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ARTHROPOD MANAGEMENT & APPLIED ECOLOGY

Amplicon Sequencing of Plant Material Links Cotton Fleahopper to Host Plants

Lindsey C. Perkin*, Kristin L. Hamons, Charles P.-C. Suh, and Gregory A. Sword

ABSTRACT

The cotton fleahopper (CFH), *Pseudatomoscelis seriatus* (Reuter), is an early season cotton pest that feeds on cotton terminals resulting in flower abortion, irregular plant growth, and delayed plant maturity. The CFH has been documented on over 160 host plants across 35 families. Identification of host plants was accomplished through observed presence on a plant in the field and/or controlled feeding studies under lab conditions. Because the CFH is a generalist, these results may not accurately represent the plants used by the CFH under natural conditions. We used amplicon sequencing to identify plant material potentially ingested by CFH nymphs. Control samples consisted of CFHs fed in the laboratory on horsemint, *Monarda spp.* Nymphs were also collected using a sweep net from fields dense with horsemint, croton (*Croton spp.*), or fields of mixed plant composition. We detected the correct plant family in control samples. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) results from the sweep net samples categorized hits into seven different plant families, one of which may be a new feeding host for CFH. Based on these findings, amplicon sequencing may be useful to further understand the complex ecology of the CFH, which may ultimately improve management strategies for CFH.

The cotton fleahopper (CFH), *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae), uses piercing-sucking mouthparts to probe individual plant cells and extract intercellular contents causing the cells to die. It is an early season cotton pest that damages tender, fleshy new plant growth, including developing pre-floral buds (referred to as squares) and terminals of cotton (King and Cook, 1932; Almand et al., 1976). This feeding can lead to square abortion, irregular plant growth, and delayed plant maturity (Almand et al., 1976; Parker et al., 2008).

In 1999, the CFH was considered the most important economic pest of cotton in the United States (Williams, 2000). More recently, the CFH has consistently ranked between fourth and sixth most important among cotton pests (Williams, 2000-2017), despite cotton not being a preferred host (Reinhard, 1926; Holtzer and Sterling, 1980). The CFH is found throughout most of the U.S. but tends only to be an economic pest of cotton in Texas, Oklahoma, and Arkansas (Barman et al., 2013). The CFH is a multivoltine insect (Parencia, 1978), feeding and reproducing on many different seasonal hosts throughout the year. Three host plants are generally thought to play key roles in its ecology as a pest where they co-occur in the cotton growing regions of Texas: horsemint (*Monarda spp.*), cotton (*Gossypium hirsutum*), and croton (*Croton spp.*). In the spring, CFH nymphs emerge from overwintered eggs in the terminal ends of croton to feed and develop on the plants within 30 m (Hixon, 1941). Subsequent adults seek out preferred host plants such as horsemint to feed and reproduce before infesting cotton later in the growing season (Hixon, 1941; Almand, 1976). However, this may not always be the case; it is possible for the first generation of adults arising from diapausing eggs to directly infest cotton under some environmental conditions (Hamons, 2018, unpublished data).

The combined effort of many feeding studies, accomplished mainly through field observation and/or controlled feeding studies under lab conditions, resulted in a compendium of over 160 host plants across 35 families that may serve as hosts for the CFH (Schuster et al., 1969; Snodgrass, 1984; Fletcher, 1940; Esquivel, 2005; Esquivel and Esquivel, 2009). However, not all of these plants co-occur, so these results may not accurately represent host plants utilized by the CFH under natural conditions in cotton growing regions such as the Brazos River Bottom production area of Texas.

L.C. Perkin* and C.P.-C. Suh, USDA-ARS, College Station, TX; K.L. Hamons and G.A. Sword, Texas A&M University, Dept. of Entomology, College Station, TX.
*Corresponding author: lindsey.perkin@usda.gov

Studies have demonstrated the possibility to identify host plant families consumed by insects based on analysis of plant DNA found in the insect gut (Jurado-Rivera et al., 2009; Avanesyan, 2014; Wang et al., 2017; Briem et al., 2018; Fourie et al., 2022; Cooper et al., 2021). Wang et. al. (2017) used a PCR-based approach to distinguish between two host plants consumed by *Apolygus lucorum* (Heteroptera: Miridae). Kheirodin et al. (2021) used a similar approach to develop sequence-specific primers for 14 important crop plants. While these methods can accurately detect target host plants, it is not able to identify non-target hosts. Other studies used a sequencing approach to amplify DNA from the insect gut to successfully identify host plants, but these studies involved dissections of the gut from a large chewing, or piercing-sucking insect, or whole-body DNA extraction from beetles, a sponging-feeding insect, and sap-feeding insects (Jurado-Rivera et al., 2009; Avanesyan, 2014; Briem et al., 2018; Cooper et al., 2021; Fourie et al., 2022). While this technique is well developed, we are unaware of an example illustrating its use in a small nymph with piercing-sucking mouthparts. The CFH has an adult body size of approximately 3 mm long (Parker, 2008), which makes inferences of direct feeding in the field and DNA extraction in the laboratory challenging. In the current study, we tested whether amplicon sequencing with universal plant primers could identify host plants potentially consumed by the CFH. We present a list of host plants detected from CFH nymphs from controlled laboratory feedings on horsemint, target-specific collections of individuals from horsemint and croton in the field, as well from individuals collected throughout a field with a mixed, known composition of plants. This approach should provide insight into CFH nymph host utilization, which may be used to improve management strategies for this pest.

MATERIALS AND METHODS

Research Fields. Research fields were selected in the Brazos River Bottom near Snook, Texas. One field (CR; 30.6130, -96.4929) contained croton, a member of the Euphorbiacea, that is used by the CFH as an overwintering host for diapausing eggs. A second field (HM; 30.5579, -96.4071) was dense with horsemint, a member of the Lamiaceae and preferred spring host. The HM field served as a source of springtime nymphs and horsemint plants were used as controls. The field was also sampled with a sweep net to collect nymphs feeding primarily on horsemint (see descriptions below). A third field (MC; 30.5638, -96.5153) had a mixed composition of plants with minimal horsemint in the spring and served as a source of CFHs allowed to feed on multiple hosts.

Field plant species composition was assessed in the MC field by counting and identifying plants in 30, 1-m^2 plots randomly selected across the field within a 30 m x 30 m area within which the emerging CFHs were sampled during April 2018. Plants that could not be identified by vegetative characteristics were revisited later when the plants were flowering. To determine if our sampling method captured all or most of the plant diversity in the MC field, we plotted the cumulative number of plant species by the number of observed plots. All plant diversity in the field was considered captured when the cumulative number of identified species within a plot plateaued across samples.

Insect Collections. CFH nymphs were targeted in this study due to their inability to readily disperse and, as a result, this minimized the chance of movement from other areas, which would confound the results. CFH nymphs were aspirated directly from horsemint growing in the HM field in June 2019 and transferred to blooming horsemint in the laboratory. The plants were transplanted from the field to pots and cleaned (e.g., spiders and other insects removed). After at least three days of feeding on the "clean" horsemint, actively feeding nymphs (third to fifth instar) were preserved in 100% ethanol to serve as a positive control. We refer to these insects as HM lab samples.

Experimental insect nymphs were collected from all three research fields by sweeping plants with a 40-cm diameter sweep-net. Ten sweep samples (sweeping left and right considered one) were collected at each field while walking diagonally across the field. Samples were collected from the HM field on three days throughout May and June 2019 to include insects primarily feeding on horsemint. CFH nymphs were collected from the CR field in August 2019 when croton was still blooming and attractive to CFHs. Starting on 3 March 2018, CFH nymphs were collected with a sweep net weekly from the MC field to gather CFH nymphs feeding on a variety of host plants. Sampling in the MC field continued until

May 2018 when adults were more abundant than nymphs. This resulted in a total of ten collection dates and allowed for the collection of CFHs that may have fed on a variety of springtime host plants as they became available over the season. Sweep net samples from each field were emptied into separate plastic bags and stored in a cooler with ice blocks until returning to the lab where they were immediately placed in a -20°C freezer. Within 24 h, CFHs were transferred to vials of 100% ethanol labeled with the date and location of collection and stored at -80 °C until DNA extraction.

Molecular Analysis. CFH nymphs were gently removed from ethanol vials with forceps and blotted on a Kimwipe (Kimberly-Clark, Irving, TX) to remove excess ethanol before being placed separately in tubes for DNA extraction. Samples consisted of 85 nymphs collected from the MC field (MC sweep), 19 collected from the HM field (HM sweep), 12 collected from the CR field (CR sweep), and 16 from the HM field that were used as positive controls (HM lab) for a total of 132 samples. DNA was extracted from individual whole bodies of all 132 CFH nymphs using the DNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and quantified using the DNA ONE fluorophore kit (proprietary information) on a Quantus fluorometer (Promega, Madison, WI). Of those, 96 samples had enough DNA (at least 12 ng total DNA) to progress to library preparation using the Illumina 16S Metagenomic Sequencing Library protocol (https://www.illumina.com/). The minimum amount of DNA was predetermined based on the optimal DNA input for the library protocol. The protocol was followed with modifications as described below. First, plant chloroplast-specific primers were designed to amplify the *rbcLa* loci with the Illumina adapter sequence added to the 5' end (forward 5' TCGTCGGCAGCGTCAGATGTGTATTAGAGACAG [ATG TCA CCA CAA ACA GAG ATC AAA GC] and reverse 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG [GTA AAA TCA AGT CCA CCR CG-]). The plant specific primers, *rbcLa-F and rbcLa-R,* were chosen from a test of four primer sets, including those amplifying *matk* and *trnL*, because it had higher amplification in our samples and amplified more consistently than the others (data not shown). PCR specifications were modified by increasing the number of amplification cycles to 45. The thermocycler conditions were as follows: 95 °C for 3 min, 45 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, a final extension step at 72 °C for 5 mins and hold at 4 °C. Amplification of the correct product size (approximately 600 bp) and sufficient quantity was verified using a TapeStation 4200 with DNA1000 tapes (Agilent, Santa Clara, CA). Specifications were met for 60 of the 96 samples, and library preparation proceeded with the 60 samples and consisted of two main steps: PCR clean-up with AMPure XP beads (Beckman Coulter, West Sacramento, CA) and the addition of sample-specific index adaptors (Nextera XT adaptors; Illumina, San Diego, CA). A random selection of samples was tested on the TapeStation to ensure products were of the correct size (approximately 675 bp) and all samples were quantified using a fluorescence-based, double stranded DNA specific dye (DNA ONE fluorophore kit) on a Quantus fluorometer (Promega, Madison, WI). Libraries and a PhiX control (5% spike-in) were then diluted to 4 pM, pooled, and denatured prior to sequencing on an Illumina MiSeq using a 600-cycle kit (2x300 bp; Illumina, San Diego, CA).

Data that passed quality control (17.2 million reads from 41 nymphs) were separated by index and trimmed of adaptors using Trimmomatic 0.38 (Bolger et al. 2014). Forward and reverse reads from each sample type (HM lab, HM sweep, CR sweep, MC sweep) were combined and filtered to only include reads in the expected range of 290 to 301 bp. This resulted in three samples from HM lab, nine samples from HM sweep, two samples from CR sweep, and 27 samples from MC sweep. The resulting sequences for each sample were aligned to the NCBI Viridiplantae (green plants) database using the BLASTn program in OmicsBox (BioBam, Valencia, Spain). The data were filtered again to only include sequences that aligned to the *rbcLa* gene, had a BLAST hit with an E-value $\leq 10e^{-15}$, and were limited to the top BLAST hits that comprised the majority of hits for each sample. The top BLAST hit refers to the best match based on lowest E-value and longest matching alignment length. The resulting hits (species level) were then extrapolated to the family level because most of the plant species in the sampled fields were not found in the NCBI database. Extrapolating to the family level provided a more conservative, but less taxonomically precise, view of the plants potentially utilized by the CFH.

RESULTS

Sequencing resulted in a total of 15 GB of data or approximately 40 million total reads. However, 57% of reads had a quality score < Q30 and were removed from the data set. Further filtering (described in Methods) resulted in a total of 22,952 sequences from three CFH nymphs fed horsemint (Lamiaceae) in the lab (HM lab), 97,318 sequences from nine CFH nymphs sweep-netted from the horsemint field (HM sweep), 20,767 sequences from two nymphs sweep-netted from the croton (Euphorbiaceae) field (CR sweep), and 368,158 sequences from 27 CFH nymphs sweep-netted from the field of mixed composition (MC sweep).

Horsemint Lab, Horsemint Sweep, and Croton Sweep. The CFH nymphs fed horsemint in the lab served as the positive control and basis for filtering and calling top hits for the remaining samples. The top BLAST hit for each HM lab nymph was Lamiaceae, the mint family (Table 1), which accounted for 97.3% of all hits from all control samples. Of the nine HM sweep CFH nymphs, the top BLAST hits came from two different plant families, Lamiaceae and Campanulaceae (Table 1). Lamiaceae accounted for approximately 92.3% of all hits from all horsemint sweep samples. The two CR sweep nymph samples that passed all quality control measures each had Euphorbiaceae as the top hit (Table 1). This family of plants accounted for 99% of all hits from both samples.

Table 1. Top BLAST hit results for all CFH nymphs collected from controlled feeding on horsemint (HM lab), sweeps from HM field and CR field (HM sweep, CR sweep), and sweeps from the MC (MC sweep) research field. Numbers represent the number of individual CFH nymphs that had each plant as the top BLAST hit.

Family	Sample			
	HM lab	HM sweep	CR sweep	MC sweep
Asteraceae				4
Campanulaceae		1		9
Euphorbiaceae			2	
Lamiaceae	3	8		
Malvaceae				1
Melastomataceae				8
Polygonaceae				1
Rosaceae				3
Solanaceae				1

Mixed Composition Sweep. Top hits for plants detected in the 27 CFH nymphs obtained from mixed plant hosts at the MC field consisted of seven different plant families. Of the 368,158 total sequences, 33% were identified as Campanulaceae and 30% as Melastomataceae. Other families that were represented in the MC sweep samples included, Asteraceae (15%), Rosaceae (11%), Malvaceae (4%), Polygonaceae (4%), Solanaceae (4%) (Fig. 1 & Table 1).

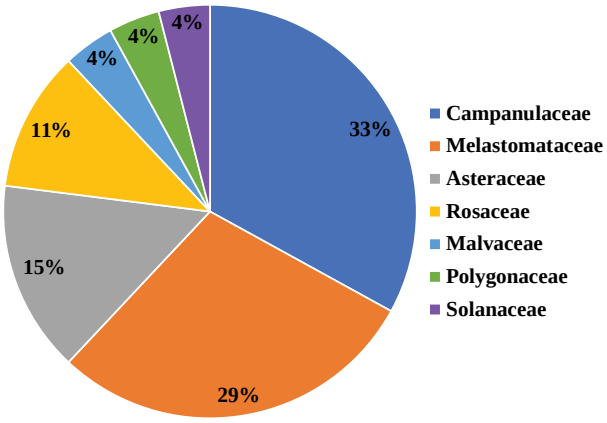

Figure 1. Plant families identified from 27 CFH nymphs sampled from a field with mixed plant composition (MC) using a sweep net from March – May 2018 in the Brazos River Bottom near Snook, Texas, USA.

Field Survey vs. Sequence Data. During the spring of 2018, plant species were surveyed in the MC field using one square-meter plots randomly placed throughout the field. Twenty-three plant species were identified in the MC field survey. The data from the survey was tested for sampling completeness by plotting the cumulative number of plant species found in each 1-m^2 plot. We found that species diversity plateaued at 12 species indicating that we identified all or most of the plant species occurring in the field (Table 2; Fig. 2). Thus, the plant species survey gave us a list of plants available to be consumed by the CFH nymphs collected from this field. The complete list of families detected in MC sweep CFHs were compared to the plants identified at the respective field (Table 2). Out of the 23 plant families positively identified as occurring in the MC field, five were represented in the sequence data. Additionally, Polygonaceae was listed as a potential CFH host plant family in Esquivel and Esquivel (2009) and was also detected in the sequencing data. Consistency between the list of surveyed plants and top BLAST hits added a level of confidence to the analysis and suggested this method is successful in detecting plant DNA from this small piercing sucking insect nymph.

Table 2. Survey of local plant composition at research fields (HM and MC) in the Brazos River Bottom near Snook, TX in the spring of 2018.

Family	Species	Common Name	Present	
			HM	MC
Amaryllidaceae	*Zephyranthes spp.*	Rain lily	N	Y
Ambrosiaceae	*Ambrosia spp.*	Ragweed	N	Y
[z]Asteraceae	*Gamochaeta pensylvanica*	Pennsylvania everlasting	N	Y
[z]Campanulaceae	*Triodanis perfoliate*	Venus' looking glass	N	Y
Caryophyllaceae	*Cerastium glomeratum*	Sticky mouse-ear chickweed	N	Y
Crassulaceae	*Centella asiatica*	Pennywort	N	Y
Euphorbiaceae	*Croton spp.*	Woolly croton	Y	Y
Fabiaceae	*Medicago lupulina*	Black medic	N	Y
Geraniaceae	*Geranium carolinianum*	Carolina geranium	N	Y
Lamiaceae	*Lamium amplexicaule*	Henbit	N	Y
Lamiaceae	*Monarda spp.*	Horsemint	Y	N
[z]Malvaceae	*Modiola caroliniana* (L.) G.	Carolina bristlemallow, Don bristlemallow	N	Y
Onagraceae	*Oenothera laciniata*	Cutleaf evening primrose	N	Y
Oxalidaceae	*Oxalis stricta*	Common yellow oxalis	N	Y
Plantaginaceae	*Veronica arvensis*	Corn speedwell	N	Y
Ranunculaceae	*Ranunculus repens*	Creeping Buttercup	N	Y
[z,y]Rosaceae	*Rubus trivialis*	Dewberry	Y	Y
Rubiaceae	*Galium aparine*	Catchweed bedstraw	Y	Y
[z]Solanaceae	*Solanum elaeagnifolium*	Silverleaf nightshade	N	Y

[z] **Plant families that were detected in the CFH sweep-samples from field with mixed plant composition.**

[y] **Plant family that was present in the MC field but not documented in the random plots due to growth habits along the fence and field management practices.**

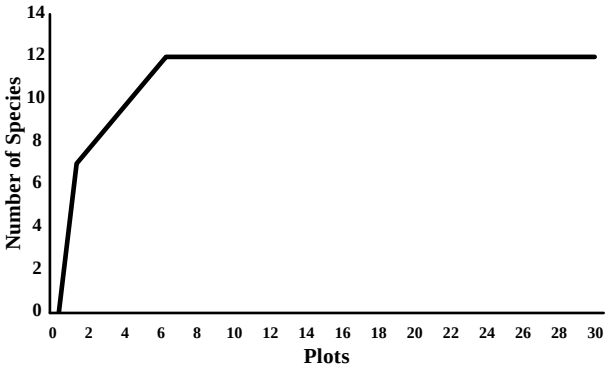

Figure 2. Cumulative plant species per plot for the MC field surveyed from March – May 2018.

DISCUSSION

The CFH is a generalist insect with over 160 host plants across 35 families listed in the literature as potential feeding hosts. However, these documented host plants may not accurately represent plants the CFH feeds on under natural conditions. Thus, we conducted a study to determine if amplicon sequencing of plant DNA from CFH nymphs collected from the Brazos River Bottom production area of Texas could give insight into the plants consumed by CFH in this region. However, not all potential host plants are represented at the species level in the current BLAST database, so identification of unknown plant sequences from CFH individuals was limited to the family level. Additionally, we cannot completely rule out the possibility of surface contamination of CFH individuals by environmental plant DNA during the collection process. The small size and delicate nature of the CFH nymph made pre-DNA extraction rinses or dissections difficult. Therefore, nymphs were only rinsed in an ethanol bath and carefully blotted on a Kimwipe (Kimberly-Clark, Irving, TX). Future work to perfect this strategy in the CFH will include a more robust insect surface cleaning strategy such as described in Greenstone et al. (2012) to make sure the plant DNA amplified is from the insect gut and not its surface.

In the control (HM lab) and targeted sweep-net samples, (HM sweep and CR sweep), the correct plant family was identified as the top BLAST hit for all samples except for one. This sample was from HM sweep and the top BLAST hit was to Campanulaceae. Although horsemint (Lamiaceae) was targeted when sweep-netting, it is possible to have collected a nymph that was feeding on another plant family such as Campanulaceae, the bellflower family, which is a common plant in the research area.

The MC field served as a test to determine if unknown plants could be identified from CFHs collected in a mixed plant community. Of the 23 plant families positively identified as occurring in the MC field, five were represented in the CFH gut sequence data, including Polygonaceae which was listed as a potential CFH host plant family in Esquivel and Esquivel (2009). Campanulaceae was identified in the field and accounted for 33% of the sequencing data, but no plants from this family have been previously reported in the literature as a host plant of the CFH. The Campanulaceae species identified in the habitat survey was Venus' Looking Glass (*Triodanis perfoliata*) which is endemic to Texas and common in the Brazos River Bottom area where these samples were collected. Thus, Venus' Looking Glass is most likely the Campanulaceae hit detected in our samples.

The second highest hit in the sequence data was Melastomataceae, but this plant was not observed in the field during the surveys. However, it is possible that a Melastomataceae species was growing inconspicuously with only vegetative foliage and was undetected during the survey. Nevertheless, a species in the Melastomataceae family that is native and common in the research area is *Rhexia mariana,* commonly called meadow beauty. *R. mariana* has been documented blooming in the area as early as July and late as September (iNaturalist https://www.inaturalist.org). Considering the fields were surveyed in spring and early summer, this plant may have been overlooked among the vegetation because it wasn't yet blooming.

Other families that were detected in the sequencing data albeit at lower levels included Asteraceae, Rosaceae, Solanaceae, Polygonaceae, and Malvaceae. All of these had representative species that were found both in the survey data and the literature. Asteraceae hosts identified in the literature include wild lettuce, *Latuca virosa,* and pyrethrum daisy, *Chrysanthemum cinerariifolium*. These exact species were not noted in the field, but Pennsylvania everlasting (*Gamochaeta pensylvanica*) is a common Asteraceae for the region and was present. Dewberry (*Rubus trivialis*), a Rosaceae, was noted growing along the edge of the MC field either on shrubs or fences, however, it was not detected in the plot samples. It may be that CFH nymphs fed on dewberry plants before moving into the interior of the field. Silverleaf nightshade (*Solanum elaeagnifolium*), a Solanaceae, grows in abundance in the fields, but at the time of the survey only a few were beginning to emerge. Polygonaceae was identified from the BLAST as *Rumex vesicarius,* commonly known as Butter Dock. Although not directly observed in the MC field, *Rumex spp.* are listed in the compendium as hosts for the CFH in Texas and documented to be available during our sampling period (Esquivel and Esquivel 2009; Reinhard, 1927).

Malvaceae, the mallow family, includes species of cotton as well as other native weeds including Carolina bristlemallow (*Modiola caroliniana*) which was identified in the MC field and known to grow throughout the region (Table 2). Given the timing of sweep sampling and the distance of cultivated cotton from the research field (two km), it is unlikely that any of the Malvaceae hits were from commercial cotton. Although flightless nymphs were sampled for this study, the possibility of wind-assisted dispersal or movement on animals (e.g., cattle) was considered. The closest commercial cotton field is approximately two km to the southwest which does not follow the prevailing wind. Moreover, the research field where the samples were collected is protected from wind-born CFHs by a dense line of trees and shrubs. Thus, we believe the Malvaceae hits in the MC field sweep samples were more likely from wild-growing native mallow plants and not commercial cotton.

While the top BLAST hit was used for the analysis presented in the results, all of the CFH nymphs collected in the MC field possessed more than one plant family. In most cases the top BLAST hit encompassed the vast majority of sequences; however, there were six individuals with almost equal abundance of another plant species. For example, four samples with the top BLAST hit Rosaceae, Melastomataceae, and Malvaceae also had a secondary BLAST hit for Campanulaceae. Likewise, one sample with top BLAST hit Campanulaceae had an equal secondary BLAST hit to Rosaceae. And lastly, one sample with top BLAST hit Asteraceae had a secondary hit to Euphorbiaceae. This suggests that

although nymphs are considered relatively immobile, they can move from plant to plant to some degree and may indicate that the CFH is truly a generalist at the individual level, and able to feed on the tender new growth of many plants surrounding its emergence site.

Additionally, our study detected two plant families previously unreported as hosts, Melastomataceae and Campanulaceae, that may serve as a potential reservoir host for the CFH. These new insights on the feeding habits of the CFH suggest the potential for undetected reservoirs in the spring when horsemint is unavailable or unattractive, that can lead to infestation in cotton. The methods described herein will be useful in answering questions concerning pest management strategies of the CFH including the identification of host plants capable of sustaining populations just prior to infesting cotton. Additionally, demonstrating this method on a small piercing-sucking insect shows potential for it to be applied beyond the scope of the CFH and to other small pests with similar feeding habits.

CONCLUSIONS

Our study demonstrates that it is possible to identify DNA from plants potentially ingested by CFH nymphs to at least the family level. Plants identified include previously documented host families (Lamiaceae, Euphorbiaceae, Polygonaceae, Asteraceae, Rosaceae, Malvaceae, and Solanaceae) and two new plant families (Melastomataceae and Campanulaceae). Until this study, plant hosts of the CFH were identified by visual observation in-field or captive-feedings in a lab setting, neither of which can confirm that the nymphs fed on those plants. By collecting field specimens, we were able to identify plant hosts likely consumed by the CFH under natural field conditions in the Brazos River Bottom region of TX. This ability, in turn, should help to identify the possible sources of CFH infestations in cotton. Ultimately, insight into the feeding habits of the CFH may help shape alternative management strategies for this pest, particularly those aimed at manipulating non-cotton hosts to reduce the numbers of CFHs infesting cotton fields. Moreover, this method of host identification can be applied beyond the scope of the CFH to other small hemipteran piercing-sucking agricultural pests whose feeding ecology in the field is notoriously difficult to study.

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DISCLAIMER

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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