Plant DNA Detection from Grasshopper Guts: A Step-by-Step Protocol, from Tissue Preparation to Obtaining Plant DNA Sequences

Author(s): Alina Avanesyan
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Protocol Note

Plant DNA detection from grasshopper guts: A step-by-step protocol, from tissue preparation to obtaining plant DNA sequences[1]

Alina Avanesyan[2,3]

[2]Department of Biological Sciences, University of Cincinnati, 614 Rieveschl Hall, Cincinnati, Ohio 45221-0006 USA

- *Premise of the study:* A PCR-based method of identifying ingested plant DNA in gut contents of *Melanoplus* grasshoppers was developed. Although previous investigations have focused on a variety of insects, there are no protocols available for plant DNA detection developed for grasshoppers, agricultural pests that significantly influence plant community composition.
- *Methods and Results:* The developed protocol successfully used the noncoding region of the chloroplast *trn*L (UAA) gene and was tested in several feeding experiments. Plant DNA was obtained at seven time points post-ingestion from whole guts and separate gut sections, and was detectable up to 12 h post-ingestion in nymphs and 22 h post-ingestion in adult grasshoppers.
- *Conclusions:* The proposed protocol is an effective, relatively quick, and low-cost method of detecting plant DNA from the grasshopper gut and its different sections. This has important applications, from exploring plant "movement" during food consumption, to detecting plant–insect interactions.

Key words: grasshoppers; insect gut content; plant DNA barcoding; trophic interactions.

Knowledge of the diet of generalist insect herbivores is critical for understanding insect feeding preferences regarding different plants, as well as for detecting and predicting plant–insect interactions in natural communities. This becomes especially important when the insects of interest are agricultural pests, such as grasshoppers. Grasshoppers cause significant damage to crops and rangelands resulting in serious economic losses in the United States and worldwide. For example, in 17 western U.S. states, grasshoppers annually consume 25% of available rangeland forage, which averages about $1 billion per year (Hewitt and Onsager, 1983). Because of their important role in accelerating nutrient cycling, grasshoppers can influence plant community composition and, in particular, alter the abundance and species richness of plant species (Belovsky and Slade, 2000). Consequently, knowledge of the feeding preferences of grasshoppers can be important for control efforts and effective restoration of damaged areas (Branson and Sword, 2009).

The first step in any study on feeding preferences of insect herbivores is an accurate confirmation of food that is consumed. Among various techniques available for food identification (including direct observation, feeding trails, and microscopic gut content analysis), PCR assays have been shown to be an accurate and relatively quick method for detecting ingested plants, features that are especially important for large-scale studies (e.g., Jurado-Rivera et al., 2009; Garcia-Robledo et al., 2013). In particular, plant DNA sequences extracted from insect gut contents can provide information about insect feeding choices occurring under natural conditions, which can be hidden from direct observations of insects on plants, or may contradict feeding preferences of insects observed in laboratory feeding trials (e.g., Garcia-Robledo et al., 2013). Therefore, potentially erroneous plant–insect interactions can be corrected.

Previous studies on plant DNA detection from insect guts have been conducted on beetles (e.g., Jurado-Rivera et al., 2009; Wallinger et al., 2013), moths (Miller et al., 2006), flies (Junnila et al., 2011), and hemipterans (Matheson et al., 2008), but only Matheson et al. (2008) included one grasshopper in their study, dissecting it 4 h post-ingestion (PI). Studies that used small insects or insect larvae often obtain whole-body DNA extracts (e.g., Staudacher et al., 2011). The extraction of plant DNA from relatively large insects is complicated by the presence of excessive amounts of nontarget DNA of the herbivore; in this case, isolating the digestive system and preventing contamination of gut contents with possible plant material from the outside surface of the insect (e.g., Matheson et al., 2008) is critical for increasing the yield of target plant DNA. Grasshoppers that reach large sizes as adults are among the most important agricultural pests, with enormous economic costs (Hewitt and Onsager, 1983); therefore, information about their food consumption and, in particular, on tissue preparation and subsequent detection of plant DNA from their gut contents is much needed.

In addition, the availability of a protocol for plant DNA extraction from different parts of an insect gut has many advantages in terms of exploring new aspects of herbivore feeding, and is especially useful for insects of relatively large size. It can allow the researcher to "follow" the plant DNA during food consumption and, for example, (1) to determine the approximate time of food consumption from its location in each compartment of the insect digestive system, or in the case of mixed diet, (2) to infer the sequence of ingestion of different plant species.

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[3]Author for correspondence: alina.avanesyan@gmail.com

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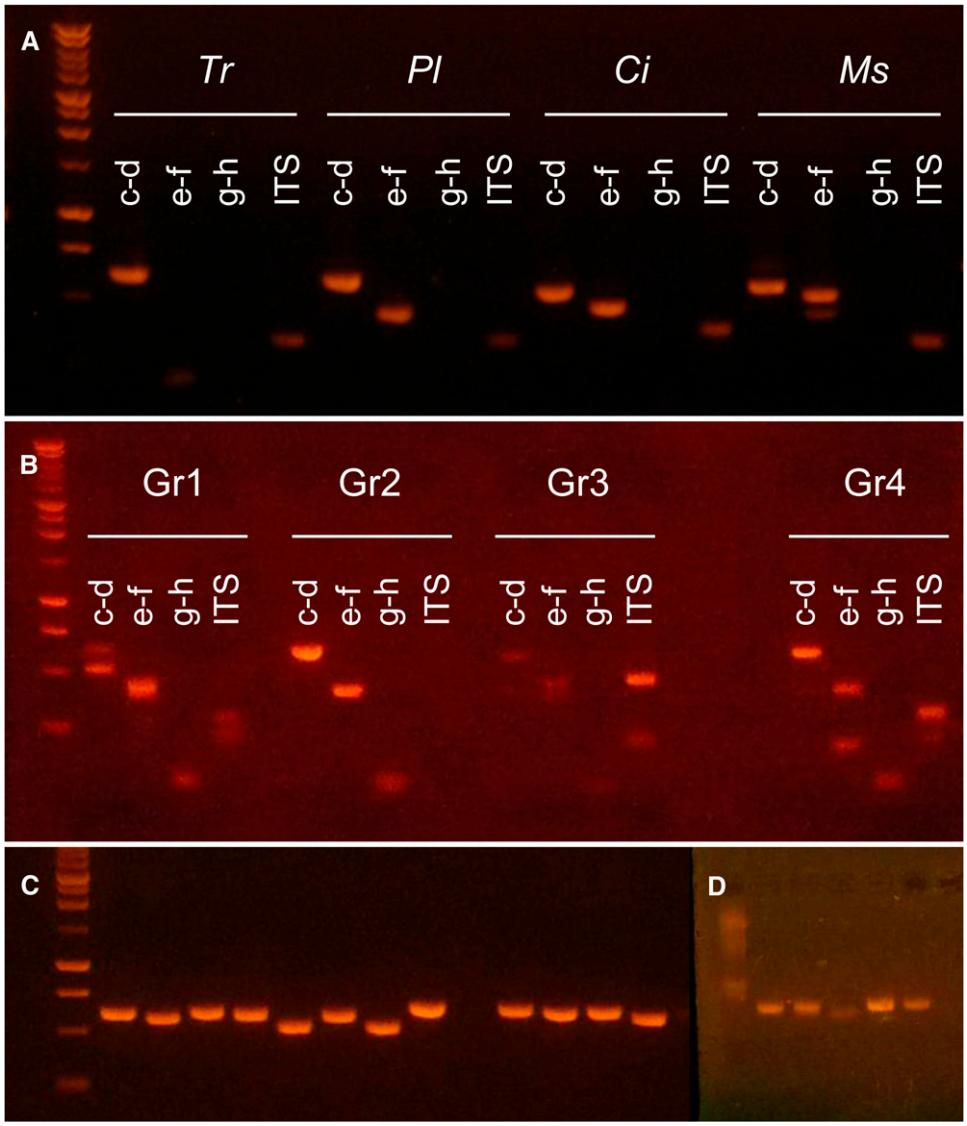

Fig. 1. PCR amplification of three fragments of the *trn*L gene (primers *c-d*, *e-f*, and *g-h*) and the ITS gene from four test plants (A) and from ingested plants within gut contents of four grasshopper individuals (B). *Ci*: *Cichorium intybus*; *Ms*: *Miscanthus sinensis*; *Pl*: *Plantago lanceolata*; *Tr*: *Trifolium repens*. Each group of four lanes for each plant species represents one plant individual. Gr1: *Melanoplus differentialis*, Gr2: nymph *Melanoplus* spp. grasshopper; Gr3-4: *M. femurrubrum* grasshoppers; M: molecular marker (1-kb DNA ladder). Each group of four lanes for each grasshopper species represents one individual. Primers *c-d* successfully amplified fragments of the chloroplast *trn*L (UAA) gene in several other test plants (C) and in ingested plants in gut contents of several nymph individuals of the *Melanoplus* spp. grasshoppers (D). Each lane represents a different plant individual of Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families (C) and a different grasshopper individual (D).

This study provides, for the first time, (1) an optimized step-by-step protocol for DNA extraction and PCR assay for detecting plant food in grasshoppers; (2) evidence of detectability of ingested plant DNA in nymphs and adult grasshoppers via feeding experiments; and (3) a step-by-step protocol for dissection and plant DNA detection in different sections of

Table 1. The feeding experiments used in the study for plant DNA detection from grasshopper gut contents.

Grasshopper species	Life stage	Weight (g), mean ± 1 SE	Type of feeding experiment	Plant species used for feeding	Total time of feeding	Tissues for DNA extraction
Melanoplus spp.	Nymph	0.11 ± 0.02	Choice	*Bouteloua curtipendula* *Bothriochloa bladhii*	3.5 h	Whole body
Melanoplus differentialis	Adult	1.66 ± 0.27	Choice	*Bouteloua curtipendula* *Bothriochloa bladhii*	3.5 h	Foregut and combined midgut+hindgut separately
Melanoplus femurrubrum	Adult	0.36 ± 0.01 0.35 ± 0.02	Choice No-choice	Plant mixture *Bothriochloa bladhii*	2 d 3.5 h	Whole gut

grasshopper guts to follow up the digestive pathway through the gut.

METHODS AND RESULTS

Sample collection—Adult *Melanoplus femurrubrum* and *M. differentialis* grasshoppers (Acrididae: Orthoptera), and nymphs of *Melanoplus* spp. grasshoppers (i.e., *M. differentialis* and *M. bivittatus*) were collected at the Western Maryland Research and Education Center (Keedysville, Maryland, USA) and the Cincinnati Center for Field Studies (New Haven, Ohio, USA). In addition, 40 different plant species of Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families were collected from the study plots and used for the feeding experiments described below. Among these species, *Trifolium repens* L., *Cichorium intybus* L., *Plantago lanceolata* L., and *Miscanthus sinensis* Andersson were used for testing primers; voucher specimens for these plants (AA-0001, AA-0002, AA-0003, and AA-0004, respectively) have been deposited at the herbarium of the University of Cincinnati (CINC). Furthermore, *Bouteloua curtipendula* (Michx.) Torr. and *Bothriochloa bladhii* (Retz.) S. T. Blake used in the feeding experiments described below were grown at the University of Cincinnati greenhouse from seeds obtained from Prairie Moon Nursery (Winona, Minnesota, USA) and Plant World Seeds (Newton Abbot, Devon, United Kingdom), respectively.

Protocol development—To first obtain plant DNA in grasshopper guts, a step-by-step protocol was developed. Following are the most important steps of this protocol; more details are provided in Appendices 1 and 2.

Step 1: Dissection and tissue preparation—After collection, grasshoppers' bodies and plant leaves (1–2 leaves from each plant species) were immediately frozen separately at −20°C. On the day of dissection, four frozen grasshoppers (two adult *M. femurrubrum*, one adult *M. differentialis*, and one nymph) were removed from the freezer and immediately rinsed with 70% ethanol to wash off all possible large, nonhost plant debris from the exterior of the insects. The grasshopper tissues were relatively soft and easy to dissect, so additional time for thawing was not needed. The hind legs and wings were then removed using fine forceps and fine scissors from a standard dissecting set. The exoskeleton of each grasshopper was then cut along the side and the digestive system was extracted. Whole guts were then stored in 1.5 mL microcentrifuge tubes with 70% ethanol overnight before the DNA extraction (Appendix 1, Video 1).

Step 2: DNA extraction—Plant DNA was extracted from four samples of grasshopper gut contents and from *T. repens*, *C. intybus*, *P. lanceolata*, and *M. sinensis*, representing grasshopper host plants (prepared in Step 1 above); both plants and grasshoppers were collected from the same study plot. DNA extraction was conducted with QIAGEN DNeasy Plant Mini Kit (cat. no. 69104; QIAGEN, Culver City, California, USA) according to QIAGEN guidelines. Although this kit is generally used for DNA extraction from standard plant tissue, the kit was recommended by QIAGEN Technical Service as useful for isolating plant material inside the insect gut. After isolation, DNA from plants and grasshopper guts was stored at −20°C for further PCR amplification.

Step 3: Primer testing and PCR amplification—DNA barcodes amplifying the chloroplast *trn*L (UAA) gene and the nuclear ITS 1-2 region were chosen for screening of plant DNA obtained from grasshopper guts because these primers proved successful for detecting ingested plant DNA in a wide range of insect herbivores (e.g., Jurado-Rivera et al., 2009; Staudacher et al., 2011; Pumarino et al., 2011). In contrast, primers suggested by Matheson et al. (2008) that targeted the *rbc*L region did not work in initial screens for this study and were not pursued further. Four sets of universal primers were tested separately on plants and grasshopper gut contents: three sets for noncoding regions of the chloroplast *trn*L (UAA) gene (Taberlet et al., 1991, 2007) and one set for the nuclear ITS region (White et al., 1990). The primer mix was prepared for each primer pair (2 μM of each forward and reverse primer). Each PCR reaction (of 10 μL volume) consisted of the following: 5 μL of QIAGEN Master Mix (QIAGEN), 1 μL of primer mix, 3.8 μL of dH_2O, and 0.2–0.3 μL of DNA. Although other PCR-based protocols sometimes use larger amounts of DNA (e.g., Matheson et al., 2008), the smaller amounts used here were sufficient, as evidenced below. Samples were amplified under the following thermocycler conditions: denaturation of 95°C for 15 min; followed by 35 cycles of 95°C for 15 s, 57°C for 90 s, and 72°C for 60 s; followed by a final extension of 60°C for 30 min. PCR products were then separated in a 1% agarose gel and visualized under a UV transilluminator (Fig. 1A–B).

Step 4: DNA sequencing and final primer selection—To confirm the presence and identity of plant DNA isolated from grasshopper guts, PCR products obtained from grasshoppers and from known plant species (from Step 1 above) were sequenced using Sanger sequencing at the Beckman Coulter Genomics facility (Danvers, Massachusetts, USA). Sequences were then edited in BioEdit (Hall, 1999) and BLASTed against the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/genbank/)

Video 1. Demonstration of grasshopper dissection detailing the procedure for isolating the gut and preparing the foregut and combined midgut+hindgut parts for DNA extraction. This video is an MP4 file and can be viewed here with QuickTime or Windows Media Player, or can be viewed from the Botanical Society of America's YouTube channel.

Table 2. Plant DNA detectability in grasshopper gut contents across several time intervals post-ingestion in different grasshopper species. A single grasshopper was tested for each time point in all experiments.

Grasshopper species	Life stage	Type of feeding experiment	Time intervals post-ingestion (h)[a]									
			0	1	2	3	4	6	8	10	12	22
Melanoplus spp.	Nymph	Choice	+		+		+	+	+	+	+	
Melanoplus differentialis	Adult	Choice	+	+		+						+
Melanoplus femurrubrum	Adult	Choice	+		+		+	+	+	—	—	
		No-choice	+		+		+	+	+	+	+	

Note: + = plant DNA was successfully amplified and sequenced; — = plant DNA was not detected.
[a] Empty cells indicate the cases where data was not available for this specific time interval.

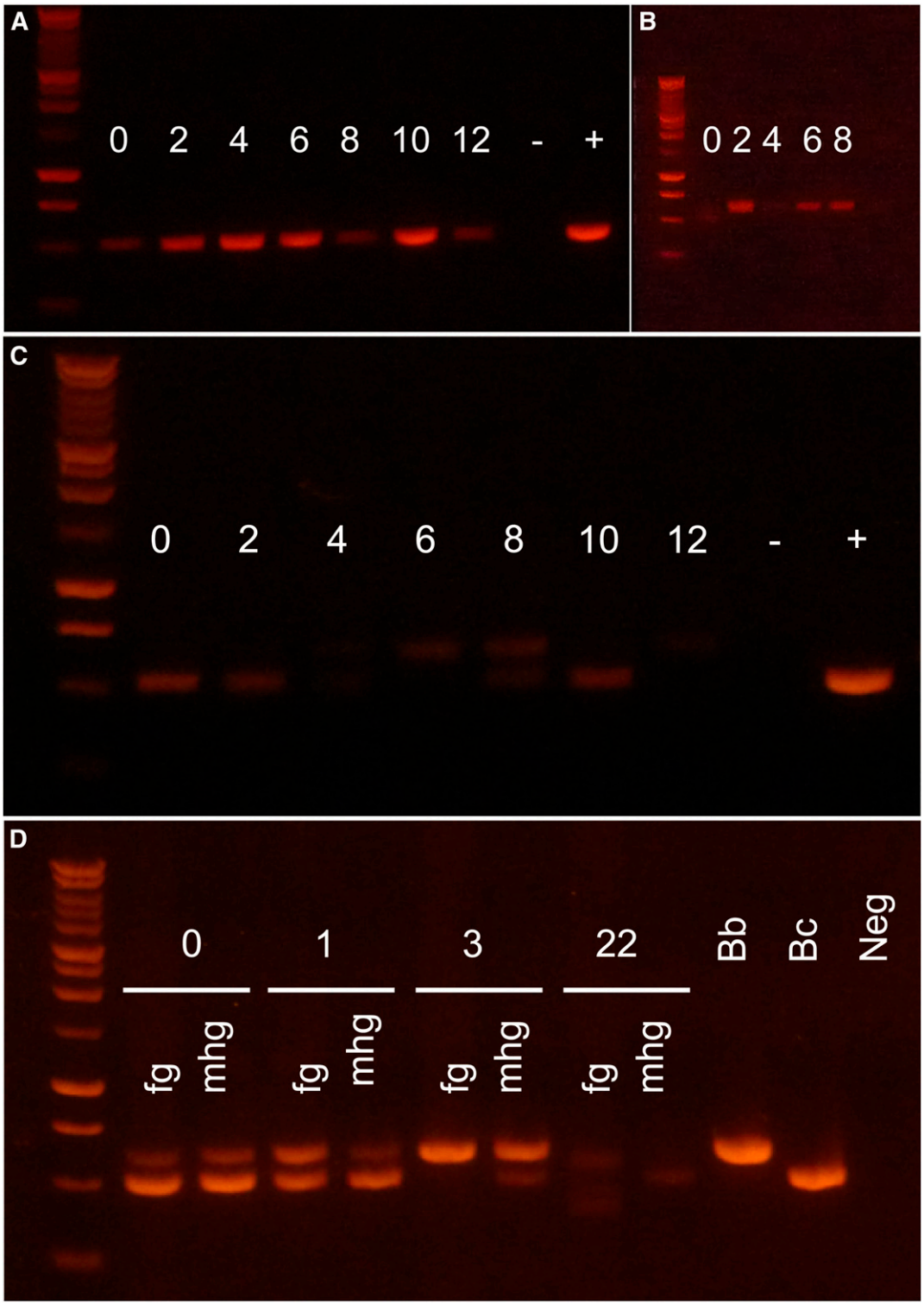

Fig. 2. PCR amplification of the *trn*L gene at different time intervals post-ingestion (PI) after feeding experiments with grasshoppers. The numbers correspond to hours post-ingestion (h PI). One grasshopper individual has been dissected at each time point. "-": negative control (DNA from grasshopper's leg muscle tissue); "+": positive control (plants offered for feeding). Each lane (A–C) represents a different grasshopper individual. (A) No-choice feeding experiment with adult *Melanoplus femurrubrum* grasshoppers and *Bothriochloa bladhii* plants. The plant DNA was present in the grasshopper guts up to 12 h PI. (B) Choice feeding experiment with adult *M. femurrubrum* grasshoppers and a mixture of plants. The plant DNA was present in the grasshopper guts up to 8 h PI. (C) Choice feeding experiment with nymph *Melanoplus* spp. grasshoppers. The plant DNA was present in the grasshopper guts up to 12 h PI. (D) Choice feeding experiment with adult *M. differentialis* grasshoppers. Bb: *Bothriochloa bladhii* (positive control 1); Bc: *Bouteloua curtipendula* (positive control 2); Neg: negative control; fg: foregut; mhg: combined midgut+hindgut. Each of the two lanes (foregut and combined midgut+hindgut) at each time point represents the same grasshopper individual. The plant DNA was present in both foregut and combined midgut+hindgut parts up to 22 h PI.

for plant identification using 98–100% match identity. Following Chen et al. (2010), the quality of sequences for both plants and grasshopper gut contents was estimated using CodonCode Aligner 4.2.5.0 (CodonCode Corporation, Centerville, Massachusetts, USA) for low, middle, and high quality levels. The highest-quality sequences (quality values higher than 30) were observed for primers *c-d* (Taberlet et al., 1991); consequently, these primers were chosen to demonstrate the utility of this protocol.

To confirm the utility of primers *c-d* for a wide range of grasshoppers' potential host plants and grasshopper gut content samples, DNA extraction, amplification, and sequencing were repeated with the remaining 36 collected plant species of Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families and also with 26 nymphs of the *Melanoplus* spp. grasshoppers collected from the same study plots. High-quality sequences with quality values higher than 30 (Chen et al., 2010) were obtained for all 36 study plants (100%, $P < 0.0001$, binomial test) and for 18 out of 26 (69%) grasshopper guts ($P = 0.03$, binomial test). For this analysis, grasshopper gut contents with only single plant DNA were considered. Thus, these results demonstrated that the 500-bp region of the chloroplast *trn*L (UAA) gene, amplified by primers *c-d*, can be reliably detected in grasshopper guts and their potential host plants (Fig. 1C–D).

***Testing the protocol*—**To further demonstrate the effectiveness of this protocol and to determine how long plant DNA remains detectable in the digestive system of grasshoppers of different sizes, three choice experiments and one no-choice feeding experiment with *Melanoplus* grasshoppers were conducted (Table 1, Appendix 2). In no-choice experiments, grasshoppers were fed a single plant species, while in choice experiments grasshoppers were provided with two or more plant species. Grasshoppers were originally collected in the field and their weights ranged from 0.11–1.66 g. Following Siemann and Rogers (2003), grasshoppers were starved for 24 h prior to all feeding experiments to make sure that no previously digested plants were present in the gut. Nymph grasshoppers (Experiment 1, choice) and adult *M. differentialis* grasshoppers (Experiment 2, choice) were offered leaves from both *Bouteloua* and *Bothriochloa* grasses for 3.5 h, *M. femurrubrum* grasshoppers were fed a mixture of plants for two days (Experiment 3, choice), and additionally, another group of *M. femurrubrum* grasshoppers were fed leaves from *Bothriochloa bladhii* grass for 3.5 h (Experiment 4, no-choice). After feeding, grasshoppers were transferred to new containers that did not contain food. Grasshoppers were then frozen separately at −20°C at several time intervals after feeding (one grasshopper at each time point); plant DNA was then extracted and sequenced from each grasshopper using the protocol described above.

The results demonstrated that plant DNA can be detectable up to 12 h PI in the guts of nymph *Melanoplus* spp. grasshoppers (Table 2, Fig. 2A) and adult *M. femurrubrum* grasshoppers (Table 2, Fig. 2B, C), as well as up to 22 h PI in *M. differentialis* grasshoppers, which were the largest in size (Table 2, Fig. 2D). Because of the difference in size and, consequently, weight of grasshoppers, the DNA extraction step (Step 2) of the protocol was adjusted. To meet the requirements for sample weight according to the QIAGEN kit (≤100 mg wet weight), the following were used in this study: whole bodies of nymph grasshoppers, whole guts of *M. femurrubrum* grasshoppers, or two parts of a gut of *M. differentialis* grasshoppers (foregut and combined midgut+hindgut, Appendix 1).

Choice feeding experiments with *M. differentialis* grasshoppers were also used to illustrate the utility of the proposed protocol for detection of plant DNA in different parts of the grasshopper digestive system. In this case, the tissue preparation step (Step 1) of the described protocol was also adjusted: after isolating the grasshopper gut from the body, the foregut and combined midgut+hindgut parts were separated (Appendix 1, Video 1). These parts were then stored separately in 70% ethanol, and plant DNA was then extracted from each section of the digestive system. The results of PCR amplification and obtained sequences of ingested plant DNA demonstrated that a researcher can "follow" the plant DNA in the process of food consumption up to 22 h PI and can make conclusions about the feeding behavior of an insect—specifically, on the order of ingested plants. For example, in this study, the pattern of PCR amplification for foregut and combined midgut+hindgut sections at 3 h PI (Fig. 2D) suggested that *M. differentialis* grasshoppers consumed different plant species sequentially, and did not switch often between grasses offered in the choice experiments.

CONCLUSIONS

Considering the high agricultural significance of grasshoppers (e.g., Hewitt and Onsager, 1983) and their impact on plant communities (e.g., Belovsky and Slade, 2000), there is a major need for an effective protocol for detecting grasshopper interactions with host plants. The utility of the chloroplast *trn*L (UAA) gene for detecting plant DNA from some coleopteran species has been demonstrated in similar studies (e.g., Jurado-Rivera et al., 2009; Staudacher et al., 2011). The developed protocol also demonstrated the utility of the chloroplast *trn*L (UAA) gene for PCR-based work with grasshoppers; 500-bp fragments of ingested plant DNA were successfully amplified and sequenced within grasshopper guts across multiple time intervals post-ingestion. The developed protocol was also effective for detecting plant DNA from different sections of grasshopper guts, which has not yet been reported as previous studies on large insects used whole guts for plant DNA extraction (e.g., Matheson et al., 2008).

The protocol described here has many applications. For example, researchers can sacrifice a small subsample of grasshoppers to accurately determine the time of starvation needed to make sure that no other previously digested plant fragments are present in gut contents. In addition, researchers can follow the "movement" of plant DNA during the food consumption process to better understand the feeding behavior of insect herbivores.

The main advantages of this protocol are as follows: (1) it includes a relatively quick DNA extraction step (less than 3 h); (2) it results in high resolution of the *trn*L gene for plant identification at the genus and, often, at the species level; and (3) it capitalizes on the low cost of PCR and sequencing procedures, which are advantageous for small laboratories without access to next-generation sequencing technologies. Potential difficulties of using this protocol include the following: (1) occasionally low resolution of the *trn*L in species discrimination (three out of 40 cases in this study), and (2) detection of multiple plant DNA in some gut contents (six out of 26 samples in this study). When critical, the former can be addressed by amplifying additional loci; the latter requires additional molecular techniques, such as cloning (Garcia-Robledo et al., 2013), or less labor-intensive methods, such as computational analysis of mixed sequencing chromatograms (Kommedal et al., 2008; Chang et al., 2012). Overall, this is a convenient protocol for detecting plant–insect interactions, and although it was developed specifically for grasshoppers, it can potentially be extended to other plant and insect species to explore different aspects of insect herbivory.

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Appendix 1. Protocol for dissecting grasshoppers and tissue preparation. Developed by A. Avanesyan. The details for isolating a gut and preparing foregut and combined midgut+hindgut parts are presented in Video 1.

Part I. Isolation of grasshoppers' guts (for *M. femurrubrum* grasshoppers*; Fig. A1A–D, Video 1):

1. Take a frozen grasshopper from the freezer and rinse it with 70% ethanol.
2. Use forceps and scissors to carefully remove hind legs and wings.
3. Put the grasshopper on its side and use insect pins to anchor it to the dissecting pad.

Fig. A1. Basic steps of dissecting grasshoppers and preparing their guts: removing hind legs and wings (A–C); cutting the exoskeleton along the side and pulling out the digestive system (D); separation of foregut and combined midgut+hindgut parts (E); storing different parts of the gut in 70% ethanol (F). Step F is not needed if the dissection is immediately followed by DNA extraction. (Images by A. Avanesyan; Video 1.)

4. Use scissors to cut the exoskeleton along the side. Start with the last segment of the abdomen and move slowly toward the head.
5. Carefully pull out the digestive system (if dissecting a female, remove bright yellow ovaries and fat bodies from the abdomen).
6. Place the whole gut in a 1.5-mL microcentrifuge tube with 70% ethanol and store it overnight before the DNA extraction. Skip this step if you immediately proceed with DNA extraction (rinse the gut with 70% ethanol for 10 s).

Part II. Preparing foregut and combined midgut+hindgut parts (for *M. differentialis* grasshoppers; Fig. A1E–F, Video 1):

1. Place an isolated gut on the dissecting pad (Fig. A1E).
2. Review a scheme of the internal structure of the grasshopper to match the main parts of the digestive system (Fig. A2).
3. Find the border between foregut and combined midgut+hindgut parts (Fig. A1E).
4. Use a scalpel to separate foregut and combined midgut+hindgut parts.
5. Place foregut and combined midgut+hindgut parts separately in 1.5-mL microcentrifuge tubes with 70% ethanol and store them overnight before the DNA extraction (Fig. A1F). Skip this step if you immediately proceed with DNA extraction (rinse the parts of the gut with 70% ethanol for 10 s).

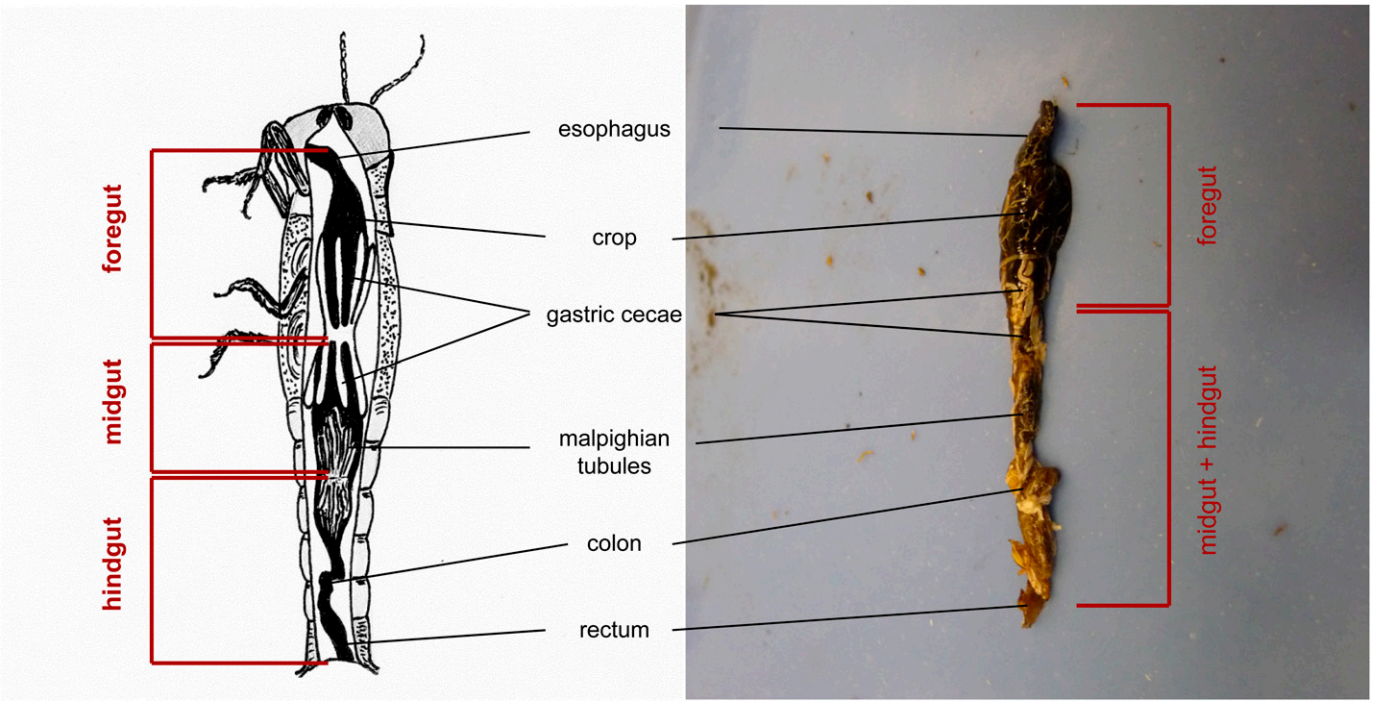

Fig. A2. Scheme of a grasshopper's digestive system (on the left) and corresponding parts in the gut removed from a dissected grasshopper (on the right). (Images by A. Avanesyan.)

Supply checklist:

Small vinyl dissecting pad (11 3/4 × 8 in; Carolina Biological Supply Company, Burlington, North Carolina, USA)

Standard dissecting set (fine scissors, straight, 4 1/2 in; fine forceps, straight, 4 1/2 in; fine forceps, curved, 4 1/2 in; scalpel)

Insect pins (black enamel insect pins, size 2, pkg. of 100; BioQuip Products Inc., Rancho Dominguez, California, USA)

70% ethanol

Microcentrifuge tubes (1.5 mL, Fisherbrand, cat. no. 05-408-129; Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Scheme of internal structure of a grasshopper (Fig. A2)

*Use the whole body of a nymph grasshopper (due to its size) in DNA extraction. Remove hind legs if necessary.

Appendix 2. Protocol for feeding experiments. Developed by A. Avanesyan.

Note: All grasshoppers were starved for 24 h prior to all feeding experiments, which consisted of the following four types.

I. Feeding experiment with nymph grasshoppers:

1. Place 12* nymph grasshoppers individually in small plastic containers.
2. Clip equal number of leaves (~0.3 g total weight) from plants that will be offered to the grasshoppers. For example, in this study, *Bouteloua curtipendula* and *Bothriochloa bladhii* plants were used.
3. Put leaves together and wrap the clipped ends of leaves with moist filter paper.
4. Place leaves on the bottom of each container and let grasshoppers feed for 3.5 h.
5. Randomly choose seven nymphs that ate the most leaf tissue and place them separately in new containers. Other grasshoppers should be continued to be maintained in the laboratory for other feeding experiments.
6. Randomly take one of the seven selected grasshoppers, put it in a plastic bag, and freeze it immediately at −20°C.
7. Freeze each of the rest of the selected grasshoppers at 2, 4, 6, 8, 10, and 12 h post-ingestion (PI) at −20°C in separate plastic bags.
8. Freeze samples of leaf tissue (~2.5 cm^2) from both plant species at −20°C for genetic analysis.

II. No-choice feeding experiments with adult *M. femurrubrum* grasshoppers:

1. Place 12 grasshoppers individually in small plastic containers.
2. Clip equal number of leaves (~0.3 g total weight) from *Bothriochloa bladhii* plants.
3. See steps 3–8 above (from feeding experiment with nymph grasshoppers).

III. Choice feeding experiments with adult *M. femurrubrum* grasshoppers:

1. Place seven grasshoppers in the same aluminum cage.
2. Prepare a mixture of plants† collected on the study plot and place them in a glass vial with water.
3. Place the vial with plants in the cage with grasshoppers.
4. Let grasshoppers feed on this mixture of plants for two days.
5. Randomly select one grasshopper, put it in a plastic bag, and freeze it immediately at −20°C.
6. Remove the other grasshoppers from the cage and place them separately in small plastic containers.
7. Freeze each of the rest of the grasshoppers at 2, 4, 6, 8, and 10 h PI at −20°C in separate plastic bags.

IV. Feeding experiment with adult *M. differentialis* grasshoppers:

1. Place six grasshoppers individually in small plastic containers.
2. See steps 2–6 above (from feeding experiment with nymph grasshoppers).
3. Freeze each of the rest of the grasshoppers at 1, 3, 8, 10, and 22 h PI at −20°C in separate plastic bags. (Two grasshoppers in the study did not eat, so the other four grasshoppers were frozen at 0, 1, 3, and 22 h PI at −20°C.)

Supply checklist:

Plastic containers (7 × 4.5 × 5 in; All Living Things Critter Totes, PetSmart, Inc., Phoenix, Arizona, USA)

Aluminum cage (16 × 16 × 20 in; Repti Breeze Aluminum Screen Cage, Zoo Med Laboratories, Inc., San Luis Obispo, California, USA)

Small Ziploc plastic bags for freezing

* Seven nymphs were actually frozen for the DNA extraction; a minimum of seven nymphs (12 were used in this study) need to be used in the feeding experiments in case some nymphs do not eat. There can be any number of extra grasshoppers.

† In this study, several collected plants of the Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families were used. To simulate natural feeding in the field, plant stems with leaves were placed in a glass vial with water to keep plants hydrated. The vial with plants was then placed in the cage with grasshoppers.