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PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency

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Abstract

We used polymerase chain reaction to determine whether *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) DNA was present in the guts of larvae and adult males and females of the generalist predator *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae). The predators were fed *Ostrinia nubilalis* egg masses and allowed to digest at either 20 °C or 27 °C for time spans ranging from 0 to 12 h. Four primer pairs, specific for *O. nubilalis* were developed, using a nuclear ribosomal RNA sequence including part of the 18S gene, the complete internal transcribed spacer (ITS-1) region and part of the 5.8S gene. These primers amplified four sequences that were 492, 369, 256 and 150 base pairs long. We found a significant negative effect of time since feeding on the number of bands that could be detected. The shortest fragment was detected for the longest time after feeding (up to 12 h). We found no effect of predator weight, sex, developmental stage, or meal size on the time course over which bands of varying lengths could be detected.

Keywords: *Coleomegilla maculata*, digestion, gut contents, *Ostrinia nubilalis*, PCR, predation

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Introduction

Identification of the gut contents of predatory insects can provide important information on trophic relationships and insights into the dynamics of predator–prey interactions. However, determining the diet of predatory insects in the field can be complicated, in part because both prey and predator are small and often cryptic. Techniques for identifying predator gut contents include direct observations (Carter & Dixon 1982; Carter *et al*. 1984; Legaspi *et al*. 1996; Heimpel *et al*. 1997; Munyaneza & Obrycki 1998), microscopic analysis of predator gut contents (Walker *et al*. 1988; Aussel & Linley 1994; Powell *et al*. 1996; Sleaford *et al*. 1996; Triltsch 1997), the development of prey-specific protein antibodies (Greenstone & Hunt 1993; Hagler *et al*. 1995; Powell *et al*. 1996; Hagler & Naranjo 1997; Symondson *et al*. 1997; Hagler 1998; Agustí *et al*. 1999b; Symondson *et al*. 1999a), and prey-specific electrophoretic analyses of predators including polymerase chain reaction (PCR)-based assays (Houck *et al*. 1991; Powell *et al*. 1996; Agustí *et al*. 1999a, 2000; Zaidi *et al*. 1999; Chen *et al*. 2000).

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Each of these methods has strengths and weaknesses and is appropriate in various contexts. Direct observations of predators in the field can be difficult and time consuming, but important behavioural data can be obtained during the observations (Kareiva & Odell 1987; Völkl 1992). Microscopic analyses of gut contents can be achieved with a minimum of technical expertise and equipment, but is only possible for chewing predators that ingest relatively large prey fragments (Aussel & Linley 1994; Powell *et al*. 1996; Triltsch 1997). Immunological assays can be sensitive, but are time consuming and expensive to develop (Greenstone 1996). They can be used to determine the absence or presence of prey in the gut, with an accuracy that depends on factors including temperature, meal size, time since feeding, resistance of the target epitope to digestion and predator species (Hagler *et al*. 1997; Symondson *et al*. 1997, 1999b; Hagler 1998). Appropriate antibodies have been developed for some insect predator–prey systems, but for ladybeetles and some of their prey, detection times averaged less than 1 h (Hagler *et al*. 1997; Hagler 1998).

Here, we describe a PCR-based method for determining whether a coccinellid beetle, *Coleomegilla maculata* De Geer, has fed on one of its prey species, the European

corn borer, *Ostrinia nubilalis* (Hübner), and to estimate the time since last feeding. Our strategy to estimate the time since the last meal is based on the digestion time of different fragment lengths of prey DNA within the predator's gut. This work builds upon a number of recent studies on the analysis of predator gut contents using PCR (Agustí *et al*. 1999a, 2000; Zaidi *et al*. 1999; Chen *et al*. 2000). In their studies, Agustí *et al*. (1999a, 2000) used sequence characterized amplified regions (SCARs), derived from randomly amplified polymorphic DNA (RAPD) bands. This method involves DNA amplification using RAPD primers of prey and predator DNA, followed by the selection of a band that is present in the prey but not the predator. This is followed by re-amplification and cloning of the selected sequence, and the development of prey-specific primers for the detection of prey DNA in the predator gut. Using this method, Agustí *et al*. (1999a) developed three primer pairs that amplified fragments of 1100, 600 and 254 bp from *Helicoverpa armigera* individuals that were ingested by the predator *Dicyphus tamaninii*.

In other studies using PCR for the analysis of gut contents, Zaidi *et al*. (1999) and Chen *et al*. (2000) argued that detection of prey DNA in the gut of predators is most likely to be successful if the genes that are amplified are present in multiple copies, and if sequences are relatively short. Zaidi *et al*. (2000) fed laboratory-reared mosquito larvae to carabid beetles. The primers that were used detected α-esterase genes, which are present in multiple copies in the mosquito DNA, but not in the carabid. They amplified PCR products of 146 bp and 263 bp. Both Agustí *et al*. (1999) and Zaidi *et al*. (1999) found that the shortest sequences were detectable for the longest period of time after feeding. In their study, Chen *et al*. (2000) used the mitochondrial COII gene that is present in multiple copies in each cell. They were able to distinguish between different species of aphids in coccinellid and lacewing guts. Two predator species were used in the study: the lacewing *Chrysoperla plorabunda* (Fitsch) and the ladybird beetle *Hippodamia convergens* Guerin. An effect of sequence length on detectability half-life of the primers was found in the former, but not in the latter predator species.

Here, we develop a method to estimate time since feeding by using primer sets that amplify fragments of different lengths that each have a characteristic detection time. The primers we used are derived from ribosomal RNA genes, which are present in multiple copies in the genome of insects (Hoy 1994; p. 72).

Materials and methods

The system

Coleomegilla maculata commonly occurs throughout central and eastern North America, where it is a native species. Its diet is diverse and it can complete development on aphids, insect eggs of different species and pollen (Andow 1990, 1996; Munyaneza & Obrycki 1998). It is one of the most important predators of *Ostrinia nubilalis* egg masses in corn (Andow 1990). *O. nubilalis* was first introduced to North America from Europe in 1917 (Baker *et al*. 1949) and is now one of the most abundant pests of corn in northcentral North America (Andow 1996).

Rearing of the insects

Coleomegilla maculata individuals that were used in this experiment were taken from a colony that had been reared in our laboratory for less than 1 year. The colony was kept at a 16 : 8 light to dark regime at 23 °C. The beetles were fed an artificial diet, based on chicken liver with added nutrients (diet no. 7 in Atallah & Newsom 1966, without antibiotics), and provided with distilled water, added to open, cotton-filled 0.5 mL microcentrifuge tubes. Individual beetles were kept in separate Petri dishes, and eggs were removed to minimize cannibalism.

O. nubilalis egg masses were obtained from a colony that was reared at the Insect Ecology Laboratory at the University of Minnesota. Rearing procedures were modified from Leahy & Andow (1994). Egg masses were laid on wax paper and were removed from the paper before they were offered to coccinellids.

Feeding experiments

Two feeding experiments were carried out. For the first, fourth instar larvae and adult male and female *C. maculata* were used. The second experiment involved only adult males and females. The two experiments were carried out in the same manner. Before each feeding experiment, individuals were starved for 48 h. Water was provided for each individual in a 0.5-mL microcentrifuge tube with moist cotton wool. The weight of each beetle was determined to the nearest 0.1 mg just before the experiment.

During the experiments, each individual was allowed to feed on a single 3-day-old *O. nubilalis* egg mass for a maximum of 30 min. The number of eggs that were eaten was recorded. After feeding, individuals were allowed to digest their meals for time-spans ranging from 0 to 12 h, with intervals of 1 h for time periods up to 6 h, and intervals of 2 h between 6 and 12 h. During digestion, individuals were kept at either 20 °C or 27 °C. They were then frozen and stored in 70% ethanol at –20 °C until DNA extraction.

DNA extraction

DNA was extracted from whole insects. We used a protocol that was modified from Bender *et al*. (1983). Individual insects were ground in 1.5 mL microcentrifuge tubes using

sterile plastic pestles, in 100 µL grinding buffer (recipe in Bender *et al*. 1983). An additional 100 µL of grinding buffer was used to rinse any remaining insect material from the pestle. After grinding, the samples were incubated at 65 °C for 30 min. To each tube 28 µL potassium acetate (8 M) was added and the samples were incubated on ice for 30 min. The samples were centrifuged for 15 min at 16 000 ***g***, and the supernatant was incubated with 200 µL 100% ethanol for 5 min at room temperature and centrifuged again for 15 min. The supernatant was removed, and the remaining pellet of DNA was washed with 100 µL 70% ethanol and again with 100% ethanol. Between washes, samples were centrifuged for 2 min. After removing the ethanol, the sample was dried at 65 °C for 5–10 min and resuspended in 100 µL Tris-EDTA (pH 8.0) buffer.

PCR

For detection of *O. nubilalis*, a DNA sequence was used that included the partial sequence of the nuclear 18S ribosomal RNA gene, the complete sequence of the internal transcribed spacer (ITS-1), and a partial sequence of the 5.8S ribosomal RNA gene (Marçon *et al*. 1999). The sequence described by Marçon *et al*. (1999) was 463 base pairs long (Fig. 1). We used a set of four primers that were designed to amplify four sequences of different length. The amplified sequences were expected to be 100, 220, 343 and 463 base pairs long (Fig. 1). In addition to the *O. nubilalis* primers, a conserved insect primer derived from a mitochondrial 12S rRNA sequence of *Drosophila yakuba* Burla was used to check for the presence of DNA in the sample (primer no. 1: 12Sai, in Noda *et al*. 1997). Amplifications were performed in 45.5 µl of 1× buffer (0.25 mM each dNTP and 5 mM $MgCl_2$), 2 µl of primer mix adjusted to 20 mM in each reaction tube, 0.5 µl *Taq* (i.e. 2.5 units) (Promega), and 2 µl DNA sample. One milligram of bovine serum albumin (BSA) per ml of 1 buffer was added to reverse inhibition of the PCR by the presence of melanin (Giambernardi *et al*. 1998).

The thermocycling program consisted of an initial step of 30 s at 94 °C, followed by 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C. These last three steps were repeated 30 times and followed by a step of 5 min at 72 °C, after which the machine cooled down to 4 °C. PCR products were electrophoresed at 95 V in a 1.2% agarose gel for approximately 1 h. The primers were tested on 20 *C. maculata* individuals that had not fed on *O. nubilalis* egg masses to ensure that no predator DNA was amplified with these primers.

Test for cross-reactivity

We tested for cross-reactivity of our primers with three potential prey species of *C. maculata*. The species tested were south-western corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae), corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), and green peach aphids, *Myzus persicae* (Sulzer) (Homoptera: Aphididae), all of which are readily fed upon by *C. maculata*.

We extracted DNA from *D. grandiosella* and *H. zea* eggs, using the extraction protocol that was used for the feeding experiments. For each extraction, 15 blackheaded (mature) eggs that were frozen at −80 °C were used. For each of these two species there were 10 replicates. A PCR was performed on each of these extractions, using the four primer sets and the conserved primer, as described above.

To test cross reactivity with *M. persicae*, and to test at the same time whether the presence of aphids inhibits PCR amplification of *O. nubilalis* DNA, we presented aphids and *O. nubilalis* egg masses at the same time to 15 adult beetles and recorded how many eggs and how many aphids were eaten by each beetle within 2 h. Two other beetles were fed only aphids. Beetles were kept at 23 °C from feeding until freezing. After 2 h, all beetles were frozen at −80 °C. DNA was extracted from these 17 individuals as described above, and in addition DNA was extracted from five frozen aphids and a frozen mix of aphids and an *O. nubilalis* egg mass. A PCR was performed on all

1	**tacacaccgc ccgtcgctac taccga**ttgg aatgatttag tgaggtcttc ggaccgacag
61	ccggtggctt accggccgtc ggcgttgctg ggaagttgac caaacttgat catttagagg
121	**aagtaaaagt cgtaacaagg** tttccgtagg ggaacctgcg gaaggatcat taacgtgtac
181	gttcacatgt gatacacaag **tgtcatgtgg gaatgatcat** acaataatcc agacacaaaa
241	caaaagttct gatcgaagga aggtctcgtt taattatgag atcgttcgac gattggcgtc
301	gtacgatatt ctacgtgtac agattgaaaa tccgcgatct cagtagtttt cgtacgttcg
361	taaaatgaca aattcatgcg gttgaaaatt gtatattttt cagacattta taaaattatt
421	attattattg tttatttata ac**cactataa accattaccc tgg**

Fig. 1 *Ostrinia nubilalis* 18S ribosomal RNA gene, partial sequence (bp 1–163); internal transcribed spacer 1, complete sequence (bp 164–442); and 5.8S ribosomal RNA gene, partial sequence (bp 443–463) (Marçon *et al*. 1999). Base pair sequences that were used as forward primers are printed in bold, base pair sequences that were used as reverse primers are underlined.

extractions, using the four primer sets and the conserved primer as described above.

Results

The DNA sequences that were amplified appeared to differ slightly from the sequence that was described by Marçon *et al*. (1999). The estimated lengths of the sequences were longer than expected (Fig. 2). This was unexpected, since Marçon *et al*. (1999) compared populations from Europe (Italy) and North America (Nebraska) and found no difference in sequence between those populations. However, intraspecific variation in length and sequence of the ITS-1 region has been found in studies of other insects (Vrain *et al*. 1992; Wesson et a. 1992; Cherry *et al*. 1997).

A total of 94 *Coleomegilla maculata* individuals were analysed: 18 adult males, 19 adult females and 19 fourth instar larvae in the first experiment, and 19 adult males and 19 adult females in the second experiment. Larvae were not included in the general analysis, but instead were analysed separately. Variables that could possibly account for differences in digestion rate, and thus detectability of bands, were sex, predator weight, time since feeding, temperature and the number of eggs eaten, which varied between 5 and 27 for adults (mean = 14.55, SEM = 0.51) (Table 1), and between 8 and 25 for larvae (mean = 13.5, SEM = 0.94).

A multiple regression analysis was performed to determine the effects of experimental replicate, digestion time (time from feeding until freezing), temperature, sex, meal size and predator weight on the number of bands that could be detected. No effect of experiment was detected, and therefore the data from both experiments were pooled for the remaining analyses. Time after ingestion and temperature both had a significant effect on the number of bands that could be detected (Table 2). For the larvae, only time had an effect on the number of bands detected. The relationship between time and number of bands detected was best described by a linear regression for the larvae ($R^2 = 0.81$, $P < 0.0001$). The equation for the regression line was: number of bands = $4.00 - (0.392 \times \text{time})$.

Although temperature had an effect on the number of bands that could be detected in adults, separate regressions for each temperature of the relationship between time and number of bands had 95% confidence intervals

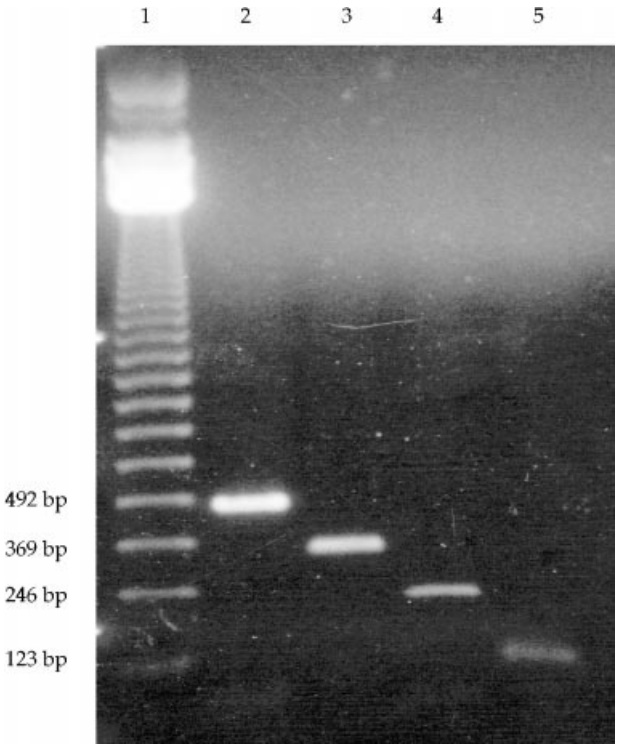

Fig. 2 Ethidium-bromide-stained agarose gel of PCR products from a *Coleomegilla maculata* larva, frozen immediately after feeding on an *Ostrinia nubilalis* egg mass. Lane 1 shows the DNA ladder, lanes 2–5 show the DNA fragments amplified by the primer sets. Estimated lengths of fragments: lane 2, 492 bp; lane 3, 369 bp; lane 4, 256 bp; lane 5, 150 bp (note that these fragment lengths differ from the expected lengths). None of these primers amplified *C. maculata* DNA.

Table 1 Mean weight of *Coleomegilla maculata* adult males and females, and mean number of eggs eaten (mean ± SE)

Expt	Sex	Individual weight (mg)	Number of eggs eaten
1	male (n = 18)	10.5 ± 0.44 (a)	14.0 ± 0.80
1	female (n = 19)	12.7 ± 0.68 (b)	15.68 ± 1.24
2	male (n = 19)	12.4 ± 0.36 (b)	12.47 ± 0.76
2	female (n = 19)	14.5 ± 0.27 (c)	16.0 ± 1.07

An ANOVA showed differences in weight between treatments. A comparison of means for each pair of treatments (Tukey–Kramer $\alpha = 0.05$) indicates which treatments differ. For each variable, mean values for groups that are indicated by a different letter are significantly different. The number of eggs eaten did not differ between groups.

Table 2 Results of multiple regression on the number of bands detectable in *Coleomegilla maculata* adults

Factor	d.f.	Sum of squares	F ratio	Prob. > F
Time since feeding	1	64.886	63.386	< 0.0001
Temperature	1	6.1627	6.0202	0.0169
Experiment	1	0.7168	0.7002	0.4058
Sex	1	1.5958	1.5589	0.2164
Beetle weight	1	0.5727	0.5595	0.4572
Eggs eaten	1	0.0682	0.0666	0.7972

Time since feeding and temperature had a significant effect on the number of bands detected. There was no difference between experiments.

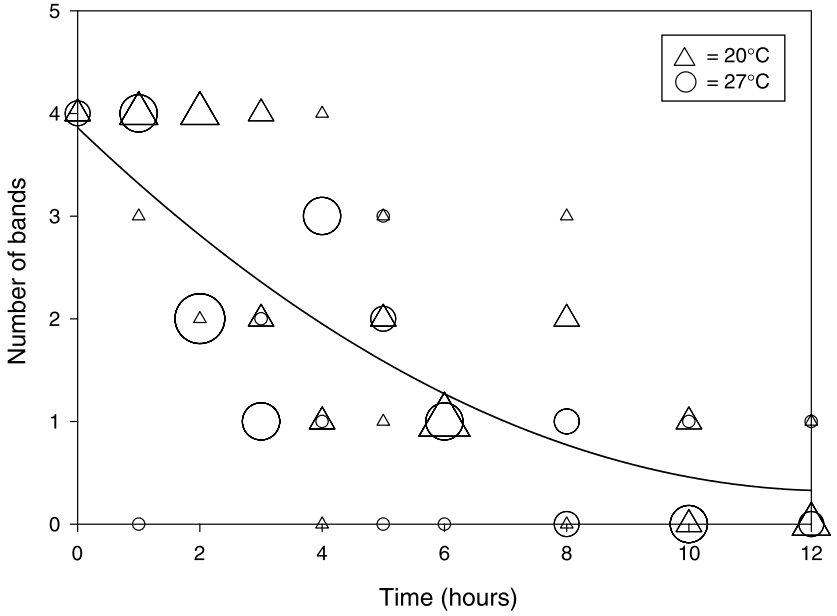

Fig. 3 Polynomial regression of time after feeding on the number of bands that is detected. The two temperatures (indicated by different symbols) are analysed together, since the slope of separate regression lines is not significantly different. The equation of the regression lines is given by: number of bands = 3.86183 – (0.56947 × time) + (0.02292 × time^2). The size of the data points indicates the number of observations (*Coleomegilla maculata* individuals) that is represented by each point in the graph. Total number of observations for 20 °C is 38 and for 27 °C is 37. For each time after digestion there were four observations per temperature, except for the 12-h period at 27 °C (three observations). Because temperature was not an applicable factor for the 0 h time period, the total number of observations for this group was four.

Table 3 Bands that are visible in each individual predator, at various times after feeding and at two different temperatures for adult males and females in both experiments

	Expt 1 at 20 °C		Expt 2 at 20 °C		Expt 1 at 27 °C		Expt 2 at 27 °C	
Time (h)	male	female	male	female	male	female	male	female
1	1,2,3,4	1,2,3,4	2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	—
2	3,4	1,2,3,4	1,2,3,4	1,2,3,4	3,4	3,4	3,4	3,4
3	3,4	3,4	1,2,3,4	1,2,3,4	4	4	4	4
4	4	4	—	1,2,3,4	2,3,4	2,3,4	4	2,3,4
5	4	3,4	2,3,4	3,4	2,3,4	3,4	—	3,4
6	4	4	4	4	4	4	—	4
8	3,4	—	2,3,4	3,4	—	—	4	4
10	4	—	—	4	—	4	—	—
12	—	—	—	4	*n.a.*	—	—	4

1 = 492 bp band, 2 = 369 bp band, 3 = 256 bp band, 4 = 150 bp band.

for intercepts and slopes that overlapped. The two temperatures were therefore pooled in regression analyses of number of bands and time. For adult beetles, the relationship between the number of bands detected and the time since feeding was best described by a quadratic function ($R^2 = 0.55$, $P < 0.0001$; P for time < 0.0001, P for (time)^2 = 0.0169). As time span increased, the number of bands detected decreased (Fig. 3). The shortest sequence was visible for the longest time after ingestion (Table 3). The maximum time that this band could be detected was longer than would be expected if the digestion rate were constant (linear).

To estimate the time since feeding for field-caught predators, we calculated the proportions of individuals with one, two, three, or four bands for each time span, and the average time span after which each number of bands was detected (Table 4). Our experiment showed that if one band is present, this is always the shortest sequence, if two bands are present these are always the two shortest sequences, etc. We calculated the average time span associated with each number of bands by using weighted proportions of beetles producing all given number of bands:

$$\text{average time} = \sum_{t=1}^{T}\left[Pt \Big/ \left(\sum_{t=1}^{T} P \right) \right]$$

where t is the number of hours since feeding, T is the number of time periods > 1 (nine in our case), and P is the time-specific proportion of beetles producing the specified number of bands (Table 4).

We used standard bootstrapping procedures (Efron & Tibshirani 1993) to obtain 95% confidence intervals for each time average (Table 4). Three different groups can be distinguished, based on the confidence limits (Table 4): group 1, four bands present (average 1.83 h); group 2, two or three bands present (averages 4.0 and 4.43 h); and group 3, one band present (average 6.41 h).

Table 4 The proportion of individuals in which one, two, three, or four bands were amplified at each time interval, and the weighted average number of bands for each time interval

	4 bands	3 bands	2 bands	1 band
1 h	0.75	0.125	0	0
2 h	0.375	0	0.625	0
3 h	0.25	0	0.25	0.5
4 h	0.125	0.375	0	0.375
5 h	0	0.25	0.5	0.125
6 h	0	0	0	0.875
8 h	0	0.125	0.25	0.25
10 h	0	0	0	0.375
12 h	0	0	0	0.25
Average time span (h)	1.83	4.43	4.0	6.41
95% CI	1.36–2.29	3.00–5.78	3.08–4.89	5.46–7.35

The 95% confidence intervals are each based on 1000 bootstrap iterations.

Cross-reactivity test

The conserved primer (12Sai, in Noda *et al*. 1997) amplified DNA from each of the species tested, showing that the extractions were successful in all cases. The *Ostrinia nubilalis* primer sets did not amplify DNA from any of the 10 extractions of *Helicoverpa zea* eggs, but two of the primer combinations did amplify DNA fragments from the more closely related *Diatraea grandiosella*. The sequences that were amplified were many times larger than the sequences that were amplified from *O. nubilalis*, and thus could easily be distinguished (Fig. 4).

None of the primer sets we used to detect *O. nubilalis* DNA in the gut amplified aphid DNA. For all beetles that were observed to eat *O. nubilalis* eggs and aphids, at least two bands were detected. No bands were detected in beetles that were observed to feed only on aphids or that were fed only aphids. No DNA was amplified from aphids alone, using the *O. nubilalis* primers. The presence of aphids did not inhibit the amplification, since DNA could be amplified from a mix of aphids and eggs, and from beetles that ate both aphids and eggs (Table 5).

Discussion

We showed that detectability of prey DNA at different times after ingestion of prey is dependent on fragment size, and that this relationship is negative and nonlinear. Detectability does not seem to be influenced by meal size, predator weight, or predator sex and stage. All individuals used in this study ate more than five eggs, and it is possible that a meal size smaller than this would influence the detectability. A higher temperature resulted in an increased rate of digestion, as has been shown before in hemipteran predators (Hagler & Cohen 1990) and tsetse flies (Loder *et al*. 1998).

Specificity of the primers

The sequence that was amplified incorporated the nuclear ribosomal ITS-1 region. This has been found to be highly variable between species, and even between populations,

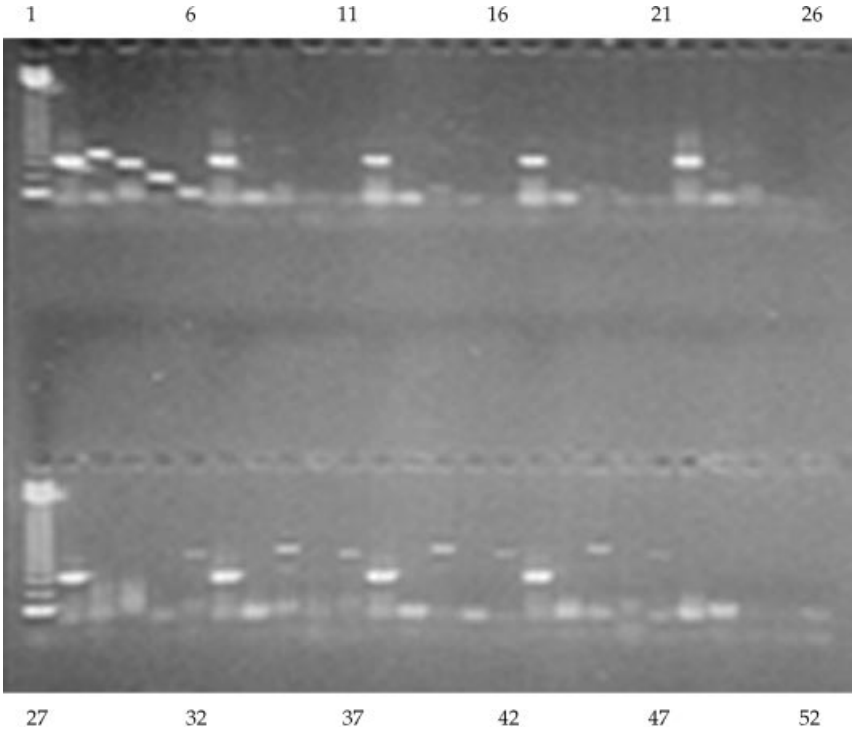

Fig. 4 Ethidium-bromide-stained agarose gel showing the banding pattern for *Ostrinia nubilalis* eggs with the conserved primer (lane 2), and with the primers amplifying 492 bp (lane 3), 369 bp (lane 4), 256 bp (lane 5) and 150 bp (lane 6). The following four samples are *Helicoverpa zea* eggs, and after these four samples of *Diatraea grandiosella* eggs, followed by a negative control (lanes 48–52). Lanes 7, 12, 17, 22, 28, 33, 38 and 43 show bands for the conserved primer. Lanes 1 and 27 show the 123 bp ladder.

Table 5 Results of a cross-reactivity test of the primer sets with *Ostrinia nubilalis* egg masses and green peach aphids

Treatment	No. of eggs	No. of aphids	Bands
2 h feeding	15	0	1, 2, 3, 4
2 h feeding	18	0	2, 3, 4
2 h feeding	7	2	1, 2, 3, 4
2 h feeding	8	1	1, 2, 3, 4
2 h feeding	14	1	1, 2, 3, 4
2 h feeding	3	2	1, 2, 3, 4
2 h feeding	11	1	1, 2, 3, 4
2 h feeding	10	2	2, 3, 4
2 h feeding	18	4	2, 3, 4
2 h feeding	2	2	2, 3, 4
2 h feeding	16	2	3, 4
2 h feeding	0	1	—
2 h feeding	0	1	—
2 h feeding	0	3	—
2 h feeding	0	3	—
2 h feeding	0	3	—
2 h feeding	0	5	—
Frozen aphids	0	5	—
Aphids + eggs	22	5	1, 2, 3, 4

especially in restriction fragment length polymorphism (RFLP) analyses (Vrain *et al*. 1992; Cherry *et al*. 1997; Marçon *et al*. 1999). The overall length and restriction fragment sites of the ITS-1 region differed substantially between *Ostrinia nubilalis* and the closely related species *Ostrinia obumbratalis* (Lederer) and *Diatraea grandiosella* Dyar (Marçon *et al*. 1999).

The two longest sequences amplified in this study incorporate part of the 18S and 5.8S sequences and the complete ITS-1 sequence; the primers used to amplify this sequence are part of the 18S and 5.8S sequence. It is possible that these primers would amplify sequences from other related species, although we found no amplification in aphids or coccinellids. If sequences are amplified from other species, the size of the sequence can be expected to differ from the sequence that is amplified from *O. nubilalis* due to the large variation in sequence and length of the ITS-1 sequence (Marçon *et al*. 1999).

For two potential prey species, green peach aphid and corn earworm, we have shown that our primers do not amplify DNA. Aphids constitute a large portion of the diet of coccinellids in the field (Triltsch 1997), and corn earworm eggs are also a known prey of *Coleomegilla maculata* (Cottrell & Yeargan 1998a, 1998b). For southwestern cornborer, a species that is closely related to the European cornborer, only two of the primer combinations amplified a DNA sequence. The sequences that were amplified were both much larger than any of the sequences that were amplified from European cornborer. Therefore southwestern cornborer could be easily distinguished from European cornborer if it were present in the gut of *C. maculata*. Furthermore, because the sequences that are amplified in this species are long, they would probably not be present in the gut for a long period after feeding. Before using this method in the field, it is however, useful to test additional potential prey species that occur in the area of interest.

Use of PCR to estimate predation rate in the field

Agustí *et al*. (1999a; 2000), Zaidi *et al*. (1999), and Chen *et al*. (2000) used PCR to detect prey remains in the gut of predators. In both studies, Agustí *et al*. (1999a, 2000) used SCARs from a RAPD band. SCAR primers for the focus prey was chosen by screening this and other potential prey species, as well as the predator, with several random primers. The bands that were present only in the species of interest were chosen, reamplified, cloned and sequenced to develop the species-specific SCAR primers. A cloning step is necessary because bands that are produced by RAPD primers may actually not be single bands, and cloning will determine if an observed band consists of one or multiple sequences (N. Agustí, personal communication).

Agustí *et al*. found that a longer sequence could be detected for a shorter time than a shorter sequence. They did not attempt to determine exactly for how long each sequence could be amplified. They froze predators either at 0 or at 4 h after feeding, and found that the shorter sequence was amplified from both treatments and the longer sequence only from the first.

Zaidi *et al*. (1999) used mosquito larvae that were fed to carabid beetles. They amplified two sequences of different lengths from multiple-copy esterase genes that are present in the genome of the mosquito. The prey they used would not be fed on by carabids in a natural environment, but it was used because the primers were already available. Zaidi *et al*. argued that multiple-copy genes are more likely to be detected in a gut content analysis using PCR than are single-copy genes. They amplified DNA from carabids fed on two different strains of mosquitoes, and found that detection times for both sequences differed between strains. They also found that the shorter sequence could be detected for at least 28 h after feeding. As in our study, no effect was found of meal size on detectability.

Chen *et al*. (2000) used a COII sequence, a mitochondrial gene that is present in multiple copies in each cell, to develop primers for six different species of aphids. Using PCR, they were able to identify these different species in the guts of predators. The primer pairs they used were designed to amplify fragments between 77 and 386 bp. They found an effect of sequence length on detectability over time in the lacewing, but not in the coccinellid predator they used. They attribute this finding to the narrow range of sequences that was amplified. However, the range of sequences that was amplified in our study is comparable to the range of sequences used by Chen *et al*. (2000).

In addition to detecting prey DNA as in the previous studies, we tried to estimate time since feeding by using a set of fragments that can be detected for different lengths of time. This method can be applied as a tool for estimating predation rate for field-caught predators. By amplifying the shortest DNA sequence, which is known to be detectable for up to 12 h, a first estimate can be given of the number of predators that had a meal in that period of time. The next shortest sequence, that we found to amplify up to 5 h after a meal, can then be used to give a more accurate estimate of the time since feeding. Sequences increasing in length can be used, until the fragment is no longer detectable. A minimum and a maximum time since feeding can thus be estimated.

We expect that the average time of digestion will often be lower in the field than under our laboratory conditions. Predators may encounter partially eaten egg masses or be partially satiated and therefore ingest smaller amounts of prey material than in the laboratory. Also, in our experiment we used mature eggs that contained fully formed embryos. Younger eggs may contain less DNA by virtue of their earlier developmental stage. For these reasons, the time-values that we assigned to particular combinations of bands in the laboratory may overestimate digestion time in the field.

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