

Rapid screening of invertebrate predators for multiple prey DNA targets

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Abstract

DNA-based techniques are providing valuable new approaches to tracking predator–prey interactions. The gut contents of invertebrate predators can be analysed using species-specific primers to amplify prey DNA to confirm trophic links. The problem is that each predator needs to be analysed with primers for the tens of potential prey available at a field site, even though the mean number of species detected in each gut may be as few as one or two. Conducting all these PCRs (polymerase chain reactions) is a lengthy process, and effectively precludes the analysis of the hundreds of predators that might be required for a meaningful ecological study. We report a rapid, more sensitive and practical approach. Multiplex PCRs, incorporating fluorescent markers, were found to be effective at amplifying degraded DNA from predators' guts and could amplify mitochondrial DNA fragments from 10+ species simultaneously without 'drop outs'. The combined PCR products were then separated by size on polyacrylamide gels on an ABI377 sequencer. New primers to detect the remains of aphids, earthworms, weevils and molluscs in the guts of carabid predators were developed and characterized. The multiplex-sequencer approach was then applied to field-caught beetles, some of which contained DNA from as many as four different prey at once. The main prey detected in the beetles proved to be earthworms and molluscs, although aphids and weevils were also consumed. The potential of this system for use in food-web research is discussed.

Keywords: Aphid, earthworm, mollusc, multiplex PCR, prey biodiversity, *Pterostichus melanarius*

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Introduction

Molecular analyses of predator–prey interactions provide a range of new opportunities for addressing previously intractable ecological questions (see Symondson 2002). This applies particularly to invertebrate systems where direct observation is usually impossible because the organisms

involved are, for example, small, cryptic, subterranean, operating under a closed canopy of vegetation, nocturnal, aquatic or possess a combination of these characteristics. The main advantage of *post-mortem* analyses of the gut contents of predators is that, up until the point when the predator is captured, the ecosystem under study is undisturbed (Sunderland 1988). With some predators it is possible to simply identify hard, undigested remains in the gut (Ingerson-Mahar 2002). However, this inevitably misses many trophic links (Dennison & Hodkinson 1983), particularly the remains of prey lacking hard parts such as slugs, and the technique cannot be used on the majority of invertebrate predators which are primarily fluid feeders.

A polymerase chain reaction (PCR)-based approach can overcome such difficulties (Symondson 2002), having the

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major advantage that it, unlike monoclonal antibody-based techniques, can be used, potentially, to study the prey range of predators, and not simply the range of predators feeding on a target prey. The problem, which this study addresses, is that each predator must be assayed for each prey that may or, in the majority of instances, may not have been eaten. In an environment containing, for example, 40+ potential prey, DNA from the guts of the predators would need to undergo PCR in 40 separate reactions (using a range of PCR conditions), even though the mean number of prey remains in the guts of such predators may be just one or two. All 40 PCR products would have to be assayed on agarose gels in a lengthy process. It would, in consequence, not be practical to screen the hundreds of predators that may be needed for a meaningful ecological study.

The aim of this work was to develop and test a PCR-based rapid screening system capable of being used to study the responses of generalist predators to prey diversity in the field and dynamic changes in prey numbers and relative density over time. Such a PCR approach could expand the potential of molecular detection into areas such as food-web research, which is currently limited by a lack of suitable techniques for integrating generalist predators into food webs. To date, the only published invertebrate gut content studies to have used PCR to measure predation on naturally occurring prey populations directly in the field have been those of Agustí *et al.* (2003a), who studied prey choice by spiders feeding on three species of Collembola and Dodd *et al.* (2003), who studied carabids feeding on slugs in arable crops. In both cases the number of prey targets was small and therefore conducting separate PCRs for each prey was feasible. Such screening is equivalent to field studies in which prey are analysed using two or more prey-specific monoclonal antibodies (e.g. Hagler & Naranjo 1994; Dodd 2004).

The technique described here uses a single multiplex PCR, in which DNA from a range of prey species can be amplified simultaneously. The use of fluorescently labelled PCR primers enables, in theory, simultaneous detection of all targets with a highly sensitive DNA sequencer-based detection system. The method is a modification of a technique pioneered in the field of population biology, to score size variation in VNTR markers (e.g. Galan *et al.* 2004; Toonen *et al.* 2004; Vaughan & Russel 2004). While multiplexing has been around for some time (Luikart *et al.* 1999), the incorporation and optimizations of numerous primer sets in a single PCR have been made much simpler recently by the development of multiplex kits. We wished to establish whether this approach could be used to rapidly and accurately amplify and detect multiple prey species in the guts of field-caught predators. Although multiplexing has been used to separate species (Hare *et al.* 2000; Hinomoto *et al.* 2004), its ability to do so when applied to the degraded,

semidigested DNA extracted from the guts or faeces of predators was untested. Before doing so we needed to develop and characterize a range of new species- and group-specific markers for prey encountered by carabid beetles on an arable field site.

To analyse the gut contents of predators, PCR primers should ideally target short sequences of multiple-copy DNA (Symondson 2002). For this study we therefore designed primers targeting the cytochrome *c* oxidase I and *12S* genes that amplified DNA fragments < 300 bp. We needed to test whether, using multiplex PCR, such markers would be able to detect degraded prey DNA in predator gut samples for extended periods following ingestion without preferential amplification or drop out of prey targets, and to validate the system by applying it to a subset of predators collected from the field.

Materials and methods

DNA extraction

DNA was extracted from a range of relevant invertebrate species from our field site at IACR-Long Ashton Research Station, Bristol, including earthworms (*Aporrectodea caliginosa*, *Aporrectodea longa*, *Aporrectodea rosea*, *Allolobophora chlorotica*, *Lumbricus castaneus*, *Lumbricus festivus*, *Lumbricus rubellus*, *Lumbricus terrestris* and *Octolasion cyaneum*), aphids (*Aphis fabae*, *Myzus persicae*, *Megoura viciae*, *Metopolophium dirhodum*, *Rhopalosiphum padi* and *Sitobium avenae*), molluscs (*Arion distinctus*, *Arion hortensis*, *Arion intermedius*, *Candidula intersepta*, *Deroceras reticulatum* and *Vallonia pulchella*) and a weevil (*Sitona* sp.). Prior to DNA extraction, the earthworms were starved for 48 h to allow soil to pass through their gut. DNA was extracted using either a salt precipitation method modified by Collins *et al.* (1987) from Livak (1984) or a DNeasy Tissue Kit (QIAGEN), following the manufacturer's instructions. DNA extracted was diluted 1 : 9 in ultra pure H₂O before PCR amplification. DNA was extracted from the guts of beetles used in feeding trials (see below) and field-caught beetles using a modification of the DNeasy Kit method. Beetles were thawed to room temperature and the foregut was removed then weighed and homogenized in a 1 : 19 w:v ratio with 1 × phosphate buffered saline (PBS) pH 7.4. The homogenate was then centrifuged at 8000 g for 15 min at room temperature and then the majority of the supernatant was transferred to a clean 1.5 mL Eppendorf tube and stored at -20 °C for subsequent analysis using enzyme-linked immunosorbent assay (ELISA). Analysis of these samples using monoclonal antibodies targeting slugs is reported elsewhere (Dodd *et al.* 2003; Dodd 2004). The particulate remains and up to 80 µL of the supernatant were retained for DNA extraction using the QIAGEN DNA Mini Kit in accordance with manufacturer's instructions.

PCR screening and primer design

'Universal' primers are available for the amplification of mitochondrial DNA (mtDNA) genes from almost any invertebrate (Simon *et al.* 1994; Folmer *et al.* 1994), facilitating the screening of suitable regions from both predator and prey species from which prey-specific primers can be designed. These primers were employed to characterize the 12S rRNA region of earthworms and molluscs (SR-J-14233 and SR-N-14588, Simon *et al.* 1994) and the COI gene of aphids and the weevil [C1-J-1718 (or Mt-6 designed for phytophagous beetles) and C1-N-2191, Simon *et al.* 1994]. Each PCR was carried out in 25 µL, containing 50–100 ng of template DNA; 1 U *Taq* polymerase (Invitrogen); 0.5 µM of each primer; 20 mM (NH₄)₂SO₄; 75 mM Tris-HCl, pH 8.8; 0.01% (v/v) Tween 20; 2 mM MgCl₂; 0.2 mM dNTPs (ABgene). The PCRs were carried out in a GeneAmp 9700 thermocycler (Applied Biosystems). For the earthworms, the PCR cycling conditions used were 94 °C for 4 min followed by 30 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 75 s and a final cycle of 72 °C for 10 min. For the aphids conditions were 94 °C for 4 min followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 75 s and a final cycle of 72 °C for 10 min. Products were purified using a Turbo GeneClean Kit (Q-BIOgene), following the manufacturer's instructions. Sequencing reactions were carried out via the manufacturer's instructions using Big-Dye Terminator mix, version 3 (PE-Applied Biosystems) in both forward and reverse orientations.

Sequences were aligned using both SEQUENCHER and CLUSTAL, and primers designed either manually or using AMPLICON software (Jarman 2004). General group-specific primers were developed for earthworms and aphids. Specific primers were designed for *Sitona* sp., *M. persicae*, *A. fabae*, *M. viciae*, *C. intersepta* and *V. pulchella*. Previously described primers were used for the molluscs *Arion* spp. and *D. reticulatum* (Dodd 2004) and some aphids (*R. padi* and *S. avenae*, Chen *et al.* 2000). One primer in each pair (usually forward) was labelled with either 6-FAM, HEX or TET (Table 1). All of the primers were empirically tested for cross-reactivity with DNA from a broad range of other potential prey species. The robustness of the multiplex system was also tested to assess whether, when combinations of targets were coamplified, any amplicon 'drop out' occurred. This was tested by mixing DNA from target species in a range of combinations, including mixing all targeted species and coamplifying them in single PCR.

Feeding trials

Separate feeding trials were performed using the ground beetle *Pterostichus melanarius* and four different prey species (the aphid *S. avenae*, the earthworm *A. chlorotica*, and the slugs *A. hortensis* and *D. reticulatum*). *P. melanarius* were

obtained by pitfall trapping and maintained individually under controlled conditions (16 °C and a 16 : 8 h L:D cycle) in perforated plastic pots (9 × 6 cm) containing c. 2 cm of sphagnum moss peat. They were fed weekly on blowfly larvae (*Calliphora vomitoria*). Those selected for feeding trials were starved for 14 days prior to the start of the experiment. Beetles were transferred to 9 × 1.5 cm Petri dishes containing moistened filter paper and allowed to feed *ad libitum* on the respective prey for two hours. These were freshly killed *S. avenae*, *A. chlorotica* or slugs (*D. reticulatum* and *A. hortensis*). For earthworms and slugs there was no restriction on the quantity that could be eaten during this period. However, for the aphid feeding trial the beetles were provided with just three early instar *S. avenae*. Only beetles that were observed to feed were retained. After this time, all beetles were placed in clean plastic pots. At set time periods beetles were removed and killed by freezing at –80 °C. The total length of time post feeding was established from preliminary feeding trials and was generally for 48 h, but extra intervals were added for earthworms (72 and 96 h) and aphids (72 h). Thus, for example, from the end of the feeding period, batches of beetles fed on earthworms were killed after 2, 4, 8, 16, 24, 32, 48, 72 and 96 h. As the beetles could have ingested the slug meals at any point during the two-hour feeding period, the midpoint (after 1 h) was considered as the mean time of consumption and therefore 1 h has been added to each time interval in the subsequent regression analyses (Symondson & Liddell 1996). Samples of five to 10 beetles were assayed for each time period. Each trial incorporated negative controls (beetles starved for at least 2 weeks).

Linear regression analysis was used to determine the rate of decay within the guts of the beetles of the earthworm, *S. avenae*, *A. hortensis* and *D. reticulatum* amplicons. Detection half-lives (T_{50}) were calculated from the regression equations. For the *D. reticulatum* feeding trial, analysis of covariance (ANCOVA) was used to determine the relationship between decay rates and provision of alternative prey. Data were transformed as necessary to log_e and square roots.

Field samples

As well as controlled feeding experiments, the multiplex system was used to analyse the gut contents of 50 *P. melanarius*, caught by dry pitfall trapping from a crop of field beans at IACR-Long Ashton, Bristol during June–September 2001. This field was the site of a long-term field study of the effects of cultivation and straw disposal techniques on invertebrate populations (Kendall *et al.* 1995). Detailed studies have been made of interactions between *P. melanarius* and slugs at the same site (Symondson *et al.* 1996, 2002). A description of the long-term field treatments and trapping protocol can be found in Dodd (2004). Mesh inserts were used in the traps to reduce predation of

Table 1 Details of primer sequences (5'–3'), fluorescent labels and expected product size for primers used in multiplex analyses

Species	Name	Forward primer	Name	Reverse primer	Label	Size	References/GenBank Accession nos
Earthworms	185F†	TGTGTACTGCCGTCGTAAGCA	14233R	AAGAGCGACGGCGATGTGT	FAM	225–236	See below (note b)
<i>Sitona</i> sp.	W139F	AGCAAATATCGCACATGAAGG	W289R‡	AAGAGGTGTCCGATCAAAGG	TET	151	AJ865012
<i>Megoura viciae</i> *	MvF	AGGATCTCCTCCTCCTGCTG	MvR‡	TCAAATTCACCTTTTCCATGA	FAM	148	AJ865011
<i>Sitobion avenae</i> *	EgaCOII‡	TATTTGAACTACAACCTCCTC	EgaCOIIR1	AGTTTTATGTCTACTTCAATTAAA	TET	231	Chen <i>et al.</i> (2000)
<i>Metopolophium</i>	BCoACOII F3‡	TTCGACTCTTAATTCATCA	BCoACOII R1	GGATTGCATCAATTTTAATAGCTAAA	TET	78	Chen <i>et al.</i> (2000)
<i>Dirhodum/Rhopalosiphum padi</i>							
<i>Myzus persicae</i>	MpF	TTGATACAAGATTGGGTCACCT	MpR‡	CCAAATCCCTTTATTTCCATG	TET	160	AJ865009
<i>Aphis fabae</i>	Af-F‡	GAATTGGATCTCCTCCTCCTG	Af-R	GAGCAATTAATTTTGTGTGCACT	HEX	212	AJ865010
<i>Vallonia pulcinella</i>	VpF‡	CCATGAGCTCTCAACATGAG	VpR2	CATGAATATTAGTTTGCACCTTG	FAM	117	AJ865014
<i>Deroceras reticulatum</i>	Dr11F‡	CTATACACAATTTTAAATAAG	DRF29RC	GCTTCTGGTTTATCTATATTTGGT	FAM	109	Dodd (2004) AY423668
<i>Candidula intersepta</i>	Ci1F‡	GTCATGGCGGTAACCTTTAA	CiAR	CGTCTGCCGACGACGGCATAAC	FAM	137	AJ865013
<i>Arion</i> sp.							
<i>A. hortensis</i>	Ai1F‡	CACATAAATGATAGTCACC	AR2R	ATACTTACAAGTCCATCTTT	FAM	208–221	Dodd (2004) 208 AY423632
<i>A. intermedius</i>						216	AY423667
<i>A. distinctus</i>						221	AY423654
Aphid	Aph14F‡	AATCAAATAAATGTTGATA	Aph236R	TCAATTTTAGGRGCAATTAA	TET	242	

*Primers for *Megoura viciae* and *Sitobion avenae* not included in multiplex (see text); †GenBank Accession nos for individual species of earthworm were: *Lumbricus castaneus* AJ865001, *Lumbricus rubellus* AJ865002, *Lumbricus festivus* AJ865008, *Apporectodia longa* AJ865003, *Apporectodia caliginosa* AJ865004, *Apporectodia rosea* AJ865005, *Octolasion cyanium* AJ865006, *Allolobophora chlorotica* AJ865007; ‡Fluorescently labelled primers.

smaller invertebrates (aphids) by beetles within the traps and the traps painted with Fluon (Whitford Plastics) to prevent escape. Traps were set in the evening and the beetles collected early the following morning. All beetles were rapidly frozen and stored at -80°C until ready for dissection (as described above). The field-collected beetles were analysed to test the ability of the multiplex system to detect predation on the targeted range of invertebrates. Ecological data were also obtained from the field site, where there was detailed monitoring of invertebrate densities over 2 years. The data from this complex long-term field experiment, along with the results of analyses of many hundreds of beetles using the multiplex approach over 2 years, will be published elsewhere, along with full details of the cropping system and other biotic and abiotic variables.

PCR analysis

A single multiplex PCR was optimized to amplify 12 of the 14 invertebrate-specific amplicons [general earthworm, *M. persicae*, *A. fabae*, *M. dirhodum*/*R. padi*, general *Arion* (three species), *C. intersepta*, *V. pulchella*, *D. reticulatum* and *Sitona* sp.]. Amplifications were performed in $6.25\ \mu\text{L}$, containing $0.75\ \mu\text{L}$ of extracted gut DNA, $1\times$ Multiplex PCR master mix (QIAGEN) and $0.2\ \mu\text{M}$ of each of the primers. PCR cycling conditions were 95°C for 15 min followed by 40 cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 90 s, and a final cycle of 72°C for 10 min. Primers for the aphids *S. avenae* and *M. viciae* could not be integrated into the multiplex. The *S. avenae* amplicon migrates in parallel with the earthworm amplicon, and could not be reliably scored. This was because the very strong signal from the blue FAM-labelled earthworm amplicons caused 'pull up' of the green TET label on the *S. avenae* amplicon. Primers for *M. viciae* showed some cross reactions with other aphid species using a T_a of 57°C . These were therefore amplified individually in $6.25\ \mu\text{L}$ consisting of $0.75\ \mu\text{L}$ of extracted DNA, 20 mM $(\text{NH}_4)_2\text{SO}_4$; 75 mM Tris-HCl, pH 8.8; 0.01% (v/v) Tween 20; 2 mM MgCl_2 ; 0.2 mM dNTPs (ABgene), 1 Unit Taq polymerase (Invitrogen), and $0.5\ \mu\text{M}$ of each primer. PCR was carried out using the following protocol: 95°C for 5 min followed by 35 cycles of 94°C for 45 s, T_a for 45 s, 72°C for 75 s, and a final cycle of 72°C for 10 min (T_a : 60°C for *S. avenae* and 65°C for *M. viciae*). Known positive controls were also run alongside the samples being tested to ensure the results were not biased by PCR failure. PCR products ($1\ \mu\text{L}$) were mixed with $2.5\ \mu\text{L}$ of gel loading mix [66.4% formamide (Sigma), 17.6% loading buffer and 16% GENESCAN-350 (TAMRA) size standard (both Applied Biosystems)] and denatured at 90°C for 2 min. Aliquots ($0.8\ \mu\text{L}$) were separated on 5% denaturing polyacrylamide gels, using 36 cm well-to-read gel plates, on an ABI Prism 377 DNA sequencer (Applied Biosystems) running GENESCAN software. Electropherograms were analysed and scored

using GENOTYPER version 2.5 (Applied Biosystems); all samples generating >100 fluorescent units were deemed to be positive. Because commercially available internal standards do not ordinarily allow safe discrimination of fragments varying in size by a single base pair (Dupuy & Olaisen 1997), accurate scoring of earthworm amplicon was achieved by running an allelic ladder in two lanes on each gel. This ladder was created using four earthworm amplicons of known size from (227, 230, 233 and 236 bp) and sequence, then the electropherograms were used as a floating window within GENOTYPER to reliably score amplified earthworm DNA.

Results

New primers

Sequences, fluorescent labels and expected product sizes (bp) for each set of primers employed in this study are listed in Table 1. The majority of primers were species specific, the exceptions being the *Rhopalosiphum padi*/*Metopolophium dirhodum* primer pair, those for the *Arion* group of slugs and for the earthworms. To score both *R. padi* and *M. dirhodum*, a single primer pair was used to amplify a 78 bp product from both species, thus they were inseparable by molecular means. In the case of the *Arion* spp. primers, insertions and deletions within the 12S gene ensured that there were species-specific size differences, such that the three commonest species from the field could be identified using only a single pair of primers (Dodd 2004). Similarly, the earthworm amplicon displayed size polymorphism among individuals. However, unlike the *Arion* group, this variation was not species-specific, and variation occurred both within and between earthworm species, revealing a total of 12 different sized amplicons at our field site (range 225–236 bp). As a consequence, amplicon size, rather than species, was used as the unit of earthworm diversity.

All primers were tested for cross-amplification of DNA from other potential prey species, including all those species for which we were developing primers plus representatives of other groups (e.g. Diptera) and the carabids themselves. In all cases, primers were found to be specific to the species or group for which they were designed. When the multiplex was simultaneously tested against multiple species in a range of combinations, the results consistently showed no preferential amplification, or amplicon dropout, and the system identified all targets.

Decay rate experiments

Regression analyses showed that the detection periods and rates of decay of DNA from four different prey within the guts of *Pterostichus melanarius* varied considerably. Data were transformed as necessary to maximize R^2 values

and hence predictive power. The data from the *Deroceras reticulatum* feeding trial appeared to show that subsequently feeding beetles with nontarget prey prolonged the detection period. The regression equations for proportions testing positive against time (h) were $\log_e y = 4.74 - 0.0315x$ (R^2 83.4%, $P < 0.001$) when fed slugs only and $\log_e y = 4.82 - 0.0255x$ (R^2 83.4%, $P < 0.001$) when refed with alternative prey. The calculated detection half-life (T_{50}) for beetles not fed alternative prey was 26.3 h, but when refed on alternative prey it was 35.6 h. However, comparison of these regression lines using analysis of covariance showed that neither the slopes nor the y axis intercepts were significantly different ($P > 0.05$). Regression equations for the other three feeding trials were: the slugs *Arion hortensis* ($\log_e y = 4.6611 - 0.0242x$, R^2 57%, $P < 0.001$), the earthworms (*Allolobophora chlorotica*) ($y = 131.26 - 0.9181x$, R^2 95%, $P < 0.001$) and the aphids *Sitobium avenae* (square root $y = 9.7344 - 0.8552$ square root x , R^2 75%, $P < 0.001$). Earthworm DNA was detectable in the guts of *P. melanarius* for an extended period, with a T_{50} of 88.5 h for *A. chlorotica*. The T_{50} for the other slug in the feeding trials, *A. hortensis*, was very similar to that for *D. reticulatum* at 31.0 h. For the aphid *S. avenae* T_{50} was much shorter, at 9.7 h, probably reflecting the smaller quantities consumed. To optimize the fit, regression analyses excluded all of the short-time periods (except the last) that gave 100% detection.

Field-caught beetles

The multiplex detection system was also tested on 50 field-caught *P. melanarius*. The aim for this techniques study was primarily to demonstrate the ability of the multiplex system to detect multiple prey in field-collected beetles. These were randomly selected from the much larger number of beetles currently being analysed from the field experiment (the full results, when available, will be published elsewhere in a study comparing prey remains in beetles guts with dynamic changes in prey densities over 2 years). Of the 50 beetles analysed, prey remains were identified in 80%. Ten beetles gave no amplification for any of the 14 prey species for which we screened. Between one and four prey species were amplified from individuals among the remaining beetles, with the majority (48%) having consumed a single prey species. A total of seven prey species were found in the 50 beetles screened (Fig. 1). Twenty beetles tested positive for earthworm, with two testing positive for two earthworm amplicons. Of these 20 earthworm positives, six of the 12 characterized amplicons were identified (see Fig. 2).

Discussion

These results demonstrate the significant benefits of the multiplex approach over existing methods of prey detection.

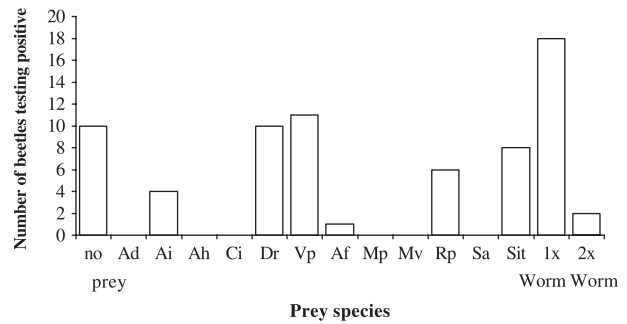


Fig. 1 Number of *Pterostichus melanarius*, from a random sample of 50 field-caught beetles, testing positive for each prey species. Abbreviation for prey are: Ad – *Arion distinctus*, Ah – *Arion hortensis*, Ai – *Arion intermedius*, Ci – *Candidula intersecta*, Dr – *Deroceras reticulatum*, Vp – *Vallonia pulchella*, Af – *Aphis fabae*, Mp – *Myzus persicae*, Mv – *Megoura viciae*, Rp/Md – *Rhopalosiphum padi*/*Metopolophium dirhodum*, Sa – *Sitobium avenae*, Sit – *Sitona* sp. and worm – earthworm. 1x worm denotes beetles testing positive for a single earthworm amplicon, 2x worm denotes those testing positive for two earthworm amplicons.

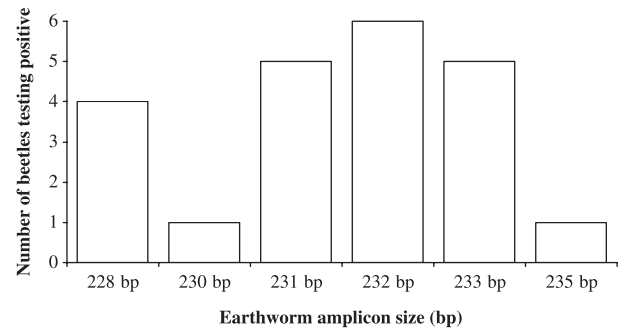


Fig. 2 Number of field-caught beetles testing positive for each of six earthworm amplicon sizes.

To date, most DNA-based predation studies have used prey-specific PCRs and agarose gel electrophoresis to identify single prey items from predators, although Dodd *et al.* (2003) and Dodd (2004) used a singleplex fluorescently-labelled PCR approach to identify slug remains within the guts of carabid predators. In order to apply this to a generalist predator such as *Pterostichus melanarius*, where multiple prey items could be present in the gut, a lengthy process of multiple PCRs and agarose gel electrophoresis would be required. However, the approach described here obviates this by using a single multiplex PCR, where targets for numerous species can be amplified simultaneously. The use of fluorescent markers has the important additional advantage of improving sensitivity. It was found that once DNA had been extracted from the predator gut samples, a single operative could multiplex 3×96 samples per day per PCR machine with a single 96-well block, and then run 192 samples per day through an ABI377 sequencer. Software then enabled all the positive peaks for

each sample (above 100 fluorescent units) to be recorded in a database. Where PCR conditions for a particular primer pair cannot be incorporated in the multiplex with the rest of the primers, the PCR products can still be pooled and run through the sequencer together (Dodd 2004). The time needed to screen each predator for multiple targets is, in consequence, much faster than with any other currently available system, including ELISA using multiple monoclonal antibodies.

No loss of sensitivity was found at any stage, and indeed the extreme length of the detection period for prey DNA following ingestion by the predators (40% still positive for earthworm DNA after 96 h digestion) is considerably longer than that recorded in any previous study. Comparable studies using invertebrate predators were able to detect prey DNA in 50% of predators after 5 h (Asahida *et al.* 1997), 45% after 4 h (Agustí *et al.* 1999), 100% after 28 h (Zaidi *et al.* 1999), 50% after 9 h (Chen *et al.* 2000), 50% after 10 h (Hoogendoorn & Heimpel 2001), 55% after 24 h (Agustí *et al.* 2003b), 100% after 24 h (Agustí *et al.* 2003a) and up to 100% after 24 h (Sheppard *et al.* 2004). Although in the current experiment detection periods for aphids and slugs were shorter than those for earthworms, they were still comparable with those achieved in earlier predation studies.

The sequences in the mtDNA genome that were suitable for designing specific primers proved to be interesting. Choice of mitochondrial region will depend on the levels of variation found within and between the potential prey species to be analysed. Simon *et al.* (1994) found that the evolutionary rates for the third domain of the 12S rRNA (the region utilized here for earthworms and molluscs) were different depending on the taxa examined. They found that the vast majority of sites in Domain III of *Drosophila* where apparently invariant. For earthworms and molluscs we found the opposite, with high levels of variation present between species. For molluscs, levels of variation were higher for 12S than for COI (Dodd 2004). For the *Arion* group of slugs a number of species-specific indels were present in the 12S gene that allowed the three commonest *Arion* species to be identified using a single primer pair. The 12S rRNA region was chosen for the earthworms for a number of reasons. Attempts to use universal COI primers resulted in amplification of only a subset of the species targeted, implying high levels of interspecific variation at this gene. Sequence data for the 12S rRNA gene, particularly at the loop domains, showed high levels of sequence diversity among, and in some cases within, species. This variability meant potential species-specific primers in loop domains would be subject to low selection pressure, raising the likelihood of mutations that would result in false negatives. Therefore, a single primer pair was developed within a stem domain of the gene that consistently amplified DNA from all earthworms tested. The sequence heterogeneity was then used as a measure of earthworm

diversity, whereby a series of indels resulted in a total of 12 different sized amplicons (range 225–236 bp). Few of these amplicon sizes proved to be specific to a single earthworm species. As a consequence, amplicon size, rather than species, was used as the unit of earthworm diversity. Though not ideal, this approach had the additional benefit that earthworms that had not been characterized would also be amplified. For the other invertebrates, the COI gene provided enough variation that species-specific primers could be developed.

The only other amplicon that coamplified another species was that for the aphids *Rhopalosiphum padi* and *Metopolophium dirhodum* (Chen *et al.* 2000). Attempts were made to design species-specific primers for these two aphids, but insufficient sequence heterogeneity existed for cross amplification to be excluded. Should significant numbers of these aphid species be found together (fortunately not the case in our field study) then new primers would have to be designed.

Although the full results of the long-term field study over two years will be published elsewhere, the field data for the 50 samples reported here were revealing. Prey were identified in 80% of the predators. Overall, 48% contained the remains of a single prey species but up to four separate prey items were identified within some individual gut samples. Among the 50 beetles, seven prey DNA targets were identified. Forty percent of beetles had consumed one or more of six identified earthworm amplicons, but the long detection period for earthworms would be expected to lead to high numbers of positives, not necessarily making these the most important prey. An earlier study on another field site at Long Ashton Research Station found that 36% of *P. melanarius* contained earthworm proteins, identified using an earthworm-specific monoclonal antibody (Symondson *et al.* 2000). Next to earthworms molluscs proved to be the main prey detected, especially *Deroceras reticulatum* and *Vallonia pulchella*, confirming that these beetles are significant mollusc predators (reviewed in Symondson 2004). The remaining prey detected were aphids and weevils. The former are eaten by *P. melanarius* when they fall from the crop to the ground (e.g. Sunderland & Vickerman 1980; Sunderland *et al.* 1987), although this carabid has been observed to climb up to aphid colonies in the laboratory (Snyder & Ives 2001). *Sitona* weevils were consumed by 16% of beetles, suggesting that they too were a relatively important prey item in bean crops. Prey choice, however, can only be fully assessed in relation to relative and absolute prey densities, which change over time. We cannot preclude the possibility that some prey were consumed as a result of scavenging or secondary predation. Earlier work using antibodies suggests that the latter may be an insignificant source of error (Harwood *et al.* 2001), but the former could generate false positives (Calder *et al.* 2005).

At this stage in the development of molecular markers for ecological studies, a major proportion of research effort has to go into the sequencing of prey genes and the design of primers. However, as GenBank and other databases grow these markers will be widely available. At that point researchers will be able to simply survey the fauna at their field sites, order the appropriate primers and start multiplexing and screening on a sequencer. We predict that this approach will prove to be the method of choice in future studies but may be superseded eventually by microarray technologies.

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