ASSESSING MUTUALISMS AND POPULATION GENETICS IN AN INVASIVE ANT AND APHID

A Dissertation

by

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ABSTRACT

When invasive species arrive to new locations, they do not arrive alone. Invasive species come with a symbiotic entourage and can establish new interactions involving both microscopic and macroscopic organisms in their introduced region. These symbiotic interactions, specifically mutualisms, may ecologically facilitate invasive insect establishment. In addition, population genetic composition of these introduced organisms can influence how they modulate both biotic and abiotic factors. The invasive sugarcane aphid (*Melanaphis sacchari* Zehntner) that feeds on sugarcane, was recently reclassified by my research group and I as sorghum aphids (*Melanaphis sorghi* Theobald) when it is found feeding on sorghum plants. This aphid was first reported in the continental United States in 2013. Another invasive insect, tawny crazy ants (*Nylanderia fulva* Mayr) were first reported in the United States in 1997 in Florida and 2002 in Texas. Since genetics and symbiosis can influence the pestiferous nature of invasive insects, this research focused on three different aspects of insect population genetics and symbiosis. First, the microbial composition of aphids feeding on sugarcane or sorghum was examined across the US. Bacteria that are not normally associated with aphids were identified (e.g., *Citrobacter* spp.), while facultative symbionts (e.g., *Regiella*, *Hamiltonella, Serratia*) commonly found in other aphids were not detected. Next, the interaction between the invasive aphid and ant were examined in greenhouse conditions. Although neither have evolutionary history together, ants tended aphids and

aphids presented ants with honeydew. In some instances, this interaction increased biomass when compared with aphids in the absence of ants. Lastly, the fine-scale population genetic structure of tawny crazy ants was assessed using High Throughput Sequencing (HTS) to compare Single Nucleotide Polymorphisms (SNPs) among collections in the US (Texas, Louisiana, Alabama, Mississippi, Georgia, and Florida) and South America (Colombia, Peru, and Argentina). HTS generated thousands of fine-scale molecular markers, allowing for the identification of genetic structure among geographically distinct collections in the US. Integrating both population genetic and microbial analyses can aid in understanding the evolutionary ecology of recently invasive pests. This knowledge can then be used to promote novel approaches toward sustainable pest management.

DEDICATION

To the inner dream of following one's passion. There is no one set path, as each journey is unique. Harness wanderlust as an outlet to decompress, feel joyful, and gain insight. Then through persistence, determination, love, and support it can become a reality.

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Chapter II is in preparation for manuscript submission. The co-authors include myself and my collaborators Antonino Malacrinò and Raul F. Medina.

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NOMENCLATURE

HTS	High throughput sequencing
OTU	Operational Taxonomic Unit
SCAs	Sugarcane aphids
TAMU	Texas A&M University
TCAs	Tawny crazy ants

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CHAPTER I

INTRODUCTION

When invasive insects arrive to a new location, they do not arrive alone. Insects harbor on and within their bodies a complex community of microorganisms (i.e. bacteria, fungi, protists), referred to collectively as a microbiome (Poulsen & Sapountzis 2012). In addition to microbial symbionts, the population genetic structure of propagules can influence the formation of symbiotic interactions and other biologically relevant traits such as stress tolerance. Understanding the establishment of invasive insects and their subsequent symbiotic interactions requires integrating both microbial composition and population genetic assessments.

This research focused on two invasive insect pests, sorghum aphids (*Melanaphis sorghi* Theobald) and tawny crazy ants (*Nylanderia fulva* Mayr, TCAs), both of which are reported in numerous states within the US (Kumar et al. 2015, Singh et al. 2004). These invasive insects have cost millions of dollars in losses and pest control efforts. For example, sorghum aphid outbreaks have cost $31.60 million in the Lower Rio Grande Valley in Texas (Zapata et al. 2016a). Similar estimates have been reported in other parts of Texas and Louisiana (Kerns 2015, Villanueva et al. 2014) and in other sorghum producing regions of the US. On the other hand, TCAs have economic costs equated to that of red imported fire ants in Texas (Wang et al. 2016) with damage estimates of $581 million in urban areas in 1998 and $90 million in agricultural areas in 1999 (Salin et al. 2000).

The Role of Microbes in Modulating Invasive Insect Interactions

Recent studies have shown that bacterial symbionts can allow some species to become pestiferous and/or invasive. For example, fungal symbionts (*Leptographium procerum*) in red turpentine beetles (*Dendroctonus valens* LeConte) allow these insects to better colonize and kill host trees in their introduced range (Lu et al. 2010). Specific microbes or microbial communities may also allow insects to colonize novel host plant species by enhancing their ability to resist natural enemies or through better nutrient acquisition (Brady & White 2013, Ferrari et al. 2004, Henry et al. 2013, Medina et al. 2011, Oliver et al. 2010). For example, the invasive kudzu bug (*Megacopta cribraria* Fabricius) in the US is able to feed on soybean because of its association with a bacterial strain of Candidatus Ishikawaella capsulata (Brown et al. 2014, Hosokawa et al. 2007, Kikuchi & Yumoto 2013). Similarly, *Regiella insecticola* presence increased the fecundity of some pea aphid (*Acyrthosiphon pisum* Harris) genotypes when feeding on clover (such as white clover *Trifolium repens* L.) (Oliver et al. 2010).

The sudden 2013 outbreak of an invasive aphid on US sorghum (*Sorghum bicolor*) was hypothesized to have resulted from a potential host shift of the sugarcane aphid (*Melanaphis sacchari*) from sugarcane to sorghum, since sugarcane aphids have been present in the continental US since 1977 on sugarcane (*Saccharum officinarum*). Since changes in the bacterial composition harbored by insects can allow them to use novel host plants (Brown et al. 2014, Hosokawa et al. 2007), it is possible that a change in the sugarcane aphid bacterial microbiota may have allowed it to become a pest in

grain sorghum. Therefore we characterized the sugarcane aphid bacterial microbiota before and after the 2013 pest outbreak and assessed whether a change in the bacterial microbiota of sugarcane aphids resulted in a host shift from sugarcane to sorghum. Although there were differences in microbial abundances of aphids on grain sorghum and sugarcane (Holt et al. 2020), in 2021 my colleagues and I determined that the 2013 aphid pest outbreak on sorghum was the result of the introduction of a different aphid species, the sorghum aphid (*Melanaphis sorghi* Theobald), and not a host shift in sugarcane aphids (*Melanaphis sacchari* Zehntner) (Nibouche et al., 2021). The variation that I found in the microbiota composition harbored by aphids that feed on sugarcane versus grain sorghum corroborates our characterization that the US sorghum pest outbreak was caused by sorghum aphids.

Invasive Insects Can Form Novel Symbiotic Interactions

In addition to the roles that microbial symbionts play in invasive species, the development of novel symbiotic interactions in invaded environments can also influence establishment. This can result through enhanced protection from natural enemies or from increased population growth. For instance, hemipterans tended by ants can receive protection from natural enemies, while ants can receive a carbohydrate rich resource (Feng 2015, Flatt & Weisser 2000, Helms & Vinson 2002, Stadler & Dixon 2017), which can result in increased colony growth for both insects.

Although ant-hemipteran interactions can be beneficial for the insects, they can have negative consequences for both natural and agro-ecosystems. These negative impacts can range from increased pest populations to increased plant damage. Population growth of newly invasive insects can aid with establishment in a new location or aid invasive insects to geographically expand their current distribution. In addition, plant damage can result from hemipterans consuming a greater amount of phloem in the presence of ants, thus removing more plant nutrients along with honeydew deposition that grows sooty mold on leaves and decreases photosynthesis, both of which can result in decreased plant health and reduced crop yield. For example, the invasive Argentine ant (*Linepithema humile* Mayr) tending a membracid (*Vanduzea segmentata* Fowler) was reported to contribute to the host range expansion of this sap-sucking pest (Harvey & Wheeler 2015). Similarly, after TCAs were introduced into Colombia they tended honeydew producing mealybugs in the genus *Antonina* causing an outbreak of this hemipteran in pastures and grasslands, thus reducing the foraging quality for cattle (Zenner de Polania 1990). In Florida, invasive TCAs have also been observed building shelters to protect honeydew producers from predators and parasitoids causing increases in hemipteran pest numbers (Sharma et al. 2013). These examples demonstrate that invasive ants can establish symbiotic interactions with hemipterans, even those with which they have no evolutionary history, and that these interactions can result in disruption of ecosystems or economic losses.

The overlap of the geographic distributions of sorghum aphids and TCAs in the southern US provides an opportunity for these species to engage in symbiotic

interactions. In particular, interactions between TCAs and sorghum aphids in agro-ecosystems planted with sorghum could cause a devastating synergism. Such a synergism could lead to an exponential increase in sorghum aphid numbers, with subsequent damage increases to sorghum. The likelihood of symbiotic interactions between sorghum aphids and tawny crazy ants was quantified **before** a widespread problem developed. The symbiotic interaction of sorghum aphids and TCAs, both recently invasive, is alarming and could result in increases in both aphid and ant biomass. The interaction between these two invasive pests should continue to be monitored for how this may impact aphid damage or yield loss to grain sorghum crops.

Implementing Fine Scale Molecular Markers in Recently Invasive Insects

Lastly, the population genetic structure of propagules can also influence establishment (Handley et al. 2011). Argentine ants (*Linepithema humile* Mayr) in the US were reported to have reduced genetic variation when compared with populations at their center of origin (Suarez et al. 2008), which likely allowed for super colony formation and in turn facilitated widespread invasion in the US (Tsutsui et al. 2001). While propagule introductions can result in genetic bottlenecks, multiple introductions can result in genetic variation among invasive species rather than the formation of super colonies. This was the case with red imported fire ants (*Solenopsis invicta* Buren) in Australia, where populations separated by 30 km were found to be genetically distinct (Henshaw et al. 2005). Although TCAs have been reported in Florida since 1953, it was not until 2002 that TCAs were reported as a major pest in Texas. Since 2002, this

invasive ant has been reported as a pest in additional states (i.e. Louisiana, Mississippi, Alabama, and Georgia). While broad-scale molecular markers were used to assess TCAs population genetic structure (Eyer et al. 2018), the fine-scale population genetic structure of tawny crazy ants was still unknown. This research used thousands of fine-scale molecular markers (single nucleotide polymorphisms or SNPs) to identify fine-scale population genetic structure based upon the geographic location of TCAs. Thus the population genetic structure of TCAs more closely resembles the genetically diverse assemblage as in the red imported fire ants in Australia rather than the super colony of Argentine ants in the US.

Overall, this research assessed how both microbiota and fine-scale population genetic composition modulate the interactions of two invasive species, sorghum aphids and tawny crazy ants. The combination of microbial and population genetic information can be used to assess factors that promote invasive insect establishment. In addition to better understanding the evolutionary ecology of these invasive pests, locally tailored management plans should take into account genetically and microbially distinct invasive insect pest populations.

CHAPTER II

DIFFERENCES IN MICROBIOTA BETWEEN TWO MULTILOCUS LINEAGES OF THE SUGARCANE APHID (*MELANAPHIS SACCHARI*) IN THE CONTINENTAL UNITED STATES*

The sugarcane aphid (SCA), *Melanaphis sacchari* (Zehntner) (Hemiptera: Aphididae), has been considered an invasive pest of sugarcane in the continental United States since 1977. Then, in 2013, SCA abruptly became a serious pest of U.S. sorghum and is now a sorghum pest in 22 states across the continental United States. Changes in insect-associated microbial community composition are known to influence host-plant range in aphids. In this study, we assessed whether changes in microbiota composition may explain the SCA outbreak in U.S. sorghum. We characterized the SCA bacterial microbiota on sugarcane and grain sorghum in four U.S. states, using a metabarcoding approach. In addition, we used taxon-specific polymerase chain reaction (PCR) primers to screen for bacteria commonly reported in aphid species. As anticipated, all SCA harbored the primary aphid endosymbiont *Buchnera aphidicola*, an obligate mutualistic bacterial symbiont.

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* Reprinted with permission from "Differences in Microbiota Between Two Multilocus Lineages of the Sugarcane Aphid (*Melanaphis sacchari*) in the Continental United States" by Jocelyn R. Holt, Alex Styer, Jennifer A. White, J. Scott Armstrong, Samuel Nibouche, Laurent Costet, Antonino Malacrinò, Josephine B. Antwi, Jason Wulff, Gary Peterson, Neal McLaren, and Raul F. Medina. 2020. *Annals of the Entomological Society of America.* 113(4): 257–265.

Interestingly, none of the secondary symbionts, facultative bacteria typically associated with aphids (e.g., *Arsenophonus*, *Hamiltonella*, *Regiella*) were present in either the metabarcoding data or PCR screens (with the exception of *Rickettsiella* and *Serratia*, which were detected by metabarcoding at low abundances <1%). However, our metabarcoding detected bacteria not previously identified in aphids (*Arcobacter*, *Bifidobacterium*, *Citrobacter*). Lastly, we found microbial host-associated differentiation in aphids that seems to correspond to genetically distinct aphid lineages that prefer to feed on grain sorghum (MLL-F) versus sugarcane (MLL-D). [1]

Since the publication of this paper, the invasive aphids reported in 2013 which were identified as MLL-F, have now been identified as a separate species called sorghum aphids (*Melanaphis sorghi* Theobald), while those present on sugarcane before the pest outbreak, identified as MLL-D, and in some sugarcane fields after the pest outbreak are sugarcane aphids (*Melanaphis sacchari* Zehntner) (Nibouche et al., 2021).

The sugarcane aphid (SCA), *Melanaphis sacchari* (Zehntner) (Hemiptera: Aphididae), is a globally distributed species that feeds on members of Poaceae including sugarcane, sorghum, rice, millet, corn, and wild grasses (Singh et al. 2004). In most parts of the world (e.g., Asia, Australia, the Americas, and Africa), SCA is considered a pest of sorghum (Singh et al. 2004). Its center of origin is currently unknown, but it is hypothesized to be either in central or northern Africa or in Asia (Nibouche et al. 2014). In regions of the world where both sugarcane (*Saccharum officinarum* and *Saccharum* spp. Linnaeus [Poales: Poaceae]) and grain sorghum (*Sorghum bicolor*, L. Moench [Poales: Poaceae]) are grown, SCA is reported to have higher abundances on grain sorghum than on sugarcane and exhibits preference for sorghum over sugarcane (Nibouche et al. 2015). SCA was first reported on sugarcane in the continental United States in Florida during 1977 (Mead 1978) and in Louisiana during 1999 (White et al. 2001). Despite the presence of SCA on commercial sugarcane, SCA was not a sorghum pest in the continental United States (Mead 1978, Hall 1987, Denmark 1988, Armstrong et al. 2015, Medina et al. 2017) until spring 2013 when SCA was reported on grain sorghum (Bowling et al. 2016). SCA damage to sorghum is caused by feeding activity and honeydew production, which combined can decrease crop yields and harvesting efficiency (Bowling et al. 2016, Zapata et al. 2016). Damage estimates in U.S. grain sorghum range from 20 to 100% crop loss (Villanueva et al. 2014, Kerns 2015, Zapata et al. 2016) plus the additional financial burden incurred by pest management efforts (Zapata et al. 2016).

Considering the fact that SCA was already present in U.S. sugarcane, a host-switch could have occurred due to a change in SCA's symbiotic bacteria. Symbiotic bacteria are a part of an insect's microbiota defined as a collection of microorganisms (e.g., bacteria, fungi, protists, and viruses) contained within and on the surface of an insect host. Similar to the important role microbiomes play in humans (Hartstra et al. 2015, Findley et al. 2016, Marchesi et al. 2016), the bacterial composition in aphids can influence their health, resource use, and vector potential (Oliver et al. 2003, Oliver et al. 2010, Lukasik et al. 2013). Symbiotic bacteria may influence insect host-range through nutritional supplementation (Hosokawa et al. 2007) or by helping their insect hosts withstand plant defenses (Adams et al. 2013, Ceja-Navarro et al. 2015, Hammer and Bowers 2015). For example, when the kudzu bug (*Megacopta cribraria*) invaded the United States, it was able to switch from kudzu to soybean because of its association with a bacterial strain of the obligate symbiont (one required for the insect's survival) Candidatus Ishikawaella capsulata (Hosokawa et al. 2007, Brown et al. 2014). Similarly, facultative bacteria (potentially beneficial to the insect, but not essential for survival) in the genus *Arsenophonus* improve cowpea aphids' (*Aphis craccivora*) fitness on locust plants (Wagner et al. 2015), whereas *Regiella insecticola* increases pea aphid fecundity on clover (Leonardo and Muiru 2003, Tsuchida et al. 2004).

Most research on aphid bacterial composition has been conducted on a few well studied species, such as the pea aphid and cowpea aphid through the use of polymerase chain reaction (PCR) with taxon specific primers (Chen et al. 1996, Darby et al. 2001, Simon et al. 2003, Brady and White 2013, Brady et al. 2014). However, it is

important to understand the bacterial composition of nonmodel organisms as they may differ in symbiont composition and those symbionts' biological functions. Although the use of PCR to detect specific taxa remains an effective method for detection of well-known symbionts (e.g., *Hamiltonella defensa*, *Serratia symbiotica*, and *Regiella insecticola*), it requires prior knowledge of the bacterial taxa and taxon-specific DNA sequences to be used for detection (Munson et al. 1991, Sandstrom et al. 2001, Russell et al. 2003, Oliver et al. 2006).

Another approach called barcoding uses general or universal PCR primers to amplify common genomic regions from a variety of organisms. After using barcoding to sequence single fragments of DNA or RNA, the nucleotide composition can be used as a proxy for organism identification. The advent of high-throughput sequencing (HTS) technologies, with the initial large data generating technologies referred to as next-generation sequencing (NGS), allow for the massive parallel sequencing of short DNA fragments pushing the boundaries of DNA barcoding and allowing the reconstruction of entire communities of organisms (Abdelfattah et al. 2018). With metabarcoding, a combination of PCR identification and high throughput sequencing, most of the bacteria harbored by an insect can be identified without any prior knowledge of what an insect may harbor and without the need to cultivate thousands of bacterial colonies or to clone thousands of DNA fragments (Mardis 2008, Malacrinò 2018).

Some of the earliest studies of aphids using 454 pyrosequencing were done on the microbial symbionts in cowpea aphids (Brady and White 2013), soybean aphid (Bansal et al. 2014), and pea aphids (Russell et al. 2013, Gauthier et al. 2015). Recently,

metabarcoding has been used to identify bacteria that were not previously associated with aphids. Metabarcoding has allowed for the identification of potential symbionts that might otherwise go unnoticed with the screening of only specific symbionts (Bansal et al. 2014, Gauthier et al. 2015, Jousselin et al. 2016, Fakhour et al. 2018).

The objective of our study was to characterize the SCA bacterial microbiota from aphids collected from sorghum and sugarcane using PCR and metabarcoding. In addition, we sought to determine whether a change in SCA microbiota supports a host plant shift, from sugarcane to grain sorghum.

Methods

Field Collections

Specimens of sugarcane aphid were collected from grain sorghum and sugarcane in four different states (i.e., Florida, Alabama, Louisiana, and Texas) in the United States between 2014 and 2015 and from sorghum in South Africa in 2014 (Table 1). Aphids within each state were collected on both grain sorghum and sugarcane from as many counties as possible, collecting specimens from fields at least 1 km apart from each other to minimize the chance of sampling siblings. We also added SCA samples from Louisiana sugarcane collected between 2007 and 2009, before the 2013 SCA invasion on sorghum in United States (Table 1). Aphids were killed in 95% ethanol and stored at 4°C.

Table 1 Number of sugarcane aphids that were pooled for each host plant and state combination used in microbial analyses. Reprinted from Differences in microbiota between two multilocus lineages of the sugarcane aphid (*Melanaphis sacchari*) in the continental United States.

Host Plant	Location and Year	Number of SCA pooled
Grain Sorghum		
	Alabama 2014	18
	Lab Colony 2015 – 2017	25
	Lab Colony 2015 (non-surface sterilized)	30
	Florida 2014	24
	Florida 2015	30
	Louisiana post 2013	17
	17	20
	South Africa 2013	19
	Texas 2013	21
Sugarcane		
	Alabama 2014	8
	Florida 2013 (nonsurface sterilized)	9
	Florida 2014	21
	Louisiana 2007-2009	7
	Louisiana 2015	20
	Texas 2015	20

Within each county, each aphid was collected at least 1 km away from each other (except for lab colony aphids). In total, 14 pooled samples were used for analysis.

DNA Extraction

Nymphs and apterous adults from each combination of host plant species and location (Table 1) were pooled together and surface sterilized before DNA extraction (Meyer and Hoy 2008, Medina et al. 2011), yielding a total of 14 samples (each containing an average of approximately 20 individuals). DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) following the standard protocol recommended by the manufacturer. DNA concentration and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Aliquots from those same samples were also used for both taxon-specific PCR and metabarcoding.

PCR for Specific Aphid Symbionts

We used taxon-specific PCR primers to screen for nine bacterial genera found in other aphid species. These bacterial genera included: *Arsenophonus*, *Cardinium*, *Hamiltonella*, *Regiella*, *Rickettsia*, *Rickettsiella*, *Serratia*, *Spiroplasma*, and *Wolbachia* (Fukatsu et al. 2001, Russell and Moran 2006, Oliver et al. 2010, Brady and White 2013). PCR reactions were run on a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) in a total volume of 10 μl containing: 2.5 mM MgCl2, 10X Taq reaction buffer (NEB), 2.5 mM dNTPs (Omega Bio-Tek, Norcross, GA, USA), 5 µM of forward and reverse primers, 0.1 μl of 5U/μl Taq DNA Polymerase (NEB, Ipswich, MA), and 2 μl of DNA template. The list of taxon-specific primers and annealing temperatures can be found in Supp Table 1 (online only). All diagnostics included positive (i.e., symbiont-positive

specimens known to host the bacteria of interest) and negative controls (i.e., nuclease-free water). PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium, Fremont, CA) under UV light. For samples that produced products of the expected size, we re-ran PCRs with a total volume of 25 μl and either purified the product using the GenCatch PCR Cleanup Kit (Epoch Life Sciences, Missouri City, TX) or in the case of double bands appearing on a gel, the band of interest (i.e., a band matching the correct size compared to the ladder) was excised and purified using the GenCatch Gel Extraction Kit (Epoch Life Sciences, Missouri City, TX). Purified products were sent to an offsite facility for Sanger sequencing (GENEWIZ, South Plainfield, NJ). Resulting sequences were searched by MegaBLAST in the GenBank database default parameters, and only sequences returning ≥97% similarity to the expected bacterial genus were considered for inclusion in bacterial presence analyses.

16s Metabarcoding

DNA samples were sent to the Molecular Research DNA Lab (MR. DNA, Shallowater, TX) for metabarcoding analyses targeting the bacterial V3-V4 16S rRNA bacterial region (Herlemann et al. 2011, Su et al. 2016). Samples were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA) using the MiSeq Reagent Kit v3 300PE chemistry. Together with experimental samples, we submitted two negative controls to identify potential environmental bacterial contaminants (Salter et al. 2014). One of the negative controls consisted of a sterilized water sample run with all the chemicals and the same protocol used for DNA extraction without any aphid DNA

(Salter et al. 2014, Malacrinò et al. 2018). The second negative control consisted of a single pool of all the last wash liquids obtained from the surface sterilization of insects, which was used for the identification of bacteria likely to be potential surface contaminants.

Data Analysis

Data handling was carried out using QIIME 1.9 (Caporaso et al.2010, Caporaso et al. 2012), quality-filtering reads (Phred ≥ 25), binning OTUs using open-reference OTU-picking through UCLUST algorithm, and discarding chimeric sequences discovered with USEARCH 6.1 (Edgar 2010). OTUs were taxonomically assigned through the BLAST method using Greengenes database for 16S rRNA (Caporaso et al. 2012). The OTU table was then filtered to remove all singletons, OTUs coming from amplification of chloroplast DNA, and those clearly belonging to contaminants (i.e., *Gardnerella*, *Granulicatella*, *Haemophilus*, *Leptotrichia*, *Prevotella*, *Ruminococcu*s , *Staphylococcus*, and *Streptococcus*). The two negative controls used in this study clustered apart from the aphid samples and allowed us to further clean our dataset from contaminants. Using a statistical approach to discover potential contaminants (Davis et al. 2018; Raw reads are available at NCBI SRA under accession number PRJNA419038), we further removed 30 OTUs from our samples. In addition, we subtracted from each sample the quantity of OTU reads found in both control types we used, under the assumption that they could be surface contaminants. Since each sample had a different sampling depth and library size, before subtraction we normalized counts

using the Variance Stabilizing Transformation algorithm from DESeq2 package (Love et al. 2014) and removed any batch effects using package limma (Ritchie et al. 2015). Negative values were then converted to zero, and we performed another round of singleton removal.

Differences in the structure of microbial communities between aphids collected from different host plants was assessed through a PERMANOVA analysis (999 permutations) using host plant as a fixed variable and sampling location to stratify permutations as a random variable to account for geographical variability. Distances between samples were calculated through a weighted UniFrac matrix and then visualized using Non-Metric Multidimensional Scaling (NMDS). Differences in relative abundance of each bacterial genus between samples from different host plants were tested through a mixed-effect model (host plant as fixed effect, and location as random variable) with lmer function (lme4 R package), using False Discovery Rate (FDR) correction for multiple comparisons. All analyses were performed using R statistical software (R Core Team 2013) with the packages vegan (Dixon 2003), phyloseq (McMurdie and Holmes 2013), and picante (Kembel et al. 2010).

Results

Bacterial Identification with PCR

PCR analyses did not detect bacterial symbionts commonly reported in other aphid species (i.e., *Arsenophonus*, *Hamiltonella*, *Regiella*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, and *Wolbachia*) see the example for *H. defensa* (Supp Fig. S2 (online

only)). These bacteria were also not identified using metabarcoding, with the exception of *Rickettsiella* and *Serratia*, which were also detected in low abundances using metabarcoding.

Microbiota and Host-Association with Metabarcoding

In total, 1,081,493 reads (with a sample average of 86,565 ± 18,914 SE paired end reads) were obtained from Illumina MiSeq, which clustered into 267 OTUs. Through a multivariate approach, we found host-associated differentiation of bacterial communities between aphids collected on sugarcane and grain sorghum ($F_{1, 12} = 4.73$; $P = 0.009$). The differentiation of the microbial community harbored by SCA clustered by host plant, which is visible in the NMDS plot (Fig. 1).

As anticipated, the majority of the bacterial reads belonged to the obligate aphid bacterial symbiont *Buchnera*. The raw unweighted percentages for *Buchnera* averaged between 90 and 99%, which overpowers the signal of less prominent bacteria if uncorrected. Consequently, all values are reported in weighted relative abundances. *Buchnera* composed a greater proportion of the bacterial community in aphids feeding on sorghum than those collected on sugarcane ($F_{2,12} = 10.58$; $P = 0.006$; 41.4 ± 6.9% and 29.7 ± 6.1%, respectively). In addition, metabarcoding detected bacteria (with abundances >1%) belonging to nineteen different genera in twelve orders (Supp Table 2 [online only]). A significantly greater proportion of Arcobacter sequences were detected in SCA associated with sugarcane than in SCA associated with sorghum ($F_{2,12} = 12.73$; $P < 0.01$; Fig. 2, Supp Table 2 [online only]). In contrast, a greater proportion

of Citrobacter sequences were detected in SCA associated with sorghum than in SCA associated with sugarcane ($F_{2,12} = 7.03$; $P = 0.02$; Fig. 2, Supp Table 2 [online only]). Some aphid-associated bacteria occurred at a low abundance of reads and included *Acidovorax*, *Lactobacillus*, *Ralstonia*, *Rickettsiella*, and *Serratia* (Supp Table 2 [online only]). The sample from Louisiana sugarcane collected during 2007–2009 lacked *Citrobacter* and *Serratia*, while hosting *Rickettsiella* (Supp Table 3 [online only]).

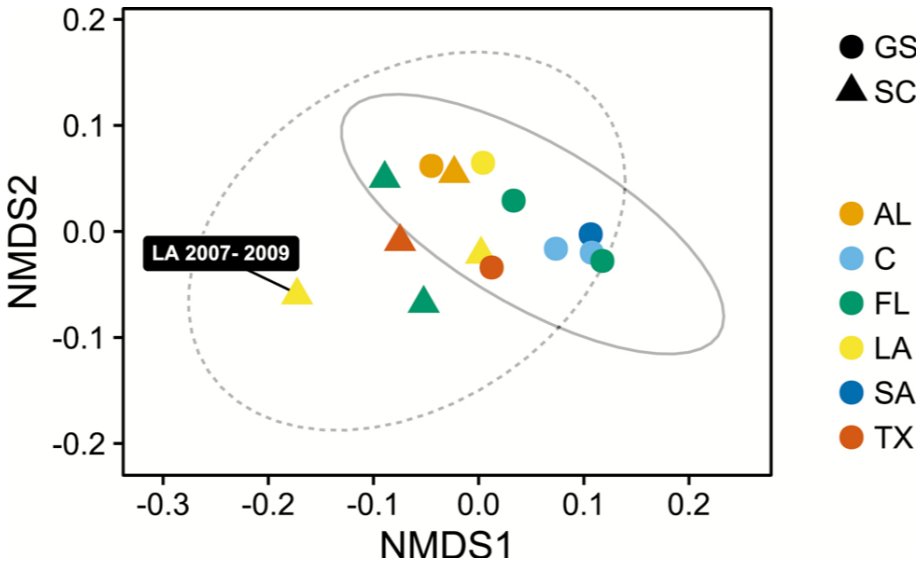

Figure 1 NMDS (Non-Metric Multidimensional Scaling) analysis showing host-associated clustering in bacterial composition (PERMANOVA: $F_{1,12} = 4.73$, $P = 0.009$). Samples collected before the pest outbreak, are marked with an a box labeled 2007–2009. The grain sorghum samples are surrounded by a solid ellipse while the sugarcane samples are surrounded by a dashed ellipse. Host plants: GS (grain sorghum) and SC (sugarcane). Locations: AL (Alabama), C (laboratory colony collected from Texas), FL (Florida), LA (Louisiana), SA (South Africa), and TX (Texas). Reprinted from Differences in microbiota between two multilocus lineages of the sugarcane aphid (*Melanaphis sacchari*) in the continental United States.

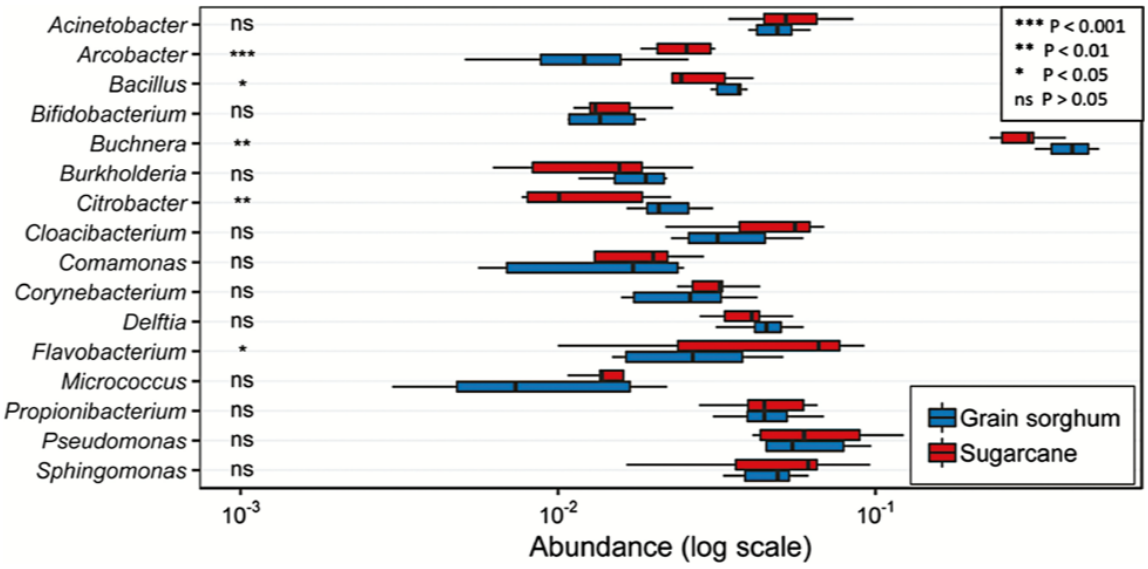

Figure 2 Relative abundance of bacterial taxa in SCA collected from grain sorghum and sugarcane. Three bacterial genera (i.e., *Arcobacter, Buchnera*, and *Citrobacter*) showed significantly different abundances between aphids on different host plants. ns = $P > 0.05$; *$P = 0.02$; **$P < 0.01$. Reprinted from Differences in microbiota between two multilocus lineages of the sugarcane aphid (*Melanaphis sacchari*) in the continental United States.

Discussion

Advances in technology have allowed us to go from the identification of bacteria through classical microbiology techniques (Escobar-Zepeda et al. 2015) to the use of PCR to screen for specific bacterial taxa (Haynes et al. 2003, Vorburger et al. 2009, Ferrari et al. 2011, Brady et al. 2014) and now to the ability to screen for entire microbiomes using high throughput sequencing without any prior knowledge of their composition. Our approach using taxon-specific PCR and metabarcoding allowed us to

screen for bacteria previously reported in aphids, while enabling us to identify other potential bacteria that SCA harbors.

The use of PCR confirmed that sugarcane aphids harbor few commonly known aphid bacterial symbionts, with the exception of the obligate symbiont *Buchnera aphidicola* (Buchner, Munson et al. 1991; Enterobacterales: Enterobacteriaceae) and facultative symbionts *Rickettsiella* and *Serratia* (identified in low abundances <1% with metabarcoding). Using metabarcoding we identified novel bacteria not previously reported in aphids (*Arcobacter*, *Bifidobacterium*, *Citrobacter*). In addition, metabarcoding allowed us to identify significantly higher abundances of bacteria in aphids from sugarcane (i.e., *Arcobacter*) when compared with aphids in grain sorghum (i.e., *Buchnera* and *Citrobacter*). A reduction in the abundance of the obligate symbiont *Buchnera aphidicola* in the presence of other bacteria is anticipated, as was shown with quantitative PCR in pea aphids that exhibited reduced *Buchnera* titers used to measure relative abundances in the presence of the secondary symbiont *Rickettsia* (Sakurai et al. 2005).

Bacterial symbionts can facilitate nitrogen use, breakdown sugar, and degrade pesticides in their insect hosts (Anderson et al. 2013, Ben-Yosef et al. 2014, Cheng et al. 2017). We identified *Bifidobacterium* in SCA collected from grain sorghum and sugarcane, a bacterium that it is known to break down carbohydrates in both insects and humans (Killer et al. 2009, O'Callaghan and van Sinderen 2016, Alberonia et al. 2019) and in SCA may play a role in processing phloem. In addition, two bacteria associated

with detoxification in other insects were found. For example, *Citrobacter* is associated with increased insecticide resistance in the tephritid fruit fly *Bactrocera dorsalis* (Hendel) (Cheng et al. 2017). Similarly, bacteria in the genus *Pseudomonas* are known to detoxify caffeine in coffee berry beetles *Hypothenemus hampei* (Ceja-Navarro et al. 2015) and may have a similar detoxifying potential in SCA.

Some bacteria may be biologically relevant even when found in low abundances (Stouthamer et al. 2018). While a relative abundance <1% may reflect some sequencing error, our use of stringent quality control filtering helped increase the reliability of low abundance reads (Bokulich et al. 2013). Both *Rickettsiella* spp. and *Serratia* spp. are reported to have biologically relevant functions at low abundances in other aphids (Enders and Miller 2016). Interestingly, *Rickettsiella*, which is known to alter body color in pea aphids (Tsuchida et al. 2010), was found in SCA and if similar in function, might influence the attractiveness of SCA to natural enemies (i.e., predators and parasitoids). SCA also harbored *Serratia*, which has been reported to provide heat tolerance in other aphids (Russell and Moran 2006, Oliver et al. 2010).

In addition to the potential symbionts mentioned above, we detected potential plant pathogens associated with members of the grass family (Poaceae) that include *Acidovorax* spp., *Corynebacterium* spp. and *Ralstonia* spp. The *Acidovorax* spp. group has been reported to cause tissue browning in some plants (Xie et al. 2011) and red stripe disease in infected sugarcane plants (Girard et al. 2014, Santa Brigida et al. 2016, Yonzone and Devi 2018). Similarly, *Corynebacterium* spp. is known as an animal

and plant pathogen (Christie et al. 1991, Barba et al. 2015), with green peach aphid reported as capable of transmitting this bacterium to potatoes resulting in ring rot (Christie et al. 1991), whereas *Ralstonia* spp. is known to cause plant wilting and death in numerous agricultural crops (Álvarez et al. 2010, Meng 2013). Further investigation is required to determine whether the strains of these bacteria found in SCA are plant pathogens and if SCA is capable of their transmission.

While the bacteria reported above have been identified in other insects and some have known functions, other bacteria that we identified that have been reported from other insects require further investigation (i.e., *Acinetobacter*, *Arcobacter, Bacillus*, *Burkholderia*, *Cloacibacterium, Delftia, Flavobacterium*, *Propionibacterium,* and *Sphingomonas* (Srivastava and Rouatt 1963, Kikuchi et al. 2005, Konig 2006, Xiang et al. 2006, Killer et al. 2009, Leroy et al. 2011, Malhotra et al. 2012, Morales-Jiménez et al. 2012, Cakici et al. 2014, Montagna et al. 2014, Ceja-Navarro et al. 2015, Meirelles et al. 2016, Meriweather et al. 2016, Segata et al. 2016, Cheng et al. 2017, Duguma et al. 2017, Luna et al. 2018). For example, *Flavobacterium* has been reported from pea aphids (Srivastava and Rouatt 1963), and while members of the order Flavobacteriales are reported to provision their insect hosts with nutrients (Wu et al. 2006, Bennett et al. 2014, Rosas-Pérez et al. 2014), it is unknown whether this bacteria would have a similar role in SCA. Similarly, *Micrococcus* in the European corn borer has potential contributions to gut enzymatic activity (Vilanova et al. 2012); however, its potential function in SCA is not known.

Interestingly, we did not detect bacterial symbionts that are well known from previous aphid studies with either PCR or metabarcoding. For example, we did not detect any reads associated with *Hamiltonella defensa,* which in another study was reported as a byproduct of bacterial read filtering from high-throughput sequencing of SCA (Harris-Shultz et al. 2017). We conclude that the presence of these other symbionts may be rare or completely absent in SCA populations in the United States.

We originally predicted that a change in the SCA microbiome composition might be responsible for SCA switching from sugarcane to grain sorghum resulting in the pest outbreak in 2013. While our results show host-associated bacterial differentiation in SCA collected from sugarcane and sorghum, these differences seem to correspond to the characterization of two genetically distinct aphid Multilocus Lineages or MLLs (Nibouche et al. 2018). These genetically distinct MLLs differ in host plant use: MLL-D prefers to feed on sugarcane and has likely been present in the United States since the 1970s while MLL-F prefers to feed on sorghum and was only recently detected in the United States (Nibouche et al. 2015, Nibouche et al. 2018). Therefore, the sudden SCA outbreak was likely caused by the introduction of a sorghum adapted strain of SCA, which also has host-associated bacterial differences, and not by the sudden acquisition or loss of a specific bacterial symbiont. Analysis of sugarcane aphids from Louisiana grain sorghum and sugarcane before and after the pest outbreak show an increase in the sorghum adapted MLL-F in sugarcane (Nibouche et al. 2018). In SCA, sugarcane populations had greater abundances of *Arcobacter* and a lower abundance of *Citrobacter* when compared to samples collected on sorghum. In fruit flies that

harbor *Citrobacter* these bacteria enhance detoxification that enables resistance to insecticides (Cheng et al. 2017), which could have a similar function in SCA. The function of *Arcobacter* is still unknown despite its presence in multiple insect species. In addition, bacteria such as *Citrobacter* and *Serratia* (lacking in samples collected before SCA invasion on U.S. sorghum) were detected in aphids from both grain sorghum and sugarcane after 2013, suggesting that the sorghum adapted MLL-F aphids are spilling over into sugarcane. Alate aphids can be transported by wind to new locations and host plants (Wiktelius 1984, Irwin et al. 1988, Loxdale et al. 1993, Mann et al. 1995), which may explain why some SCA samples collected from sugarcane after 2013 (in Alabama, Florida, and Louisiana) have microbial compositions that cluster closer to SCA collected on grain sorghum. Our findings suggest a spillover of SCA from sorghum to sugarcane likely due to dispersal. Although changes in the microbiota composition between SCA before and after 2013 are unlikely to have caused the SCA outbreak in sorghum, the bacteria that we identified may play important roles in SCA.

Microbial organisms, such as bacteria, fungi, and viruses may play important roles in the management of insect pests (Lacey et al. 2001, Glare et al. 2012, Lacey et al. 2015). We currently know that some microorganisms influence the effectiveness of pest control strategies. For example, pea aphid association with specific strains of *Hamiltonella defensa* or *Regiella insecticola* confers increased resistance against parasitoid attacks (Oliver et al. 2005, Oliver et al. 2009, Vorburger et al. 2009), thus reducing the effectiveness of biological control. In contrast, pea aphids harboring some strains of *Serratia symbiotica* can be killed with lower doses of insecticide from different

chemical classes including a neonicotinoid, carbamate, ketoenol, and organic thiophospahate (Skaljac et al. 2018). Thus, in theory we could manipulate the microoganisms associated with insect pests, either by adding or eliminating microbes that benefit or harm pest control efforts. We currently use entomopathogens as biopesticides and manipulating symbiotic microorganisms would help expand pest control. The manipulation of pests' microbiotas as part of IPM may significantly reduce runoff and off-target effects associated with chemical control (Chandler et al. 2011, Dara 2017, Lenteren et al. 2018) of some insect pests. The ability to incorporate relevant genes in insects and plants using novel gene editing methods (e.g., CRISPR-Cas9) provides another promising application for characterizing and manipulating pest insects' microbiota. Future approaches to genetic pest control may benefit not only from altering insect genes and their expression (e.g., through gene editing or RNAi for targeted gene silencing), but also by incorporating microorganisms' genes to enhance IPM.

Our research team plans to continue examining the role(s) that SCA bacteria may play in this insect pest. Future research will include assessing the prevalence of these bacteria among aphids in different locations as well as the potential detoxification role of *Citrobacter* in SCA. As was seen in pea aphids where their microbial composition influenced stress tolerance or fecundity (Oliver et al., 2010), these potential bacterial roles in SCA need to be characterized. In addition, screening of low abundance bacteria in different aphid populations, will allow us to determine transmission rates of bacteria in SCA. Last, further research into the potential role that microbes play in the attraction

of natural enemies or mutualists to SCA is needed and could provide valuable insight for the use of biological control.

Overall, we found that populations of SCA in the continental United States contain only two of the well-studied facultative symbionts reported in aphids. It is important to note that HTS allowed us to identify bacteria in SCA, which had not been known to be associated with aphids, but have been hypothesized to act as potential symbionts in other insects. In addition, we found evidence of host-associated differentiation in SCA microbiota. This study provides a foundation for understanding the bacterial composition of SCA, which can be used to better inform Integrated Pest Management (IPM) of this pest in grain sorghum by providing novel targets for control.

Electronic Data Access

Raw data can be found on NCBI under accession number PRJNA578411. Scripts for data analysis are available through GitHub at: https://github.com/amalacrino/SCA_microbiota.

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CHAPTER III

QUANTIFYING THE FREQUENCY OF INVASIVE ANTS TENDING INVASIVE APHIDS

The establishment of novel symbiotic interactions between introduced species in an invaded location may facilitate invasion success. Tawny crazy ants (*Nylanderia fulva* Mayr) are known to be opportunistic tenders of honeydew producing insects and their novel symbiotic interactions have exacerbated agricultural damage in some regions of the world where they have invaded. Although the invasive sorghum aphid (*Melanaphis sorghi* Theobald) was first reported as a pest in the continental United States as recent as 2013, tawny crazy ants (TCAs), which invaded Texas in the early 2000s, are already tending these aphids in field and greenhouse settings. This study quantified the tending frequency of TCAs along with sorghum aphid biomass in the presence and absence of ants in greenhouse conditions. Aphid clone colonies were established from aphids collected from three different host plants (i.e., sugarcane, grain sorghum, or Johnson grass). Quantification of invasive ant-aphid interactions in greenhouse conditions showed that despite a lack of evolutionary history together, TCAs have developed a tending interaction with sorghum aphids in Texas sorghum. Although this relatively new symbiotic interaction did not significantly increase overall aphid biomass, aphids were observed excreting honeydew after being antennated by TCAs. Thus, it is recommended to monitor this novel interaction between TCAs and sorghum aphids in field conditions due to its potential to increase aphid populations and sorghum plant injury.

When invasive species arrive to a new location, they may establish symbiotic interactions (i.e., mutualism, parasitism, or commensalism) with other organisms. In particular, novel mutualisms with invasive species may ecologically facilitate an invasive species' fitness and range expansion (Prior et al. 2015; Wilder et al. 2011; Zhou et al. 2012). For example, after the invasive mealybug (*Phenacoccus solenopsis* Tinsley) arrived in China, it established a new interaction with the native ghost ant (*Tapinoma melanocephalum* Fabricius) in less than five years (Feng et al. 2015). This symbiotic interaction resulted in the ghost ant defending the mealybug against native parasitoid wasps in exchange for honeydew (Feng et al. 2015) benefiting population growth of both species (Zhou et al. 2014). As a result, invasive mealybugs are likely to cause greater injury to cotton and hibiscus plants in the future.

In some scenarios non-indigenous insects or those that have become invasive can facilitate or promote secondary invasions. This phenomenon is referred to as invasional meltdown (Simberloff & Holle 1999). For example, on Christmas Island, yellow crazy ants (*Anoplolepis gracilipes* Smith) tended invasive scale insects (*Tachardina aurantiaca* Cockerell), which resulted in ants forming supercolonies, potentially because ants received abundant carbohydrate-rich honeydew. In addition, the killing of land crabs by these ants then created enemy free space that facilitated the invasion of an exotic land snail (Green et al. 2011). While initially land snail presence has only resulted in a slight reduction of leaf litter in the rainforest (O'Loughlin & Green 2017), over time land snails could disrupt nutrient cycling. Similarly, the spread of invasive argentine ants (*Linepithema humile* Mayr) across the US was likely facilitated by the consumption of

honeydew produced by native membracids (Harvey & Wheeler 2015). Despite the lack of evolutionary history between these two insects, this tending interaction resulted in damage to both urban and wildland systems (Menke et al. 2018). Therefore, these symbiotic interactions, many of which are beneficial for invasive insects, can facilitate ecosystem disruption and thus future potential invasions. Additional investigations into potential invasional meltdown (O'Loughlin & Green 2017, Pearson et al. 2018) could provide insight into improving invasive species management through the development of predictive models for the identification and monitoring of ecosystem variables likely to be disrupted by the introduction of invasive species over time.

Potential host-plant adaptation or more broadly, any local adaptation can also influence ant-aphid symbiotic interactions. In some instances, host plants may play a role in attracting ant tenders. For example, both black garden ants (*Lasius niger* L.) and black bean aphids (*Aphis fabae* Scopoli) preferred tansy host plant genotypes that emitted chemical volatile blends including eucalyptol and terpineol, and ant attractiveness to these volatile blends brought ants in contact with aphids, which facilitated ant-aphid interactions (Weisser 2019). Local adaptation may also influence ant tending rates. Such was the case with cowpea aphids (*Aphis craccivora* Koch*)* collected from Otsu and Saga in Japan, which are approximately 700 km apart. In a controlled lab experiment, cowpea aphids from Otsu were tended more frequently by ants than aphids collected from Saga (Katayama et al. 2013). Thus, aphids and ants adapted to different geographic locations or host plants may experience different tending interaction intensities.

In this study, we examined tending interactions between the recent invasive tawny crazy ants (*Nylanderia fulva* Mayr) and sorghum aphids (*Melanaphis sorghi* Theobald). While sorghum aphids have already resulted in significant economic damage to grain sorghum crops (Villanueva et al. 2014, Zapata et al. 2016b) a potential mutualism between ants and these invasive aphids could worsen pest injury, by increasing aphid populations and resulting in increased aphid phloem consumption. Although there are many potential ant species that could tend the sorghum aphid, this research focused on tawny crazy ants (TCAs) because they are known to be opportunistic tenders of honeydew-secreting hemipterans, which can result in decreased plant health and increased hemipteran population growth. In Florida TCAs were first reported as a pest around the 1990s, and have opportunistically tended both native (e.g., willow aphids, juniper aphids, psyllids, cottony maple scale, kermes scale) and invasive (e.g., citrus whitefly, cowpea aphids, mealybugs) hemipterans (Sharma et al. 2013). As TCAs have continued to expand their geographic range, they now occur in many of the same geographic regions as invasive sorghum aphids (Nibouche et al. 2021).

A symbiotic interaction between TCAs and sorghum aphids may be beneficial to these insect pests and increase their numbers in sorghum fields. This dynamic has been reported for TCAs in other scenarios. For example, in Colombia, TCAs opportunistic tending of mealybugs (*Antonina* sp.) caused grasslands to dry out and reduced their foraging value for cattle (Zenner de Polania 1990). Similarly, higher sugarcane aphid presence on sugarcane was reported in Ecuador, where large TCA populations tended these aphids (Pazmino-Palomino et al. 2020). Previously, aphid injury towards

sugarcane was considered low and aphid population sizes were controlled by parasitoid wasps and other natural enemies. However, the tending by TCAs reduced natural enemy presence (Pazmino-Palomino et al. 2020), and resulted in increased sugarcane aphid populations. In these instances, interactions between two invasive pests ecologically facilitated aphid fitness and reduced crop health.

Sorghum aphids, which are now distributed across the continental US (Nibouche et al., 2021), have been observed being tended by red imported fire ants (*Solenopsis invicta* Buren), acrobat ants (*Crematogaster* sp. Lund), and TCAs in sorghum fields already (Wright et al. in review, and Holt personal observations). Akin to fire ants, invasive TCAs continue to expand in geographic range and are opportunistic tenders lacking evolutionary history with a variety of hemipterans in the invaded regions of the US that they occupy. Despite the recent introduction of TCAs that were reported as a pest in Florida in the 1990s and in Texas during the 2000s (MacGown 2015, Meyers & Gold 2008) and sorghum aphids being reported in the US in 2013, these insects have a symbiotic interaction where ants antennate aphids for honeydew and aphids often excrete honeydew after being antennated. The present study quantified the tending of invasive TCAs to sorghum aphids. In addition, we assessed whether tending influenced aphid biomass and examined whether aphids collected from different host plants differ in ant tending frequency or biomass.

Methods

Insect Collections and Rearing

Sorghum aphids (*Melanaphis sorghi*) from grain sorghum, Johnson grass, and sugarcane were used in these experiments (Supplemental Table 3.1). Aphids collected on sugarcane in the continental US after the sorghum aphid invasion in 2013 were determined to be predominately sorghum aphids (Nibouche et al. 2021); this is potentially due to sorghum aphids blowing over onto sugarcane plants. Aphids collected from different host plant species were kept separate inside fine mesh cages. Aphid clone lines were generated using single parthenogenetic females, to ensure genetic homogeneity within each colony.

All plants used in this study (to rear aphids and for experiments) belonged to the susceptible grain sorghum variety, DELKALB® DKS 4420 (Bayer, St. Louis, MO). Plants grown from seeds were reared in the greenhouse in 6 inch round pots (McConkey, Sumner, WA) filled with Jolly Gardener Pro Line C/25 soil (Jolly Gardener, Atlanta, GA) and allowed to grow for 2 to 4 weeks before they were used in experiments. All plants used for rearing aphids were of similar size (>20 cm tall).

The tawny crazy ants (*Nylanderia fulva*) used in these experiments were collected from field sites in Texas (i.e., Bryan and Austin). A total of eight colonies were collected from Bryan and one colony from Austin. Population genetic structure analyses of Texas TCAs used in these experiments showed that they were genetically similar (Holt Chapter IV). Ant colonies were kept following a slightly modified version of Drees and McDonald's rearing protocol (Drees 2012, McDonald et al. 2013). Each ant

colony was kept in a five-gallon bucket filled with the soil surrounding each colony at the time of collection, with the inside rim lined with corn starch to prevent ants escaping. Each bucket was supplied weekly with a whole cricket and with two vials, one containing reverse osmosis water and the other one containing an artificial nectar solution (100 g sucrose: 90 g glucose: 53 g fructose); vials were replenished as needed. Ants were dripped and aspirated out of collection buckets for the experiment. Each collection bucket was split into two or more sub-colonies that included 500 ant workers and 1 ant queen. Tawny crazy ant worker numbers are based upon those used in Katayama et al. (2013). Any remaining ants were left in the original collection colony for future studies. One day before the experiment, artificial nectar was removed from all ant colonies to be used, to promote foraging behavior for honeydew (Katayama et al. 2013). A new water vial and cricket were provided to ants for the experiment.

Ant-Aphid Tending Setup

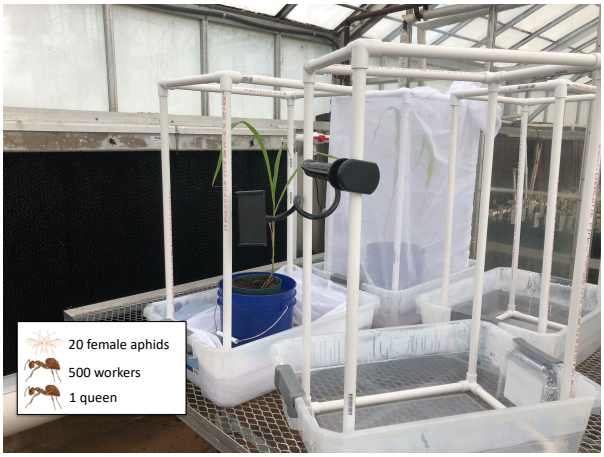
Plant cages were built using PVC pipe (1.27 cm diameter) with a rectangular shape (dimensions 25.4 L x 43.18 W x 76.2 H cm), and mesh bags composed of 100% polyester white voile (86.36 cm tall x 182.88 cm long) were used to enclose the PVC frame. Plant cages built to enclose each experimental unit were placed inside a Fluon (Insect-a-Slip Barrier by BioQuip, Rancho Dominguez, CA) coated container (Sterlite 58 Quart Storage Box dimensions 59.69 L x 42.88 W x 31.11 H cm). As an additional barrier to prevent ants from escaping, the table legs of the greenhouse benches were placed into a container of mineral oil (Equate Mineral Oil, Al Ahmandi, Kuwait).

A total of 20 individual aphids from a clone line were transferred onto plants with a damp paintbrush. Ten aphids were placed on the stem and 10 were placed on the 3rd leaf from the bottom of each sorghum plant used in experiments. While the same clone was used for a pair of experimental units (i.e., a plant with aphids and ants and a plant with aphids without ants), not all experimental pairs were exposed to the same clone. Aphids used in experiments represented eight clonal lines: four clones from Johnson grass, two from sorghum, and two from sugarcane. Each aphid clone was used at least once for an observation period (Supplemental Table 3.1).

In the case where aphids were placed with ants, aphids were allowed to acclimate between two to four hours before adding an ant sub-colony (500 workers + 1 queen). Each new ant sub-colony was placed inside a 1-gallon bucket (Lowes, Mooresville, NC) filled ~ 90% with soil (Jolly Gardener Pro Line C/25, Atlanta, GA) centered around a 6 inch round pot (McConkey, Sumner, WA) that contained a sorghum plant with 20 aphids. The pot nested inside the bucket was placed inside of a PVC frame with a mesh cage that was tied at the top, and then this was placed inside a large plastic container coated with Fluon at the top rim (Figure 3.1). There was a total of 13 experimental units that had aphids in the presence of ants and 13 experimental units that had aphids in the absence of ants.

Figure 3.1 Observation setup for ant-aphid interaction experiments. Experimental units with ants consisted of a sorghum plant with 20 reproductive female aphids, 500 ant workers and 1 ant queen. Experimental units without ants consisted of a sorghum plant with 20 reproductive female aphids only. The picture

shows a mesh bag lowered, as it would be during an observation. When experimental units were not under observation, the mesh was raised and tied closed.



Ant-Aphid Observations

Observations were conducted the day after ants and aphids were placed together to allow the insects to acclimate. During ant tending observations, the mesh cages were lowered for each set of aphids with ants and aphids without ants. Observations were conducted between 8 AM and 2:00 PM, from May to October. Each plant with ants and aphids was observed for tending behavior (i.e., ants antennating aphids) on the leaf and the stem and was recorded for a total of 10 minutes [based on (Katayama et al., 2013)]. The order in which experimental units were observed was randomized each day. Observations for tending behavior for each experimental unit were conducted for five consecutive days and then these tending numbers were averaged. After the fifth day, the sorghum plant was cut at the base, placed in a large zippered plastic bag, and stored in the freezer. Aphids on these plants were later used in aphid biomass assessments.

Measuring Aphid Biomass

Sorghum plants were removed from the freezer and aphids were collected from the observation area using a paint brush. Aphids were placed into a glass vial with acetone. All vials were labeled with the experiment number, whether ants were present or absent, and whether aphids came from an observation area (i.e., leaf or stem position) or the rest of the plant. Aphid biomass was measured by removing the aphids from acetone filled vials with a paint brush then placing them into a pre-weighed aluminum weigh boat (approximately 1.27 cm diameter by 0.635 cm deep) and allowing them to dry out. Once aphids dried, the weigh boats with aphids were placed onto a precision balance (Mettler Toledo UMX2, Columbus, OH). Aphid biomass (mg) from the entire plant was logged for each experimental unit. Weighed aphids were returned to their vials which were kept as vouchers.

Data Analysis of TCA Tending and Aphid Biomass

Ant tending averages and aphid biomass were analyzed using a linear mixed model in R v. 4.1.0 with the package lme4 (Bates et al. 2015). Data were tested for normality by examining the qqplots. The random effect variable "year" was originally accounted for in a mixed effect ANOVA model, it was removed if the residual equaled zero (Davis 2018). The package dbplyr/dplyr was used, which allowed for data processing (Wickham et al. 2018). The package car was used to run an ANOVA and obtain p values (Fox & Weisberg 2019). Tukey pairwise comparisons were conducted to discern differences in aphid biomass using the packages emmeans and multcompView

(Graves et al. 2019, Lenth et al. 2021). If any interaction or fixed effect in the above analyses was not significant, it was removed from the model and re-run with the remaining effects.

The general behavior of aphids in the presence of ants was observed to assess how this symbiotic interaction occurred on plants. In addition, ant tending frequency towards aphids on leaves and stems together, the stem only, and leaf only was assessed. Tending frequency was analyzed using a one sample t-test with a population mean (μ) of zero (CRAN, Gerald 2018). A two-way ANOVA was used to analyze whether the position on a plant that an aphid was placed, either stem or leaf (henceforth referred as position), whether host plant species from which aphids were collected (henceforth referred to as host plant), and whether position by host plant interaction had an impact on ant tending frequency. Aphid biomass from observation sites was analyzed with a three-way ANOVA by comparing position (i.e., stem vs leaf), with host plant, with whether ants were present or absent (hereafter referred to as ant presence), along with the interaction among position, host plant, and ant presence. Next, aphid biomass from the entire plant was analyzed with a two-way ANOVA and whether host plant, whether ant presence, and whether the interaction between host plant and ant presence effected total aphid biomass.

Results

Sorghum Aphid Honeydew Excretion Behavior

Sorghum aphids generate copious amounts of honeydew, which was observed being flicked away from the insect when it was not tended by ants (https://youtu.be/VJcEpk5jXJw). However, after being antennated by TCAs, sorghum aphids were observed modifying this behavior to excrete honeydew for ant consumption (https://youtu.be/H8jD5YkBkpM).

Invasive Ants Tend Invasive Aphids

TCAs were observed tending sorghum aphids on sorghum plants. The average tending of sorghum aphids by TCAs was significantly greater than zero for the entire plant ($t = 4.03$, $df=25$, $p = 0.0005$). When tending was analyzed by position, TCAs were found to tend sorghum aphids on stems ($t = 4.26$, $df=12$, $p = 0.001$) significantly more than zero, however no significant tending was detected for aphids on leaves ($t = 1.74$, $df=12$, $p = 0.12$).

Tending frequency by TCAs was different based upon whether the aphids were positioned on the stem or leaf. Aphids positioned on stems were tended more than aphids positioned on leaves ($F = 7.9$, $df = 1$, $p = 0.01$; Figure 3.2). Host plant did not significantly influence tending average ($F = 0.21$, $df = 2$, $p = 0.81$).

Aphid Biomass

Aphid biomass differed based upon whether aphids were positioned on the stem or leaf. Aphids on stems had greater biomass than aphids positioned on leaves ($F = 4.93$, $df = 1$, $p = 0.031$; Figure 3.3). However, host plant did not significantly contribute to the difference between stem and leaf aphid biomass ($F = 1.10$, $df = 2$, $p = 0.34$) nor did the presence of ants ($F = 0.89$, $df = 1$, $P = 0.35$). When total aphid biomass was analyzed (i.e., aphids from the entire plant) no effect of host plant was detected ($F = 3.52$, $df = 2$, $p = 0.05$; Figure 3.5). Similarly, total aphid biomass was not significantly increased in the presence of ants ($F = 1.15$, $df = 1$, $p = 0.29$) even though TCAs were observed tending aphids (Figure 3.4).

Figure 3.2 Invasive tawny crazy ants tend invasive sorghum aphids. Tending was greater for aphids on stems than on leaves ($F = 7.9$, $df = 1$, $P = 0.01$). The bar graphs show the ant tending average with error bars for SE of stem (5.6) and leaf (3.3) samples both with $n = 13$.

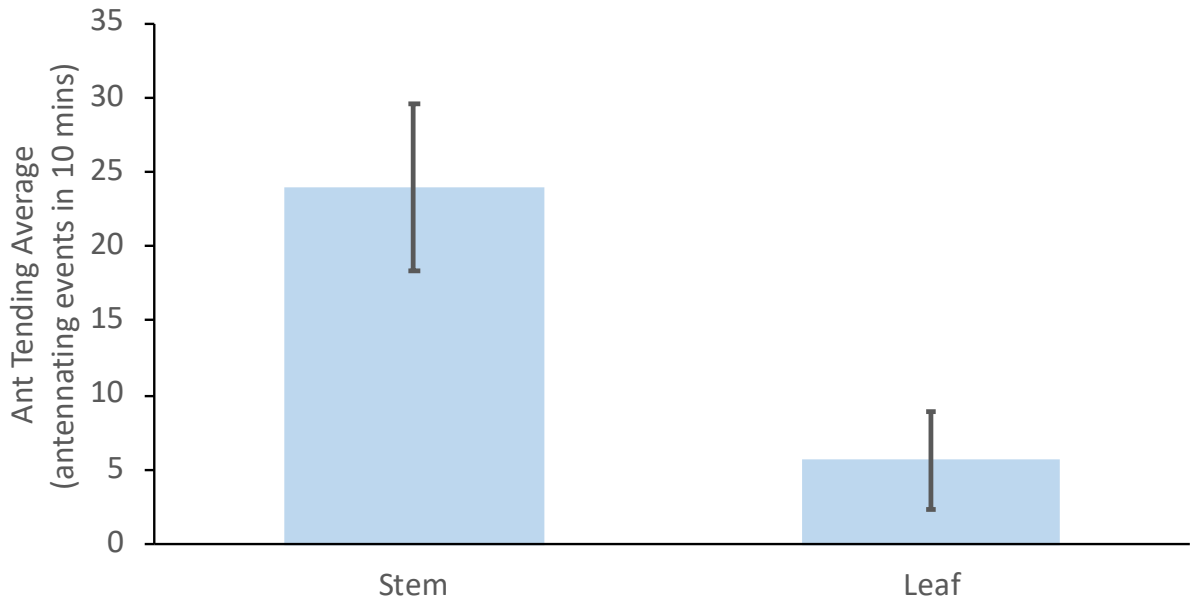


Figure 3.3 Aphid biomass was greater for aphids on stems than on leaves (F = 4.93, df = 1, *P* = 0.031). The bar graphs show average aphid biomass on different positions of sorghum plants with the SE of stems (0.65) versus leaves (0.34) for *n* = 13 samples.

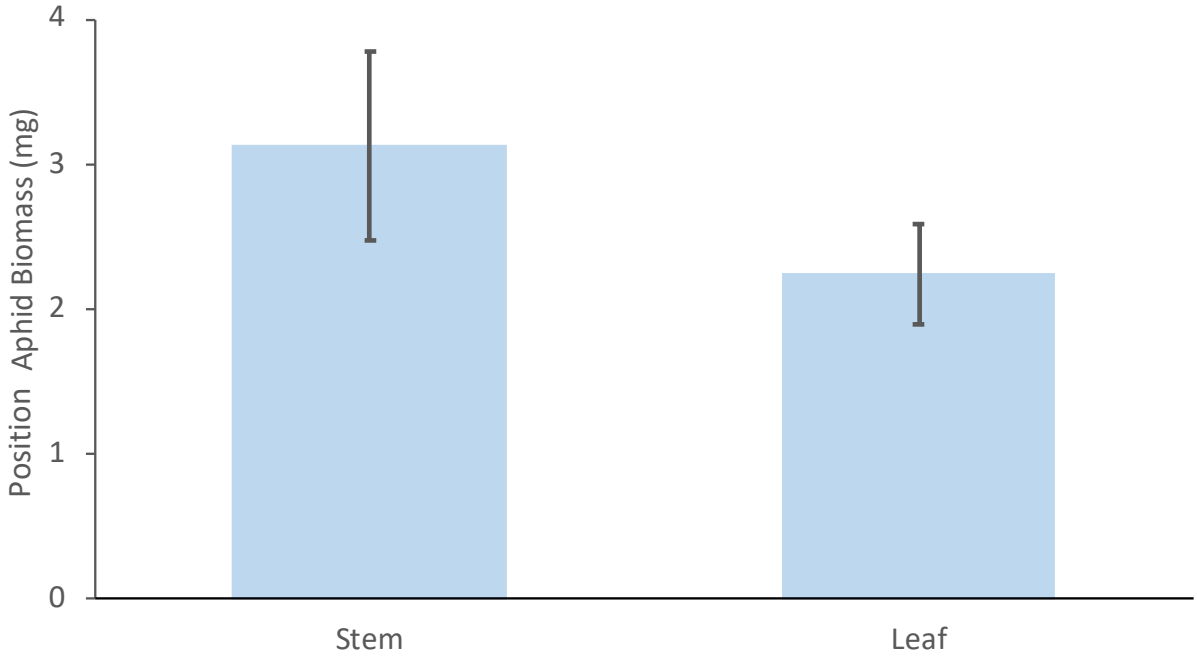


Figure 3.4 The total biomass of aphids in the presence of ants was not significantly different from aphids in the absence of ants (F = 1.15, df = 1, p = 0.29). Aphids in the presence of ants averaged 6.5 mg (n = 13, SE = 1.03), while aphids in the absence of ants averaged 5.3 mg (n = 13, SE = 0.69).

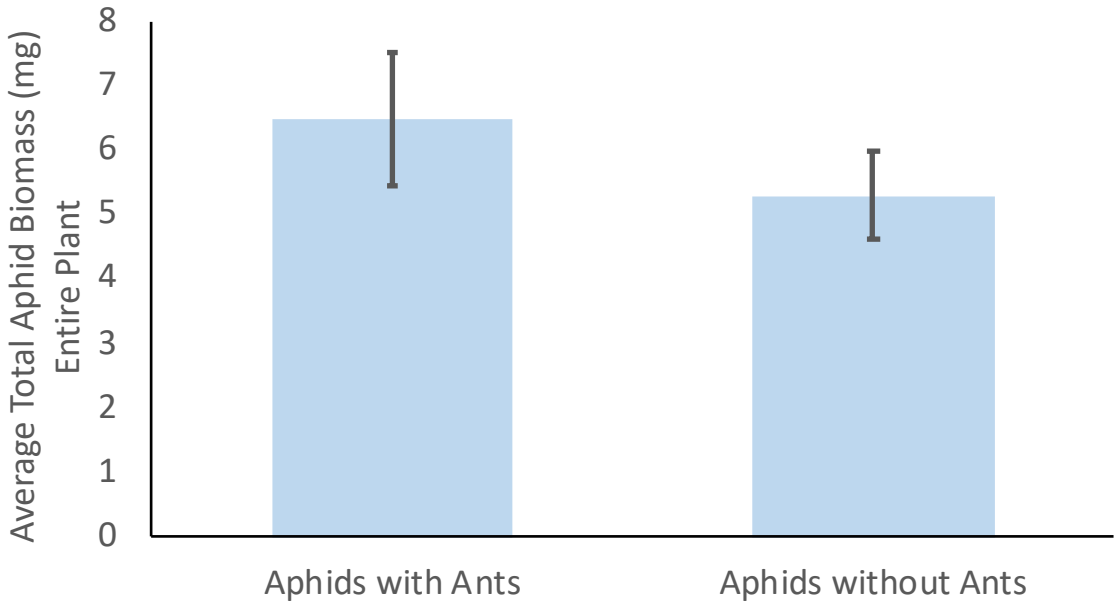
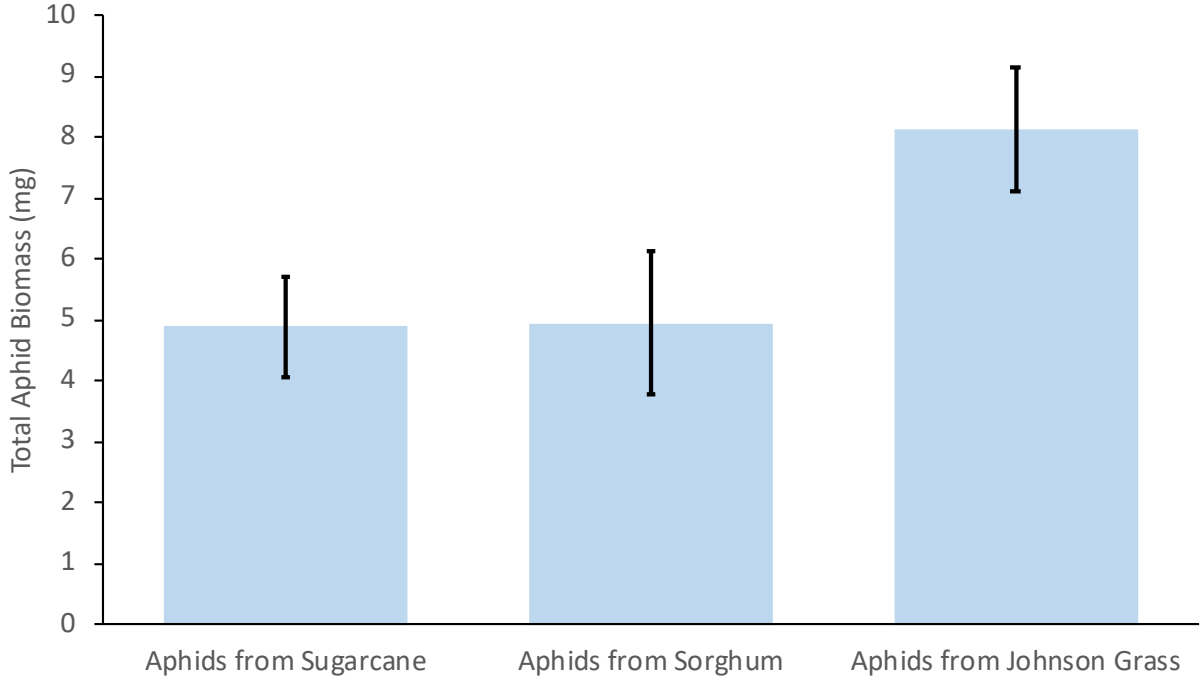


Figure 3.5 There was no effect of host plant from which aphids were originally collected on total aphid biomass (F = 3.52, df = 2, *P* = 0.05) when aphids were raised on grain sorghum. Although aphids collected from Johnson grass (*n* = 8, SE = 1.46) had slightly greater biomass, this was not significantly different from aphids collected from either sorghum (*n* = 6, SE = 0.95) or sugarcane (*n* = 12, SE = 0.58).



Discussion

While invasive species can bring a suite of microscopic symbiotic interactions (i.e., their microbiota) to invaded locations, the establishment of new symbiotic interactions, particularly mutualisms, may ecologically facilitate establishment. We found that despite a lack of evolutionary history between two relatively recent invasive pests, sorghum aphids reported in 2013 and TCAs reported in 2002, TCAs tended sorghum aphids. In addition, sorghum aphids modified their behavior from flicking

honeydew to secreting honeydew after being antennated by ants in their invaded habitats. Similar to other reports of TCAs being opportunistic tenders (Sharma et al., 2013), this was also the case for TCAs tending sorghum aphids.

In this study, the position of aphids on a plant was found to influence TCA tending rate. Higher tending occurred when aphids were located on the stem of a plant than higher up on the plant (Figure 3.2). This difference in tending could have been influenced by ants scouting and finding aphids more easily when they were located on the stem compared with aphids higher up on the leaf.

This study did not find that TCA tending increased aphid biomass during short term greenhouse experiments. However, it is possible that a study of longer duration or with aphids on plants in the field could result in aphid biomass increases in the presence of ants. Interestingly, aphids collected from Johnson grass had a slightly greater biomass at the end of the experiment, although it was not significantly different from aphids collected on sugarcane or sorghum (Figure 3.5). These findings contrast with other studies that found significantly reduced survivorship and reproduction for sorghum aphids on different host plants (Nibouche et al. 2015, Paudyal et al. 2019). While host plant did not have an effect on aphid biomass or ant tending, host plant should still be taken into consideration when assessing the impact of ant presence or tending on aphid biomass.

The number of aphids on a plant can potentially impact ant-aphid symbiotic interactions. In this study, tending was observed with an initial placement of twenty aphids at the beginning of the experiment. In sorghum fields, it was reported that red

imported fire ants (*Solenopsis invicta* Buren) increased sorghum aphid numbers when they were at low numbers (Wright 2021). Further investigation into how aphid numbers affect ant tending is needed to understand ant and sorghum aphid dynamics.

The intensity of recently developed symbiotic interactions can be influenced by factors, such as temperature, humidity, or impacts of climate change. It is therefore important to consider how climate change could over time result in an intensification of the interaction between tawny crazy ants and sorghum aphids. For example, under enhanced atmospheric carbon dioxide conditions the mutualism between cowpea aphids (*Aphis craccivora* C.L. Koch) and invasive yellow crazy ants (*Anoplolepis gracilipes* F. Smith) intensified with increased aphid honeydew production (Kremer et al. 2018). Under continued changing climatic conditions sorghum aphids might similarly produce more honeydew, which could result in the greater attraction of ants, and an increased interaction intensity between sorghum aphids and TCAs.

Shifting the focus from sorghum aphid pests to ants, there remain unanswered questions as to the impact of tending on TCAs. The potential impact of honeydew on the presence of ants or of carbohydrate availability on colony growth should be further investigated. For instance, invasive fire ants (*Solenopsis invicta* Buren) were reported to increase in colony growth in the presence of cotton aphids (*Aphis gossypii* Glover) feeding on cotton plants (Wilder et al. 2011). With an increase in ant colony growth, this could result in further displacement of native arthropod species or an exacerbation of invasive hemipteran damage. While our research does not suggest that an invasional meltdown from the interaction between tawny crazy ants and sorghum aphids is likely at

present, potential interactions between TCAs and other hemipterans may produce different results. Therefore, further quantitative research on how TCA tending may influence colony growth or increases in biomass for ants and/or hemipterans is recommended.

Although insect species may be recently reported as invasive, this research verifies that symbiotic interactions can develop within a short timeframe. The symbiotic interaction between sorghum aphids and TCAs happened approximately in four years. Continued monitoring of this symbiotic interaction is recommended, as it is possible that in agricultural or natural ecosystems, ant tending by tawny crazy ants, red imported fire ants, or other ants could cause sorghum aphid population increases. The potential factors influencing the symbiotic interaction between invasive TCAs and sorghum aphids is complex. The dynamics of sorghum aphid and TCA interaction can also be influenced by factors such as honeydew concentration or chemical composition (Wright 2021), or honeydew bacterial composition (Holt et al. in progress). Disentangling the potential biologically relevant factors that influence ant-aphid symbiotic interactions may aid with future pest management efforts.

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CHAPTER IV

FINE SCALE POPULATION GENETIC STRUCTURE OF TAWNY CRAZY ANTS (*NYLANDERIA FULVA*; MAYR: HYMENOPTERA: FORMICIDAE) IN THE US

Tawny crazy ants, (*Nylanderia fulva* Mayr), which are native to South America, were first reported in the continental United States (US) in 1931, but it was not until the 1990s in Florida and 2000s in Texas that this ant was considered as a serious pest. Tawny crazy ants (TCAs) are currently considered an invasive pest in six US states and their invasion success is attributed in part to their unicolonial nature and omnivorous diet. A limited number of broad-scale molecular markers (e.g., COI, EF1α-F1, EF1α-F2, CAD, and microsatellites) have shown little genetic differentiation among TCA populations across their geographic distribution in the US. Using High Throughput Sequencing (HTS) we assessed the fine-scale genetic variation of TCA populations across its geographic distribution in the US. We detected fine-scale genetic variation among populations in different geographic locations within the US (i.e., Texas, Louisiana, Alabama, Mississippi, Georgia, and Florida). Our results underscore that increasing the number of markers used in population genetics studies allows for the detection of fine-scale genetic variation among recently introduced populations.

Genetic differentiation of recently invasive insects is often reported as lacking. However, microevolutionary changes in both native and pest species have been reported to happen in as little as 10 to 50 years (Comerford et al. 2021, Koch et al. 2020). Relatively recent introductions of invasive insect species (50 years or less) provide an opportunity to characterize the level of genetic differentiation among different geographic locations. This information can then be used to increase monitoring at points of entry, determine centers of origin, and determine if genetically differentiated invasive pest populations differ in traits relevant to their control. In this study, we characterized the population genetic structure of tawny crazy ants (*Nylanderia fulva* Mayr) in the US.

Invasive TCAs were first identified in Texas in 1938 and later in Florida in 1953 (Klotz et al. 1995, Trager 1984). This species has potential points of origin in the Brazilian Mata Atlântica (Atlantic Coastal Forest) and along the Paraná and Uruguay rivers (Darocha et al. 2015). TCAs are considered pest species in urban, agricultural and wildland areas (LeBrun et al. 2013, Zenner de Polania 1990, Zenner de Polania & Martinez Wilches 1992). After the initial reports and identification of TCAs in the US, little to no information was recorded about their impacts to urban and ecological settings or geographic expansion, suggesting that these first reported propagules may have failed to establish. TCAs were then reported in large numbers in Florida hospitals in the 1990s, and subsequently during a 2002 outbreak in Houston at NASA, and the greater Houston metro area (MacGown 2015, Meyers & Gold 2008). After the 1990s, TCAs expanded their geographic distribution and, in addition to Texas and Florida, are now reported in Alabama, Georgia, Louisiana, and Mississippi (MacGown 2015). Because TCAs are a

recent invader, microevolutionary forces shaping their adaptation to a new environment may not have had enough time to leave a signature widely across their genome.

Previous population genetic studies of TCAs have used molecular markers suitable for detection of broad-scale genetic differentiation (e.g., COI, EF1α-F1, EF1α-F2, CAD, argK, and microsatellites) and not surprisingly, found a lack of genetic differentiation among TCA populations in the US. The most recent TCA population genetics study used 13 microsatellite markers that characterized TCAs in the US as unicolonial, failing to detect differences in population genetic structure from five different states (i.e., Texas, Louisiana, Mississippi, Georgia, and Florida). In contrast, within their native range in South America, significant population genetic structure was detected (Eyer et al. 2018).

The choice of molecular markers used to detect genetic variation in invasive insects should be informed by the influence that microevolutionary forces (i.e., genetic drift, gene flow, selection, time spent in reproductive isolation, and mutation rates) can have on population genetic structure. In particular, gene flow and effective population sizes, will impact the number of molecular markers needed to characterize invasive species' population genetic structure (Medina et al. 2006). The resolution of any population genetic structure characterization will vary depending on the number of molecular markers used. That is, the use of broad-scale markers (e.g., using a single marker such as Cytochrome Oxidase I, COI, or tens to a hundred molecular markers such as microsatellites) will result in lower resolution while the use of fine-scale markers (e.g., using hundreds to thousands of molecular markers such as amplified fragment

length polymorphisms, AFLPs, or single nucleotide polymorphisms, SNPs) will result in higher resolution.

Broad-scale resolution is useful for differentiating among populations that have experienced reproductive isolation for a relatively long time, allowing for the independent accumulation of genetic variation over time. At one end of the spectrum a single diagnostic marker (i.e., nuclear or mitochondrial) is sufficient to differentiate among populations of the same species that have been isolated for many generations, or among organisms that now constitute cryptic species (Feder et al. 1988). Such is the case for differentiating between kissing bugs that can transmit Chagas disease to people, from other cryptic reduviid species that transmit pathogens to wild animals (Pavan et al. 2013). In another example, the use of EF1-α along with morphometric data allowed for the identification of cryptic aphid species that occur on different host plants (Nibouche et al. 2021). In a contrasting scenario, broad-scale resolution markers may not accurately characterize genetic differences among recently introduced propagules that derive from the same source and colonize geographically separated locations or among those that are experiencing moderate or high levels of gene flow, yet are in the process of locally adapting to their invaded habitats. In these situations, a small number of markers do not provide enough power to discern genetic differences, however minor that may still be biologically significant. In these scenarios, fine-scale molecular markers are needed to increase the power to tease apart potential genetic differentiation.

Fine-scale markers allow for differentiation among populations that may have low genetic diversity due to a recent invasion or ongoing gene flow (Kliman & Sheehy

2008, Lemopoulos et al. 2020, Morin et al. 2004). The use of fine-scale resolution markers allows for the detection of genetic differences among populations where broad-scale markers fail to detect variation (Andrews et al. 2016, Morin et al. 2004, Peterson et al. 2012, Sunde et al. 2020). For example, in native populations of geographically isolated American lobsters (*Homarus americanus* H. Milne-Edwards), the use of microsatellite markers failed to detect population genetic structure between the northern and southern range, while the use of 8,144 SNPs (single nucleotide polymorphisms) allowed for the detection of significant genetic differences between these populations (Benestan et al. 2015). Similarly, DAPC analysis of the yellow fever mosquitoes (*Aedes aegypti* L.) with 18,147 SNPs showed clear delineation of populations in Asia and Australia when compared with using 8 microsatellite markers (Rašić et al. 2014); which also allowed for the detection of gene flow barriers. In whitefly (*Bemisia tabaci* complex Gennadius) populations, analysis of 38,041 SNPs identified gene flow due to global trade, which was not previously detected with 10 microsatellite markers (Elfekih et al. 2018, Thierry et al. 2015). Thus, the higher power of fine-scale resolution markers allows for exploration of potential genetic differentiation among recently introduced insects, while providing a high ability to discern potential differences among different collection locations or ongoing gene flow among locations.

Since TCAs are recent invaders in the US, we used HTS to generate thousands of sequences to assess fine-scale resolution among TCA populations. Fine-scale genetic characterization of TCAs may inform practices aimed to reduce inadvertent transportation of this pest into novel locations. In addition, assessing genetic variation

among populations may allow for the identification of traits correlated with pest potential and improve the precision of pest control methods. In this study, our objective was to detect whether there were fine-scale genetic differences among geographically isolated populations of TCAs in the US.

Methods

Sample Collection

TCA workers were collected from across the US (i.e., Alabama, Florida, Georgia, Louisiana, Mississippi, and Texas) and internationally (i.e., Argentina, Colombia, and Peru) (Table 4.1). To maximize detection of potential genetic variation within each state and country, when possible, worker ants were collected at least 1 km apart from each other (Supplemental Table 4.1). Worker ants (female and diploid) were collected from across its geographic distribution. In addition, when possible, workers were collected from three different locations within each state or country. Ants were preserved in 95% ethanol and labeled with collection site information, collection date and the collector's name. Ants were vouchered in the Medina lab at Texas A&M University, College Station.

DNA Extraction

DNA was extracted from individual worker ants using a PureGene Kit (QIAGEN, Valencia, CA, USA). The quantity and purity of DNA was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and

a PicoGreen dye assay on a NanoDrop Fluorospectrometer (NanoDrop Technologies, Inc., DE). In some instances, multiple workers from the same collection locality were submitted to enhance the likelihood of obtaining good quality sequences back. DNA samples were submitted to Texas A&M University AgriLife Genomics and Bioinformatics Service (TXGen, College Station, TX). Samples with good quantities and quality of DNA (i.e. 20+ ng/uL DNA concentrations on Nanodrop, a Genomic Quantity Number (GQN) 2+ on PicoGreen with some exceptions, and samples that contained average genomic fragment lengths at or above 10000 bp (indicating low shearing) underwent library preparation. All samples were prepped at the same time and each sample was given a combinatorial dual index with the i5 index joined to the EcoRI cut site and the i7 index added to each sample pool via PCR following the dual ligation ddRAD and ddRADseq protocols (Emel et al. 2021, Yang et al. 2020). High-throughput sequencing was done using Illumina NovaSeq 6000 with the restriction enzymes EcoRI and NIaIII.

Data Analysis and Single Nucleotide Polymorphism Identification of Ants

An average of 3.8 million reads with phred scores ≥ 24 (an average sequence phred score of 35) were obtained for each individual TCA worker, for a total of 98 samples, with known geographic locations. Sequence reads were filtered for quality using a MultiQC tool to summarize all Phred scores, which met a minimum score of 20 for each sample (Puritz et al. 2014). Reads were demultiplexed using process radtags in STACKS, providing both restriction enzymes (--index-index -e ecoRI –renz_2 nlalll)

and quality filtering (size of sliding window, -w 0.15 ; Phred score, -s 20). Reads were mapped onto the tawny crazy ant (*Nylanderia fulva*) reference genome (NCBI BioProject PRJNA517949 by Kranti Konganti and Aaron Tarone) following the STACKS protocol (Catchen et al. 2013, Rochette & Catchen 2017). The demultiplexed reads were run through the reference map pipeline (comprised of gstacks and populations). The marukilow model was used to call variants and genotypes in gstacks. The populations program was run with TCA split into 8 geographic locations (Argentina, Colombia + Peru, Texas, Louisiana, Florida, Georgia, Alabama, and Mississippi) and only the first SNP in a locus was kept (--write-single-snp) (Pearman et al. preprint) and a single representative for overlapping sites for reference aligned reads was enabled (--ordered-export). Samples from Peru and Alabama contained only 2 individuals, although in other studies this was sufficient for accurately detecting genetic differences. Settings for the populations program included filtering loci that were shared by 80% or more of samples within a population (--min-samples-per-pop; -r 0.8), a minor allele frequency of 2% (–min-maf 0.02), and loci shared by a minimum of 2 populations (--min-populations; -p 2) were kept for analysis, with a maximum observed heterozygosity of 70% (--max-obs-het 0.7) for the metapopulation, and a minimum percentage of individuals across populations as 0% (--min-samples-overall; -R) (Dussex et al. 2015, Graham et al. 2020, O'Leary et al. 2018, Paris et al. 2017, Rochette & Catchen 2017). The populations program in STACKS was used to generate population genetic summary statistics (Table 4.1) (Catchen et al., 2013).

Evaluating Genetic Relationships

The populations data file generated by STACKS was used to assess population genetic variation in STRUCTURE (Pritchard et al. 2000). STRUCTURE runs were done for the aforementioned eight ant collection locations from the introduced and native ranges plus an additional three potential populations (K = 1 – 12). Each run had a 10,000 burnin with 10,000 iterations for MCMC (Markov Chain Monte Carlo) and was replicated 10 times for each value of K (Hubisz et al. 2009, Pless et al. 2017). The number of putative populations in the data was analyzed with three different programs, including the R program Pophelper 2.3.1 (http://www.royfrancis.com/pophelper/articles/index.html) for all 10 runs of K 1 to 12, Structure Harvester Web with five runs per value of K from 1 to 10., and with the packages poppr 2.9.3, ape, and magrittr in R to run a BIC (Bayesian Information Criterion) (Earl & vonHoldt 2012, Francis 2017, Kamvar 2014, López-Uribe 2019). The estimated number of populations was determined by assessing peak ΔK values (Evanno et al. 2005, Francis 2017).

An Analysis of MOlecular VAriance (AMOVA) in R was used to analyze population genetic statistics (Excoffier et al. 2019), on the genepop file. The following packages were run in R adegenet vcfR, poppr, genepop, ggplot2 for 999 permutations (Ginestet 2011, Jombart 2008, Jombart & Ahmed 2011, Kamvar et al. 2014, Knaus & Grunwald 2017, Raymond & Rousset 1995). Sample collections were analyzed following guidelines and from Population genetics in R

(https://grunwaldlab.github.io/Population_Genetics_in_R/TOC.html). The missing loci parameter was set to ignore (missing = "ignore").

Similarity of SNPs among samples was calculated using a DAPC (Discriminant Analysis of Principal Components) with the adegenet program (Jombart 2008, Jombart 2021).

Table 4.1. Number of TCA samples from US and International collection locations along with summary statistics for loci. The inbreeding coefficient (Fis) was low except for ants sampled from Columbia and Peru. n = number of individuals, V = variant sites that are polymorphic in at least one collection location, % poly = percent polymorphic sites, SNPs = polymorphic sites within a collection location, P = average frequency of the most common allele, Ho = observed heterozygosity, He = expected heterozygosity, π = average nucleotide diversity, Fis = inbreeding coefficient.

Collection Region	***n***	**V**	**% poly**	**Polymorphic Sites/SNPs**	**Private Alleles**	**P**	**Ho**	**He**	**π**	**Fis**
US										
Alabama	2	15595	3.81	4714	53	0.922	0.140	0.108	0.1298	-0.0176
Florida	6	18563	7.1	8695	128	0.911	0.154	0.129	0.1382	-0.0340
Georgia	6	20335	6.9	8914	168	0.910	0.155	0.130	0.1426	-0.0273
Louisiana	7	19936	7.4	9242	176	0.909	0.152	0.132	0.1425	-0.0210
Mississippi	6	20468	7.0	8679	176	0.912	0.148	0.126	0.1379	-0.0202
Texas	58	19104	10.7	13288	550	0.913	0.142	0.131	0.1325	-0.03021
South America										
Argentina	10	19073	10.4	12674	1974	0.867	0.189	0.191	0.2001	0.0330
Columbia & Peru	(2 +1) = 3	14081	4.6	5871	2532	0.878	0.109	0.163	0.1961	0.1627
Total Individuals	98									

Results

Data Filtering

A total of ~745 million reads were retained after using the process radtags filtering step in the software STACKS version 2.53 (Catchen et al. 2013, Rochette et al. 2019). There were between 4.4 to 12 million reads per sample. To reduce linked alleles, only one SNP per locus was used for data analysis. This resulted in 98 individuals with 24,341 SNPs among eight geographic collections.

Population Genetic Analysis

The number of putative populations estimated by ΔK peaked at 5 with a second peak at 7 (Figure 4.1). Ants clustered in seven genetically differentiated populations according to their geographic origin (Figure 4.2).

Population genetics statistics for each collection location are provided in Table 4.1.The observed heterozygosity (Ho) was slightly higher for collection locations compared with the expected heterozygosity (He) for ants (Table 4.1). The number of private alleles was highest for ants sampled from Columbia and Peru (2,532), while ants from Alabama in US had the lowest number (53) of private alleles (Table 4.1). Most TCAs have little genetic differentiation (Hartl & Clark 1997) ($F_{st} < 0.05$) among geographic regions (Table 4.2), which, given the time since invasion, is not surprising. There were two samples that suggest ongoing introductions or gene flow, such as sample 2 from Louisiana that is genetically similar to Argentina and sample 78 from Texas that

is genetically similar to Alabama. The inbreeding coefficient was low for most sampled populations, except for samples collected from Columbia and Peru (Table 4.1).

A DAPC (Discriminant Analysis of Principle Components) showed that TCA in both South America and the US had genetically differentiated clusters based upon geographic collection location (Figure 4.3, Table 4.2). Ants collected from Argentina clustered separately from those collected in Colombia and Peru. Within the US, ants collected from each state clustered separately (Figure 4.4).

An AMOVA on collection locations for TCAs showed that there was significant variation among populations, with the components of variance broken into variations among populations at 23.05% ($DF = 7$, $phi = 0.22$, $p < 0.001$) of the variability, while variation within collection locations accounted for 77.88% ($DF = 90$, $phi = 0.23$, $p < 0.05$) of the variability, and variation between individuals within a population was not identified at -0.93% ($DF = 98$, $phi = -0.01$, $p > 0.5$). Similarly, pairwise F_{st} values revealed genetically distinct clusters associated among collection locations (Table 4.2).

Figure 4.1. Structure Harvester results showing a peak of ΔK at 5 and another peak at 7 putative populations.

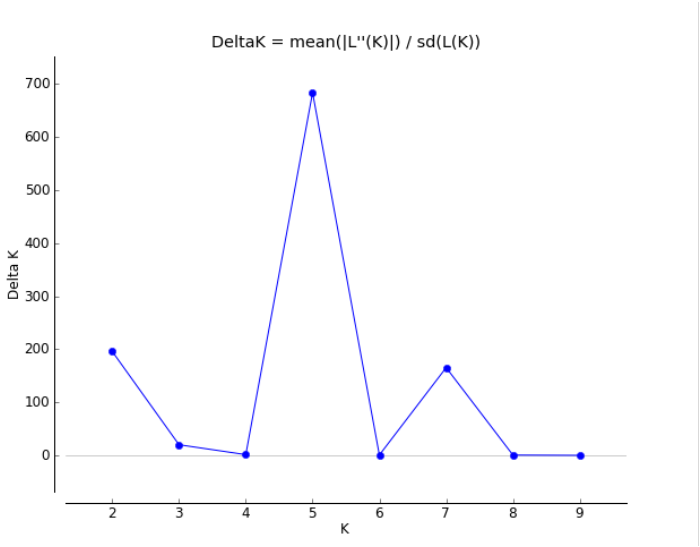


Figure 4.2. Structure plot showing geographically differentiated populations of TCAs within the US. The colors show seven genetically differentiated clusters based upon geographic location. There are two samples that may have been introduced to new locations, such as sample 2 from Louisiana that clusters with Argentina and sample 78 from Texas that clusters with Alabama. The geographic collection location is provided at the top of the structure plot. The numbers at the bottom of each bar corresponds to each individual sample used in the STRUCTURE analysis (Supplemental Table 4.1).

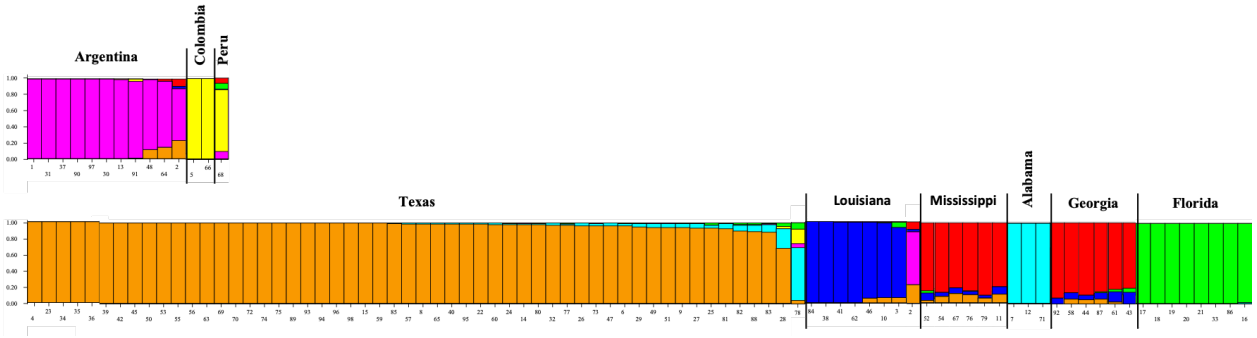


Table 4.2. Pairwise Fst values among each collection location for TCAs. Samples from South America show strong genetic differentiation from US samples. *** = very great, ** = great, * = moderate, and $^{+}$ = little genetic differentiation.

Collection Region	1 Argentina	2 Louisiana	3 Texas	4 Colombia & Peru	5 Alabama	6 Florida	7 Georgia & Mississippi
1 Argentina	–						
2 Louisiana	0.1369*	–					
3 Texas	0.1802**	–0.000212^{+}	–				
4 Colombia & Peru	0.447***	0.5619***	0.601***	–			
5 Alabama	0.1097*	–0.003787^{+}	0.003148^{+}	0.5232***	–		
6 Florida	0.1467*	0.003673^{+}	0.01078^{+}	0.5692***	0.003804^{+}	–	
7 Georgia & Mississippi	0.157**	0.007253^{+}	0.0064^{+}	0.5794***	0.00998^{+}	0.0205^{+}	–

Figure 4.3. DAPC analysis in R. The colored squares represent each geographic region from which samples were collected in South America and the US.

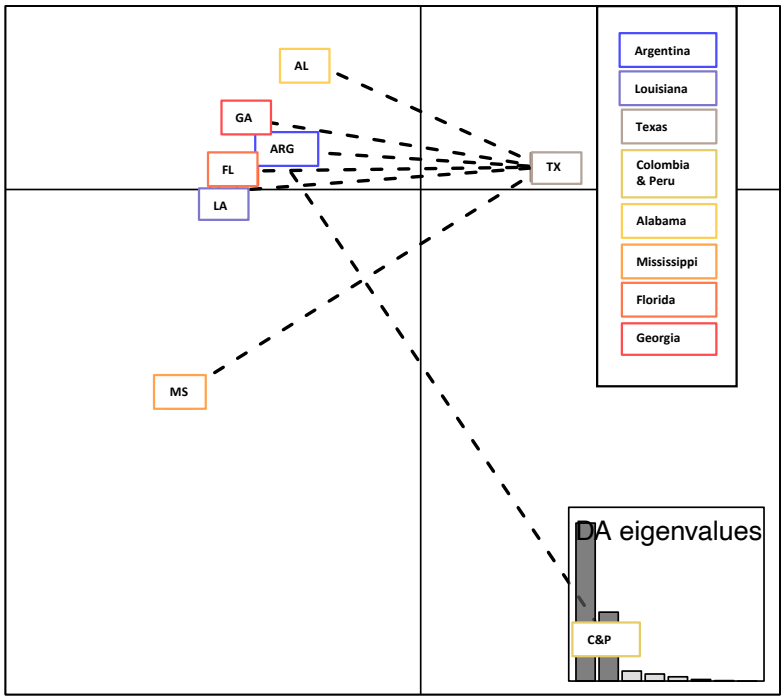
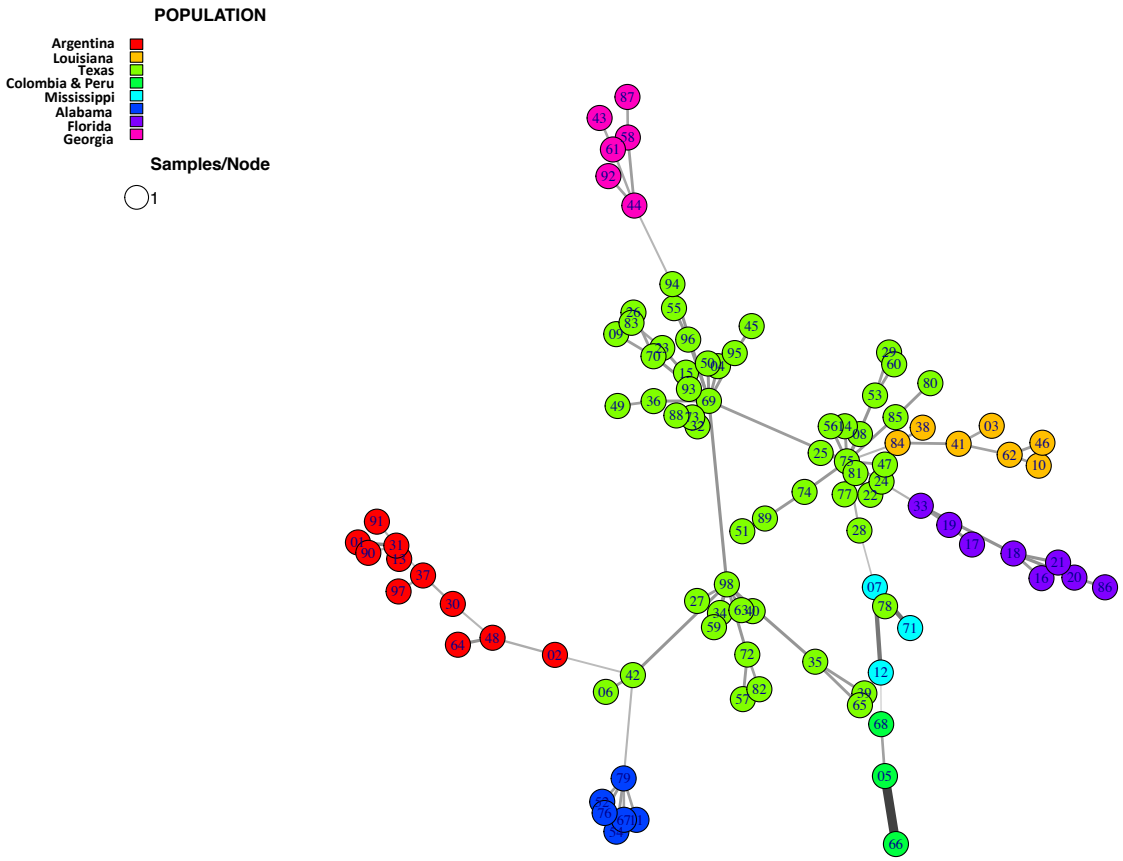


Figure 4.4. Minimum spanning network among TCA collections. The colors represent geographic collection locations. Each circle represents an individual ant. The numbers inside circles refer to individual specimen collection information (Supplemental Table 4.1).



Discussion

Our research shows that invasive TCAs within the US form five genetically differentiated populations, which could result from multiple independent introductions into the US, ongoing gene flow among geographic regions, or differentiation post introduction. While evolutionary processes can occur over long time scales, there are instances in which easily discernable morphological or molecular changes have occurred rapidly. For instance in less than fifty years, soapberry bugs feeding on invasive plants in north America evolved decreased beak length associated with feeding on smaller seeds compared with the longer beaks required to feed on larger seeds from native plants (Carroll & Boyd 1992). Furthermore, soapberry bugs in Texas and New Mexico were recently reported as undergoing host-associated differentiation on Mexican buckeye (Comerford et al. 2021), which may have been facilitated by the presence of invasive golden rain trees. Similarly, invasive spotted-wing drosophila (*Drosophila suzukii* Matsumura) is estimated to have colonized the Hawaiian archipelago in the 1980s and shows morphological differentiation with larger wing size at higher elevation along with corresponding genetic differentiation among different island populations (Koch et al. 2020). Our findings with 24,341 SNPs (i.e., fine-scale markers) are in contrast with recent research that identified TCAs in the US as a superclone using microsatellite markers (Eyer et al. 2018).

Our results are not surprising, as the use of more molecular markers often provides finer scale resolution that may otherwise remain undetected. Several studies have shown the emergence of population genetic structure at greater resolution. For

example, the genetic characterization of yellow fever mosquito (*Aedes aegypti* L.) populations in California, using SNPs generated from HTS, identified previously undetected distinct Northern and Southern populations, suggesting independent introduction events and limited admixture (Pless et al. 2017). Thus, when some recently invasive species are reported as having low genetic variation, it is important to determine how much of this reporting may be due to the use of molecular markers designed to detect only broad-scale genetic variation.

The population genetic structure of TCAs in the US may have resulted from an influx of invasive insect propagules arriving directly to their current locations, such as ants located in Florida and Texas (Figure 4.2). In addition, it is possible that populations of TCA from Texas were transported to other states, such as Alabama, where they have differentiated over time (Figure 4.2, Figure 4.4). Ants in Mississippi and Georgia seem to have been colonized by genetically similar propagules, that may have originally been from Texas, given the closer clustering of these samples to Texas versus South America (Figure 4.3 and Figure 4.4). Since the invasion of these genotypes in the US, current sampling does not suggest that there is much introgression from South America. It is possible then that the populations in each state are locally adapted, which could result in immigrant inviability (Nosil et al. 2005).

Distinct genotypes may interact in invaded regions in ways dissimilar to their native habitats (Cohen & Privman 2019, Bermond et al. 2012). While transportation of TCA propagules can happen via contaminated soil, wood (i.e., logs or pallets), or vegetation (Zenner de Polanía 2019), the population structure revealed by this study

suggests that is likely infrequent among states (Figure 4.2). However, it is important to continue monitoring the spread of these ants as widespread transportation increases the likelihood for interactions among invasive populations that could result in admixture or hybridization events with genotypes that do not normally mate (Anderson et al. 2018, Corrêa et al. 2019, Su et al. 2017). Such was the case, when admixture between rice adapted and corn adapted strains of diamond back moths occurred in conjunction with insect dispersal, which maintained alleles for insecticide resistance in the surrounding geographic areas (Arias et al. 2019). Similarly, when the native corn earworm (*Helicoverpa zea* Boddie) mated with invasive cotton bollworms (*Helicoverpa armigera* Hübner) in Brazil, this resulted in hybrid offspring that were more resistant to pesticide applications than the native corn earworms (Valencia-Montoya et al. 2019). Thus, admixture in invaded regions could result in novel adaptations, which might influence invasive pest behaviors and management.

While fine-scale resolution can discern low levels of genetic differentiation among recently introduced populations or those that experience moderate levels of gene flow, the upfront financial costs can be higher than screening samples with already developed broad-scale resolution markers. In addition, genomic material (e.g., DNA) for HTS requires a higher quality standard, meaning low shearing and high genomic quantities, when compared with using specific broad-scale resolution markers such as microsatellites. Thus, the use of fine-scale resolution markers is better suited for analyses where the information needed cannot be obtained with broad-scale resolution

markers, such as when discerning populations with low genetic differentiation or recent reproductive isolation.

While our study shows the use of fine-scale molecular markers can unveil population genetic structure among recently invaded propagules, continued periodic genetic characterization of invasive pests can provide information on how microevolutionary forces impact populations. For example in varroa mites (*Varroa destructor* Anderson & Trueman and *Varroa jacobsoni* Oudemans), parasites of honeybees, positive selection in genes associated with chemosensory detection, molting and reproduction (Techer et al. 2019) was detected, and this selection may allow parasites to become locally adapted to honeybees inhabiting different geographic locations. In addition, further studies should characterize how genetically distinct invasive TCA pest populations differ in traits such as vector competency, insecticide susceptibility, and variation in other traits that are potentially relevant to pest management (Medina 2012, Pless et al. 2017, Suarez et al. 2008). For instance, genetically distinct populations of green peach aphids (*Myzus persicae* Sulzer), Mediterranean fruit flies (*Ceratitis capitata* Wiedemann), and mountain pine beetles (*Dendroctonus ponderosae* Hopkins) have all been shown to vary in their susceptibility to pesticides and stress tolerance (Dowle et al. 2016, Singh et al. 2021, Weldon et al. 2018). Although propagules of an invasive insect such as TCAs may look morphologically similar when compared to those in different geographic locations, genetic differentiation among populations can influence pest traits (e.g., stress tolerance,

pesticide resistance, symbiotic interactions) and thus the ability to effectively manage these pest populations (Bernaola & Holt, 2021).

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CHAPTER V

CONCLUSIONS

Invasive species are responsible for damages and negative economic impacts to agriculture, urban landscapes, and human and animal health. While invasive insect parasites of the same species that are located in different geographic regions or consume different resources are often treated as the same, both the microbial and genetic composition of the insect host and of the invading population can modulate the expression of key traits and modify ecological interactions of invasive populations with other organisms in the invaded areas. This research stresses the importance of integrating both genetic and microbial characterizations of invasive species to better understand how these components affect invasive populations' establishment and symbiotic interactions.

While invasive insect populations belonging to the same species may morphologically look the same, the microbial composition of different insect populations can influence invasive pest traits, such as stress tolerance or resource use. Therefore, characterizing the microbial composition of invasive insects should become routine for understanding invasive pest establishment, pest potential, and pest management approaches. Through this research, host associated differences in microbial abundances among aphids feeding on grain sorghum and sugarcane were detected within a recent invasive species (Holt et al. 2020). In addition, the use of high throughput sequencing in this research detected bacterial symbionts not previously identified in aphids (e.g., *Arcobacter*, *Bifidobacterium*, and *Citrobacter*). The roles of these bacteria in modulating

sorghum-sugarcane aphid interactions is still unknown, but additional investigation could provide clues into whether these microbes provide a benefit to invasive aphids. These findings also align with my research groups' findings that the aphid reported during the 2013 pest outbreak in US sorghum is the sorghum aphid (*Melanaphis sorghi* Theobald), while the aphid that has been reported since the 1970s in Florida and later in Louisiana sugarcane fields is a separate species identified as the sugarcane aphid (*Melanaphis sacchari* Zehntner) (Nibouche et al. 2021).

In addition to characterizing the microbial symbionts of invasive insects, identifying and quantifying the macroscopic symbiotic interactions of invasive pests can inform the evolutionary ecology of insects in introduced environments. While symbiotic interactions, and in particular mutualisms, are often thought of as occurring among organisms sharing a relatively long evolutionary history, I provide evidence that it is possible for novel symbiotic interactions to occur relatively fast, potentially facilitating invasive species establishment and proliferation. In the case of tawny crazy ants, which are reported to be opportunistic tenders, these ants interact with a variety of hemipterans in invaded environments. In addition, these ants have been reported to exacerbate hemipteran damage to forage grass and cultivated sugarcane (Pazmino-Palomino et al. 2020), underscoring the importance of identifying novel symbiotic interactions with invasive insects. The findings in this dissertation suggest that the novel symbiotic interaction between tawny crazy ants and sorghum aphids should continue to be monitored for agricultural and ecological impacts. Information on the novel symbiotic interactions with invasive insects should be incorporated into pest management

approaches, which could reduce the exacerbation of pest damage caused by the establishment of novel symbiotic interactions.

My population genetic analysis of tawny crazy ants shows that genetic differentiation can be detected in relatively recent invasions, between approximately 20 to 35 years. The advent of fine-scale molecular markers increases the power to detect potential genetic differentiation among recently invasive insects. While genetic differentiation is reported to happen in a few insects within a 10 to 50 year timeframe or a relatively small number of generations (Comerford et al. 2021, Koch et al. 2020), this phenomenon is likely more common than has been previously reported with broad scale molecular markers. In addition, understanding the population genetic structure and potential gene flow among populations may aid with establishing protocols that reduce continued inadvertent transportation and allow for proactive management or modeling of long term effects. The idea that invasive populations show no to very low genetic differentiation on recently invaded regions will continue to shift as more studies implement the use of fine-scale molecular markers and continue to find population genetic differences that broad scale molecular markers, such as microsatellites and COI, were unable to detect.

This research provided valuable information about two relatively recent invasive insect pests to the US and also revealed opportunities to further this knowledge. With regard to the microbial composition of sorghum aphids, further investigation into the potential role of *Citrobacter sp.* (which has not previously been reported in aphids) in detoxifying insecticides in aphids could be similar to the role this bacterium performs in

fruit flies (Cheng et al. 2017). The symbiotic interaction between two recently invasive pests, sorghum aphids and tawny crazy ants, is ideal to further investigate the role of symbiotic interactions in facilitating invasive insect establishment, both at macroscopic as well as at microscopic levels. In addition, incorporating fine-scale population genetic analyses that has the power to identify genetic differentiation among recently invasive insects is important for understanding how populations may differ in pestiferous traits or the potential role these genetic differences may play in the symbiotic interactions insects establish.

While introductions of invasive insect populations of the same species are often treated similarly with regard to management plans, this research shows that a one-size-fits-all approach is likely to fail due to potential variation in biological traits due to genetic differentiation and variation in microbiota composition among populations of even recently reported invasive insects. This research highlights that modern pest management strategies should incorporate more targeted approaches than the ones currently in used, such as tactics that consider the microbial composition and local adaptation of invasive insect populations. The path forward in targeted or precision pest management includes incorporating both tailored approaches for invasive insect populations, while continuing to promote sustainable practices (Bernaola & Holt 2021).

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APPENDIX A

SUPPLEMENTAL MATERIALS

Supplemental Table 3.1 Aphid collection information for the ant aphid interaction experiment.

Host Plant	County/Parish	State
Sugarcane	NA	Louisiana
Sugarcane	Iberville Parish	Louisiana
Sorghum	Weslaco	Texas
Sorghum	Franklin	Louisiana
Johnson grass	Brazos	Texas
Johnson grass	Brazos	Texas
Johnson grass	Robertson	Texas
Johnson grass	Travis	Texas

Supplemental Table 4.1 Tawny crazy ant samples for population genetic analysis.

Structure and DAPC Sample Label	Location City	State/Country	County/Parish/Province
71	Theodore	Alabama	Mobile County
12	Theodore	Alabama	Mobile County
7	Theodore	Alabama	Mobile County
64	Buenos Aires	Argentina	Buenos Aires
31	Camping area to 5 km from Aristóbulo del Valle	Argentina	Misiones
1	Camping area to 5 km from Aristóbulo del Valle	Argentina	Misiones
90	El Dorado, near junction Rt. 12 and Rt. 17	Argentina	Misiones
30	Gualeguay entrance	Argentina	Entre Ríos
48	Hurlingham, FuEDEI laboratory	Argentina	Buenos Aires
13	Junction Rt. 11 and Rt. 12, El Alcazar	Argentina	Misiones

Structure and DAPC Sample Label	Location City	State/Country	County/Parish/Province
91	Rt. 12, Shell gas station, between Alcazar and Puerto Iguazú	Argentina	Misiones
37	Rt. 14, Juan Pujol	Argentina	Corrientes
97	Rt. 14, Villa San José	Argentina	Entre Ríos
66	El viajano, Bella Vista	Colombia	Santiago de abajo
5	El viajano, Bella Vista	Colombia	Santiago de abajo
18	likely close to Winter Garden	Florida	na
19	likely close to Winter Garden	Florida	na
21	likely close to Winter Garden	Florida	na
20	Likely close to Winter Garden	Florida	na
17	Powerline Road, Lithia	Florida	Hillsborough County
33	SE Regional Park, Boca Raton	Florida	Palm Beach County
86	Winter Garden	Florida	Orange County
16	Lithia	Florida	Hillsborough County
43	Albany	Georgia	Dougherty County
44	Garden City, Port of Savannah	Georgia	Chatham Co.
58	Garden City, Port of Savannah	Georgia	Chatham Co.
61	Garden City, Port of Savannah	Georgia	Chatham Co.
87	Garden City, Port of Savannah	Georgia	Chatham Co.
92	Garden City, Port of Savannah	Georgia	Chatham Co.
46	Baton Rouge	Louisiana	East Baton Rouge Parish
62	Baton Rouge	Louisiana	East Baton Rouge Parish
38	by Lafayette Memorial Park Cemetary	Louisiana	Lafayette Parish
84	N. Sterling, Lafayette	Louisiana	Lafayette Parish
10	N. Sterling, Lafayette	Louisiana	Lafayette Parish
41	Vermilion	Louisiana	Vermilion Parish
2	Vermilion	Louisiana	Vermilion Parish
3	Vermilion	Louisiana	Vermilion Parish
52	Ocean Springs	Mississippi	Jackson Co.
54	Ocean Springs	Mississippi	Jackson Co.
67	Ocean Springs	Mississippi	Jackson Co.

Structure and DAPC Sample Label	Location City	State/Country	County/Parish/Province
76	Ocean Springs	Mississippi	Jackson Co.
79	Ocean Springs	Mississippi	Jackson Co.
10	Ocean Springs	Mississippi	Jackson Co.
68	Yurimaguas	Peru	na
63	Austin	Texas	Travis County
69	Austin	Texas	Travis County
77	Austin	Texas	Travis County
8	Austin	Texas	Travis County
24	by MET Center & Airport	Texas	Travis County
95	Brazoria Co.	Texas	Brazoria County
34	Bryan	Texas	Brazos County
72	Bryan	Texas	Brazos County
74	Bryan	Texas	Brazos County
82	Bryan	Texas	Brazos
28	Bryan	Texas	Brazos
98	Bryan	Texas	Brazos
13	Bryan, Golden Eagle Dr	Texas	Brazos County
14	Bryan, Golden Eagle Dr	Texas	Brazos County
22	Bryan	Texas	Brazos
29	Buda	Texas	Hays County
32	by Buescher State Park, Bastrop	Texas	Bastrop County
49	by Buescher State Park, Bastrop	Texas	Bastrop County
65	by Buescher State Park, Bastrop	Texas	Bastrop County
23	by Buescher State Park, Bastrop	Texas	Bastrop County
53	College Station by Baylor Scott & White (BSW)	Texas	Brazos County
35	College Station by BSW	Texas	Brazos County
39	College Station by BSW	Texas	Brazos County
78	College Station by BSW	Texas	Brazos County
80	College Station by BSW	Texas	Brazos County
85	College Station by BSW	Texas	Brazos County
42	Columbus	Texas	Colorado Co.

Structure and DAPC Sample Label	Location City	State/Country	County/Parish/Province
50	Conroe	Texas	Montgomery County
55	Conroe	Texas	Montgomery County
40	Conroe	Texas	Montgomery County
36	East Columbia	Texas	Brazoria County
45	East Columbia	Texas	Brazoria County
47	East Columbia	Texas	Brazoria County
59	East Columbia	Texas	Brazoria County
81	East Columbia	Texas	Brazoria County
57	El Campo	Texas	Hillje, Wharton County
26	Weslaco, Estero Llano Grande Valley State Park (SP)	Texas	Hidalgo Co.
70	Estero Llano Grande Valley SP	Texas	Hidalgo Co.
9	Estero Llano Grande Valley SP	Texas	Hidalgo Co.
96	Estero Llano Grande Valley SP	Texas	Hidalgo Co.
25	Estero Llano Grande Valley SP	Texas	Hidalgo Co.
6	Estero Llano Grande Valley SP	Texas	Hidalgo Co.
27	La Marque	Texas	Galveston County
88	La Marque	Texas	Galveston County
4	La Marque, Carbide Park	Texas	Galveston County
94	Richmond	Texas	Fort Bend County
93	Richmond	Texas	Fort Bend County
56	Rosharon	Texas	Brazoria County
75	Rosharon	Texas	Brazoria County
73	San Antonio	Texas	Bexar County
83	San Antonio	Texas	Bexar County
51	Spring/The Woodlands	Texas	Montgomery County
60	Spring/The Woodlands	Texas	Montgomery County
89	Spring/The Woodlands	Texas	Montgomery County