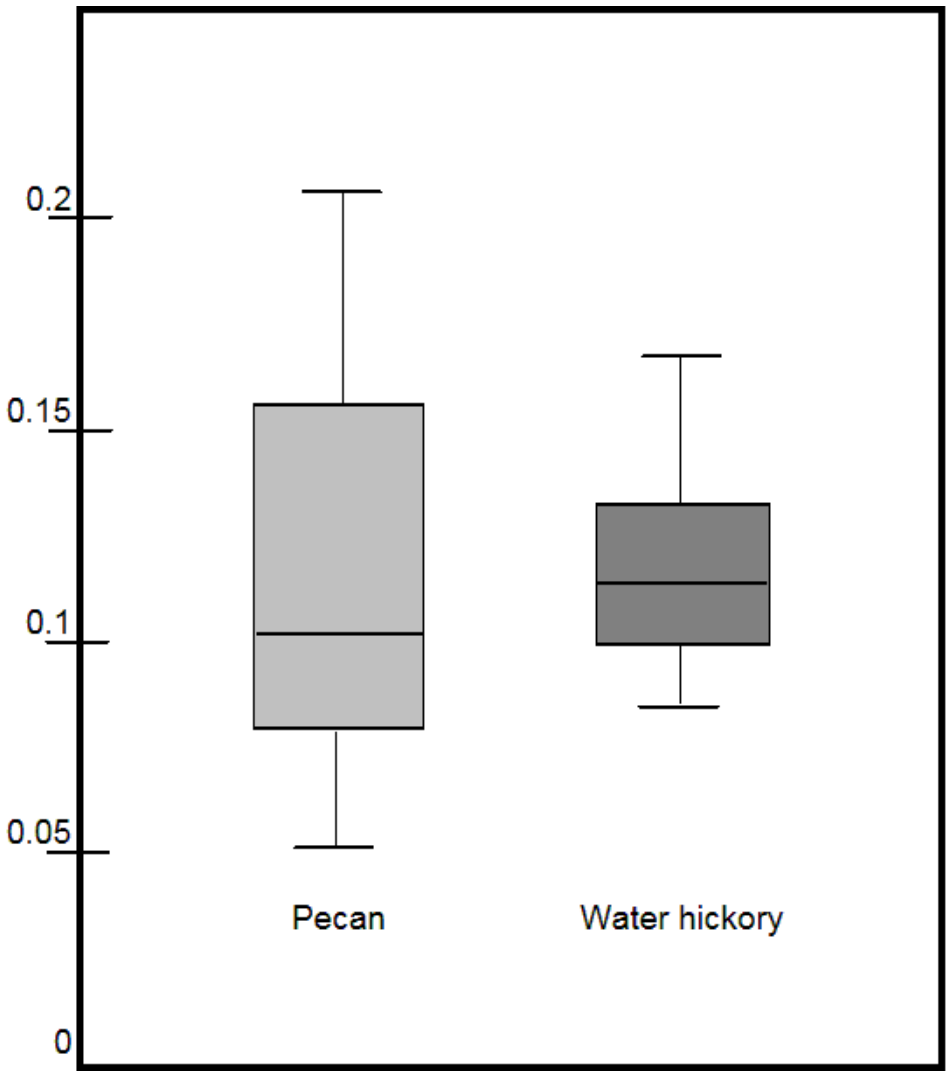
Site	Location (degrees decimal)	Tree Species	# Trees Sampled
Texas A&M University Campus	96.349 W 30.615 N	water hickory	3
		pecan	3
Somerville Wildlife Management Area	96.742 W 30.318 N	water hickory	3
		pecan	1
USDA-Pecan Genetics	96.434 W 30.517 N	pecan	3
Wolf Pen Creek	96.301 W 30.621 N	pecan	3
Fort Boggy State Park	95.979 W 31.189 N	water hickory	3

Folin-Denis assay

The Folin-Denis assay was run on June 9 and June 10, 2008. 1:200 dilutions of each sample were submitted in duplicate to a Folin-Denis assay for total phenolics and read with a microplate reader (BioRad, Hercules, CA) with a 725nm filter. If the %CV (concentration variance) of a sample was higher than 10%, the sample was re-analyzed. If not, the concentrations of duplicate samples were averaged to give a consensus concentration of total phenolics. Known concentrations of tannic acid, 0-70µg/mL, were used as a standard. The final sample concentrations were expressed in mg/ml and were adjusted using the slope and intercept of the standard curve, the sample dilution concentration (1:200), the final concentrated sample volume (0.24mL) and the conversion factor, 0.001 mg/µg. Sample concentrations were then divided by the corresponding sample dry mass to give a proportion expressed in mg/mg tannic acid equivalents. The raw tannic acid equivalents data is given in Appendix B.

Data analysis

Data were non-normal and remained untransformed. Kruskal-Wallis tests (Sokal & Rohlf, 1995) were used to test for differences among sites (5 sites) and differences among trees (19 trees). A Mann-Whitney U test was used to test for differences in total phenolics between the two tree species. For the Mann-Whitney U test, the singleton pecan tree from Somerville Wildlife Management Area was removed to obtain equal sample sizes.



Appendix 1, Figure 2: Total phenolics as a proportion of leaf mass (mg/mg) for Pecan and Water hickory. The Mann-Whitney U statistic is not statistically significant ($p>0.1$). Box plots indicate 10^{th}, 25^{th}, 75^{th}, and 90^{th} percentiles.

Results

There were no differences in total phenolics between tree species ($0.2 > P > 0.1$) (Appendix 1, Figure 2). There were, however, differences among both sites and trees ($P<0.05$).

Discussion

Insect herbivores must be able to deal with different plant chemicals to grow and survive (Berenbaum, 1981, Zangerl & Berenbaum, 1993). Thus, different plant chemical environments are expected to exert different selective pressures on insect herbivores (Berenbaum & Zangerl, 1998, Awmack & Leather, 2002). We wanted to test for divergent plant chemistry as a possible explanation for differential fitness of yellow pecan aphid on pecan and water hickory (Chapters II and III). Such an explanation remains a possibility, but not with regard to the quantity of total phenolics.

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APPENDIX B

TOTAL PHENOLICS DATA FOR PECAN AND WATER HICKORY FOLIAGE

Appendix B Table: Corrected proportion of total phenolics expressed as mg Tannic Acid Equivalents per mg dried leaf tissue.

Species	Site	Tree	Leaf	Phenolics
water hickory	Texas A&M University Campus	1	1	0.121
water hickory	Texas A&M University Campus	1	2	0.101
water hickory	Texas A&M University Campus	1	3	0.093
water hickory	Texas A&M University Campus	1	4	0.099
water hickory	Texas A&M University Campus	1	5	0.1
water hickory	Texas A&M University Campus	1	6	0.107
water hickory	Texas A&M University Campus	1	7	0.104
water hickory	Texas A&M University Campus	1	8	0.139
water hickory	Texas A&M University Campus	1	9	0.093
water hickory	Texas A&M University Campus	1	10	0.098
water hickory	Texas A&M University Campus	2	1	0.203
water hickory	Texas A&M University Campus	2	2	0.132
water hickory	Texas A&M University Campus	2	3	0.167
water hickory	Texas A&M University Campus	2	4	0.141
water hickory	Texas A&M University Campus	2	5	0.19
water hickory	Texas A&M University Campus	2	6	0.15
water hickory	Texas A&M University Campus	2	7	0.132
water hickory	Texas A&M University Campus	2	8	0.179
water hickory	Texas A&M University Campus	2	9	0.138
water hickory	Texas A&M University Campus	2	10	0.121
water hickory	Texas A&M University Campus	3	1	0.092
water hickory	Texas A&M University Campus	3	2	0.083
water hickory	Texas A&M University Campus	3	3	0.103
water hickory	Texas A&M University Campus	3	4	0.086
water hickory	Texas A&M University Campus	3	5	0.107
water hickory	Texas A&M University Campus	3	6	0.073
water hickory	Texas A&M University Campus	3	7	0.082
water hickory	Texas A&M University Campus	3	8	0.073
water hickory	Texas A&M University Campus	3	9	0.102
water hickory	Texas A&M University Campus	3	10	0.084
water hickory	Somerville Wildlife Management Area	4	1	0.119

Appendix B Table continued: Corrected proportion of total phenolics expressed as mg Tannic Acid Equivalents per mg dried leaf tissue.

Species	Site	Tree	Leaf	Phenolics
water hickory	Somerville Wildlife Management Area	4	2	0.104
water hickory	Somerville Wildlife Management Area	4	3	0.149
water hickory	Somerville Wildlife Management Area	4	4	0.116
water hickory	Somerville Wildlife Management Area	4	5	0.121
water hickory	Somerville Wildlife Management Area	4	6	0.119
water hickory	Somerville Wildlife Management Area	4	7	0.105
water hickory	Somerville Wildlife Management Area	4	8	0.21
water hickory	Somerville Wildlife Management Area	4	9	0.142
water hickory	Somerville Wildlife Management Area	4	10	0.121
water hickory	Somerville Wildlife Management Area	5	1	0.127
water hickory	Somerville Wildlife Management Area	5	2	0.125
water hickory	Somerville Wildlife Management Area	5	3	0.134
water hickory	Somerville Wildlife Management Area	5	4	0.113
water hickory	Somerville Wildlife Management Area	5	5	0.115
water hickory	Somerville Wildlife Management Area	5	6	0.122
water hickory	Somerville Wildlife Management Area	5	7	0.097
water hickory	Somerville Wildlife Management Area	5	8	0.214
water hickory	Somerville Wildlife Management Area	5	9	0.131
water hickory	Somerville Wildlife Management Area	5	10	0.115
water hickory	Somerville Wildlife Management Area	6	1	0.158
water hickory	Somerville Wildlife Management Area	6	2	0.096
water hickory	Somerville Wildlife Management Area	6	3	0.101
water hickory	Somerville Wildlife Management Area	6	4	0.104
water hickory	Somerville Wildlife Management Area	6	5	0.187
water hickory	Somerville Wildlife Management Area	6	6	0.119
water hickory	Somerville Wildlife Management Area	6	7	0.115
water hickory	Somerville Wildlife Management Area	6	8	0.111
water hickory	Somerville Wildlife Management Area	6	9	0.122
water hickory	Somerville Wildlife Management Area	6	10	0.134
water hickory	Fort Boggy State Park	7	1	0.122
water hickory	Fort Boggy State Park	7	2	0.121
water hickory	Fort Boggy State Park	7	3	0.099
water hickory	Fort Boggy State Park	7	4	0.119
water hickory	Fort Boggy State Park	7	5	0.089
water hickory	Fort Boggy State Park	7	6	0.139
water hickory	Fort Boggy State Park	7	7	0.122

Appendix B Table continued: Corrected proportion of total phenolics expressed as mg Tannic Acid Equivalents per mg dried leaf tissue.

Species	Site	Tree	Leaf	Phenolics
water hickory	Fort Boggy State Park	7	8	0.136
water hickory	Fort Boggy State Park	7	9	0.103
water hickory	Fort Boggy State Park	7	10	0.112
water hickory	Fort Boggy State Park	8	1	0.098
water hickory	Fort Boggy State Park	8	2	0.07
water hickory	Fort Boggy State Park	8	3	0.07
water hickory	Fort Boggy State Park	8	4	0.074
water hickory	Fort Boggy State Park	8	5	0.099
water hickory	Fort Boggy State Park	8	6	0.1
water hickory	Fort Boggy State Park	8	7	0.099
water hickory	Fort Boggy State Park	8	8	0.092
water hickory	Fort Boggy State Park	8	9	0.534
water hickory	Fort Boggy State Park	8	10	0.106
water hickory	Fort Boggy State Park	9	1	0.122
water hickory	Fort Boggy State Park	9	2	0.107
water hickory	Fort Boggy State Park	9	3	0.096
water hickory	Fort Boggy State Park	9	4	0.086
water hickory	Fort Boggy State Park	9	5	0.124
water hickory	Fort Boggy State Park	9	6	0.107
water hickory	Fort Boggy State Park	9	7	0.122
water hickory	Fort Boggy State Park	9	8	0.16
water hickory	Fort Boggy State Park	9	9	0.173
water hickory	Fort Boggy State Park	9	10	0.209
pecan	Texas A&M University Campus	10	1	0.156
pecan	Texas A&M University Campus	10	2	0.139
pecan	Texas A&M University Campus	10	3	0.166
pecan	Texas A&M University Campus	10	4	0.171
pecan	Texas A&M University Campus	10	5	0.157
pecan	Texas A&M University Campus	10	6	0.222
pecan	Texas A&M University Campus	10	7	0.238
pecan	Texas A&M University Campus	10	8	0.206
pecan	Texas A&M University Campus	10	9	0.187
pecan	Texas A&M University Campus	10	10	0.154
pecan	Texas A&M University Campus	11	1	0.114
pecan	Texas A&M University Campus	11	2	0.122
pecan	Texas A&M University Campus	11	3	0.106

Appendix B Table continued: Corrected proportion of total phenolics expressed as mg Tannic Acid Equivalents per mg dried leaf tissue.

Species	Site	Tree	Leaf	Phenolics
pecan	Texas A&M University Campus	11	4	0.144
pecan	Texas A&M University Campus	11	5	0.113
pecan	Texas A&M University Campus	11	6	0.133
pecan	Texas A&M University Campus	11	7	0.106
pecan	Texas A&M University Campus	11	8	0.103
pecan	Texas A&M University Campus	11	9	0.155
pecan	Texas A&M University Campus	11	10	0.185
pecan	Texas A&M University Campus	12	1	0.13
pecan	Texas A&M University Campus	12	2	0.135
pecan	Texas A&M University Campus	12	3	0.151
pecan	Texas A&M University Campus	12	4	0.199
pecan	Texas A&M University Campus	12	5	0.144
pecan	Texas A&M University Campus	12	6	0.169
pecan	Texas A&M University Campus	12	7	0.137
pecan	Texas A&M University Campus	12	8	0.16
pecan	Texas A&M University Campus	12	9	0.234
pecan	Texas A&M University Campus	12	10	0.206
pecan	Somerville Wildlife Management Area	13	1	0.125
pecan	Somerville Wildlife Management Area	13	2	0.1
pecan	Somerville Wildlife Management Area	13	3	0.092
pecan	Somerville Wildlife Management Area	13	4	0.089
pecan	Somerville Wildlife Management Area	13	5	0.042
pecan	Somerville Wildlife Management Area	13	6	0.099
pecan	Somerville Wildlife Management Area	13	7	0.106
pecan	Somerville Wildlife Management Area	13	8	0.137
pecan	Somerville Wildlife Management Area	13	9	0.08
pecan	Somerville Wildlife Management Area	13	10	0.1
pecan	USDA Pecan Genetics	14	1	0.083
pecan	USDA Pecan Genetics	14	2	0.092
pecan	USDA Pecan Genetics	14	3	0.086
pecan	USDA Pecan Genetics	14	4	0.081
pecan	USDA Pecan Genetics	14	5	0.096
pecan	USDA Pecan Genetics	14	6	0.093
pecan	USDA Pecan Genetics	14	7	0.095
pecan	USDA Pecan Genetics	14	8	0.097
pecan	USDA Pecan Genetics	14	9	0.127

Appendix B Table continued: Corrected proportion of total phenolics expressed as mg Tannic Acid Equivalents per mg dried leaf tissue.

Species	Site	Tree	Leaf	Phenolics
pecan	USDA Pecan Genetics	14	10	0.101
pecan	USDA Pecan Genetics	15	1	0.069
pecan	USDA Pecan Genetics	15	2	0.111
pecan	USDA Pecan Genetics	15	3	0.213
pecan	USDA Pecan Genetics	15	4	0.102
pecan	USDA Pecan Genetics	15	5	0.093
pecan	USDA Pecan Genetics	15	6	0.093
pecan	USDA Pecan Genetics	15	7	0.079
pecan	USDA Pecan Genetics	15	8	0.129
pecan	USDA Pecan Genetics	15	9	0.098
pecan	USDA Pecan Genetics	15	10	0.081
pecan	USDA Pecan Genetics	16	1	0.122
pecan	USDA Pecan Genetics	16	2	0.063
pecan	USDA Pecan Genetics	16	3	0.062
pecan	USDA Pecan Genetics	16	4	0.296
pecan	USDA Pecan Genetics	16	5	0.303
pecan	USDA Pecan Genetics	16	6	0.247
pecan	USDA Pecan Genetics	16	7	0.088
pecan	USDA Pecan Genetics	16	8	0.046
pecan	USDA Pecan Genetics	16	9	0.052
pecan	USDA Pecan Genetics	16	10	0.105
pecan	Wolf Pen Creek Park	17	1	0.015
pecan	Wolf Pen Creek Park	17	2	0.166
pecan	Wolf Pen Creek Park	17	3	0.118
pecan	Wolf Pen Creek Park	17	4	0.28
pecan	Wolf Pen Creek Park	17	5	0.312
pecan	Wolf Pen Creek Park	17	6	0.057
pecan	Wolf Pen Creek Park	17	7	0.046
pecan	Wolf Pen Creek Park	17	8	0.093
pecan	Wolf Pen Creek Park	17	9	0.043
pecan	Wolf Pen Creek Park	17	10	0.161
pecan	Wolf Pen Creek Park	18	1	0.071
pecan	Wolf Pen Creek Park	18	2	0.058
pecan	Wolf Pen Creek Park	18	3	0.081
pecan	Wolf Pen Creek Park	18	4	0.079
pecan	Wolf Pen Creek Park	18	5	0.098

Appendix B Table continued: Corrected proportion of total phenolics expressed as mg Tannic Acid Equivalents per mg dried leaf tissue.

Species	Site	Tree	Leaf	Phenolics
pecan	Wolf Pen Creek Park	18	6	0.093
pecan	Wolf Pen Creek Park	18	7	0.052
pecan	Wolf Pen Creek Park	18	8	0.087
pecan	Wolf Pen Creek Park	18	9	0.176
pecan	Wolf Pen Creek Park	18	10	0.032
pecan	Wolf Pen Creek Park	19	1	0.052
pecan	Wolf Pen Creek Park	19	2	0.048
pecan	Wolf Pen Creek Park	19	3	0.055
pecan	Wolf Pen Creek Park	19	4	0.024
pecan	Wolf Pen Creek Park	19	5	0.064
pecan	Wolf Pen Creek Park	19	6	0.081
pecan	Wolf Pen Creek Park	19	7	0.077
pecan	Wolf Pen Creek Park	19	8	0.058
pecan	Wolf Pen Creek Park	19	9	0.031
pecan	Wolf Pen Creek Park	19	10	0.03

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