

DESIGN AND ASSESSMENT OF PRIMERS TO DETECT INSECT PESTS OF *BRASSICA* CROPS IN THE GUT CONTENTS OF ARTHROPOD PREDATORS

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ABSTRACT: Techniques based on the polymerase chain reaction (PCR) have been shown as powerful tools for ecological studies of predator-prey interactions. Species-specific primers, which are designed to amplify target prey DNA from the gut contents of generalist predators, can potentially be used to develop highly specific and sensitive assays. We developed species-specific primers from the cytochrome oxidase subunit I (COI) gene for six insect pests of *Brassica* crops (*Plutella xylostella*, *Pieris rapae*, *Hellula hydralis*, *Helicoverpa punctigera*, *Brevicoryne brassicae*, and *Myzus persicae*). Specificity tests confirmed each primer pairs specifically amplifies prey DNA without cross-reactivity to predators or other non-target species, which are commonly found in the same habitats. These molecular markers also allow amplification of a very small amount of target DNA in the presence of substantially greater amounts of predator DNA. Although multiplexing of primers could potentially be used to detect the presence of multiple prey species in a single assay, the sensitivity of it compare with singleplex PCR was lower. Here we show that these primers are specific and sensitive and can be applied in ecological studies of predator-prey interactions in the field.

KEY WORDS: Molecular markers, *Brassica* pests, PCR, cytochrome oxidase subunit I, predation.

One of the most difficult aspects of studying invertebrate predators is assessing their feeding behaviour under natural conditions. Knowledge of prey items and rates of predation are important in determining the effects of predators on prey populations and are especially critical in evaluating the effectiveness of a predator as a biological control agent (Hayes & Lockley, 1990). Unfortunately, detection of predation is technically difficult because prey and predator are relatively small and cryptic in most cases (Greenstone, 1996; Naranjo & Hagler, 1998). In order to overcome these difficulties, biochemical techniques have been developed and used to identify prey species in predators' diets. Monoclonal and polyclonal antibodies (Symondson, 2002) and enzyme-electrophoresis (Traugott, 2003) can be used to determine which prey has been consumed by a predator. However, amplification of specific prey DNA using the polymerase chain reaction has proven to be more specific and sensitive in detecting prey remains. It can be used to specifically detect small amounts of prey DNA in the gut contents of invertebrate predators (Hoogendoorn & Heimpel, 2001; Symondson, 2002; Agusti et al., 2003a; Harper et al., 2005; Juen & Traugott, 2005).

PCR-based techniques are rapidly replacing other molecular techniques because molecular biology facilities are widely available and prey-specific primers

can be used in different contexts once they have been designed. This technique has been successfully used for detection of a variety of prey remains in predators' gut contents (Zaidi et al., 1999; Chen et al., 2000; Hoogendoorn & Heimpel, 2001; Agusti et al., 2003a; Agusti et al., 2003b; Harper et al., 2005; Juen & Traugott, 2005; Read et al., 2006; Hosseini et al., 2006b, 2008).

Brassica vegetables and Oilseeds are economically important crops; approximately 3.1 and 26.1 million ha respectively were grown worldwide in 2004 (Food and Agriculture Organization of the United Nations, 2007). These crops are associated several destructive and widespread insect pests. Total damage caused by these pests is substantial; for example management costs for *P. xylostella* alone were estimated at US\$ 1 billion annually in 1997 (Shelton et al., 1997). In Australia, the pests of *Brassica* crops include the lepidopterans *Plutella xylostella* (L.), *Pieris rapae* (L.), *Hellula hydralis* Guenee and *Helicoverpa punctigera* Wallengren, and two aphids, *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer). Integrated pest management (IPM) systems and the use of biological control methods are preferred approaches to controlling these pests over insecticides due to the prevalence of insecticide resistance in diamondback moth (Shelton et al., 1997).

Generalist predators can play a major role in the control of agricultural pests (Symondson et al., 2002). Studies showed *Brassica* crops have a rich fauna of predators. Shelton et al. (1983) reported on extensive ground-dwelling predators in cabbage fields in central New York State, and Schmaedick & Shelton (2000) have documented a list of predators associated with *P. rapae* in cabbage fields of New York State. Hosseini et al. (2006a) reported a range of predators associated with *Brassica* pests in South Australia. The impact of predators on pests of *Brassica* crops has not been thoroughly studied and therefore their potential in suppression of major pests of *Brassica* has not been elucidated. Hooks et al. (2003), found broccoli plants protected by birds and spiders as predators sustained less damage from caterpillars and the plants had greater productivity compared to control plants. In cage exclusion experiments, between 7% and 81% of immature stages of *P. xylostella* were lost due to predation (Wang et al., 2004). Subsequent extensive research (Furlong et al., 2004) confirmed estimated losses of *P. xylostella* due to predation of between 2%-85%. Hence, for this one insect, predators are known to cause considerable mortality at times. It is essential to develop a reliable technique to evaluate the diets of key predatory species in order to understand their role in suppressing pests like *P. xylostella*.

Ma et al. (2005) developed a species-specific marker for *P. xylostella* based on the internal transcribed spacer (ITS-1) of the ribosomal gene. This specific primer pair was used to detect prey in the gut contents of two polyphagous predators, *Nabis kinbergii* and *Trochosa exopolita* (reported as *Lycosa* sp.). In this pilot study of predation of diamondback moth on cauliflower and broccoli farms near Virginia, South Australia, the remains of *P. xylostella* were detected in the gut contents of both species of field-collected predators.

As a mitochondrial gene, it occurs as multiple copies in each cell (Hoy, 2003), which increases the likelihood of successful amplification of prey residues in the predators' gut contents. This gene is a protein-coding gene that has a high level of interspecific variability (Zhang & Hewitt, 1996), which allows closely related species to be separated. Therefore, in the current study, cytochrome oxidase subunit I (COI) was selected as a potentially diagnostic gene.

The aims of the present study were: 1) to develop species-specific primers for each of the six major pests of *Brassica* crops; 2) to test their specificity and sensitivity against non-target species and other common predators (A false

positive results can affect the interpretation of predation, especially when generalist predators can feed on a wide range of prey species (Sunderland, 1975); thus the specificity and sensitivity of each primer set are critical and have to be tested before any application in field studies); and 3) to test the potential of multiplexing of two primer pairs to assess for simultaneous detection of DNA from two different target prey species.

MATERIALS AND METHODS

Sample collection

Six species of crop pests of *Brassica* spp. and predators and non-predatory arthropods were collected from different farms in South Australia, but mainly from Pitchford's broccoli farm (Currency Creek, South Australia) during 2004 and 2005 [Table 1; e.g. *P. xylostella*, *P. rapae*, *H. hydralis*, *H. punctigera*, *B. brassicae*, *M. persicae*, *Nabis kinbergii* (Reuter), *Hippodamia variegata* (Goeze), *Trochosa exopolita* (L.Koch) and *Oechalia schellenbergii* (Guerin-Meneville)]. In addition, *P. xylostella* and *P. rapae* were obtained from a laboratory culture. Collection was done by hand, vacuum sampler or insect net for day active arthropods and with a headlamp at night for wolf spiders (Wallace, 1937). Identification of species (pests and predators) was done morphologically following the most current relevant taxonomic keys and confirmed by comparison to identified specimens in the Waite Insect and Nematode Collection (Waite Campus, The University of Adelaide, South Australia). Wolf spider samples were identified by Volker W. Framenau (Department of Terrestrial Invertebrates, Western Australian Museum, Perth, WA). Generally, collected samples were preserved either in 70% ethanol or stored at -20°C for future molecular work. Some live predators were kept individually in a plastic cup (7.5 cm diam. × 4.5 cm) provided with a piece of wet cotton wool in the laboratory at room temperature for subsequent feeding experiments.

DNA extraction

DNA of individual specimens was extracted from legs in order to avoid contamination with gut contents but DNA of predators used for feeding trails was extracted from the whole body. Each sample was homogenised in 1.5 ml microcentrifuge tubes using a clean and sterile plastic pestle in 400 µl homogenisation buffer (10mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1% SDS). After adding 5 µl proteinase K (20 µg/µl) (Sigma), samples were incubated at 56°C for 15 min. For removal of RNA, 0.5 µl RNase-A (10mg/ml) (Sigma) was added and the tube was incubated at 37°C for 15 min. Extraction was done once with Phenol/Chloroform. DNA was precipitated by adding two volumes of cold ethanol (kept at -20°C overnight) and 0.2 volume of 5 M sodium chloride and then stored at -20°C for 15 min. After centrifugation at 13000 g for 15 min, the pellet was washed with 70% ethanol and then dried at 65°C for 5 min. Pellets were resuspended in 200 µl of TE (10mM Tris-HCl pH 8.0, 1mM EDTA) and resulting DNA suspensions were stored at -20°C. DNA of six species of *Brassica* plants was extracted by the same Phenol/Chloroform method from the leaves (Table 1).

Preliminary studies showed DNA extracted by Phenol/Chloroform method from the whole bodies of spiders could not be reliably amplified by PCR. Therefore DNA from spiders was extracted using a method that incorporated silica (Hosseini et al., 2007).

DNA concentration was measured for all DNA extracts by NanoDrop (ND-1000 spectrophotometer; NanoDrop technologies Inc., Wilmington, DE, USA) according to the manufacturer's manual.

PCR and sequencing

Two universal primers, C1-J-1718 as forward and C1-N-2191 as reverse primer which they have proved to work very well with all insects tested (Simon *et al.*, 1994), were used to amplify a portion of the mitochondrial cytochrome oxidase subunit I (COI) gene of the six species of *Brassica* pests. Amplification was performed in 50 µl total volume of reaction buffer containing 150 µM dNTPs (Fisher Scientific Inc., USA), 2 mM MgCl₂, 0.4 µM each primer, 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia) and 8 µl of DNA template (20-40 ng). The reaction mix was put into a 0.2 ml PCR tube and amplification was performed in a PTC-200 thermocycler (MJ Research, MA, USA) with the following temperature profile: 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final elongation at 72°C for 5 min.

PCR products were separated by electrophoresis on a 1.8% agarose gel in TAE or TBE containing ethidium bromide for DNA staining. COI specific fragments of each species were sliced from the agarose gel and purified using a Perfectprep® Gel Cleanup kit (Eppendorf AG, Hamburg, Germany), following the manufacturer's instructions. Sequencing reactions were carried out via the manufacturer's instructions using ABI PRISM® Big-Dye Terminator Cycle Sequencing ready reaction mix version 3 (Applied Biosystems, Foster City, California, USA) in both forward and reverse orientations.

Sequence alignment and primer design

COI fragments were sequenced from two individuals per species in both forward and reverse directions. Sequencing results were reviewed by the CHROMAS program (version 1.45)(<http://www.technelysium.com.au>) and SeqEd Version 1.3 (Macintosh) and edited manually for each species separately. The Basic Local Alignment Search Tool (BLAST) was used to compare the similarity of nucleotide sequences with sequences present in GeneBank database (<http://www.ncbi.nlm.nih.gov/blast/>). All edited sequences were aligned using GENEDOC (<http://www.psc.edu/biomed/genedoc>). Pairs of primers were designed for each of the six species according to their sequence variations, especially in regions that were unique to each species. Primer design guidelines proposed for the design of efficient and specific primers by Innis & Gelfand (1990) and Saiki (1990) were followed. The primer-primer interactions were analysed using the program "Oligonucleotide Properties Calculator" (<http://www.basic.northwestern.edu/biotools/oligoalc.html>). Primers were synthesized by Geneworks, Adelaide, South Australia. For optimisation of each primer pair, a gradient PCR program was performed by using gradient thermocycler (PTC-200) with the following temperature profile: 35 cycles at 94°C for 30 sec, 53°C as the lower temperature and 65°C as the higher temperature for 30 sec, 72°C for 60 sec. A first cycle of denaturation was carried out at 95°C for 2 min and a last cycle of extension was performed at 72°C for 5 min.

Singleplex and multiplexing of PCR primers

Singleplex PCR was performed in 25 µl total volume of reaction buffer containing 150µM dNTPs (Fisher Scientific Inc., USA), 2 mM MgCl₂, 0.4 µM each of primer pairs for each species (Table 2), 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia), 1× of reaction buffer provided by manufacturer and

4 µl of DNA template. To evaluate the ability of multiplex PCR for simultaneous detection of two target prey DNA, a multiplex PCR procedure was developed and optimised to multiplex the primer pairs DBM-F-2/DBM-R1-1 and HH-F-1/HH-R-1 (Table 2) for *P. xylostella* and *H. hydralis*, respectively. These primers have the same annealing temperature but each pair can amplify fragments of different size. Based on a 25 µl reaction volume, the multiplex PCR reaction mix consisted of same materials and concentrations as singleplex, where 0.4 µM each of the *P. xylostella* primers, 0.2 µM each of the *H. hydralis* primers and 0.8 µg/µl BSA (Promega, Madison, USA) were used in multiplexing reaction. The cycling conditions for singleplex PCR were 95°C for 2 min followed by 35 cycles of 94°C for 30 sec, 30 sec at the specific annealing temperature for each specific primer pairs (Table 2), and 72°C for 1 min and final extension at 72°C for 5 min.

For multiplex PCR, annealing temperature used in cycling program was 58°C for 30 sec and the rest of conditions were the same as singleplex.

Primer specificity and sensitivity

The specificity of the primer pairs was tested separately for each of the designed primers by attempting to amplify target DNA (at least 10 individuals) from six *Brassica* pests and other arthropods, including a representative sample of most abundant Diptera, Hymenoptera, and selected arthropod predators as well as an aphid and a lepidopteran, and six species of *Brassica* crops collected from the field (Table 1). In order to test if the designed primers would amplify specific DNA in the presence of predator DNA even in high amounts, extracted DNA of each species was diluted and mixed with DNA of a selected predator (*T. expolita*). The concentration of the predator's DNA was 20 ng and constant in all mixtures, while the ratio of predator DNA to target species DNA mixtures varied (Table 3).

Specificity and Sensitivity of Multiplex PCR

Apart from testing specificity and sensitivity of primers in the singleplex PCR, separate assays were performed to test specificity and sensitivity of multiplex PCR. Primer pairs DBM-F-2/DBM-R1-1 and HH-F-1/HH-R-1 were used for multiplexing. Four different DNA template mixtures were tested in multiplex PCR: 1) a serial dilution of *P. xylostella* DNA mixture with DNA of predator, 2) a serial dilution of *P. xylostella* DNA mixture with a 0.8 ng constant DNA of *H. hydralis* and DNA of predator 3) a serial dilution of *H. hydralis* DNA mixture with a DNA of predator, and 4) a serial dilution of *H. hydralis* DNA mixture with a 1 ng constant DNA of *P. xylostella* and DNA of predator. The concentrations of serial dilutions for *P. xylostella* and *H. hydralis* DNA ranged from 16.8 ng to 2 pg and 11.6 ng to 0.02 pg, respectively and the concentration of the predator's DNA (*T. expolita*) was 20 ng and constant in all mixtures.

Detection of prey DNA in gut contents of predators

Four experiments were performed to test the ability to detect DNA from five different preys' species in the gut contents of predators. In the first experiment, three species of field-collected predators (5 specimens each of *Trochosa expolita*, *Nabis kinbergii* and *Hippodamia variegata*) were starved at room temperature for at least seven days. After this period each predator was fed a 2nd or 4th instar of *P. xylostella*. In the second experiment 5 starved *T. expolita* were fed on a 2nd instar of *P. rapae*. In the third assay 6 starved *T. expolita* were fed on a 2nd–3rd instar of *H. hydralis* and in the fourth experiment which has been done in two separate assay, starved *H. variegata* (5–6 specimens for each assay) were fed on

5-10 nymphs of *M. persicae* or *B. brassicae*. Predators were frozen at -20°C zero to two hour after consuming their prey for subsequent molecular assay.

Field samples

In order to test the ability of primers to detect predation in the field, adult of an abundant predator "*Oechalia schellenbergii*" (n=82) were collected at random from a broccoli field on a commercial vegetable farm at Currency Creek, South Australia ($35^{\circ} 41' \text{ S}$, $138^{\circ} 75' \text{ E}$). All collected specimens were kept chilled on ice until they were transferred to the laboratory and placed at -80°C overnight prior to DNA extraction and PCR using methods and conditions described before.

RESULTS

Primer design and specificity and sensitivity

The readable fragments sequence from the COI gene of each of these six species varied in length (*P. xylostella* 481 bp, *P. rapae* 478 bp, *B. brassicae* 502 bp, *H. punctigera* 514 bp, *M. persicae* 478 bp and *H. hydralis* 492 bp). Sequences were submitted to the National Centre for Biotechnology Information Genebank (<http://www.ncbi.nlm.nih.gov>; Table 2). The sequences of these species were aligned and, on the basis of diagnostic differences among sequences, one species-specific pair of primers was designed for each species. Optimised annealing temperatures ranged from 58°C to 64°C for each primer pair (Table 2). The target sequences amplified by the six primers pairs range from 200 to 307bp (Table 2, Fig. 1). Each primer pairs proved to be highly specific against non-target DNA and could amplify the expected fragment size only in the presence of the respective target species DNA (Fig. 1).

Singleplex PCR showed that the highest and lowest detection sensitivity among the designed primers belong to *H. hydralis* with 0.02 pg and *H. punctigera* with 27.3 pg (Table 3), where the key pest species "*P. xylostella*" could be detected with as little as 2 pg (Fig. 3).

Specificity and sensitivity of Multiplex PCR

Multiplex PCR with DBM-F-2/DBM-R1-1 and HH-F-1/HH-R-1 demonstrated the possibility of simultaneous detection of *P. xylostella* and *H. hydralis* (Fig. 4) and specificity against other non-target species.

Multiplex PCR showed less sensitivity compare with singleplex PCR. In the multiplex PCR assay, detection sensitivity of *H. hydralis* primers in the mixture with predator DNA was 8-fold lower while in the mixture with *P. xylostella* DNA showed a 140-fold lower sensitivity compared to singleplex PCR. Likewise, *P. xylostella* primer pairs in the mixture with predator DNA had the same sensitivity as singleplex PCR, however their sensitivity in the mixture with *H. hydralis* DNA was 8.2-fold lower than with singleplex PCR (Table 4).

Detection of prey DNA in the gut contents of predators

DNA of target pests *P. xylostella*, *P. rapae*, *H. hydralis*, *M. persicae* and *B. brassicae* (Fig. 2A) were detected in the gut contents of *T. expolita*, *N. kinbergii* and *H. variegata* (Fig. 2B).

Field samples

Results showed all designed primers can be effectively used for detection of target prey DNA in collected predators from the field. DNA from all six pests was found in the gut contents of *O. schellenbergii*, which indicates that, this species is

a polyphagous predator (Fig. 5). Fig.6 as an example shows detection of remains of four preys including *P. xylostella*, *H. Hydralis*, *P. rapae* and *B. brassicae* in 10 predator specimens collected from the field.

DISCUSSION

Species-specific molecular markers were developed for six *Brassica* pests. The designed primer pairs were tested against many possible non-target invertebrate species found in broccoli fields as well as *Brassica* plants with no amplification detected for non-target species, which indicates that these primer pairs were highly specific for the target species. In the only published study of molecular detection of *Brassica* crop pests in predators' gut contents, Ma et al. (2005) showed that using a primer set based on the ITS-1 region not only amplified target DNA but also larger non-specific fragments from *Brassica* plants.

It has been known that prey DNA in a predators' gut is fragmented by digestion enzymes and as a result the detection time of prey DNA depends on the length of the amplification product (Hoogendoorn & Heimpel, 2001; Agusti et al., 2003a). Larger fragments become undetectable in the gut more rapidly than smaller ones (Zaidi et al., 1999; Agusti et al., 2000). However, some studies showed there is no difference in detection rate within certain range of fragment lengths. For example Chen et al. (2000) found no difference in the detection rates of *S. avenae* fragments shorter than 246bp. In similar cases, studies showed there was not difference in detection rates of DNA fragments between 175 and 387 bp in feeding experiments with cockchafer prey (Juen & Traugott, 2005), and detection for a 127 bp fragment was not significantly higher than for a 463 bp fragment of the *A. solstitiale* (Juen & Traugott, 2006). For this reason in this study we aimed to identify specific sequences that are 300 bp or smaller to ensure reasonable detection times.

The sensitivity thresholds of species-specific primers in prey detection achieved by PCR is an important issue because it indicates whether a single prey consumed by a predator is sufficient for detection (e.g. Zaidi et al., 1999; Chen et al., 2000; Admassu et al., 2006). In our study a concentration-response trial of prey DNA in the presence of a constant concentration of predator DNA was performed for all six primers pairs. Detection limits ranged from 0.02 pg to 27.3 pg (Table 3). This result is comparable to the highest detection sensitivity levels achieved in a study on used to detect parasitism; Traugott et al. (2006) reported detection limits ranging between 0.6 pg and 46.8 pg of DNA from a parasitoid of *P. xylostella*. Our results showed that the presence of a second non-target DNA in the singleplex PCR does not influence the detectability of target DNA in all primers pairs examined. For example *H. hydralis* DNA was detectable in the presence of 10^6 times the concentration of *T. expolita* DNA. This enables us to use the whole body of a predator for DNA extraction without any dissection of the gut or its contents. This is a big advantage in field studies and makes the analysis of samples with small predators easier and quicker. In a study of earthworm residues in predators' gut contents by Admassu et al. (2006), sensitivity of designed primers was determined to be 0.15 ng/ μ l of earthworm DNA in the presence of 243 times the amount of predators' DNA. Likewise, Sheppard et al. (2004) showed that, despite the presence of large amounts of predator tissue, there was no evidence that non-target material could mask the detection of very small amounts of prey DNA.

Some workers used another approach to determine the sensitivity threshold of primers. For example, Agusti et al. (2003b) in their study were defined the

sensitivity of designed primers at 10^{-5} dilution of a target sample and Chen et al. (2000) detected 10^{-7} aphid equivalents of DNA. However, concentration of the extracted DNA depends on the size of sample and extraction method used therefore results will vary with smaller or larger specimens.

Our results revealed the possibility of applying multiplex PCR for simultaneous detection of at least two prey species in one reaction. This is a big advantage in increasing the efficiency of PCR amplification. However, the result indicated that the ratio of concentration among DNA of target prey is an important factor in detection of each prey in multiplexing system. On the other hand, if the concentration of *H. hydralis* is higher than *P. xylostella*, the latter prey may either not be detected or only observed as a very faint band in the gel electrophoresis. A possible explanation may be the competition among DNA of target species. For this reason sensitivity of primers in multiplex was less than singleplex PCR. This was obvious when we mixed DNA of *H. hydralis* with a constant concentration of *P. xylostella* and vice versa (Table 4). In a multiplex PCR assay with primers for *P. xylostella* and one of its parasitoids, detection sensitivity of the parasitoid primers was determined to be 4-fold lower compared to singleplex PCR, but the sensitivity of *P. xylostella* primers was not determined in the multiplex PCR reaction (Traugott et al., 2006). Harper et al. (2005) developed a multiplex PCR incorporating fluorescent markers to detect mitochondrial DNA fragments from more than 10 prey species simultaneously in the gut contents of generalist predators. However, the higher cost of techniques and equipment used is a limiting factor in the application of this method in ecological investigations. Moreover the sensitivity of the multiplex systems was not tested with varying ratios of prey DNA to check the effect on detection of each species.

Competition between DNA from different target species is a known phenomenon in multiplex PCR (Markoulatos et al., 2002). For example, in a study on simultaneous detection of four bacterial pathogens (Stralin et al., 2005), when there was a clear dominant band in the gel electrophoresis from one pathogen, a relatively weaker PCR band was identified for the pathogen with the lower concentration. Consequently a strong band for one pathogen in the gel electrophoresis may be associated with a decreased sensitivity for detection of other species by multiplex PCR. Therefore, we suggest further investigation should be done to check the real sensitivity of each primer in multiplex PCR before any application in the ecological studies.

This study has demonstrated that species-specific primers targeting six pests of *Brassica* crops can be used for ecological studies of *Brassica* pests and to screen field-caught predators for prey species consumption. This study showed that *Oeochalia schellenbergii* collected from the field is a polyphagous predator and could be considered as a potential predator of pests in *Brassica* crops. This fact has previously been reported in other crops systems that this species feed on a wide range of immature stages of moths, beetles, sawflies and weevils (Mensah, 1999; Copper, 1981; Cordingly, 1981; Awan, 1985a,b; 1988; 1990) and other coleopteran pests (Edwards & Suckling, 1980). Multiplexing of primers has potential for application in ecological investigations but further studied is needed to establish the sensitivity of the method in each case.

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LITERATURE CITED

- Admassu, B., Juen, A. & Traugott, M.** 2006. Earthworm primers for DNA-based gut content analysis and their cross-reactivity in a multi-species system. *Soil Biology and Biochemistry*, 38: 1308-1315.
- Agusti, N., De Vicente, M. C., & Gabarra, R.** 2000. Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology*, 9: 263-268.
- Agusti, N., Shayler, S. P., Harwood, J. D., Vaughan, I. P., Sunderland, K. D., & Symondson, W. O. C.** 2003a. Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. *Molecular Ecology*, 12: 3467-3475.
- Agusti, N., Unruh, T. R., & Welter, S. C.** 2003b. Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts by using COI mitochondrial markers. *Bulletin of Entomological Research*, 93: 179-185.
- Awan, M. S.** 1985a. Anti-predator plays of *Heliothis punctigera* (Lep: Noctuidae) caterpillar against the predator *Oechalia schellenbergii* (Hem.: Pentatomidae). *Australian Journal of Zoology*, 33: 885-890.
- Awan, M. S.** 1985b. Studies on the food preferences of immature stages of predacious pentatomids, *Oechalia schellenbergii* (Guerin-Meneville) and *Cermatulus nasalis* (Westwood). *Sindh University Research Journal (Sci-Ser.)*, 17: 69-74.
- Awan, M. S.** 1988. Development and mating behaviour of *Oechalia schellenbergii* (Guérin-Méneville) and *Cermatulus nasalis* (Westwood) (Hemiptera: Pentatomidae). *Journal of the Australian Entomological Society*, 27: 183-187.
- Awan, M. S.** 1990. Predation by three hemipterans: *Tropiconabis nigrolineatus*, *Oechalia schellenbergii* and *Cermatulus nasalis*, on *Heliothis punctiger* larvae in two types of searching arenas. *Entomophaga*, 35: 203-210.
- Chen, Y., Giles, K. L., Payton, M. E., & Greenston, M. H.** 2000. Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology*, 9: 1887-1898.
- Cooper, D. J.** 1981. The role of predatory Hemiptera in disseminating a nuclear polyherosis virus of *Heliothis punctiger*. *Journal of the Australian Entomological Society*, 20: 145-150.
- Cordingly, C. L.** 1981. Factors causing the mortality of the grape vine moth, *Phalaenoides glycine* Lewin (Lepidoptera: Agariidae). *Journal of the Australian Entomological Society*, 20: 33-38.
- Edwards, R. B., & Suckling, D. M.** 1980. *Cermatulus nasalis* and *Oechalia schellenbergii* (Hemiptera: Pentatomidae) as predators of eucalyptus tortoise beetle larvae, *Paropsis charybdis* (Coleoptera: Chrysomelidae), in New Zealand. *New Zealand Entomologist*, 7: 158-164.
- Food and Agriculture Organization of the United Nations.** 2007. FAOSTAT: Core production data. faostat.fao.org [accessed 6 February 2007].
- Furlong, M. J., Zuhua, S., Shijan, G., Yinquan, L., Sheng, L. S. & Zalucki, M. P.** 2004. Quantitative evaluation of the biotic mortality factors affecting diamondback moth in south-east Queensland, Australia. pp. 185-193 in Endersby NM & Ridland PM (Eds.) *The management of diamondback moth and other crucifer pests: Proceedings of the 4th International Workshop*, Melbourne, Australia, 26-29 November 2001.
- Greenstone, M. H.** 1996. Serological analysis of arthropod predation: past, present and future. In Symondson WOC (Ed.) *The Ecology of Agricultural Pests: Biochemical Approaches*. Chapman & Hall, London.
- Harper, G. L., King, R. A., Dodd, C. S., Harwood, J. D., Glen, D. M., Bruford, M. W. & Symondson, W. O. C.** 2005. Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology*, 14: 819-827.
- Hayes, J. L. & Lockley, T. C.** 1990. Prey and nocturnal activity of wolf spiders (Aranea: Lycosidae) in cotton fields in the Delta region of Mississippi. *Environmental Entomology*, 19: 1512-1518.

- 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. *Molecular Ecology*, 10: 2059-2067.
- Hooks, C. R., Pandey, R. R. & Johnson, W. M.** 2003. Impact of avian and arthropod predation on lepidopteran caterpillar densities and plant productivity in an ephemeral agroecosystem. *Ecological Entomology*, 28: 522-532.
- Hosseini, R., Keller, M. A., Schmidt, O. & Baker, G. J.** 2006a. Predators associated with crop pests of *Brassica* spp. in South Australia. 5th International Workshop on management of the Diamondback Moth and Other Insect Pests. Beijing, China Oct 24-27, 2006. Pp. 338-344.
- Hosseini, R., Keller, M. A., Schmidt, O. & Dong-Mei, L.** 2006b. Molecular detection of common pests of Brassica crops in the guts of predators. 5th International Workshop on management of the Diamondback Moth and Other Insect Pests. Beijing, China Oct 24-27.
- Hosseini, R., Keller, M. A., Schmidt, O. & Framenau, V. W.** 2007. Molecular identification of wolf spiders (Araneae: Lycosidae) by multiplex polymerase chain reaction. *Biological Control*, 40: 128-135.
- Hosseini, R., Keller, M. A. & Schmidt, O.** 2008. Molecular detection of *Plutella xylostella* (L.) (Lepidoptera; Plutellidae) in the wolf spider gut using COI mitochondrial markers. 18th Plant Protection Congress. Hamedan University, Iran 24-28 Aug.
- Hoy, M. A.** 2003. *Insect Molecular Genetics, an Introduction to Principles and Applications*, 2nd edn. Academic Press, San Diego.
- Innis, M. A. & Gelfand, D. H.** 1990. Optimization of PCRs. pp. 3-12. in Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Eds) *PCR Protocols*. San Diego, Academic Press.
- Juen, A. & Traugottm 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.
- Juen, A. & Traugott, M.** 2006. Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biology and Biochemistry*, 38: 1872-1879.
- Ma, J., Li, D., Keller, M., Schmidt, O. & Feng, X.** 2005. A DNA marker to identify predation of *Plutella xylostella* (Lep., Plutellidae) by *Nabis kinbergii* (Hem., Nabidae) and *Lycosa* sp. (Araneae, Lycosidae). *Journal of Applied Entomology*, 129: 330-335.
- Markoulatos, P., Siafakas, N. & Moncany, M.** 2002. Multiplex Polymerase Chain Reaction: A Practical Approach. *Journal of Clinical Laboratory Analysis*, 16: 47-51.
- Naranjo, S. E. & Hagler, J. R.** 1998. Characterizing and estimating the impact of heteropteran predation. pp. 170-197 in Colle, M. & Ruberson, J (Eds) *Predatory Heteroptera: their ecology and use in biological control*. Entomological Society of America, Lanham, Maryland.
- Read, D. S., Sheppard, S. K., Bruford, M. W., Glen, D. M. & Symondson, W. O. C.** 2006. Molecular detection of predation by soil micro-arthropods on nematodes. *Molecular Ecology*, 15: 1963-1972.
- Saiki, R. K.** 1990. Amplification of genomic DNA. In *PCR Protocols*. Pp. 13-20 in Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (Eds) *PCR Protocols*. San Diego, Academic Press.
- Schmaedick, M. A., & Shelton, A. M.** 2000. Arthropod predators in cabbage (Cruciferae) and their potential as naturally occurring biological control agents for *Pieris rapae* (Lepidoptera: Pieridae). *The Canadian Entomologist*, 132: 655-675.
- Shelton, A. M., Andarolo, J. T. & Hoy, C. W.** 1983. Survey of ground-dwelling predaceous and parasitic arthropods in cabbage fields in upstate New York. *Environmental Entomology*, 12: 1026-1030.
- Shelton, A. M., Perez, C. J., Tang, J. D. & Vandenberg, J. D.** 1997. Prospects for novel approaches towards management of the diamondback moth. pp 7-20 in Sivapragasm A, Loke WH, Hussan, AK & Lim GS (Eds.) *Proceedings of the Third International Workshop on the Management of Diamondback Moth and Other Crucifer Pests*. Kuala Lumpur.

Sheppard, S. K., Henneman, M. L., Memmott, J. & Symondson, W. O. C. 2004. Infiltration of alien predators into invertebrate food webs in Hawaii: a molecular approach. *Molecular Ecology*, 13: 2077-2088.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primer. *Annals of the Entomological Society of America*, 87: 651-701.

Stralin, K., Backman, A., Holmberg, H., Fredlund, H. & Olcen, P. 2005. Design of a multiplex PCR for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *Chlamydomytila pneumoniae* to be used on sputum samples. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, 113: 99-111.

Sunderland, K. D. 1975. The diet of some predatory arthropods in cereal crops. *Journal of Applied Ecology*, 12: 507-515.

Symondson, W. O. C. 2002. Molecular identification of prey in predator diets. *Molecular Ecology*, 11: 627-641.

Symondson, W. O. C., Sunderland, K. D. & Greenstone, M. 2002. Can generalist predators be effective biocontrol agents? *Annual Review of Entomology*, 47: 561-595.

Traugott, M. 2003. The prey spectrum of *Cantharis*-beetle larvae and adults in the field: an electrophoretic approach. *Pedobiologia*, 47: 161-169.

Traugott, M., Zangerl, P., Juen, A., Schallhart, N. & Pfiffner, L. 2006. Detecting key parasitoids of lepidopteran pests by multiplex PCR. *Biological Control*, 39: 39-46.

Wallace, H. K. 1937. The use of the headlight in collecting nocturnal spiders. *Entomological News*, 48: 107-111.

Wang, X. G., Duff, J., Keller, M. A., Zalucki, M. P., Liu, S. S. & Bailey, P. 2004. Role of *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) in controlling *Plutella xylostella* (Lepidoptera: Plutellidae): Cage exclusion experiments and direct observation. *Biocontrol Science and Technology*, 14: 571-586.

Zaidi, R. H., Jaal, Z. & Hawkes, N. J. 1999. Can multiple-copy sequences of prey DNA be detected amongst the gut content of invertebrate predators? *Molecular Ecology*, 8: 2081-2087.

Zhang, D. X. & Hewitt, G. M. 1996. Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution*, 11: 247-251.



Figure 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified DNA using all six pest specific primer pairs for the target species, lane 1, 100 bp molecular marker, lanes 2-3 *P. xylostella* (293 bp), lanes 4-5, *H. hydralis* (200 bp), lanes 6-7, *B. brassicae* (307 bp), lanes 8-9, *H. punctigera* (270 bp), lanes 10-11, *P. rapae* (222 bp), lanes 12-13, *M. persicae* (247 bp), lane 14, negative control (Predator's DNA).

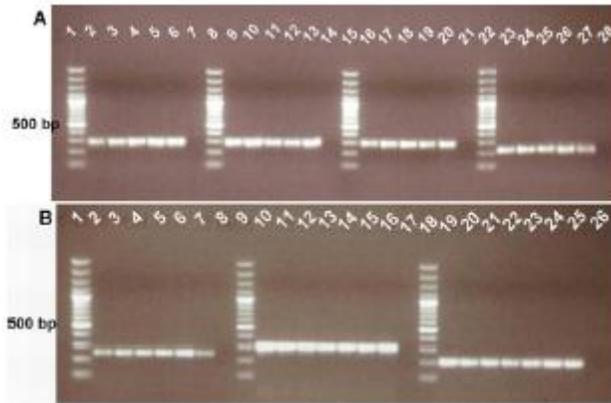


Figure 2. Detection of target prey DNA; **A**: using primer pairs DBM-F-2/DBM-R1-1 and PR-F-1/PR-R-1. from gut contents of *T. expolita*, *N. kinbergii* and *H. variegata* fed on *P. xylostella* larvae respectively, and *T. expolita* fed on *P. rapae* (five replication for each), lanes 1,8,15,22, 100 bp molecular markers, lanes 7,14,21,28, negative control. **B**: using primer pairs MP-F/MP-R, BB-F-1/BB-R and HH-F-1/HH-R-1. from gut contents of *H. variegata* fed on *M. persicae* and *B. Brassicae* and *T. expolita* fed on *H. hydralis* larvae (five replication for each), lanes 1,9,18, 100 bp molecular markers, after molecular markers first lanes are positive control, lanes 8,17,26 negative control (Predator's DNA).

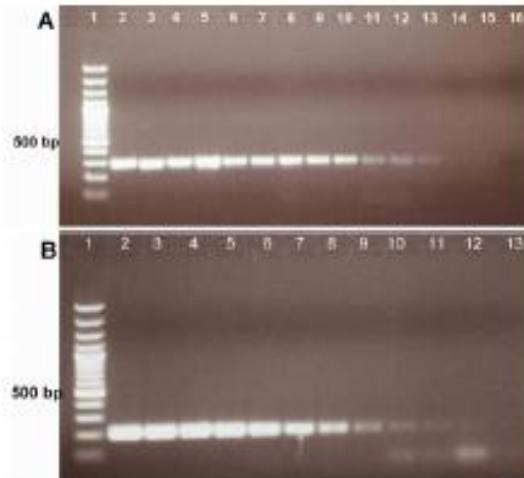


Figure 3. **A**: Concentration-response trial to determine the sensitivity of *P. xylostella* primer pairs (DBM-F-2/DBM-R1-1). lane1, 100 bp molecular marker, lanes 2-15 are, 16.8 ng, 8.4 ng, 4.2 ng, 2.1 ng, 1.05 ng, 0.526 ng, 0.262 ng, 0.131 ng, 0.065 ng, 0.032 ng, 0.0164 ng, 0.0082 ng, 0.0041 ng, 0.0028 ng (total DNA in PCR) respectively, lane 16, negative control (Predator's DNA). **B**: Concentration-response trial to determine the sensitivity of *H. hydralis* primer pairs (HH-F-1/HH-R-1). lane1, 100 bp molecular marker, lanes 2-12 are, 0.022 ng, 0.01 ng, 0.005 ng, 0.002 ng, 0.001 ng, 0.0007 ng, 0.0003 ng, 0.0001 ng, 0.00008 ng, 0.00004 ng, 0.00002 ng (total DNA in PCR) respectively, lane 13, negative control (Predator's DNA). In both experiments prey DNA was serially diluted in a constant concentration of 20 ng of DNA from *T. expolita*.

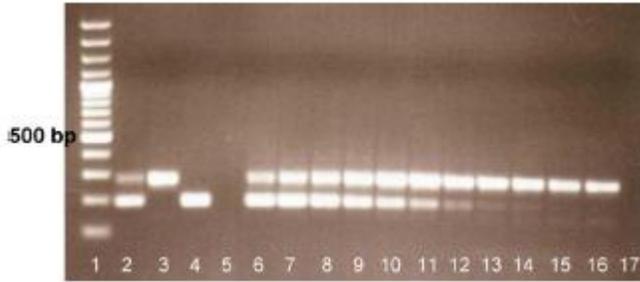


Figure 4. Concentration-response trial to determine the sensitivity of *H. hydralis* primer pairs (HH-F-1/HH-R-1) in multiplex PCR (mixture of *H. hydralis* and *P. xylostella* primers). DNA of *H. hydralis* was serially diluted in a constant concentration of 20 ng of DNA from *T. expolita* and 1 ng *P. xylostella*, lane1, 100 bp molecular marker, lanes 2-16 , 0.8, 3, zero, 0.8, empty well, 0.72, 0.36, 0.18, 0.09, 0.045, 0.022, 0.011, 0.005, 0.002, 0.001, 0.0007 ng of *H. hydralis* DNA respectively, lane 17, negative control (Predator's DNA).

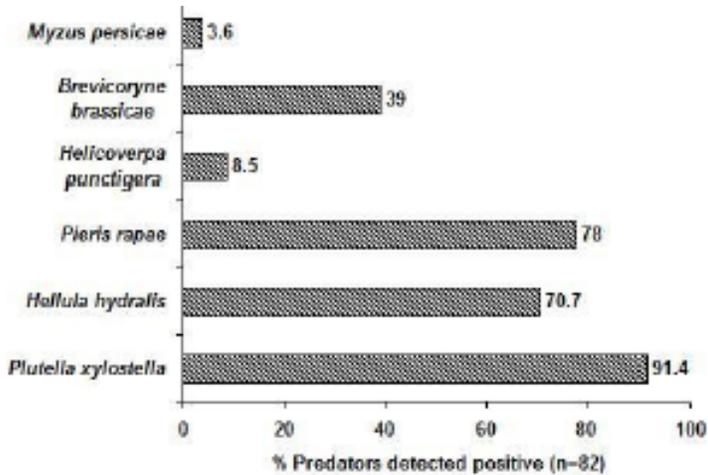


Figure 5. Percentage detection of different prey species in *Oechalia schellenbergii*. Numbers beside bars indicate percentages.

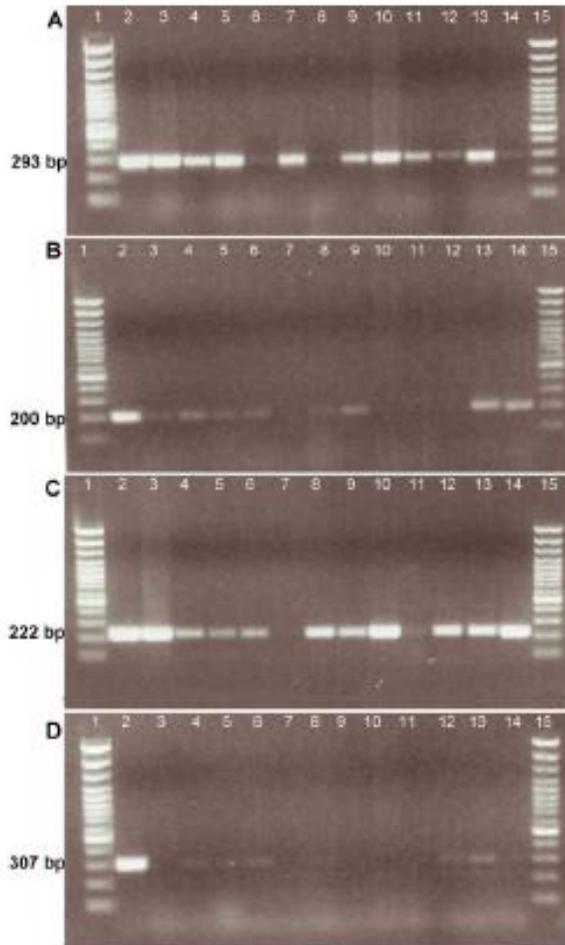


Figure 6. Detection of target prey remains in 10 specimens of *O. schellenbergii*. **A:** Detection of *P. xylostella*, , lanes 3-14, predators detected positive for remains of *P. xylostella*. **B:** Detection of *Hellula hydralis*, , lanes 3-6, 8,9, 13,14, predators detected positive for remains of *H. hydralis*, lanes 7, 10-12 no detection for remains of target prey. **C:** Detection of *P. rapae*, lanes 3-6, 8-14, predators detected positive for remains of *P. rapae*, lane 7, no detection for remains of target prey. **D:** Detection of *B. brassicae*, lanes 4-6, 12,13, predators detected positive for remains of *B. brassicae*, lanes 3, 7-11, 14, no detection for remains of target prey. Lanes 1,15 100 bp molecular marker, lane 2, positive control.

Table 1. List of arthropods and *Brassica* plants used to test PCR primer specificity.

Scientific name	Order: Family	Common name
<i>Plutella xylostella</i> (L.)	Lepidoptera: Plutellidae	Diamondback moth
<i>Pieris rapae</i> (L.)	Lepidoptera: Pieridae	Cabbage white butterfly
<i>Hellula hydralis</i> Guenee	Lepidoptera: Pyralidae	Cabbage centre grub
<i>Helicoverpa punctigera</i> Wallengren	Lepidoptera: Noctuidae	Native budworm
<i>Agrotis</i> sp.	Lepidoptera: Noctuidae	Cutworm
<i>Brevicoryne brassicae</i> (L.)	Homoptera: Aphididae	Cabbage aphid
<i>Myzus persicae</i> (Sulzer)	Homoptera: Aphididae	Green peach aphid
<i>Acyrtosiphon kondoi</i> Shinji	Homoptera: Aphididae	Bluegreen lucerne aphid
Unknown fly	Diptera	Fly
<i>Diadegma semiclausum</i> (Hellén)	Hymenoptera: Ichneumonidae	Diamondback moth parasite
<i>Cotesia plutellae</i> (Kurdjumov)	Hymenoptera: Braconidae	Diamondback moth parasite
<i>Nabis kimbergii</i> (Reuter)	Heteroptera: Nabidae	Pacific damsel bug
<i>Oechalia schellenbergii</i> (Guerin-Meneville)	Heteroptera: Pentatomidae	Predatory shield bug
<i>Creontiades dilutus</i> (Stål)	Heteroptera: Miridae	Green mirid
<i>Philonthus</i> sp.	Coleoptera: Staphylinidae	Rove beetle
<i>Diomus notescens</i> (Blackburn)	Coleoptera: Coccinellidae	Minute two-spotted ladybird
<i>Hippodamia variegata</i> (Goeze)	Coleoptera: Coccinellidae	Spotted amber ladybird
<i>Coccinella transversalis</i> Fabricius	Coleoptera: Coccinellidae	Transverse ladybird
<i>Micromus tasmaniae</i> (Walker)	Neuroptera: Hemerobiidae	Tasman's lacewing
<i>Hogna crispipes</i> (L. Koch)	Araneae: Lycosidae	Wolf spider
<i>Hogna kiyani</i> Framenau	Araneae: Lycosidae	Wolf spider
<i>Venatrix pseudospeciosa</i> Framenau and Vink	Araneae: Lycosidae	Wolf spider
<i>Lycosa godeffroyi</i> (L. Koch)	Araneae: Lycosidae	Wolf spider
<i>Venator spenceri</i> Hogg	Araneae: Lycosidae	Wolf spider
<i>Trochosa exposita</i> (L. Koch)	Araneae: Lycosidae	Wolf spider
<i>Brassica oleracea</i> var. <i>capitata</i>	Brassicaceae	Cabbage
<i>Brassica oleracea</i> var. <i>botrytis</i>	Brassicaceae	Cauliflower
<i>Brassica oleracea</i> var. <i>gongylodes</i>	Brassicaceae	Kohlrabi
<i>Brassica oleracea</i> var. <i>italica</i>	Brassicaceae	Broccoli
<i>Brassica campestris</i> var. <i>pekinensis</i>	Brassicaceae	Chinese cabbage
<i>Brassica rapa</i>	Brassicaceae	Canola

Table 2. Species-specific primer sequences designed from the COI mtDNA of six common pests of *Brassica* crops, optimal PCR annealing temperature, amplification size and GeneBank accession numbers for the COI gene fragments.

Species	Primer name	Sequence	Accession no.	Annealing temp. (°C)	Amplification size
<i>Plutella xylostella</i>	DBM-F-2	5'-TGTATTATCCTCCTTTATCTTCA-3'	AY898745	58	293
	DBM-R1-1	5'-CTCCTGCAGGATCAAAGAAG-3'			
<i>Pieris rapae</i>	PR-F-1	5'-AGTGTACCCCCCACTTTCTT-3'	AY898746	60	222
	PR-R-1	5'-ACTGGTAATGATAATAGTAAAAGT-3'			
<i>Helicoverpa punctigera</i>	HP-F	5'-CTCATGGAGGAAGATCTGTA-3'	AY898747	64	270
	HP-R	5'-CTCCTCCTCCAGCAGGAT-3'			
<i>Brevicoryne brassicae</i>	BB-F-1	5'-ITGATTACTCCCTCCATCAC-3'	AY898748	60	307
	BB-R	5'-TCCAGCTAATACTGGGAGA-3'			
<i>Myzus persicae</i>	MP-F	5'-TGATTATTACCACCCTCAT-3'	AY898749	58	247
	MP-R	5'-TGGAAATAAAGGGATTGG-3'			
<i>Hellula hydralis</i>	HH-F-1	5'-TGGTGGGAAGATCAGTTGATC-3'	DQ387063	58	200
	HH-R-1	5'-CTCCAGCTAATACTGGTAGT-3'			

Table 3. Sensitivity of all six *Brassica* pests primer pairs.

Species	Sensitivity in singleplex PCR
<i>Hellula hydralis</i>	0.02 pg
<i>Plutella xylostella</i>	2 pg
<i>Pieris rapae</i>	9.6 pg
<i>Helicoverpa punctigera</i>	27.3 pg
<i>Brevicoryne brassicae</i>	0.13 pg
<i>Myzus persicae</i>	15.6 pg

Table 4. Comparison sensitivity of *Hellula hydralis* and *Plutella xylostella* primer pairs in singleplex and multiplex in two different DNA mixtures.

Species	Sensitivity in Singleplex PCR		Sensitivity in Multiplex PCR	
	Prey's DNA mix with Predator's DNA	<i>H. hydralis</i> + <i>P. xylostella</i> DNA + Predator's DNA	Prey's DNA mix with Predator's DNA	<i>H. hydralis</i> + <i>P. xylostella</i> DNA + Predator's DNA
<i>H. hydralis</i>	0.02 pg	0.02 pg	0.16 pg	2.8 pg
<i>P. xylostella</i>	2 pg	2 pg	2 pg	16.4 pg