Available online at www.sciencedirect.com
ScienceDirect
Organisms, Diversity & Evolution 7 (2008) 294–303
ORGANISMS DIVERSITY & EVOLUTION
www.elsevier.de/ode

A PCR method for detection of plant meals from the guts of insects

Carney D. Matheson[a,*], G.C. Muller[b], A. Junnila[c], K. Vernon[d], A. Hausmann[e], M.A. Miller[e], C. Greenblatt[b], Y. Schlein[b]

[a]*Paleo-DNA Laboratory, Department of Anthropology, Lakehead University, Thunder Bay, Ontario, Canada P7B 5Z5*
[b]*Department of Parasitology, The Kuvin Center for the Study of Infectious and Tropical Diseases, The Hebrew University-Hadassah Medical School, 91120 Jerusalem, Israel*
[c]*Institute of Parasitology, Macdonald College, McGill University, 21,111 Lakeshore Rd., Ste-Anne-de-Bellevue, Montreal, Quebec, Canada H9X 3V9*
[d]*Department of Zoology, University of Queensland, Brisbane 4072, Australia*
[e]*Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München, Germany*

Received 22 June 2005; accepted 25 September 2006

Abstract

The feeding behaviour of insects is a difficult ecological interaction to study. To date, entomologists have used biochemical and molecular techniques to identify the meals of predatory insects. We present here a molecular approach to identifying the DNA of plant species in the insect gut using the ribulose bisphosphate carboxylase gene large subunit (*rbc*L). A reference collection of 23 plant species from the southern Jordan Valley, Israel, was genetically characterized and employed. Insects belonging to eight different families were collected in the field along with the plants upon which they were found. After collection and prior to analysis, these insects were isolated on the plants they were found upon in the laboratory. This was to ensure that the insects had only one plant meal in their gut, as multiple plant meals would require additional techniques like cloning. A blind study was performed, genetically confirming plant DNA to species level from the processed gut contents of the insects. All reference plant species could be differentiated using a 157 bp long fragment of the *rbc*L gene. Plant DNA was identifiable, and the meal of the respective insect was accurately determined in each case. Analyses using experimentally fed crickets, *Gryllodes hebraeus*, determined that plant DNA was still detectable by PCR up to 12 h post-ingestion. This research proposes the application of molecular techniques for the identification of herbivorous insect feeding behaviour to increase understanding of plant–insect interactions.

Keywords: DNA identification; Insect meal; Israeli flora; Israeli insects; Ribulose bisphosphate carboxylase large subunit (*rbc*L); Insect ecology

Introduction

More than half of all known insects feed upon plants and still more are associated with plants in one way or another (May 1988). Plants play an integral role in insect life cycles and are used by insects as sites for

*Corresponding author. Fax: +1 807 3438619.
E-mail address: cmatheso@lakeheadu.ca (C.D. Matheson).

1439-6092/$ - see front matter © 2007 Gesellschaft für Biologische Systematik. Published by Elsevier GmbH. All rights reserved.
doi:10.1016/j.ode.2006.09.002

feeding, mating, egg-laying, and/or refuge. Some insects use one plant species for all activities, but it is more common for insects to use different plants for different activities, producing complex patterns of activity that confound the accurate identification of insect–plant interactions (Sandholm and Price 1962; Prokopy 1976; Prokopy et al. 1984; May 1988; Mogi and Miyagi 1989). Explorations of insect–plant interactions are multi-disciplinary in nature, incorporating numerous techniques and encompassing aspects of many fields of study, including botany, entomology, ecology, behavioural studies, physiology, and biochemistry (Ehrlich and Raven 1964; Eastop 1979; Cates 1980; Hendrix 1980; Belsky 1986).

The feeding behaviour of insects has been a difficult ecological interaction to study. Previous studies have not provided enough accurate details of food sources and feeding habits of herbivorous insects that would allow a full understanding of their complex ecological interactions, for the reasons stated below. First, field studies based on observations of insects on plants cannot specifically identify the interaction which is occurring between the insect and the plant. Some of these studies of insects on plants have determined that the insects are indeed feeding (Sandholm and Price 1962; Basset 1992), but without further accurate analysis it is difficult to determine whether the insect is mono- or polyphagous. Second, biochemical and histological studies of plant content within the gut of insects (Moore et al. 1987; Schlein and Muller 1995; Johnson and Nicolson 2001), as with field observations, cannot accurately and specifically identify the plant from which the insect fed, nor when the insect fed. Other studies (Abdel-Malek and Baldwin 1961; Akey et al. 1991) have involved analysis of plant material that has been force fed to insects. Obviously, this may not indicate the preferred plant that the insect may choose to feed upon.

The direct analysis of plant material within the insect gut is an accurate approach to identifying feeding behaviours. There are many techniques available with which to study the gut contents of insects. The simplest methods involve microscopic analysis of the insect gut post-feeding. In some insect species, fed on flowers or nectar, pollen was found in the gut (Johnson and Nicolson 2001), signifying that the insect has directly fed upon plant material. Pollen morphology is species-specific, and can be used to identify the plants that the insect has consumed. In most insect species, however, the identification of plant remains is more complex, requiring dissections and stains, dyes or biochemical techniques (Schlein and Jacobson 1994; Schlein and Muller 1995).

The development of the polymerase chain reaction (PCR) and DNA sequencing, which can be used on minuscule amounts of sample, have resulted in greater sensitivity and specificity which can be especially useful when studying the feeding habits of insects. In fact, these techniques have been applied to the identification of the gut contents of numerous predatory insect species (Chen et al. 2000; Symondson 2002; Augusti et al. 2003; Juen and Traugott 2005) or to identify the blood meal in some bloodsucking arthropods (Tobolewski et al. 1992; Gokool et al. 1993; Augusti et al. 1999b, 2003; Kreike and Kampfer 1999; Zaidi et al. 1999; Chen et al. 2000; Chow-Shaffer et al. 2000; Hoogendoorn and Heimpel 2001).

It is also important to note that, although it has been demonstrated that insect–prey DNA was identifiable in the guts of different predatory insects for up to 32 h (Augusti et al. 1999a, b 2000; Chen et al. 2000), the degradation of the plant DNA through digestion or other processes in certain insects may be much more rapid and may impede molecular analyses. This phenomenon should be assessed for each species of insect under study.

In this pilot study, we addressed the following questions by analyzing a small region (157 bp) of the plant chloroplast gene *rbc*L by PCR and DNA sequencing. (1) Could the plant meal in the gut of insects be reliably and accurately detected by PCR? (2) Could DNA sequencing of the PCR product identify the species of plant on which the insect fed? (3) Is there any difference in the sensitivity of detection from insects of various sizes? (4) Is there any difference in the sensitivity of detection due to the effects of time since feeding?

Material and methods

Study area

The plants and insects were collected in the southern Jordan Valley, Israel, at an altitude of approximately −350 m, in the spring of 2002. The region belongs to the Saharo-Arabian phyto-geographical zone of desert vegetation, which spreads from the Sahara Desert to Iran (Zohary 1973). The climate here consists of a very short rainy season in winter and an extremely dry and hot summer. This site hosts a rich insect fauna that can be found on a limited number of plant species.

Insect collection

Representatives of several insect groups were caught on plants (Table 1), and were transferred in groups of about 20 specimens into square plexiglass cages of 30×30 cm, with a piece of the plant they were collected on. Different species were transported to the lab separately, within 1 h at 4 °C. Insectary conditions were 26 ± 1 °C, 60% relative humidity, and a photoperiod of

Table 1. List of insect species and their respective plant food sources

Insects		Plants
Species (family)	Common name	Species (family)
Ocnogyna loewii (Arctiidae)	Caterpillar	*Emex spinosa* (Polygonaceae)
Isophya savignyi (Tettigonidae)	Grasshopper	*Malva nicaeensis* (Malvaceae)
Anacridium aegyptium (Acrididae)	Locust	*Onopordum cynarocephalum* (Asteraceae)
Larinus onopordi (Curculionidae)	Beetle	*Onopordum cynarocephalum* (Asteraceae)
Criptocephalus sinaica (Chrysomelidae)	Beetle	*Ochradenus baccatus* (Resedaceae)
Myzus persicae (Aphididae)	Aphid	*Pergularia tomentosa* (Asclepiadaceae)
Oxyrrhachis versicolor (Cicadidae)	Cicada	*Tamarix nilotica* (Tamaricaceae)
Gryllodes hebraeus (Gryllidae)	Cricket	*Vitis vinifera* (Vitaceae)

17:7 light:dark. The insects were maintained for 8 days and provided with daily foliage of the plant species associated with their capture. After 8 days, insects were frozen at −70 °C until further use. It should be noted here that isolating the insects post-capture with the plant they were collected on reduced the chances of detecting polyphagy since the gut contents would consist of only the one species of plant. Multiple plant meals in the gut would require a modified approach using additional techniques like cloning. It was also important that selected insects differed in size in order to assess the sensitivity of this molecular approach.

Crickets (*Gryllodes hebraeus*) were collected to verify the sensitivity of the tests and used as positive controls. They were starved to remove their original gut content and experimentally fed with a plant species not locally found in the collection to control for cross-contamination. Crickets were chosen as controls because they are relatively available and have a relatively large body mass which provides ample gut content for analysis.

Gut preparations of insects for genetic analysis

Previously collected insects were submerged in a solution of 0.5% hypochlorite with 0.01 µl/ml Triton X-100 detergent, agitated gently for 1 min with forceps, and then rinsed in double distilled water (ddH20) for 1 min. This was to remove any plant debris that may have been on the outside of the insect, which could otherwise contaminate the sample during gut dissection and preparation. They were then placed on a Petridish sterilized through the flame of a Bunsen burner, and dissected with No. 5 watchmaker forceps.

The gut was dissected and its contents were analyzed for plant residue, to ensure the insects had actually fed, utilizing three techniques: a modified anthrone test, calcofluor fluorescent staining, and PCR. The extraction of DNA from the insect gut samples was conducted using at least one whole insect gut, and at maximum 20 mg of gut content sampled from larger insects, including our control and experimentally fed *G. hebraeus*. Thorax muscles and parts of the mandibles and tarsi of a fed *G. hebraeus* were also dissected and used as negative controls. These negative controls were used to assess whether the preparation and washing of the insect had successfully removed plant debris that could contaminate the surface of the insects and the preparation procedure, and were also used to ensure that the plant-specific primers did not amplify the insect DNA.

For the fluorescent staining of cellulose particles, insect guts were dissected in a fresh solution of 0.05% calcofluor (Fluorescent brightener 28, White M2R, C.I. 40622 Sigma, St. Louis, MO), mounted on microscope slides, and covered with cover slips that had been sterilized through the flame of a Bunsen burner to eliminate fluorescing particles of paper and cloth (Schlein and Muller 1995). The preparations were examined under a phase contrast microscope at a wavelength of 360–440 nm, with a Wr 2B filter (Zetopan, Reichert, Vienna) to detect calcofluor-stained cellulose particles (Kasten 1980).

To detect sucrose as a quick initial test to determine the presence of plant meals, gut samples were placed in the wells of a flat-bottomed 24-well tissue culture microplate and crushed with a sterile glass rod before adding 300 µl of reaction solution to each well. The solution contained 0.15% anthrone (Sigma, St. Louis, MO) (wt/vol) in 71.7% sulphuric acid. Reactions were examined under a dissecting microscope after incubation at 25 °C for 60 min. In the presence of sucrose the reaction liquid changed its colour from yellow to blue.

The sensitivity and effect of digestion on the detection of the plant *rbc*L genetic material from the gut contents of *G. hebraeus* was evaluated. These experiments were tested on *G. hebraeus* insects fed on grapes (*Vitis vinifera*) ad libitum, sacrificed and frozen in groups of four, at each time interval with four controls, after 0, 2, 4, 6, 8, 10, and 12 h of starvation. Afterwards, they were extracted and amplified for the presence of *V. vinifera* DNA in their gut. As a negative control, four *G. hebraeus* that had been starved for 48 h after feeding were amplified for the *rbc*L gene and the amplification products were qualitatively assessed after gel electrophoresis (Figs. 1 and 2).

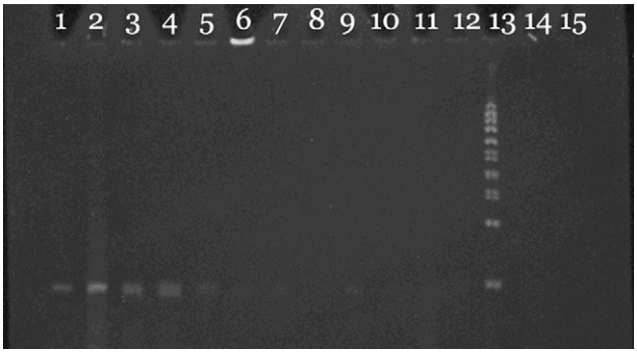

Fig. 1. A qualitative representation of PCR detection of the *rbc*L gene from insect gut contents at different periods of time post-ingestion. Lanes 1–8: crickets and the time they were allowed to digest; lanes 9–12: other insects after 4 h of digestion. Lane 1: 0h; 2: 2h; 3: 4h; 4: 6h; 5: 8h; 6: 10h; 7: 12h; 8: unfed negative control; 9: caterpillar; 10: grasshopper; 11: aphid; 12: cicada; 13: molecular marker; 14: extraction negative control; 15: PCR negative control.

Plant reference collection

Common plants to be used as reference sequences from the southern Jordan Valley, Israel, were collected and identified by traditional methods at the Israel National Herbarium, Givat Ram, Jerusalem, Israel (Table 2). The samples were dried prior to DNA extraction.

DNA extraction

The prepared gut samples were placed in a 1.5 ml tube containing 500 µl of guanidine thiocyanate solution (Boom et al. 1990), vortexed for 1 min and incubated at 56 °C for 5–8 h under gentle agitation. Samples were incubated at 94 °C for 10 min, centrifuged at 12,000 rpm for 3 min, and the supernatant transferred to another sterile 1.5 ml tube. One ml of guanidine thiocyanate solution and 10 µl of silica bead suspension were added to the sample supernatant. The tube was mixed for 20 s and placed on ice for 1 h with agitation every 15 min. The sample was centrifuged at 12,000 rpm for 30 s and the supernatant was carefully discarded. Next, 500 µl of washing buffer (2 mM Tris–HCl; pH 7.5), 10 mM EDTA (pH 8.0), and 10 mM NaCl in a 50% (v/v) water/ethanol solution (−20 °C) were added, mixed, and centrifuged for 30 s at 12,000 rpm.The supernatant was discarded. The wash buffer step was repeated until the pellet was clean. The silica pellet was then washed with 200 µl absolute ethanol as described above, and allowed to air dry. DNA was eluted with 100 µl of ddH_2O, mixed for 20 s and incubated at 56 °C for 1 h. The sample was centrifuged at 12,000 rpm for 3 min and the DNA extract was stored at 4 °C. Negative extraction controls and standard precautions used to minimise contamination were employed, including the use of sterile tubes, plugged tips, ultraviolet (UV) irradiation and 0.5% hypochlorite solutions.

DNA from reference plants was extracted and purified according to the guidelines in REDExtract-N-Amp Plant DNA extraction kit (Sigma). A leaf was first washed with ddH_2O, then sampled using a sterile hole-punch, and placed in a sterile 1.5 ml tube with 100 µl of extraction buffer. This tube was vortexed, incubated at 95 °C for 10 min, and then 100 µl of dilution solution was added. After briefly vortexing the sample, it was stored at 4 °C until its use in PCR amplification.

PCR amplification

PCR amplification was performed at the Hebrew University of Jerusalem, Israel and replicated at the Paleo-DNA Laboratory at Lakehead University in Canada. Each reaction (carried out in a 25 or 50 µl volume) consisted of 5–20 µl of resuspended DNA, 1X buffer (10 mM Tris–HCl pH 8.4 with AmpliTaq Gold (Invitrogen) or 20 mM Tris–HCl pH 8.4 with Platinum Taq (GIBCO BRL, MD)), 50 mM KCl, 2.25 mM MgCl2, 2.5 mM of each nucleoside triphosphate (dNTPs), 0.25 µM of each primer (Table 3), and 1.25 units of polymerase (AmpliTaq Gold or Platinum Taq). A PCR negative control was routinely used. Samples were amplified in a DNA thermal cycler (Biometra UNO-Thermoblock) or a Master Cycler (Eppendorf) thermal cycler using an initial denaturation of 94 °C for 3 min, followed by 45 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

PCR reactions of plant reference samples were carried out according to the REDExtract-N-Amp PCR guidelines (Sigma): 10 µl of the REDExtract-N-Amp PCR Ready Mix, 2.5 µl of each primer (0.125 µM), 1.0 µl of water, and 4.0 µl of REDExtract-N-Amp plant DNA extract were used. The PCR utilized primers for the *rbc*L

Fig. 2. Tree view representations of significant BLAST matches between eight unknown insect gut meal sequences against their plant reference sequence and GenBank public database sequences. Note: both the locust (*Anacridium aegyptium*) and beetle (*Larinus onopordi*) gut meals were identified as *Onopordum cynarocephalum* with identical trees; only one is shown. (A) Unknown from caterpillar plant meal identified as *Emex spinosa*. (B) Unknown from grasshopper plant meal identified as *Malva nicaeensis*. (C) Unknown from locust and beetle plant meals identified as *Onopordum cynarocephalum*. (D) Unknown from beetle plant meal identified as *Ochradenus baccatus*. (E) Unknown from aphid plant meal identified as *Pergularia tomentosa*. (F) Unknown from cicada plant meal identified as *Tamarix nilotica*. (G) Unknown from cricket plant meal identified as *Vitis vinifera*.

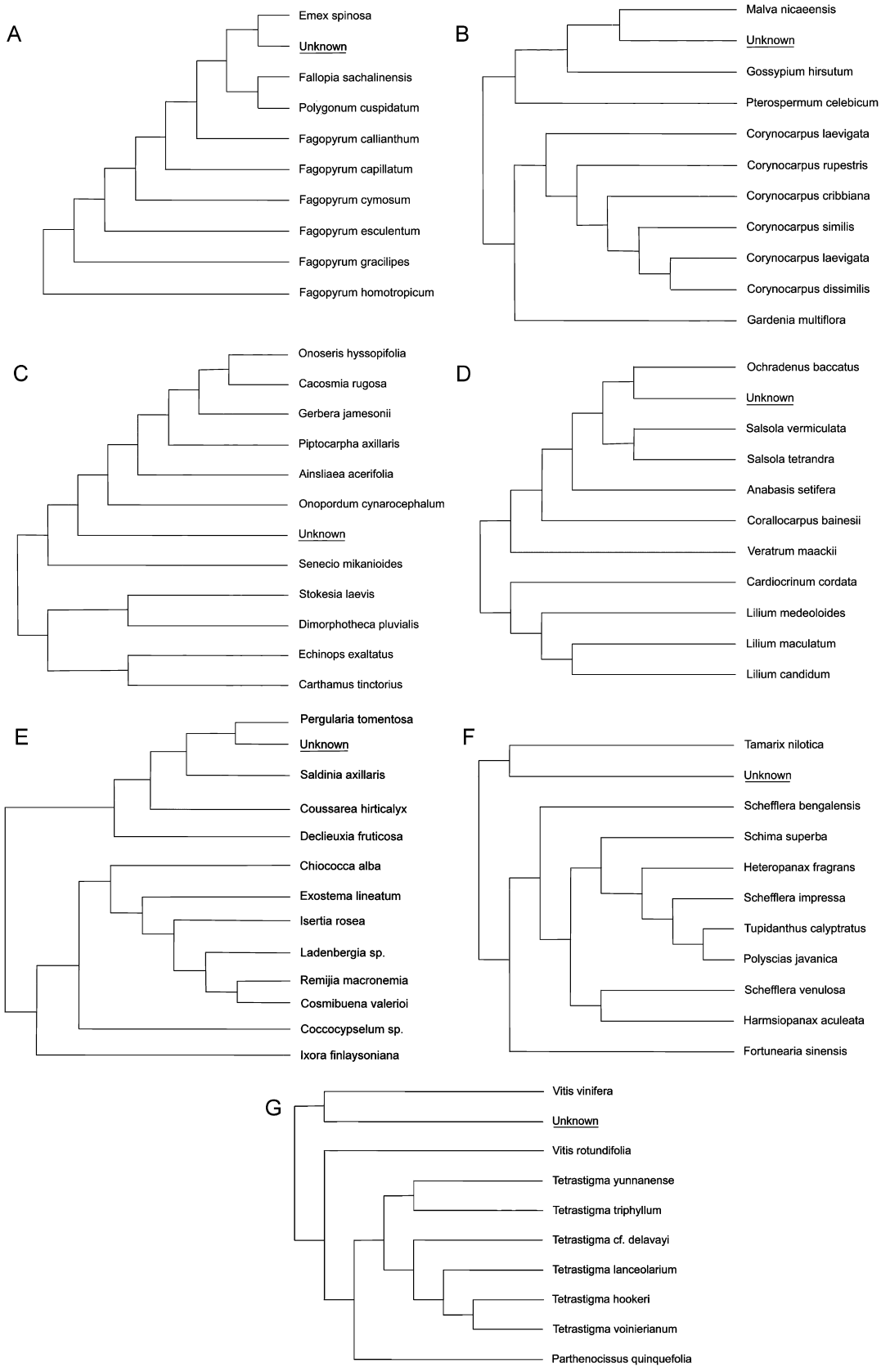
A
Emex spinosa
Unknown
Fallopia sachalinensis
Polygonum cuspidatum
Fagopyrum callianthum
Fagopyrum capillatum
Fagopyrum cymosum
Fagopyrum esculentum
Fagopyrum gracilipes
Fagopyrum homotropicum
B
Malva nicaeensis
Unknown
Gossypium hirsutum
Pterospermum celebicum
Corynocarpus laevigata
Corynocarpus rupestris
Corynocarpus cribbiana
Corynocarpus similis
Corynocarpus laevigata
Corynocarpus dissimilis
Gardenia multiflora
C
Onoseris hyssopifolia
Cacosmia rugosa
Gerbera jamesonii
Piptocarpha axillaris
Ainsliaea acerifolia
Onopordum cynarocephalum
Unknown
Senecio mikanioides
Stokesia laevis
Dimorphotheca pluvialis
Echinops exaltatus
Carthamus tinctorius
D
Ochradenus baccatus
Unknown
Salsola vermiculata
Salsola tetrandra
Anabasis setifera
Corallocarpus bainesii
Veratrum maackii
Cardiocrinum cordata
Lilium medeoloides
Lilium maculatum
Lilium candidum
E
Pergularia tomentosa
Unknown
Saldinia axillaris
Coussarea hirticalyx
Declieuxia fruticosa
Chiococca alba
Exostema lineatum
Isertia rosea
Ladenbergia sp.
Remijia macronemia
Cosmibuena valerioi
Coccocypselum sp.
Ixora finlaysoniana
F
Tamarix nilotica
Unknown
Schefflera bengalensis
Schima superba
Heteropanax fragrans
Schefflera impressa
Tupidanthus calyptratus
Polyscias javanica
Schefflera venulosa
Harmsiopanax aculeata
Fortunearia sinensis
G
Vitis vinifera
Unknown
Vitis rotundifolia
Tetrastigma yunnanense
Tetrastigma triphyllum
Tetrastigma cf. delavayi
Tetrastigma lanceolarium
Tetrastigma hookeri
Tetrastigma voinierianum
Parthenocissus quinquefolia

Table 2. List of plant species analyzed in this study

Family	Species	GenBank No.
Chenopodiaceae	*Suaeda asphaltica*	AY541059
	Suaeda fruticosa	AY545876
	Suaeda monoica	AY545877
	Suaeda palaestina	AY545897
	Anabasis setifera	AY545878
	Atriplex halimus	AY545879
	Salsola vermiculata	AY545880
	Salsola tetrandia	AY545881
Asteraceae	*Onopordum cynarocephalum*	AY545882
	Conyza dioscoridis	AY545883
	Inula crithmoides	AY545884
Asclepiadaceae	*Pergularia tomentosa*	AY545885
Ephedraceae	*Ephedra aphylla*	AY545886
Fabaceae	*Alhagi graecorum*	AY545887
Rhamnaceae	*Ziziphus spina-christi*	AY545888
Mimosaceae	*Prosopis farcta*	AY545889
Poaceae	*Phragmites australis*	AY545890
	Poa eigii	AY545891
Zygophyllaceae	*Nitraria retusa*	AY545892
Polygonaceae	*Emex spinosa*	AY545893
Malvaceae	*Malva nicaeensis*	AY545894
Resedaceae	*Ochradenus baccatus*	AY545895
Tamaricaceae	*Tamarix nilotica*	AY545896
Vitaceae	*Vitis vinifera*	AY566240

Table 3. The *rbc*L primers used to characterize plant species in this study (Poinar et al. 1998)

Name	Target region	Sequence
*rbc*L19	*rbc*L gene	5′—AGATTCCGCAGCCAC TGCAGCCCCTGCTTC—3′
*rbc*LZ1	*rbc*L gene	5′—ATGTCACCACAAAC AGAGACTAAAGCAAGT—3′

gene (Table 3) that produced an amplicon of 157 bp (Poinar et al. 1998). A total reaction volume of 20.0 µl was used with standard PCR thermal cycler parameters, with an initial denaturation of 72 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 10 min.

Amplification products were separated in a 2.0% (w/v) agarose gel and stained with ethidium bromide (prior to manual sequencing) according to standard procedures; the same products were separated on 6% polyacrylamide gels (prior to automated sequencing) and according to the same standard procedures. Sequencing was carried out by two different methods to ensure reproducibility of results. Both methods of sequencing were repeated four times.

DNA sequencing

The PCR product of interest was excised from the gel and asymmetrically sequenced using both radioactive manual sequencing (Sequenase Kit, Amersham) and automated sequencing on an ABI PRISM 3100 capillary-based sequencer. For automated sequencing on the ABI prism, the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) was used as follows: 3 µl reaction mix, 0.5 µl forward or reverse primer (concentration according to the manufacturer's instructions), 1 µl sample (concentration according to the manufacturer's instructions), and 15.5 µl of sterile water. Thermocycling conditions were as follows: 25 cycles of denaturation 96 °C for 30 s, annealing 50 °C for 15 s, extension 60 °C for 4 min.

Sequence analysis

Sequences of *rbc*L were aligned by BioEdit sequence analysis software using the Smith–Waterman local alignment algorithm option. The local alignment method is most often applied to sequence sets that are relatively small and contain few gaps. To assess whether a given alignment may be an indication for homology, it also helps to know how strong an alignment can be expected from chance alone. For this we used NCBI GenBank BLASTn (www.ncbi.nih.gov/blast) to determine % similarity among sequences generated in this study, and then compared them to those already available from GenBank (www.ncbi.nih.gov). This comparison was graphically represented using Tree View as a BLAST option. Trees were produced in the rectangular configuration using the neighbour joining method with a maximum sequence difference of 0.5. Branch lengths are not shown.

Plant species producing significant BLAST matches to our own sequences were checked against the Flora of Israel (Zohary 1966, 1972; online database, beta version© 2003–2006 http://flora.huji.ac.il), to determine their presence or absence in our study area.

Results

PCR and gel electrophoresis

Amplicons consistent in size with the target fragment of plant *rbc*L were generated by PCR from all insects and reference plants tested. There was little or no observable difference in the recovery of DNA from the plant meal of insects of different sizes as assessed by gel electrophoresis. PCR of the smallest insect analyzed, *Myzus persicae*, produced viable plant DNA in amounts quantitatively comparable to the other insects, including

the relatively large *G. hebraeus* controls. There was, however, an observable decrease through time in the intensity of *rbc*L bands from *G. hebraeus* fed on grapes (*V. vinifera*).

Sequencing results: Plant reference collection

Sequences of the *rbc*L gene fragment were obtained for all of the plants we collected in the southern Jordan Valley (Table 2). Replicate sequences of each plant species were analyzed to confirm polymorphisms. Sequences of *rbc*L obtained for the plant reference collection were unique when compared to each other (Table 4). Furthermore, a BLAST search of our reference plants against the many thousands in GenBank revealed that 23 out of 24 of our sequences were unique. The exception was *Onopordum cynarocephalum*, which was identical to *Ainsliaea acerifolia*, *Piptocarpha axillaris*, and *Gerbera jamesonii* in the short region of *rbc*L. Fortunately, these plants are not found in the southern Jordan Valley. Interestingly, our sequence of *V. vinifera* differed by one transition polymorphism (C–T) from the GenBank sequence of the same species.

Sequencing results: Insect gut meals

The DNA sequences from insect guts were BLASTed against the plant reference sequences to make an identification of the gut meal. In all cases, gut meal sequences were 100% homologous with a sequence from the plant reference collection.

To ensure that the *rbc*L region chosen would be capable of discriminating many species, gut meal sequences were also BLASTed against the GenBank public database (www.ncbi.nih.gov/blast). In all but one case (*V. vinifera*), we had been the first to submit sequence of an *rbc*L fragment of the plants collected from the southern Jordan Valley. Likewise, in all but one case (*O. cynarocephalum*) the insect gut meal sequence matched the sequence generated from our plant reference more closely than any other of the thousands of sequences publicly available. In cases where the match was close (98% or 99% identity), the closely matching plant, checked against the Israeli flora database, was not found at all in our study area.

Discussion

A selection of insects, consisting of eight families, and a variety of body masses were collected from the field. This range of insects was chosen to determine if the presence of plant meals could be detected from large and small insects alike. It was found that plant DNA could be recovered from all of the insects, even the smallest, with little or no visible difference in DNA yield. This could be attributed to the PCR plateau effect that after a certain number of cycles the reaction becomes saturated.

A series of crickets, *G. hebraeus*, large in comparison to other insects in this study, were fed and sacrificed in time intervals of 2 h (from 0 to12 h) to determine the effect of time on gut content degradation. Many phytophagous insects feed constantly or at least within relatively short periods within the same day or night. In our experiments, plant meals were still detectable 12 h after ingestion. In most cases this should be enough time to determine feeding of an insect. However, the amount of DNA recovered did decrease over this time period, as one would expect with the digestion and breakdown of the plant meal. The sensitivity of the PCR detection system indicates that this type of analytical procedure is still feasible a number of hours after the insect feeds. Once the insect is collected it can be sacrificed and stored for up to 6 months if kept at $-70\,^{\circ}C$. The rates of digestion may differ between insects. Therefore, the detection limits for the insect of study should be determined before any research commences. This study did not determine the extent of DNA recovery from insects more than 12 h after ingestion or from insects stored for more than 6 months, questions that are worth being investigated.

BLAST analysis of unidentified gut meal sequences revealed three important points. First, it is important to obtain a reference collection of plants from the area under study, even though this may become relatively expensive and time consuming, because the plant of interest may not yet have been sequenced and submitted to GenBank. This was the case for all of our eight reference plants. Second, in the case of *O. cynarocephalum*, the small region of *rbc*L sequenced here was not unique in GenBank. In this case, the other BLAST matches were from plants not in the study area, but authors of future studies are cautioned about the possibility of this result. It may be necessary to sequence additional regions of the gene, or fragments of other genes, to increase the resolving power of this method. Third, we discovered that *Vitis* spp. sequences in GenBank, including our own, were more polymorphic compared to the other plants we sequenced in this study. In fact, species belonging to the genus *Vitis* appeared in three separate branches. This was confirmed in replicate sequences and so is more likely to be the result of within-species variation rather than a polymerase generated error. The *rbc*L fragment studied herein may then, in some cases, be able to distinguish among allelic variants of the same species. However, many more sequences from members of the same species will need to be generated and analyzed to determine if this is indeed the case.

One point that deserves attention is how well this method would potentially operate on polyphagous

Table 4. Sequence polymorphisms obtained for the *rbc*L gene from the southern Jordan Valley plant reference collection

Nucleotide position (np) within chloroplast genome of *Zea mays*[a] GenBank Acc. No. X86563	5 6 9 0 5	5 6 9 0 6	5 6 9 0 9	5 6 9 1 1	5 6 9 1 2	5 6 9 1 3	5 6 9 1 4	5 6 9 1 5	5 6 9 1 6	5 6 9 1 7	5 6 9 1 8	5 6 9 2 7	5 6 9 3 0	5 6 9 3 3	5 6 9 3 5	5 6 9 3 6	5 6 9 4 5	5 6 9 4 8	5 6 9 5 1	5 6 9 5 4	5 6 9 5 5	5 6 9 5 7	5 6 9 6 0	5 6 9 6 3	5 6 9 7 4	5 6 9 7 5	5 6 9 7 6	5 6 9 7 7	5 6 9 7 8	5 6 9 7 9	5 6 9 8 0	5 6 9 8 1	5 6 9 8 2	5 6 9 8 3	5 6 9 8 4	5 7 0 0 0	5 7 0 0 3	5 7 0 0 6	5 7 0 1 1	5 7 0 1 8	5 7 0 2 0	5 7 0 2 1	5 7 0 2 4	5 7 0 2 8	5 7 0 2 9	5 7 0 3 0	5 7 0 3 1	5 7 0 3 3	5 7 0 3 6
Zea mays[a]	T	T	A	T	T	A	A	A	G	C	T	G	T	T	A	A	C	C	C	G	G	G	C	A	A	C	C	A	A	G	G	A	T	A	C	A	C	G	T	C	G	C	C	G	C	T	A
Chenopodiaceae																																																	
Suaeda asphaltica	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	T	T	T	T	-	-	T	-	-	-	-	C	-	A	-	-	-	-	-	T	-	-	A	C	T	A	-	-	A	-	-	-
Suaeda fruticosa	-	-	-	-	-	C	C	C	-	-	-	A	-	-	-	-	T	T	T	T	-	-	T	-	-	-	-	C	-	A	-	-	-	-	-	T	-	-	A	C	T	A	-	-	A	-	-	-
Suaeda monoica	-	-	-	-	-	-	-	-	-	-	A	A	-	-	-	-	T	T	T	T	-	-	T	-	-	-	-	C	-	A	-	-	-	-	-	T	-	-	A	C	T	A	-	-	A	-	-	-
Suaeda palestina	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	T	T	T	T	-	-	T	-	-	-	-	C	-	A	-	-	-	-	-	T	-	G	A	C	T	A	G	G	A	-	-	-
Anabasis setifera	-	-	-	-	-	-	-	-	-	-	-	A	-	C	-	-	T	T	T	T	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	T	-	A	C	T	A	-	-	A	-	C	-
Atriplex halimus	-	-	-	-	-	-	-	-	-	-	-	A	-	C	-	-	T	T	T	T	-	-	T	-	-	-	-	C	T	A	-	-	-	-	-	-	-	G	A	C	G	A	-	-	A	-	C	-
Salsola vermiculata	C	-	-	-	-	-	-	-	-	-	C	A	-	C	-	G	T	T	T	T	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	G	A	C	T	A	-	-	A	-	C	-
Salsola tetranda	C	-	-	-	-	-	-	-	-	-	C	A	-	C	-	-	T	T	T	T	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	G	A	C	T	A	-	-	A	-	C	-
Asteraceae																																																
Onopordum cynarocephalum	-	-	-	-	C	-	-	-	-	-	-	A	-	C	-	-	T	T	T	T	-	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	A	C	T	A	-	-	-	-	-	-
Conyza dioscoridis	-	-	-	-	A	-	-	G	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	G	-	-
Inula crithmoides	-	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	T	T	T	T	-	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	A	C	G	C	-	-	-	-	-	-
Asclepiadaceae																																																
Pergularia tomentosa	-	-	-	C	A	-	G	C	-	G	-	A	G	C	-	-	T	T	T	T	-	A	-	-	-	-	A	-	-	A	-	-	-	-	-	-	-	-	-	A	C	-	A	-	-	A	-	-	-
Ephedraceae																																																
Ephedra aphylla	-	-	-	-	A	-	-	G	C	T	-	A	-	C	-	-	T	T	T	T	-	-	T	-	-	-	-	C	T	A	-	-	-	-	-	-	T	-	G	A	C	G	A	-	-	A	-	-	-
Fabaceae																																																
Alhagi graecorum	-	-	G	-	C	-	-	-	-	-	-	A	-	-	G	-	T	T	T	T	C	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	T	A	-	-	-	-	-	G
Rhamnaceae																																																
Ziziphus spina-christi	-	-	-	-	C	-	-	-	-	-	C	A	-	-	-	-	T	-	T	T	-	C	T	-	-	-	-	-	-	A	-	-	-	-	-	G	T	-	-	A	C	T	A	-	-	-	-	-	G
Mimosaceae																																																
Prosopis farcta	-	-	G	-	C	-	-	-	-	-	-	A	-	-	-	-	T	T	T	T	-	C	T	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	A	C	T	A	-	-	-	-	-	-
Poaceae																																																
Phragmites australis	A	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	G	-	-
Poa eigii	-	-	-	-	-	C	-	-	-	-	-	A	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	-	-	-	C	-	G	-
Zygophyllaceae																																																
Nitraria retusa	-	-	-	-	C	-	-	-	-	-	C	A	-	-	-	-	T	T	T	T	-	A	T	-	-	-	-	-	-	A	-	-	-	-	-	-	T	-	A	C	-	A	-	-	A	-	C	-
Polygonaceae																																																
Emex spinosa	-	-	-	-	C	-	-	-	-	-	-	A	A	C	-	-	T	T	T	T	-	C	T	-	C	-	-	C	-	T	-	-	C	C	A	-	T	-	-	A	C	T	A	-	-	A	G	A	-
Malvaceae																																																
Malva nicaeensis	-	-	-	-	C	-	-	-	-	-	-	A	G	-	-	-	T	T	T	T	-	A	T	-	G	T	-	-	-	A	C	-	-	-	-	C	-	-	G	A	C	-	A	-	-	-	-	-	G
Resedaceae																																																
Ochradenus baccatus	C	-	-	-	C	-	-	-	-	-	C	A	-	C	-	-	T	T	T	T	-	-	T	-	-	G	G	-	-	A	-	-	-	-	-	-	-	-	-	A	C	T	A	-	-	A	-	C	-
Tamaricaceae																																																
Tamarix nilotica	-	-	-	-	C	-	-	-	-	-	-	A	-	C	-	-	T	T	T	T	C	A	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	A	C	T	A	-	-	T	-	A	G
Vitaceae																																																
Vitis vinifera ssp.[b]	-	-	-	-	C	-	-	-	-	-	C	A	-	C	-	-	T	T	T	T	-	A	T	G	-	-	-	-	-	A	C	C	-	-	-	-	T	-	-	A	C	T	A	-	-	A	-	C	-
Vitis vinifera Acc. No. AJ487011	-	-	-	-	C	-	T	-	-	-	C	A	-	C	-	-	T	T	T	T	-	A	T	G	-	-	-	-	-	A	C	C	-	-	-	-	T	-	*	*	*	*	*	*	*	*	*	*

*No sequence data for these nucleotide positions.

- Nucleotid identical with that one in the reference sequence.

[a]Reference sequence.

[b]Plant used for experimental study.

insect species. Polyphagy has been controlled for in the present study, but is likely to be commonly encountered in the field. In this case, gut meal samples should be cloned to determine the presence of one or more concomitant sequences. Given the reasonably good resolving power of this small fragment of *rbc*L, as demonstrated by BLAST analysis, we believe that the cloning approach would be sufficient to detect and identify multiple plants ingested by polyphagous insects. We do, however, suggest cloning and sequencing of additional small regions of *rbc*L or other genes to strengthen the DNA identification evidence.

The *rbc*L fragment sequenced here can serve as an informative marker in future studies of degraded DNA, that would provide information about plant species fed upon by insects, provided there is a reasonably well represented plant sequence reference collection from the area under study. Such a small region of the *rbc*L gene was chosen because in previous studies it has been shown that autolytic and hydrolytic degradation processes often reduce DNA to fragments of 250 bp or less (Pääbo et al. 2004). Also, this same region was previously used to differentiate plant material in degraded condition (Poinar et al. 1998). However, we do recommend again that additional short regions of the *rbc*L gene be sequenced to improve taxonomic resolution. In addition, sequencing of fragments from other more quickly evolving genes may be required for resolution of closely related plant species. This study demonstrates the potential use of PCR as a methodology for the identification of plant material ingested by insects.

Acknowledgements

We thank Dr. David Heller from the National Herbarium at The Hebrew University of Jerusalem, Israel, and Mr. Michael Blecher and Dr. Rueven Ortal from the Nature and Parks Authority, Israel, for assistance in the molecular identification and characterization of plant species, Drs. V. Kravchenko and V. Tchekoternov from the Zoology Department, University of Tel Aviv, for providing invaluable help in identifying the insects, the anonymous referees for valuable and constructive criticism, and Kristine Hynne for her invaluable assistance in editing this manuscript. We would also like to thank the staff of the Paleo-DNA Laboratory at Lakehead University in Canada and the staff of the Kuvin Centre at Hebrew University in Israel for their support.

References

Abdel-Malek, A.A., Baldwin, W.F., 1961. Specificity of plant feeding in mosquitoes as determined by radioactive phosphorus. Nature 192, 178–179.

Akey, D.H., Hayes, J.L., Fleischer, S.J., 1991. Use of elemental markers in the study of arthropod movement and trophic interactions. Southwest. Entomol. 14 (Suppl.), 1–87.

Augusti, N., Aramburu, J., Gabarra, R., 1999a. Immunological detection of *Helicoverpa armigera* (Lepidoptera: Noctuidae) ingested by heteropteran predetors: time-related decay and effect of meal size on detection period. Ann. Entomol. Soc. Am. 92, 56–62.

Augusti, N., de Vincente, M.C., Gabarra, R., 1999b. Development of sequence amplified characterization region (SCAR) markers of *Helioverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. Mol. Ecol. 8, 1467–1474.

Augusti, N., de Vincente, M.C., Gabarra, R., 2000. Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. Insect Mol. Biol. 9, 263–268.

Augusti, N., Unruh, T.R., Welter, S.C., 2003. Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. Bull. Entomol. Res. 93, 179–185.

Basset, Y., 1992. Host specificity of arboreal and free living insect herbivores in rain forests. Biol. J. Linn. Soc. 47, 115–133.

Belsky, A.J., 1986. Does herbivory benefit plants? A review of the evidence. Am. Nat. 127, 870–892.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M., van der Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495–503.

Cates, R.G., 1980. Feeding patterns of monophagous, oligophagous, and polyphagous insect herbivores, the effect of resource abundance and plant chemistry. Oecologia 46, 22–31.

Chen, Y., Giles, K.L., Payton, M.E., Greenstone, M.H., 2000. Identifying key cereal aphid predators by molecular gut analysis. Mol. Ecol. 9, 1887–1898.

Chow-Shaffer, E., Sina, B., Hawley, W.A., De Benedictis, J., Scott, T.W., 2000. Laboratory and field evaluation of polymerase chain reaction-based forensic DNA profiling for use in identification of human blood meal sources of *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 37, 492–502.

Eastop, V., 1979. Sternorrhyncha as angiosperm taxonomists. Symb. Bot. Uppsala 22, 120–134.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants, a study in coevolution. Evolution 18, 586–608.

Gokool, S., Curtis, C.F., Smith, D.F., 1993. Analysis of mosquito bloodmeals by DNA profiling. Med. Vet. Entomol. 7, 208–215.

Hendrix, S.H., 1980. An evolutionary and ecological perspective of the insect fauna of ferns. Am. Nat. 115, 171–196.

Hoogendoorn, M., Heimpel, G.E., 2001. PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. Mol. Ecol. 10, 2059–2067.

Johnson, S.A., Nicolson, S.W., 2001. Pollen digestion by flower-feeding Scarabaeidae: protea beetles (Cetoniini) and monkey beetles (Hopliini). J. Insect Physiol. 47, 725–733.

Juen, A., Traugott, M., 2005. Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. Oecologia 142, 344–352.

Kasten, F.H., 1980. Methods for fluorescence microscopy. In: Clark, G. (Ed.), Staining Procedures. Williams & Wilkins, Baltimore, pp. 39–103.

Kreike, J., Kampfer, S., 1999. Isolation and characterization of human DNA from mosquitoes (Culicidae). Int. J. Legal Med. 112, 380–382.

May, R.M., 1988. How many species are there on earth? Science 241, 1441–1449.

Mogi, M., Miyagi, I., 1989. Sugar feeding of *Topomyia pseudobarbus* (Diptera: Culicidae) in nature. J. Med. Entomol. 26, 370–371.

Moore, J.S., Kelly, T.B., Killick-Kendrick, R., Killick-Kendrick, M., Wallbanks, K.R., Molyneux, D.H., 1987. Honeydew sugars in wild-caught *Phlebotomus ariasi* detected by high performance liquid chromatography (HPLC) and gas chromatography (GC). Med. Vet. Entomol. 1, 427–434.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Despres, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., Hofreiter, M., 2004. Genetic analyses from ancient DNA. Annu. Rev. Genet. 38, 645–679.

Poinar, H.N., Hofreiter, M., Spaulding, W.G., Martin, P.S., Stankiewicz, A.B., Bland, H., Evershed, R.P., Possnert, G., Pääbo, S., 1998. Molecular coproscopy: dung and diet of the extinct ground sloth *Nothrotheriops shastensis*. Science 281, 402–406.

Prokopy, R.J., 1976. Feeding, mating and oviposition activities of *Rhagoletis fausta* flies in nature. Ann. Entomol. Soc. Am. 69, 899–904.

Prokopy, R.J., Roitberg, B.D., Averill, A.L., 1984. Chemical mediation of resource partitioning in insects. In: Carde, R.T. (Ed.), The Chemical Ecology of Insects. Chapman & Hall, London, pp. 301–330.

Sandholm, H.A., Price, R.D., 1962. Field observations on the nectar-feeding habits of some Minnesota mosquitoes. Mosquito News 22, 346–349.

Schlein, Y., Jacobson, R.L., 1994. Mortality of *Leishmania major* in *Phlebotomus papatasi* caused by plant feeding of the sand flies. Am. J. Trop. Med. Hyg. 50, 20–27.

Schlein, Y., Muller, G., 1995. Assessment of plant tissue feeding by sand flies (Diptera: Psychodidae) and mosquitoes (Diptera: Culicidae). J. Med. Entomol. 32, 882–887.

Symondson, W.O., 2002. Molecular identification of prey in predator diets. Mol. Ecol. 11, 627–641.

Tobolewski, J., Kaliszewski, M.J., Colwell, R.K., Oliver Jr., J.H., 1992. Detection and identification of mammalian DNA from the gut of museum specimens of ticks. J. Med. Entomol. 29, 1049–1051.

Zaidi, R.H., Jaal, Z., Hawkes, N.J., Hemingway, J., Symondson, W.O., 1999. Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Mol. Ecol. 8, 2081–2087.

Zohary, M., 1966. Flora Palaestina. Part I. Israel Academy of Sciences and Humanities, Jerusalem.

Zohary, M., 1972. Flora Palaestina. Part II. Israel Academy of Sciences and Humanities, Jerusalem.

Zohary, M., 1973. Geobotanical Foundations of the Middle East. Gustav Fischer Verlag, Stuttgart.