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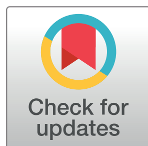

RESEARCH ARTICLE

Rapid PCR-based method for herbivore dietary evaluation using plant-specific primers

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Abstract

Polyphagous pests cause significant economic loss worldwide through feeding damage on various cash crops. However, their diets in agricultural landscapes remain largely unexplored. Pest dietary evaluation in agricultural fields is a challenging task currently approached through visual observation of plant feeding and microscopic identification of semi-digested plant material in pest's guts. While molecular gut content analysis using metabarcoding approaches using universal primers (e.g., rbcl and trnL) have been successful in evaluating polyphagous pest diet, this method is relatively costly and time-consuming. Hence, there is a need for a rapid, specific, sensitive, and cost-effective method to screen for crops in the gut of pests. This is the first study to develop plant-specific primers that target various regions of their genomes, designed using a whole plant genome sequence. We selected *Verticillium* wilt disease resistance protein (VE-1) and pathogenesis related protein-coding genes 1–5 (PR-1-5) as our targets and designed species-specific primers for 14 important crops in the agroecosystems. Using amplicon sizes ranging from 115 to 407 bp, we developed two multiplex primer mixes that can separate nine and five plant species per PCR reaction, respectively. These two designed primer mixes provide a rapid, sensitive and specific route for polyphagous pest dietary evaluation in agroecosystems. This work will enable future research to rapidly expand our knowledge on the diet preference and range of crops that pests consume in various agroecosystems, which will help in the redesign and development of new crop rotation regimes to minimize polyphagous pest pressure and damage on crops.

1 Introduction

Polyphagous mammalian pests [1] and insect pests [2] challenge the sustainability of agricultural production through direct crop damage and disease transmission [2]. Crop losses of up to 50–60% due to insect damage are reported from various parts of the world [2]. Therefore, it is of major importance to exercise all viable management options to minimize the impact of the polyphagous pest on agricultural production, which includes biological control, cultural control and habitat management strategies. Molecular gut content analysis (MGCA) is a powerful tool that broadens our understanding of trophic linkages among organisms in agroecosystems [3], which can advance these management strategies. To date, MGCA has been mostly

employed to determine the diets of generalist predators [3–5], estimate the levels of insect parasitism [6–8], investigate intraguild predation [9, 10], with an aim to find important natural enemies of insect pests in agroecosystems. To a lesser extent, MGCA has been employed to determine the diet of insect herbivores in agroecosystems [11]. In this study, MGCA is employed to evaluate herbivore diet composition in agricultural fields. Given the significant contribution of various natural enemies to pest control services in the agroecosystems, these biological control-focused efforts are justified. However, more recently, an increasing body of evidence suggests crop diversity in agroecosystems can directly contribute to pest population control [12–14], regardless of natural enemies. Therefore, determining the range of crops consumed by particular pest species can help achieve a better understanding of their dispersal patterns [15], and develop more efficient crop rotation regimes and habitat management strategies to minimize pest damage to cash crops in agroecosystems [16, 17]. Such dietary information is vital for subtropical regions since unlike areas experiencing strong seasonality or those where only a few crops are grown, subtropical agricultural landscapes contain crops that are frequently rotated throughout the year [18]. Therefore, crop rotation and the right kind of crop diversity could play an important role in polyphagous pest population regulation in subtropical regions such as Southeast USA. Altogether, polyphagous pest dietary information could be used to add the right kind of crop diversity and manipulate crop planting dates to reduce pest pressure and damage to crops in agricultural landscapes.

Several approaches have been used to explore the dietary composition of insect pests, including visual observations of pest presence and plant damage in the field, microscopic gut content evaluation, and laboratory feeding trials [19]. Laboratory feeding trials may represent unrealistic host preferences because captive insect pests may only feed on the given plant species under laboratory conditions and not natural conditions [20]. Further, understanding the diet composition of polyphagous pests in agroecosystems is a challenging task using direct observation of feeding in agricultural fields, due to the time (e.g., nocturnal) and location of pest activity (e.g., under soil feeding) [19]. At the landscape scale, several techniques including bi-directional malaise traps [21], molecular mark-recapture [22–25] and isotope techniques [25–28] have been used to monitor the movement of polyphagous pests and their natural enemies between habitats, to understand how habitat preference or habitat composition shapes pest and natural enemy movement. However, while these techniques could provide useful information regarding the movement of pests and natural enemies among crop and habitat patches in the landscape, it does not reveal the diet breadth of polyphagous pests and natural enemies, or their intention for visiting different crop patches in the landscape (e.g., to feed on insect prey or plant carbohydrate resources). Employing MGCA, however, can increase the level of accuracy of identifying ingested plant DNA in herbivores. Thus, building diagnostic MGCA for identifying plant DNA in herbivores will help resolve host- polyphagous pest interactions.

Developing PCR-based molecular gut content analysis (MGCA) is now a cost-effective strategy to study complex ecological interactions (e.g. predator-prey, herbivore-plan, pollinator-host interactions) in agroecosystems [3, 4, 29, 30]. MGCA allows researchers to obtain dietary information of pests and their natural enemies by tracking the insect and plant DNA detection frequency in natural contexts. Several techniques employ MGCA to determine polyphagous pest diets in agricultural fields, including restriction fragment length polymorphism (RFLP) [31, 32], singleplex [33] and multiplex PCR [34–37], and metabarcoding approaches [20, 38–41]. MGCA of herbivorous insects using metabarcoding with universal plant primers (e.g., rbcl and trnL) has allowed comprehensive analysis of polyphagous pest gut content to examine host plant diversity and preference [20, 38–41]. While metabarcoding is very sensitive to small degraded quantities of digested plant DNA in the polyphagous pest's

gut [19], it comes with the following dependency on bioinformatics expertise, and relatively high sequencing costs [29]. Similarly, RFLP allows the separation of several species in a sample using restriction enzymes [31, 32]. However, the technique becomes less sensitive when a high number of species are present in samples due to the presence of similar amplicon sizes within the gut content, which results in overlapping bands and detections [29]. Multiplex PCR is a powerful tool that allows rapid and precise identification of several target species in one PCR reaction. The major difficulty of the technique is designing primer mixes that are sensitive (e.g., detecting a low concentration of target DNA in the mix), specific (e.g., amplify only the target and no non-target species in the mix) and compatible with one another within the mix. However, once optimized, multiplex PCR offers a rapid and cost-effective solution for estimating dietary preferences of polyphagous pests and predators.

The plant chloroplast genome sequence is typically used for developing specific and universal plant primers for polyphagous pest dietary evaluations and plant phylogenetic analysis [29, 42–44]. However, due to low variability in the chloroplast genome sequences among the target plant species, we used a whole plant genome approach on multiple genes to develop and optimize a multiplex PCR approach for documenting plant feeding by polyphagous pests. Currently, MGCA protocols for plants are rare for crop plant detection in polyphagous pest's gut (however, see [24, 33]). While several non-crop species-specific plant primers were previously reported [45], to our knowledge, there is only one study detailing a multiplex PCR approach for crop and non-crop plant detection in the gut contents of pests [29]. Following the development of the multiplex PCR primer mixes, the same group has successfully documented herbivorous feeding frequencies of wireworms in wheat and veggie landscapes [34–37]. Using molecular dietary evaluation, Staudacher et al. [34] found a significant decrease in maize feeding and damage by wireworms in diversified cropping systems with grass and legume relative to maize monoculture, which translated to higher maize yield. This result suggests that crop diversification could result in lower pest pressure and damage to field crops, supporting the resource concentration hypothesis [14] and demonstrating how MGCA with multiplex primer sets could answer the important ecological and applied question in agricultural fields.

While these initial contributions to understanding polyphagous pest feeding are encouraging, the targeted plants are not representative of several important cash crops in agroecosystems. Here we present the first method using whole plant genome sequences to design specific primers for 14 important crop species in the world and the USA [18], that enables polyphagous pests (e.g., animal pests, birds and insect pests) dietary determination in agroecosystems. Such information can be used in association with the levels of crop and habitat diversity in the surrounding landscapes to find the best crop rotation and habitat management strategy to reduce pest damage to cash crops. Our objectives in this study were to 1) design specific and sensitive plant primers for fourteen important crop species worldwide, 2) optimize and create efficient, low-cost multiplex PCR primer mixes that are compatible, specific and sensitive towards their target plant species, 3) determine the plant DNA detectability half-life in *M. ponderosus* gut through environmentally controlled laboratory feeding trials, and 4) screen field-collected *M. ponderosus* individuals using the optimized primer mixes to determine the polyphagous pest's diet under field conditions.

2 Materials and methods

2.1 Case study

This study is part of a comprehensive project to unravel polyphagous pest diets (e.g., birds and insect pests) in the Southeast USA, and part of a large-scale landscape study to understand polyphagous pest movement across (or between) habitats within agricultural landscapes.

Given the high abundance and damage of grasshoppers to agricultural fields [46] and due to the fact that grasshoppers are considered polyphagous pests that feed on various crops [47], we used the spur-throat grasshoppers, *Melanoplus ponderosus*, as a model to test and evaluate the efficacy and specificity of the designed primers. *M. ponderosus*, was targeted to understand polyphagous pest feeding in agricultural mosaics containing cropland cover where pests may move between crops. This species was selected due to 1) its high abundance in agricultural fields in our study area, 2) the generalist diet status of grasshoppers that are known to feed on several crops and non-crops [47], and 3) worldwide there is a demand to understand grasshopper diets and food webs in agricultural and native lands.

2.2 Primer design

The most common annual crops with the highest acreage in the Southeast USA were selected for primer design (Table 1) [18]. The plant chloroplast genome sequence is commonly used for phylogenetic analysis and species identification of plants [42–44]. In this study, we first tried to use available regions from chloroplast genomes in common crops using two well-known genes (*ie*; trn-L, trn-F). However, due to the high conservation of chloroplast genomes compared to nuclear and mitochondrial genomes, and due to difficulty in finding a specific region in conserved parts of the genome in different plants that we used in this research, we were not able to find a region that could be used to design plant-specific primers. We searched the full genome sequences to find a region to design plant-specific primers to overcome this issue. For this purpose, we used the full genome sequences of 12 different plants, including *Solanum lycopersicum*, *Gossypium hirsutum*, *Phaseolus vulgaris*, *Citrullus lanatus*, *Cucumis sativus*, *Cucurbita pepo*, *Solanum melongena*, *Glycine max*, *Zea mays*, *Solanum tuberosum*, *Sorghum bicolor* and *Arachis hypogaea* (Table 1). Full genome alignments showed suitable regions to design plant-specific primers. Based on our analysis, *Verticillium* wilt disease resistance protein (VE-1) genes for *S. lycopersicum*, pathogenesis related protein-coding gene 2 (PR-2) in squash and PR-4 in all other species were chosen as good targets for plant-specific primer design. These regions allowed us to easily find species specific regions as we could see high variability in the sequences amongst different plant species used in this research. Since there are no available genomes for onion (*Allium cepa*) and sweet potato (*Ipomoea batatas*), we used the full sequences of onion as well as sweet potato's PR-5, PR-1 and PR-2 in all our alignments to find the best fragment for these plants. Whole genome sequence alignments were performed using MAFFT (Multiple Alignment using Fast Fourier Transform; [48]) using the default

Table 1. Isolate numbers and genome sequence information used for species specific multiplex primer design.

Species	GenBank Accession number	References
Solanum lycopersicum	AEKE00000000	[50]
Gossypium hirsutum	LBLM00000000	[51]
Phaseolus vulgaris	ANNZ00000000	[52]
Citrullus lanatus	VOOL00000000	[53]
Cucumis sativus	ACHR00000000	[54]
Cucurbita pepo	NHTM00000000	[55]
Solanum melongena	BAUE00000000	[56]
Glycine max	ACUP00000000	[57]
Zea mays	CABHLF000000000	[58]
Solanum tuberosum	AEWC00000000	[59]
Sorghum bicolor	ABXC00000000	[60]
Arachis hypogaea	PIVG00000000	[61]

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parameters. After choosing proper regions based on the genome alignments, we used BioEdit [49] to hand-edit the gene alignments. From the alignments, we found regions specific to each of the 14 species and could be used as a target for specific primer design. For primer design, we used CLC Genomic Workbench version 11.0.1 (Qiagen Bioinformatics, Aarhus, Denmark). Finally, we did a blast search of each primer against all other plant genomes to ensure specificity on target plants. In summary, we designed 14 pairs of species-specific primers by leveraging the available genomes of 12 plant species and gene sequences from two additional species (Table 2).

2.3 Plant preparation and DNA extraction

Fresh plant material was collected from agricultural fields and kept in a -20 freezer until extraction. Before extraction, 100 mg of plant leaf tissue was washed using 10% bleach, molecular grade H2O, and 95% ethanol to ensure no cross-plant DNA contamination was present on the leaves. Samples were frozen using liquid nitrogen and then ground to a fine powder using sterilized ceramic mortar and pestles. Then, DNA was extracted following manufacturer protocols for Qiagen DNeasy Plant Mini Kit (QIAGEN, Chatsworth, CA, USA).

Table 2. List of designed primers along with detailed information regarding their target plant species, primer sequences, expected band sizes, optimized annealing temperature (˚C), and their concentration (μM) within multiplex primer mixes.

Target plant species: common names	Primer names	Primer sequences (5'-3')	Plant genome region	Product size (bp)	Annealing temperature	Primer conc in mix (μM)	Multiplex mixes
Cucurbita pepo	CuPE-FP	GCCAAAGATTGCCAGATGGTC	PR-2 genes	115	59.86	0.2	Mix 1
Squash	CuPE-RP	CCCACATTTGAACTGCGTCA			59.05		
Allium cepa	AlCE-FP	TTGAAAACCGCTACTGGCCT	PR-5 genes	145	59.89	0.3	Mix 1
Onion	AlCE-RP	AATATACTGGGGCCGGGGA			59.76		
Glycine max	GlMA-FP	ATGCCACCAAGGCCAAGAC	PR-4 genes	200	60.61	0.2	Mix 1
Soybean	GlMA-RP	AGGGACAACCGTGTTAGCATA			59.10		
Ipomoea batatas	IpBA-FP	TTGGTTTACACGACCCGGTG	PR-1 genes	220	60.53	0.3	Mix 1
Sweet potato	IpBA-RF	TGCACCGACAAATAACAGCG			59.48		
Phaseolus vulgaris	PhVU-FP	AACACCACAGAGAGTGTTGGG	PR-4 genes	250	60.13	0.3	Mix 1
Bean	PhVU-RP	CAGCTTCGCAATACAGGTGC			59.90		
Gossypium arboretum	GoAR-FP	GGGCACTTCAAAGGAAAGCAG	PR-4 genes	270	60.00	0.2	Mix 1
Cotton	GoAR-RP	TCCAGTGTCGCAAACCACTC			60.53		
Citrullus lanatus	CiLA-FP	CTACTGGGCAAATTCTTGCGT	PR-4 genes	330	59.19	0.2	Mix 1
Watermelon	CiLA-RP	GTGAAGTATGACAAAGACATGAACA			57.81		
Solanum lycopersicum	Soll-FP	TGCACACAAACACAAGATAGAGG	VE-1 genes	380	59.19	0.2	Mix 1
Tomato	Soll-RP	TGCGAGGAAAGTCCAAAACAC			59.33		
Arachis hypogaea	ArHY-FP	GCTTACTCTCAAGTACCACACCA	PR-4 genes	405	59.99	0.2	Mix 1
Peanut	ArHY-RP	AGCTGCAGCAGATAGAAGGC			60.18		
Zea mays	ZeMA-FP	GGCGAGAGCCCCTACTAGA	PR-4 genes	180	59.85	0.4	Mix 2
Corn	ZeMA-RP	CACAAATCGCCTGCATGGTT			59.76		
Solanum melongena	SoME-FP	TGACTGGGTGCTTTGTCGAA	PR-4 genes	325	59.82	0.3	Mix 2
Eggplant	SoME-RP	CATGAGTCGGAACCTGAGCC			60.46		
Solanum tuberosum	SoTU-FP	ATGTCTTGGGATGCCGGTTT	PR-4 genes	340	59.46	0.4	Mix 2
Potato	SoTU-RP	AGTAAGGACGTTGTCCGACC			59.40		
Sorghum bicolor	SoBI-FP	GACATGCGGTACCAGTTCCT	PR-4 genes	350	59.82	0.4	Mix 2
Sorghum	SoBI-RP	CTGCCATTGTAGCATGTGACC			59.90		
Cucumis sativus	CuSA-FP	CCCCATTCTCCTCCTCCTAAC	PR-4 genes	407	58.95	0.3	Mix 2
Cucumber	CuSA-RP	TCTAGCACATCCAATCCGGC			59.89		

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2.4 Primer specificity and sensitivity testing

For in silico primer specificity testing, all primer sets were blasted against the NCBI database using Primer-BLAST with the following settings [62]: targeting organisms matching the class of plants, Insecta and spiders. We limited our search to a maximum of one mismatch to the target species and up to four mismatches to the non-target species and ignored any hit with more than four mismatches to the blasted primer sets.

Furthermore, for each primer pair, we performed in vivo specificity test in the laboratory to test the potential cross-amplification on 15 non-target plant and insect species (Table 3). The non-target plant species (e.g., plants that we did not design specific primers for) were selected based on their availability in Southeast production landscapes, which included several crops and non-crop plants (Table 3). Plants were tested using the 14 primer pairs against all non-target species to ensure that the designed primers did not amplify non-target plant DNA. In brief, each primer pair was initially tested on the target plant and then tested against the combined DNA from all non-target plants (non-target plants included and not included in our multiplex primer mixes, (Table 3)). The specificity of the primer to amplify its target DNA fragment in the multiplex primer mix was evaluated by testing the primer mix on their mixed intended target DNA (9 and 5 plant species DNA for mix 1 and 2 respectively), and target DNA within the mix of 15 non-target plants DNA's (Table 3). This was done to ensure that the primer set was able to amplify its target DNA both when only the target DNA was present and when pooled with non-target DNA samples. The sensitivity of each primer set was further evaluated by diluting the extracted plant DNA into four ten-fold serial dilutions (1:10–1:10000). All primer set sensitivity tests were done in triplicate. The primer sensitivity test was performed on individual primer sets as well as mixed primer sets to ensure primer sensitivity within the primer mix. The concentration of DNA in the diluted samples was estimated using a Spectra Max Gemini XPS microplate reader (Molecular Devices, LLC, San Jose, California, USA).

Table 3. List of target and non-target plant and insect species used to test for individual primer specificity evaluations using singleplex PCR.

Target tested	Non-target tested
Plant species tested	**Plant species tested**
Solanum lycopersicum; Tomato	*Vigna unguiculate;* Black-eye peas
Gossypium hirsutum; Cotton	*Vigna unguiculate;* Blue lake snap peas
Phaseolus vulgaris; Beans	*Abelmoschus esculentus;* Okra
Allium cepa; Onion	*Vigna unguiculate*; Zipper peas; Lady Finger peas
Glycine max; Soybean	*Phaseolus lunatus*; Butter beans
Citrullus lanatus; Watermelon	*Capsicum annum*; Jalapenos pepper; Bell pepper
Cucurbita pepo, Squash	*Cucumis melo;* Athena cantaloupe
Arachis hypogaea; Peanut	*Rudbeckia hirta*; Susan
Ipomoea batatas; Sweet potato	*Prunus persica*; Peach
Solanum melongena; Eggplant	*Clitoria ternatea*, Butter peas
Sorghum bicolor; Sorghum	*Brassica oleracea*; Collard, cabbage, broccoli
Cucumis sativus; Cucumber	*Brassica rapa*; Mustard, canola, oilseed
Solanum tuberosum; Potato	**Insect species tested**
Zea mays; Maze, corn	*Bemisia tabaci*; Whitefly adult
	Acrosternum hilare, Stink bug adult
	Melanoplus ponderosus, Grasshopper

Note: None of the non-target species tested were amplified by any of the primers under singleplex or multiplex tests of cross-reactivity

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2.5 PCR optimization and multiplex primer mix testing

The PCR reactions were carried out in a Bio-Rad C1000 Touch © Thermal Cycler (Bio-Rad, Hercules, California USA). Initial PCR's were performed using four different volumes of target DNA and PCR grade water: 1, 2, 2.5 and 3.5 μL of target DNA and 3.65, 2.65, 2.15 and 1.15 μL of PCR grade water, respectively. The best quality of bands and detection was achieved with 3.5 μL of target DNA. Hence, the PCR components were optimized as follows: 6.25 μL of 2x Qiagen Multiplex Master Mix (Qiagen, Hilden, Germany), 0.1 μL of 5xQ-solution (Qiagen, Hilden, Germany), 0.25 μL of Bovine Serum Albumin (Thermo Scientific, Waltham, MA USA), 1.15 μL of PCR grade water, 1.25 μL of primer mix, and 3.5 μL of DNA, to the total volume of 12.5 μL. The PCR reaction cycle started with an initial denaturation at 95˚C for 15 min, followed by 34 cycles of 30 s denaturation at 95˚C, 90 s annealing at 60.0˚C, 90 s of extension at 72˚C and a final extension at 72˚C for up to 10 min. The PCR products were visualized using QIAxcel Advanced Systems (Qiagen®).

Primer mixes were created and tested based on the primers target product size, compatibility, sensitivity and specificity towards their target DNA. Step-wise testing was performed to adjust the concentration of primers within each mix to ensure standardized sensitivity of all primer pairs for their target plant DNA.

2.6 Half-life detectability

2.6.1 Sample collection. For field sites, we used land associated with the College of Agriculture & Environmental Sciences (CAES) Research and Education Centers (RECs), University of Georgia, Tifton, Georgia (31˚32'05" N 83˚24'24" W). The CAES mission for the RECs is to provide experimental land for all UGA scientists and students to enhance knowledge and education of agriculture and outreach to the public. *M*. *ponderosus* individuals were collected using sweep net sampling from grassy areas, and were immediately transferred to sterile cylinder shape containers. The containers contained two square-shaped holes on the sides and transparent lead covered by mesh, to allow airflow. In the laboratory, the individual grasshoppers were provided with wet filter paper to provide humidity and stored in growth chambers (25˚C, L16: D8), and starved for 48 hours before the feeding trial. The filter papers were changed every day to maintain the humidity in the containers.

2.6.2 Experimental setting. The experiment was conducted under controlled conditions (25˚C, L16: D8). As a food source, cotton leaves were collected from the University of Georgia experimental plots at the Tifton campus within cotton fields receiving no insecticide treatments. To standardize the amount of plant material, each grasshopper was provided with a rectangular piece of cotton leaf (3 cm length * 1 cm width) and kept under continuous observation until the entire leaf was consumed. Prior to the trial, each container was randomly assigned to a time interval. All individuals were kept under continuous observation, and the exact time at which the individual consumed the leaf fragment was recorded. Seven-time intervals were tested, including: 0, 12, 24, 36, 48, 72 and 96 hours after feeding (n = 10 per time interval). Following observed consumption at each time interval, individuals were immediately transferred to sterile vials containing prechilled 95% ethanol and stored at -20˚ C until DNA extraction.

2.7 Grasshopper diets in Georgia agroecosystems

Ninety-four individual *M*. *ponderosus* were collected by sweep-net at six locations within the CAES REC near Tifton, GA. Individuals were collected from grasslands located between various agricultural crop fields (e.g., corn, cotton, peanut fields) to increase the chance of mix plant DNA diet in grasshopper guts. Immediately after collection, each individual was

transferred to individual 20 ml disposable scintillation vials (Fisher®) containing 95% ethanol and placed inside a cooler box to avoid DNA contamination and degradation. In the laboratory, the vials were kept at -20˚ C and stored until extraction.

2.8 Grasshopper preparation and DNA extraction protocol

Frozen grasshoppers from the laboratory feeding trials and the field collections were transferred to sterile Petri-dishes (60 × 15 mm, Falcon®) and air-dried for ~30 seconds. The specimens were then dissected using (sterile) surgical scissors and forceps, and the entire digestive parts (e.g., crop and colon) were transferred to individual Eppendorf 1.5 mL vials. After each dissection, the scissor and forceps were carefully washed with 10% bleach, molecular grade water, 95% ethanol and flame sterilized. The DNA was extracted from dissections using Qiagen DNA blood and tissue extraction kits (Qiagen, Hilden, Germany) according to the manufacturers protocol with slight modifications. In the final DNA extraction steps, instead of an overall 200 μL of room temperature buffer AE, 150 μL of ~62 C˚ AE buffer was used in an attempt to extract a higher concentration of the plant DNA. Each sample was then processed using the optimized multiplex PCR protocols to screen for plant DNA.

2.9 Statistical analysis

We conducted all the statistical analyses in R version 3.6.3 [63]. The detectability of cotton DNA in the gut of *M*. *ponderosus* was calculated using probit regression. Probit analysis allows identifying the time period at which DNA is traceable in the gut content of consumers [36]. The binary presence/absence data of cotton DNA in the *M*. *ponderosus* gut was the dependent variable and time intervals after leaf fragment consumption was the independent variable. We fit the binomial presence/absence cotton DNA data over six-time intervals to a probit regression model to predict the half-life (i.e. the time at which the cotton DNA is traceable with 50% probability) for cotton DNA in grasshopper's gut.

The MGCA result from the field-collected grasshopper was used to estimate the percent of grasshopper positives for each plant species and the total percentage positives for plant DNA. The percentage of *M*. *ponderosus* positives for each target plant species was estimated by dividing the number of positives for each target plant by the total number of individuals tested. Total percentage of positives for plant DNA was calculated by dividing the number of individuals positive for all plant target species by the total number of the individuals tested.

3 Results

3.1 Primer specificity and sensitivity

The in-silico primer specificity testing in NCBI Primer-Blast indicated high specificity of the designed primers to their target plants, but no non-target plant, insect or spider species. The only exception was *Solanum tuberosum* that could also hit *Capsicum annuum* with 3 mismatches. All other primers only targeted their intended plant species sequences with up to 99% similarities and no other species. In vivo primer testing confirmed this, as none of the designed primers amplified any of the 28 non-targets (e.g., 13 plants that we designed primer for in this study along with 15 additional tested plants and insects; Tables 2 and 3). All primer pairs were designed to have annealing temperatures around 60˚C, and as expected, this annealing temperature yielded the best quality bands. Further, in-vivo specificity tests indicated that the multiplex primer mixes did not amplify any of the 15 non-target plant and insect species, indicating high specificity within the mix of primers. The primer sensitivity test further showed that all designed primers were capable of amplifying their target plant DNA in

Table 4. The result of in-silico NCBI Primer-BLAST, indicating the number of matches and number of mismatches with the intended target and non-target hits, along with sensitivity of the specific primers both individually and when used in the mix estimated by Spectra Max Gemini XPS microplate reader.

Target plant species: common names	Primer names	Hits to plants	Hits to Insecta	Sensitivity single primer	Sensitivity in primer mix
Solanum lycopersicum	Soll-FP	*Solanum pennellii*, wild tomato, one mismatch, *S. lycopersicum*, no mismatch	None	0.140 DNA ng/μL	0.140 DNA ng/μL
Tomato	Soll-RP				
Gossypium arboretum	GoAR-FP	*G. arboretum*, *G. hirsutum* and *G. raimondii* with no mismatch	None	0.125 DNA ng/μL	0.125 DNA ng/μL
Cotton	GoAR-RP				
Phaseolus vulgaris	PhVU-FP	*Phaseolus vulgaris* with no mismatch	None	0.125 DNA ng/μL	0.185 DNA ng/μL
Bean	PhVU-RP				
Ipomoea batatas	IpBA-FP	None. The genome is not available in NCBI database	None	0.161 DNA ng/μL	0.122 DNA ng/μL
Sweet potato	IpBA-RF				
Allium cepa	AlCE-FP	None. The genome is not available in NCBI database	None	0.122 DNA ng/μL	0.122 DNA ng/μL
Onion	AlCE-RP				
Glycine max	GlMA-FP	*Glycine max* with no mismatch	None	0.144 DNA ng/μL	0.144 DNA ng/μL
Soybean	GlMA-RP				
Citrullus lanatus	CiLA-FP	The genome used for primer design is not available in NCBI. None	None	0.122 DNA ng/μL	0.113 DNA ng/μL
Watermelon	CiLA-RP				
Cucurbita pepo	CuPE-FP	*C. moschata* with one mismatch, and *C. pepo* with no mismatch	None	0.219 DNA ng/μL	0.113 DNA ng/μL
Squash	CuPE-RP				
Arachis hypogaea	ArHY-FP	*A. hypogaea* and *A. ipaensis* with no mismatch	None	0.131 DNA ng/μL	0.131 DNA ng/μL
Peanut	ArHY-RP				
Solanum melongena	SoME-FP	The genome used for primer design is not available in NCBI. None	None	0.180 DNA ng/μL	0.180 DNA ng/μL
Eggplant	SoME-RP				
Zea mays	ZeMA-FP	*Zea mays* with no mismatch	None	0.203 DNA ng/μL	0.203 DNA ng/μL
Corn	ZeMA-RP				
Solanum tuberosum	SoTU-FP	*Capsicum annuum* with 3 mismatch, and *S. tuberosum* with no mismatch	None	0.151 DNA ng/μL	0.151 DNA ng/μL
Potato	SoTU-RP				
Sorghum bicolor	SoBI-FP	*Sorghum bicolor* with no mismatch	None	0.210 DNA ng/μL	0.210 DNA ng/μL
Sorghum	SoBI-RP				
Cucumis sativus	CuSA-FP	Cucumis sativus with no mismatch	None	0.172 DNA ng/μL	0.219 DNA ng/μL
Cucumber	CuSA-RP				

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concentrations as low as 0.219 plant DNA ng/μL. The majority of primers were capable of amplifying target plant DNA in concentrations as low as 0.144 plant DNA ng/μL (Table 4). Corn, sorghum and bean were among the least sensitive primer pairs when tested in the mixes, and their concentration in two primer mixes was adjusted to standardized the mix. By adjusting each primer pair concentration based on the sensitivity of each primer within the mix, we standardized the mix to enhance the efficacy of each primer (Table 2). Our results further indicate that all primers within these two mixes are compatible, and can detect target DNA without masking the performance of other primers within the mixes.

3.2 Multiplex primer mixes optimization

The compatibility and sensitivity of primers were tested within various mixes to create two working multiplex PCR primer mixes (Table 2, Fig 1). Some primers were not compatible with others within the first mix, including sorghum and potato. Therefore, a second primer mix was required to effectively screen for all species. Furthermore, the expected band size for some of the primers was similar, resulting in a need to separate them in order to definitively conclude a plant species (e.g., *A. hypogaea* and *C. sativus*). Using primer sensitivity results as a

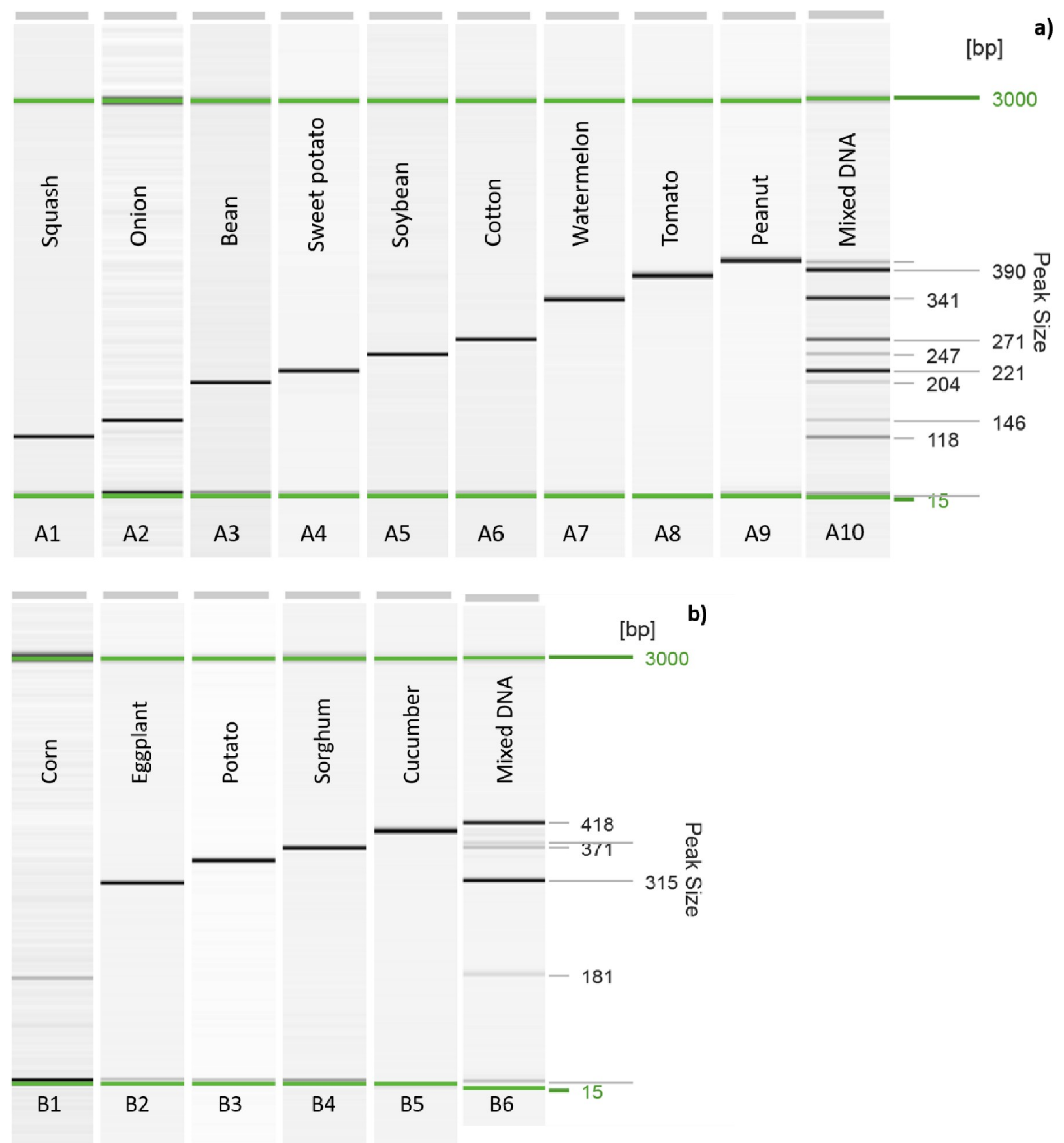

Fig 1. The amplification of the plant target DNA using two working multiplex primer mixes (primer mix 1 & 2, panel a & b) containing several species-specific primers and their associated amplicon sizes (bp). The PCR products were visualized on a QIAxcel Advanced system, and Qiagen alignment markers were employed to separate amplicon sizes ranging from 15bp to 3000bp. The plant species were ordered by expected base pair sizes, to indicate the expected ranges of band sizes using the designed primers. Panel a) consisted of the result of multiplex PCR on a single target species from left to the right A1-A9: *Cucurbita pepo*, *Allium cepa*, *Glycine max*, *Ipomoea batatas*, *Phaseolus vulgaris*, *Gossypium arboretum*, *Citrullus lanatus*, *Solanum lycopersicum* and *Arachis hypogaea*, respectively, followed by A10) their mixed target species DNA sample. Panel b) consisted of the result of multiplex PCR from left to right B1-B5 including *Zea mays*, *Solanum melongena*, *Solanum tuberosum*, *Sorghum bicolor* and *Cucumis sativus*, respectively, followed by B6) their mixed target species DNA sample.

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guide, the PCR conditions were optimized testing different concentration of primers (0.1–0.5 μM) within the mixes. The final primer mixes resulted in the efficious detections as well as yielding PCR products with the highest and most equivalent concentrations of PCR products for each primer pair in the multiplex PCR (Tables 2 and 3).

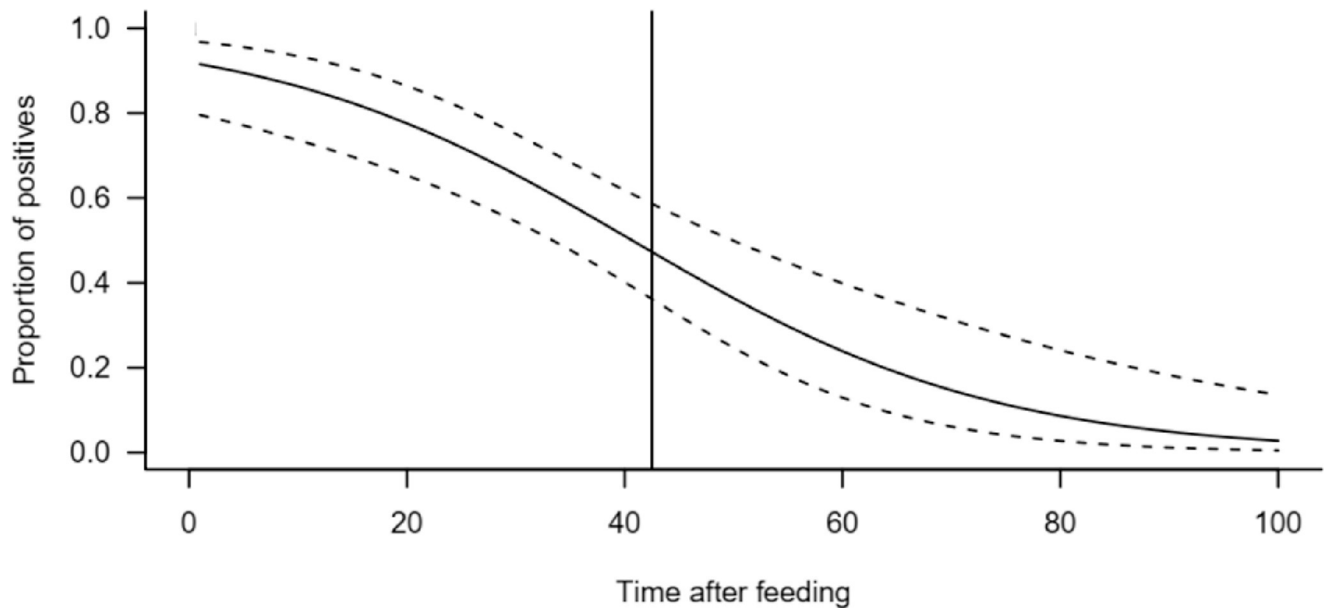

Fig 2. The proportion of *Melanoplus ponderosus* positives for cotton DNA at the intervals of 0, 12, 24, 36, 48, 72 and 96 h post-feeding, using the multiplex primer mix. The solid curve line represents the fitted probit model accompanied it's of 95% confidence intervals (dotted lines). The solid vertical line is the time at which 50% of individuals are expected to test positive for plant DNA in their gut (half-life). R statistical software was used to create this figure.

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3.3 Detectability half-life

The detectability half-life for cotton DNA in the gut of *M*. *ponderosus* was up to 42.5 h after feeding (Fig 2). The slope of the probit model significantly differed from zero (slope = -0.05999, z-value = -4.51, df = 68, P<0.0001). Similarly, the concentration of the remaining plant DNAs decreased over-time detected by the multiplex primer pair used, where the concentration of remaining cotton plant DNA in the gut of *M*. *ponderosus* dropped from 1.21 (ng/μL) at a 0 h, to lower than 0.22 (ng/μL) at 48 h (Fig 3).

3.4 Field-collected grasshopper screening

Using these two primer mixes, 94 field-collected *M*. *ponderosus* were screened, out of which 47 individuals contained DNA from one or multiple crop targets in their gut content. One individual contained DNA from 5 target species, three individuals contained DNA from four target species, three individuals had DNA from three target species, six individuals had DNA from two target plant species, and thirty-five individuals only had one target species DNA in their gut. The feeding frequency on *C*. *lanatus* was higher, followed by *G*. *arboretum*, *Z*. *mays*, *C*. *pepo*, *I*. *batatas* and *I*. *batatas* with 14.90, 13.83, 9.57, 8.51 and 6.38%, respectively. The frequency of feeding was generally low or negligible for the other seven crop plant species with about 1% of individuals being positive for these crops (Fig 4).

4 Discussion

Our results show that the two working multiplex primer mixes designed in this study are specific to their target, and are sensitive to low concentrations of their target plant DNA in the gut content of polyphagous insect pests. Furthermore, our results show that primer pairs within each mix are compatible with one another, enabling the identification of 14 plant species within the guts content of insect pests using only two multiplex PCR reactions. We also report

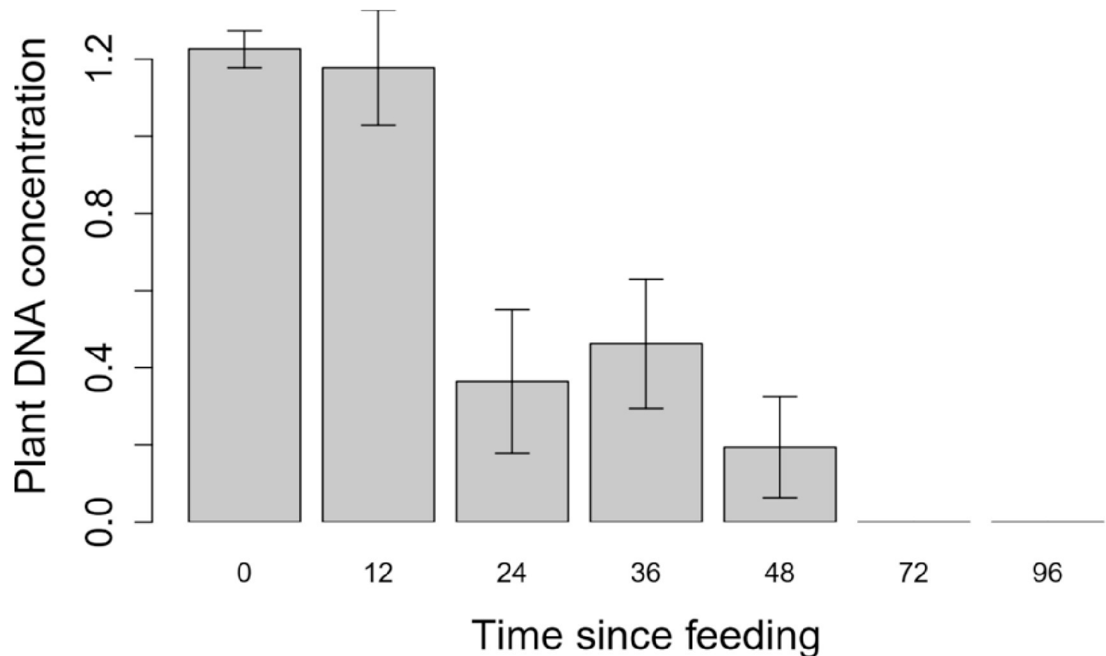

Fig 3. The concentration of remaining plant DNA ± SE in the gut of *Melanoplus ponderosus* after feeding at intervals of 0, 12, 24, 36, 48, 72- and 96-hours post-feeding, using the multiplex primer mix. R statistical software was used to create this figure.

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an optimized PCR assay condition that maximized the plant target detection within pest gut contents.

Several DNA regions have been used to date to develop specific and universal primers for the detection of animal and plant DNA in the gut of insect pests or predators [3]. Cytochrome c oxidase subunit 1 (CO1) is shown to be an excellent region for specific primer design for various insects, to screen for predator-prey interactions or estimate phylogenetic relationships [4, 64]. For plant identification, however, due to the lower rate of evolution in the CO1 region, the use of CO1 to differentiate plant species is inappropriate and impractical [65]. Hence, other DNA regions with a higher rate of evolution, such as rbcl, trnH-psbA, and ITS have been used to develop species-specific or universal plant primers for plant identification and polyphagous pest dietary evaluation [66, 67].

Given the difficulties of finding plant species-specific regions, most studies to date have used DNA metabarcoding approaches using universal rbcl and trnL primers to screen polyphagous pest diet in agroecosystems [20, 38–41, 65]. Few studies have attempted to design and report crop plant species-specific primers. Using the internal transcribed spacer (ITS) region, Pumarino et al. [33] reported a specific primer for tomato, which was used to screen omnivores for evidence of feeding on tomato plants. Similarly, Wang et al. [24] reported specific primers for cotton and mungbean from the trnL-trnF region of chloroplast DNA and used these primers to explore the movement of *Apolygus lucorum* (Heteroptera: Miridae) between cotton and mungbean fields. Currently, only one study [29], using chloroplast region (trnL-F cpDNA region) developed and reported several multiplex primer mixes for crop and non-crop plant dietary detection in insect pests, highlighting a need for additional studies. However, our study is the first to design and optimize a plant species-specific primer mix that can successfully detect 14 target crop plant DNA within two PCR reactions, which significantly eases plant molecular detection. Further, our primer mixes are the first to exclusively target

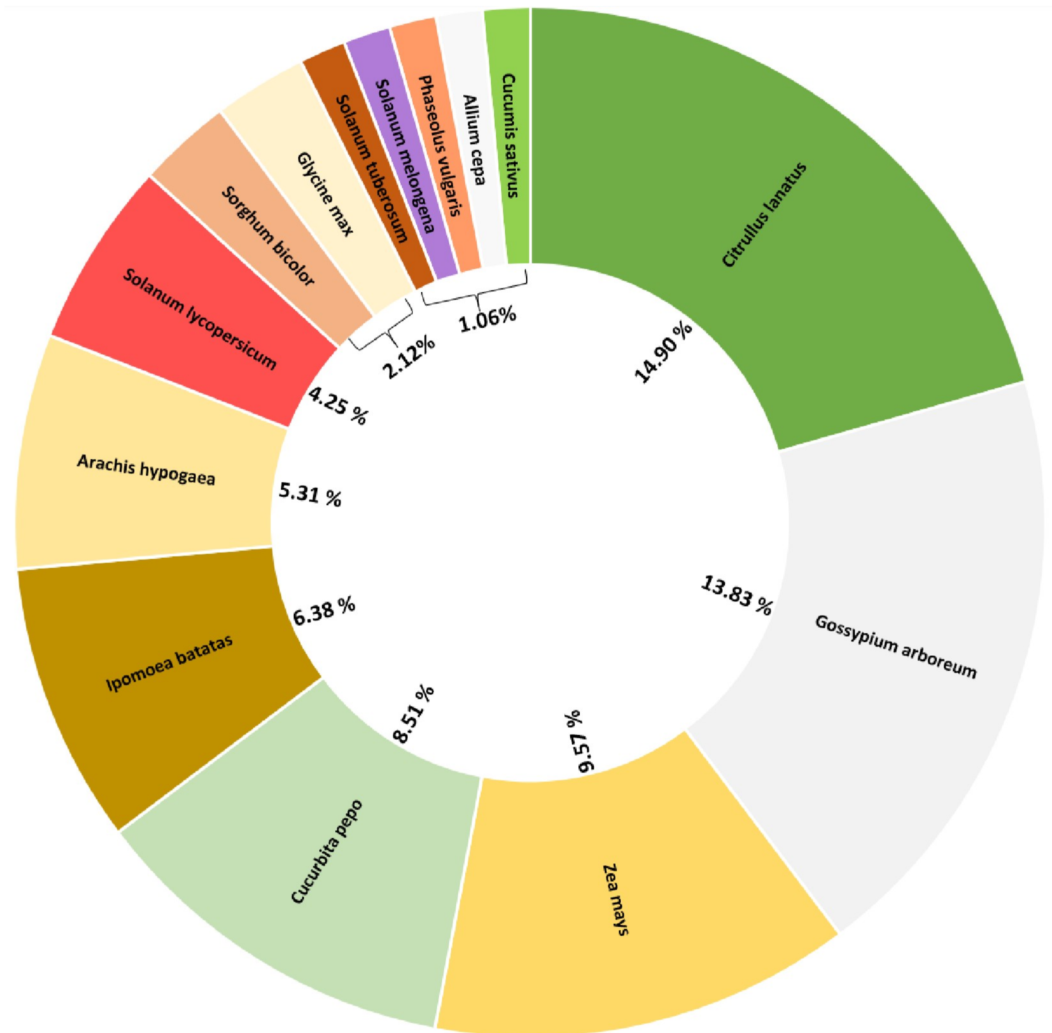

Fig 4. The frequency of feeding on 14 different crop plant species by *Melanoplus ponderosus* in South Georgia agricultural fields. The percentages of feeding on each plant species were calculated by dividing the total number of positives for each plant by the total number of tested individuals (94 individuals), multiplied by 100. The Microsoft PowerPoint program was used to create this figure.

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economically important crops in agroecosystems, which could be used to screen various polyphagous pests (e.g., birds, mammals and insects) for evidence of feeding on these important crops in agroecosystems.

To our knowledge, no study has used full genome sequences to design crop plant species-specific primers for multiplex settings. While some earlier studies used chloroplast genome sequences for phylogenetic analysis and species identification [24, 42–44], we found that the chloroplast region is too conserved for designing plant-species specific primers for most of our target plant species. Therefore, for the first time, we have explored various regions of the plant genome and designed specific crop plant primers from VE-1 genes for *S. lycopersicum*, pathogenesis related protein-coding gene 2 (PR-2) in squash, PR-5 in *A. cepa*, and PR-4 region in all other plant species. Overall, our results suggest that designing primers based on sequences from whole-genome sequences, instead of chloroplast sequences, for plant species-specific primer design is an effective alternative approach that can significantly increase the possibility

for plant-specific primer design for several plant species in agroecosystems. Our results warrant that future efforts use the full-genome approach to significantly boost the available specific primer pairs for additional plant molecular identification and dietary research in agroecosystems.

Over the years, many scientists attempt to use different approaches from restriction fragment length polymorphism (RFLP) to metabarcoding and sequencing for the identification of plant samples [31, 32, 68, 69]. However, there are some disadvantages to using these techniques. Most of the currently developed methods for the identification of plant species involve DNA sequencing [70, 71], which entails high costs. RFLP is another powerful approach to differentiate several plant species within a sample using restriction enzymes [31, 32]. The presence of a high number of species in samples can diminish RFLP usability due to the presence of similar base pairs amplicon sizes within the gut content, which result in overlapping bands and detections [29]. So, one of the advantages of our method is the efficiency of the technique in detecting and differentiating more than nine plant species simultaneously in one PCR reaction. However, one of the constraints of our technique is that the identification of different plant species would be based on the amplicon sizes. So, when designing specific primers, it is crucial to ensure that all amplicon sizes are varied with at least 10 bp in length. Also, care is advised when checking the primers against all sets of different non-target plants whose DNA might be present in the sample [72]. Overall, our developed primer mixes add a new path to the number of approaches that can be used to screen polyphagous pest diets in agroecosystems, which is specific, sensitive, rapid and cost-effective.

In this study, we designed primer mixes that successfully detected 14 plant species from the gut content of *M*. *ponderosus*, indicating a wide range of host crops this polyphagous pest consumes in the Georgia agricultural fields. The highest incidence of feeding observed was for watermelon, cotton and corn (Fig 4). Consistent with results from [47], our results confirmed that grasshoppers, such as *M*. *ponderosus*, feed on a wide range of crop plants in agroecosystems. Recent studies of grasshopper diets focus primarily on grasses and non-crop host plants [73]. Isley [47] study listed corn, cotton, sunflower, wheat, tobacco and sugarcane as potential host plants of grasshoppers in laboratory choice tests. Our results added several new host crop species such as watermelon, bean, eggplant, peanut to the diet of grasshoppers in agricultural fields. However, it needs to be pointed out that crop mixtures are frequently changing in the southeast and sub-tropical agroecosystems, and later in the season a polyphagous pest diet could certainly change depending on the type of crops available in agroecosystems. Our results further indicated that the designed primer mix can successfully be used to evaluate the diet of chewing insect pests within or between the cropping system. Hence, future studies should be conducted to test the efficacy of these primer sets for dietary detection of sucking pests. An earlier study by Wang et al. [24] reported the successful use of plant-specific primers for the detection of cotton and mungbean in the gut of *A*. *lucorum* (Heteroptera: Miridae), a sucking pest. This suggests that singleplex and multiplex PCR could be potentially used to screen sucking pest diets in agroecosystems, which warrants future studies.

Estimating the detectability half-life for prey/plant DNA in an insect gut enables standardization of the frequencies of molecular detections, and provides a window of detection for recent feeding [74]. While many studies estimated the detectability half-life for insect prey DNA in the gut of predators (reviewed by Greenstone et al., [74]), such estimation has seldom been assessed for plant DNA decay in herbivore guts [33, 34, 37]. We found that plant DNA remained detectible in grasshopper guts with a 50% probability for up to 40h post feeding, providing further support for the sensitivity of the designed primers for pest diet breadth evaluations. Pumarino et al. [33] found significant differences in the detectability half-life for tomato DNA in the gut of three insect predator and pest species, where it was shortest for the

predatory bug, *Macrolophus pygmaeus* with 5.8h, followed by herbivory moths *Helicoverpa armigera* and *Tuta absoluta* with 27.7h and 28.7h, respectively. Further, using general plant primers, Staudacher et al. [36] found significant differences in the detectability half-life for six plant species in the gut of wireworm (Agriotes), suggesting that various plant DNA decay at a different pace in the gut of the same pest species. Altogether, these two studies suggest that the half-life should be estimated for each insect predator and pest species, and should further be estimated for a different type of diet DNA (e.g., plant or insect). Therefore, future studies using the multiplex PCR primer mixes should consider estimating detectability half-life for each pest and plant species under study, to ensure a standardized frequency of detection that would result in a justifiable conclusion regarding diet share of each plant species in polyphagous pest gut content.

The understanding of polyphagous pest diets and their natural enemies in agroecosystems is of major importance for designing habitat management strategies to minimize crop damage both directly through reduction of pest preferred host plants by rotation or indirectly by the provision of carbohydrate resources for natural enemies that boost their potential and result in higher pest control services [16, 75]. Insect pest and predator dietary information can further be used to develop push-pull strategies based on pest and predator dietary preferences to further reduce damage to the cash crop by diverting pests to the trap crop, and also attract more natural enemies into the cash crop system, as shown in previous studies [76, 77]. Several landscape-scale studies have been conducted to determine the pattern of insect movement between habitats in an agricultural landscape, using bi-directional malaise traps [78], molecular mark-recapture [25] and isotope techniques [27]. These authors investigated the movement of polyphagous insect pests and generalist predators to understand what habitats could provide them with alternative resources, and act as a reservoir from which pests and natural enemies spillover into agricultural fields. However, insect movement between habitats should not be the primary means of inferring their diet in agroecosystems. Detection does not reveal the true intention of insect pest or natural enemy for visiting a habitat. Using MGCA with specific plant and insect primers significantly boosts ecologists' understanding of complex plant-pest-natural enemy interactions in agroecosystems. While the explanation for an insect pest visiting a crop field might be simple, for natural enemies it can be more complicated, as they are often omnivores and feed on both insect prey and plant carbohydrates [33]. Therefore, using MGCA can reveal the true purpose for predator movement to crop fields, by revealing their plant and insect diet in each habitat. Overall, the use of MGCA through specific plant primers designed in this study and Wallinger et al. [29], could have broad implications for insect pest management in agroecosystems and warrant future studies to fully explore and harness the knowledge acquired by employing MGCA to understand plant feeding and host use of common pests.

5 Conclusions

In this study, we report two optimized multiplex primer mixes for identifying recent feeding on plants, which could significantly ease the dietary evaluation of various polyphagous pests in agricultural environments. The designed multiplex PCR method provides rapid polyphagous pest dietary evaluation, with relatively low costs and time investment. Overall, this study provides a powerful tool to screen the diet of pests for 14 important crop plant species and exemplifies the broad dietary information a multiplex PCR approach can offer. These primer mixes also have implications in agroecological studies. Herbivore screening for recent feeding on plants can provide a cost-effective method for determining dietary breadth, tracking movement patterns between hosts in agricultural landscapes, and developing comprehensive host-

herbivore networks to aid in designing habitat management practices that minimize benefits to pests.

Supporting information

S1 File.
(ZIP)

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Methodology: Arash Kheirodin, Mohammad Sayari, Jason M. Schmidt.

Project administration: Arash Kheirodin.

Validation: Arash Kheirodin.

Visualization: Arash Kheirodin.

Writing – original draft: Arash Kheirodin.

Writing – review & editing: Arash Kheirodin, Mohammad Sayari, Jason M. Schmidt.

References

1. Latham ADM, Latham MC, Norbury GL, Forsyth DM, Warburton B. A review of the damage caused by invasive wild mammalian herbivores to primary production in New Zealand. New Zealand Journal of Zoology. 2020; 47(1):20–52.
2. Manosathiyadevan M, Bhuvaneshwari V, Latha R. Impact of Insects and Pests in loss of Crop Production: A Review. In: Dhanarajan A, editor. Sustainable Agriculture towards Food Security. Singapore: Springer Singapore; 2017. p. 57–67.
3. King RA, Read DS, Traugott M, Symondson WOC. Molecular analysis of predation: a review of best practice for DNA-based approaches. Mol Ecol. 2008; 17(4):947–63. https://doi.org/10.1111/j.1365-294X.2007.03613.x PMID: 18208490
4. Symondson WO. The molecular revolution: using polymerase chain reaction based methods to explore the role of predators in terrestrial food webs, In Gurr G. M., Wratten S., Snyder W. E.and Read M. Y. (eds.), Biodiersity and Insect Pests: key issues for sustainable management. Wiley-Blackwell, West Sussex, UK. 2012(Insect Pests: key issues for sustainable management):166–84.
5. Schmidt JM, Acebes-Doria A, Blaauw B, Kheirodin A, Pandey S, Lennon K, et al. Identifying molecular-based trophic interactions as a resource for advanced integrated pest management. Insects. 2021; 12 (4):358. https://doi.org/10.3390/insects12040358 PMID: 33923556
6. Grab H, Danforth B, Poveda K, Loeb G. Landscape simplification reduces classical biological control and crop yield. Ecol Appl. 2018; 28(2):348–55. https://doi.org/10.1002/eap.1651 PMID: 29345735

7. Gariepy TD, Messing RH. Development and use of molecular diagnostic tools to determine trophic links and interspecific interactions in aphid–parasitoid communities in Hawaii. Biol Control. 2012; 60(1):26–38.
8. Gariepy TD, Kuhlmann U, Haye T, Gillott C, Erlandson M. A single-step multiplex PCR assay for the detection of European *Peristenus* spp., parasitoids of *Lygus* spp. Biocontrol Science Technology. 2005; 15(5):481–95.
9. Traugott M, Symondson WOC. Molecular analysis of predation on parasitized hosts. Bulletin of Entomological Research. 2008; 98(3):223–31. https://doi.org/10.1017/S0007485308005968 PMID: 18439340
10. Traugott M, Bell J, Raso L, Sint D, Symondson WOC. Generalist predators disrupt parasitoid aphid control by direct and coincidental intraguild predation. Bulletin of Entomological Research. 2012; 102 (2):239–47. https://doi.org/10.1017/S0007485311000551 PMID: 22008233
11. Avanesyan A, Sutton H, Lamp WO. Choosing an effective PCR-based approach for diet analysis of insect herbivores: a systematic review. J Econ Entomol. 2021; 114(3):1035–46. https://doi.org/10.1093/jee/toab057 PMID: 33822094
12. Kheirodin A, Cárcamo HA, Costamagna AC. Contrasting effects of host crops and crop diversity on the abundance and parasitism of a specialist herbivore in agricultural landscapes. Landsc Ecol. 2020; 35 (5):1073–87.
13. Redlich S, Martin EA, Steffan-Dewenter I. Landscape-level crop diversity benefits biological pest control. J Appl Ecol. 2018; 55(5):2419–28.
14. Root RB. Organization of a plant-arthropod associations in simple and diverse habitats- fauna of Collards (Brassica-Oleracea). Ecol Monograph. 1973; 43(1):95–120.
15. Sivakoff FS, Rosenheim JA, Hagler JR. Relative dispersal ability of a key agricultural pest and its predators in an annual agroecosystem. Biol Control. 2012; 63(3):296–303.
16. Gurr GM, Wratten SD, Landis DA, You M. Habitat management to suppress pest populations: progress and prospects. Annu Rev Entomol. 2017; 62(1):91–109. https://doi.org/10.1146/annurev-ento-031616-035050 PMID: 27813664
17. Landis DA, Wratten SD, Gurr GM. Habitat management to conserve natural enemies of arthropod pests in agriculture. Annu Rev Entomol. 2000; 45:175–201. https://doi.org/10.1146/annurev.ento.45.1.175 PMID: 10761575
18. USDA. National Agricultural Statistics Service Cropland Data Layer. Published crop-specific data layer [Online]. Available at http://nassgeodata.gmu.edu/CropScape/ (accessed {12.03.2020}). USDA-NASS, Washington, DC 2019 [
19. Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P. Who is eating what: diet assessment using next generation sequencing. Mol Ecol. 2012; 21(8):1931–50. https://doi.org/10.1111/j.1365-294X.2011.05403.x PMID: 22171763
20. García-Robledo C, Erickson DL, Staines CL, Erwin TL, Kress WJ. Tropical plant–herbivore networks: Reconstructing species interactions using DNA barcodes. PLoS One. 2013; 8(1):e52967. https://doi.org/10.1371/journal.pone.0052967 PMID: 23308128
21. Samaranayake K, Costamagna AC. Adjacent habitat type affects the movement of predators suppressing soybean aphids. PLoS One. 2019; 14(6):20. https://doi.org/10.1371/journal.pone.0218522 PMID: 31211798
22. Batuecas I, Agustí N, Castañé C, Alomar O. Molecular tracking of insect dispersal to verify arthropod predator movement from an alfalfa field to a peach orchard. Biol Control. 2020:104506.
23. Hayashi M, Abe J, Owashi Y, Miura K. Estimation of movement from insectary plants to crop plants in Orius bugs (Heteroptera: Anthocoridae) by molecular gut content analysis. Appl Entomol Zoolog. 2020; 55(3):361–5.
24. Wang Q, Bao WF, Yang F, Xu B, Yang YZ. The specific host plant DNA detection suggests a potential migration of Apolygus lucorum from cotton to mungbean fields. PLoS One. 2017; 12(6):e0177789. https://doi.org/10.1371/journal.pone.0177789 PMID: 28586352
25. Agustí N, Castañé C, Fraile I, Alomar O. Development of a PCR-based method to monitor arthropod dispersal in agroecosystems: *Macrolophus pygmaeus* (Hemiptera: Miridae) from banker plants to tomato crops. Insect Science. 2020; 27(5):1125–34. https://doi.org/10.1111/1744-7917.12717 PMID: 31420911
26. Madeira F, di Lascio A, Carlino P, Costantini ML, Rossi L, Pons X. Stable carbon and nitrogen isotope signatures to determine predator dispersal between alfalfa and maize. Biol Control. 2014; 77:66–75.
27. Madeira F, di Lascio A, Costantini ML, Rossi L, Rösch V, Pons X. Intercrop movement of heteropteran predators between alfalfa and maize examined by stable isotope analysis. J Pest Sci. 2019; 92(2):757–67.

28. Di Lascio A, Madeira F, Costantini ML, Rossi L, Pons X. Movement of three aphidophagous ladybird species between alfalfa and maize revealed by carbon and nitrogen stable isotope analysis. Biocontrol. 2016; 61(1):35–46.

29. Wallinger C, Juen A, Staudacher K, Schallhart N, Mitterrutzner E, Steiner E-M, et al. Rapid plant identification using species- and group-specific primers targeting chloroplast DNA. PLoS One. 2012; 7(1): e29473. https://doi.org/10.1371/journal.pone.0029473 PMID: 22253728

30. Schmidt JM, Acebes A, Blaauw B, Kheirodin A, Pandey S, Lennon K, et al. Identifying molecular trophic interactions as a resource to advance integrated pest management. Insects. 2021;In press.

31. Bobowski BR, Hole D, Wolf PG, Bryant L. Identification of roots of woody species using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Mol Ecol. 1999; 8 (3):485–91. https://doi.org/10.1046/j.1365-294x.1999.00603.x PMID: 10199009

32. Ridgway KP, Duck JM, Young JPW. Identification of roots from grass swards using PCR-RFLP and FFLP of the plastid trn L (UAA) intron. BMC Ecology. 2003; 3(1):8.

33. Pumarino L, Alomar O, Agusti N. Development of specific ITS markers for plant DNA identification within herbivorous insects. Bulletin of Entomological Research. 2011; 101(3):271. https://doi.org/10.1017/S0007485310000465 PMID: 21092379

34. Staudacher K, Schallhart N, Thalinger B, Wallinger C, Juen A, Traugott M. Plant diversity affects behavior of generalist root herbivores, reduces crop damage, and enhances crop yield. Ecol Appl. 2013; 23 (5):1135–45. https://doi.org/10.1890/13-0018.1 PMID: 23967581

35. Wallinger C, Staudacher K, Schallhart N, Mitterrutzner E, Steiner E-M, Juen A, et al. How generalist herbivores exploit belowground plant diversity in temperate grasslands. Mol Ecol. 2014; 23(15):3826–37. https://doi.org/10.1111/mec.12579 PMID: 24188592

36. Wallinger C, Staudacher K, Schallhart N, Peter E, Dresch P, Juen A, et al. The effect of plant identity and the level of plant decay on molecular gut content analysis in a herbivorous soil insect. Molecular Ecology Resources. 2013; 13(1):75–83. https://doi.org/10.1111/1755-0998.12032 PMID: 23167731

37. Staudacher K, Wallinger C, Schallhart N, Traugott M. Detecting ingested plant DNA in soil-living insect larvae. Soil Biology and Biochemistry. 2011; 43(2):346–50. https://doi.org/10.1016/j.soilbio.2010.10.022 PMID: 21317975

38. De la Cadena G, Papadopoulou A, Maes J-M, Gómez-Zurita J. Evaluation of bias on the assessment of diet breadth of herbivorous insects using molecular methods. Insect Science. 2017; 24(2):194–209. https://doi.org/10.1111/1744-7917.12303 PMID: 26663763

39. Navarro SP, Jurado-Rivera JA, Gomez-Zurita J, Lyal CHC, Vogler AP. DNA profiling of host–herbivore interactions in tropical forests. Ecol Entomol. 2010; 35(s1):18–32.

40. Wang Q, Bao W, Zhang Q, Fu X, Yang Y, Lu Y. Host plant use of a polyphagous mirid, *Apolygus lucorum*: Molecular evidence from migratory individuals. Ecol Evol. 2019; 9(19):11518–28. https://doi.org/10.1002/ece3.5660 PMID: 31641490

41. Zhu C, Gravel D, He F. Seeing is believing? Comparing plant–herbivore networks constructed by field co-occurrence and DNA barcoding methods for gaining insights into network structures. Ecol Evol. 2019; 9(4):1764–76. https://doi.org/10.1002/ece3.4860 PMID: 30847071

42. Turktas M, Aslay M, Kaya E, Ertvçrul F. Molecular characterization of phylogeneticrelationships in Fritillaria species inferred from chloroplast trnL-trnF sequences. Turkish Journal of Biology. 2012; 36:552–60.

43. Scarcelli N, Barnaud A, Eiserhardt W, Treier UA, Seveno M, d'Anfray A, et al. A set of 100 chloroplast DNA primer pairs to study population genetics and phylogeny in monocotyledons. PLoS One. 2011; 6 (5):e19954. https://doi.org/10.1371/journal.pone.0019954 PMID: 21637837

44. Ronsted N, Law S, Thornton H, Fay MF, Chase MW. Molecular phylogenetic evidence for the monophyly of Fritillaria and Lilium (Liliaceae; Liliales) and the infrageneric classification of Fritillaria. Molecular phylogenetics and evolution. 2005; 35(3):509–27. https://doi.org/10.1016/j.ympev.2004.12.023 PMID: 15878122

45. McNickle GG, Cahill J, Deyholos M. A PCR-based method for the identification of the roots of 10 co-occurring grassland species in mesocosm experiments. J Botany. 2008; 86(5):485–90.

46. Schell SP. Large-Scale Grasshopper Infestations on North American Rangeland and Crops In: Shroder J.F., Sivanpillai R.(eds.), Biological and Environmental Hazards, Risks, and Disasters. Saint Louis, United States: Elsevier; 2015.

47. Isely FB. The Relations of Texas Acrididae to Plants and Soils. Ecol Monograph. 1938; 8(4):551–604.

48. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002; 30(14):3059–66. https://doi.org/10.1093/nar/gkf436 PMID: 12136088

49. Hall TA, editor BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT1999: Nucleic acids symposium series, 41: 95–98.
50. Sato S, Tabata S, Hirakawa H, et al. The tomato genome sequence provides insights into fleshy fruit evolution. Nature. 2012; 485(7400):635–41. https://doi.org/10.1038/nature11119 PMID: 22660326
51. Li F, Fan G, Lu C, et al. Genome sequence of cultivated Upland cotton (Gossypium hirsutum TM-1) provides insights into genome evolution. Nature Biotechnology. 2015; 33(5):524–30. https://doi.org/10.1038/nbt.3208 PMID: 25893780
52. Schmutz J, McClean PE, Mamidi S, Wu GA, Cannon SB, Grimwood J, et al. A reference genome for common bean and genome-wide analysis of dual domestications. Nature Genetics. 2014; 46(7):707–13. https://doi.org/10.1038/ng.3008 PMID: 24908249
53. Wu S, Wang X, Reddy U, et al. Genome of 'Charleston Gray', the principal American watermelon cultivar, and genetic characterization of 1,365 accessions in the U.S. National Plant Germplasm System watermelon collection. 2019; 17(12):2246–58.
54. Huang S, Li R, Zhang Z, et al. The genome of the cucumber, *Cucumis sativus* L. Nature Genetics. 2009; 41(12):1275–81. https://doi.org/10.1038/ng.475 PMID: 19881527
55. Montero-Pau J, Blanca J, Bombarely A, Ziarsolo P, Esteras C, Martí-Gómez C, et al. De novo assembly of the zucchini genome reveals a whole-genome duplication associated with the origin of the *Cucurbita* genus. Plant biotechnology journal. 2018; 16(6):1161–71. https://doi.org/10.1111/pbi.12860 PMID: 29112324
56. Hirakawa H, Shirasawa K, Miyatake K, Nunome T, Negoro S, Ohyama A, et al. Draft genome sequence of eggplant (*Solanum melongena* L.): the representative solanum species indigenous to the old world. DNA research: an international journal for rapid publication of reports on genes and genomes. 2014; 21 (6):649–60. https://doi.org/10.1093/dnares/dsu027 PMID: 25233906
57. Schmutz J, Cannon SB, Schlueter J, et al. Genome sequence of the palaeopolyploid soybean. Nature. 2010; 463(7278):178–83. https://doi.org/10.1038/nature08670 PMID: 20075913
58. Liu J, Seetharam AS, Chougule K, Ou S, Swentowsky KW, Gent JI, et al. Gapless assembly of maize chromosomes using long-read technologies. Genome Biology. 2020; 21(1):121. https://doi.org/10.1186/s13059-020-02029-9 PMID: 32434565
59. Xu X, Pan S, Cheng S, et al. Genome sequence and analysis of the tuber crop potato. Nature. 2011; 475(7355):189–95. https://doi.org/10.1038/nature10158 PMID: 21743474
60. Paterson AH, Bowers JE, Bruggmann R, et al. The *Sorghum bicolor* genome and the diversification of grasses. Nature. 2009; 457(7229):551–6. https://doi.org/10.1038/nature07723 PMID: 19189423
61. Bertioli DJ, Jenkins J, Clevenger J, et al. The genome sequence of segmental allotetraploid peanut *Arachis hypogaea*. Nature Genetics. 2019; 51(5):877–84. https://doi.org/10.1038/s41588-019-0405-z PMID: 31043755
62. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012; 13(1):134. https://doi.org/10.1186/1471-2105-13-134 PMID: 22708584
63. R Development Core Team R. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. URL http://www.R-project.org/. 2020.
64. Hebert PDN, Ratnasingham S, deWaard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc Biol Sci. 2003; 270 Suppl 1(Suppl 1):S96–S9. https://doi.org/10.1098/rsbl.2003.0025 PMID: 12952648
65. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102 (23):8369–74. https://doi.org/10.1073/pnas.0503123102 PMID: 15928076
66. Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haider N, et al. Land plants and DNA barcodes: short-term and long-term goals. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2005; 360(1462):1889–95. https://doi.org/10.1098/rstb.2005.1720 PMID: 16214746
67. Fazekas AJ, Kuzmina ML, Newmaster SG, Hollingsworth PM. DNA barcoding methods for land plants. DNA barcodes: Springer; 2012. p. 223–52. https://doi.org/10.1007/978-1-61779-591-6_11 PMID: 22684959
68. Brunner I, Brodbeck S, Büchler U, Sperisen C. Molecular identification of fine roots of trees from the Alps: reliable and fast DNA extraction and PCR-RFLP analyses of plastid DNA. Mol Ecol. 2001; 10 (8):2079–87. https://doi.org/10.1046/j.1365-294x.2001.01325.x PMID: 11555251
69. Donini P, Koebner RM, Elias ML, Bougourd SM. AFLP fingerprinting reveals pattern differences between template DNA extracted from different plant organs. Genome. 1997; 40(4):521–6. https://doi.org/10.1139/g97-068 PMID: 18464843

70. Jackson RB, Moore LA, Hoffmann WA, Pockman WT, Linder CR. Ecosystem rooting depth determined with caves and DNA. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96(20):11387–92. https://doi.org/10.1073/pnas.96.20.11387 PMID: 10500186
71. Linder CR, Moore LA, Jackson RB. A universal molecular method for identifying underground plant parts to species. Mol Ecol. 2000; 9(10):1549–59. https://doi.org/10.1046/j.1365-294x.2000.01034.x PMID: 11050550
72. Soininen EM, Valentini A, Coissac E, Miquel C, Gielly L, Brochmann C, et al. Analysing diet of small herbivores: the efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. Frontiers in Zoology. 2009; 6(1):16. https://doi.org/10.1186/1742-9994-6-16 PMID: 19695081
73. Avanesyan A. Should I eat or should I go? Acridid grasshoppers and their novel host plants: potential for biotic resistance. Plants. 2018; 7(4):83. https://doi.org/10.3390/plants7040083 PMID: 30301240
74. Greenstone MH, Payton ME, Weber DC, Simmons AM. The detectability half-life in arthropod predator-prey research: what it is, why we need it, how to measure it, and how to use it. Mol Ecol. 2014; 23 (15):3799–813. https://doi.org/10.1111/mec.12552 PMID: 24303920
75. Landis DA. Designing agricultural landscapes for biodiversity-based ecosystem services. Basic Appl Ecol. 2017; 18:1–12.
76. Khan ZR, Pickett JA. The 'push-pull'strategy for stemborer management: a case study in exploiting biodiversity and chemical ecology. In Ecological Engineering for Pest Management: Advances in Habitat Manipulation for Arthropods, ed Gurr GM, Wratten SD, Altieri MA,. Wallington, Oxon, UK: CABI; 2004. p. 155–64.
77. Cook SM, Khan ZR, Pickett JA. The use of push-pull strategies in integrated pest management. Annu Rev Entomol. 2007; 52(1):375–400. https://doi.org/10.1146/annurev.ento.52.110405.091407 PMID: 16968206
78. Samaranayake KGLI, Costamagna AC. Levels of predator movement between crop and neighboring habitats explain pest suppression in soybean across a gradient of agricultural landscape complexity. Agric Ecosyst Environ. 2018; 259:135–46.