Detection of intraguild predation between coccinellids using molecular analyses of gut-contents

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Introduction

Intraguild predation (IGP) has received greater attention in recent years because of growing evidence of the impact of predator-predator interactions on the structure of ecological communities and biological control of pests (Onzo *et al*., 2005). The release of multiple natural enemies or the increase of natural enemy diversity or density via conservation methods may enhance or disrupt pest control (Denoth *et al*., 2002; Cardinale *et al*., 2003; Aquilino *et al*., 2005). Intraguild predation (IGP) has most traditionally been viewed as a factor that can be responsible for the disruption of biological control (Rosenheim *et al*., 1995; Kester and Jackson, 1996; Snyder and Ives, 2001). However, combinations of natural enemies can suppress pests even when IGP is present (e.g. Chang, 1996; Snyder *et al*., 2004; Harvey and Eubanks, 2005). To determine why the presence of multiple natural enemies can enhance biological control in some instances and disrupt it in others, we must know first which species engage in IGP, and then, evaluate their respective impact on pest control.

One way to evaluate intraguild interactions is to use controlled laboratory studies, often in Petri dishes (e.g. Cottrell and Yeargan, 1998; Burgio *et al*., 2002). These methods do not necessarily represent authentic occurrence of natural enemies and can be usefully complemented by field cage experiments (e.g. Rosenheim *et al*., 1993; Snyder and Ives 2001; Hoogendoorn and Heimpel, 2004). Although field cage experiments are more complex with the use of host plant, they are limited in scope as well, and do not represent the actual field conditions. The principal negative factors of this method are the restrictive number of species in cages and constrained movement. All of these constraints can bias the number of negative interactions. Few studies have looked at IGP in real agroecosystems because traditional techniques are not well adapted to study interactions that are relatively rare (but see Finke and Denno, 2003; Rosenheim *et al*., 2004).

Molecular gut-content analyses are a very promising method for the quantification of IGP within the field. PCR is a powerful tool that has been used recently for this kind of study and that provides new level of resolution (e.g. Agustí *et al*., 1999, 2000, 2003; Zaidi *et al*., 1999; Chen *et al*., 2000; Hoogendoorn and Heimpel, 2001, 2002; Symondson, 2002; Dodd *et al*., 2003; Sheppard *et al.*, 2004; Harper *et al*., 2005). Limiting factors of cage studies such as movement constraints and diversity restriction are eliminated with PCR gut-content analyses conducted on field-caught predators. Real interactions between natural enemies can now be evaluated without a bias in the results. By collecting individuals in the field, it is now possible to identify species eaten by a predator without disturbing its feeding habit. Therefore, application of gut-content analyses to IGP research can provide biological control researchers with a better understanding of natural enemy interactions within a community.

In this study, we developed a molecular technique to analyze gut-contents for determination of IGP frequency between coccinellids in soybean fields throughout Québec agricultural regions. Our first objective was to develop PCR markers for the main coccinellids in Québec. Specific primers for regions of the internal transcribed spacer of the ribosomial gene complex (ITS-1) and the mitochondrial gene cytochrome oxidase I (COI) were designed for four coccinellid species (Coleoptera: Coccinellidae): *Coccinella septempunctata* Linnaeus, *Propylea quatuordecimpuntata* Linnaeus, *Harmonia axyridis* (Pallas) and *Coleomegilla maculata* lengi Timberlake. Second, we assessed the specificity of each primer pair by doing a cross-reactivity test. Finally, to be able to estimate the occurrence of IGP in the field, we did an experiment on the digestion rate of each coccinellid species. This data is of crucial importance if we want to compare IGP frequency between coccinellid species. Digestion rates can be different between species and can overestimate or underestimate the real occurrence of IGP in the field.

Materials and Methods

Study system

The soybean aphid is native of Asia and is a new pest in North America since is first detection in 2000 in Wisconsin, USA (Ragsdale *et al*., 2004). This pest has transformed the ecological communities in soybean field since its arrival. The most common predators of SBA found in agroecosystem in Quebec are the coccinellids *C. septempunctata, P. quatuordecimpunctata, H. axyridis* and *C. maculata* (Migneault *et al*., in press). Specimens were collected in soybean field with sweep netting, brought back to the laboratory and put in vials with alcohol 70%.

DNA extraction and primer design

DNA was extracted from whole larvae of second, third and fourth instars of coccinellids collected in the field using the same protocol described in Hoogendoorn and Heimpel (2001). Gene sequence in ITS-1 or COI regions were founded in Genbank for *C. septempunctata, H. axyridis* and *C. maculata*. We sequenced the region ITS-1 for *P. quatuordecimpunctata* from specimens collected in soybean fields of seven different regions in Québec in 2004. All primers were designed within these sequences with the software 'Primer 3', available on the web. Primer sequences and fragment size detected for all coccinellids species are given in table 1.

Table 1. Characteristics of each primers used for detection of coccinellids species.

Species	Region	Fragment size	Sequence
Coccinella septempunctata	ITS-1	105 bp	C7-ITS-R: AAG TTC GCT CGT CCT GGT TA C7-ITS-F: CGA AAG ACG ATC CCT ACG AA
Propylea quatuordecimpunctata	ITS-1	115 bp	P14-ITS-R: ATC GCT TTC TCC ACC TCG TA P14-ITS-F: GAT ATA TCG GCG CGT TTC TC
Harmonia axyridis	ITS-1	120 bp	Ha-ITS-R: AGG TAG CTT CAA TCG ATC GG Ha-ITS-F: AAG AGG AGA CGC CGA CCA GA
Coleomegilla maculata	COI	137 bp	Cmac-COI-R: GCC TTC TCC TTC CCT TCT TT Cmac-COI-F: AGT GAA AAT GGG CAA CAA CA

PCR amplification

We used a conserved primer derived from a mitochondrial 12S rRNA sequence of *Drosophila yakuba* Burla to check for the presence of DNA in each sample (12Sai and 12Sbi, in Noda *et al*. (1997)). Subsequently, all PCR reactions were done separatly with each primer pair. Amplifications were performed in 20.25 µl of 1× buffer (0.25 mM of each dNTP and 1.5 mM of $MgCl_2$), 2.5 µl of primer mix (20 µM), 0.25 µl of *Taq* (i.e. 1.75 units) (Promega), and 2 µl DNA sample. The thermocycling program consisted of an initial step of 30s at 94°C, followed by 30s at 94°C, 30s at 52°C, and 30s at 72°C. The three last steps were repeated 30 times and were followed by a step of 5 min at 72°C. For *H. axyridis* only, we did a hot start (i.e. first step 5 min at 94°C and then we added the *Taq)*, and all the following steps were similar, except for the annealing temperature that was at 55°C instead of 52°C. PCR products were electrophoresed at 130V in a 2% agarose gel for approximately 2 h.

We used two types of both negative and positive controls for all amplifications. The first negative control contained DNA from the head of an adult coccinellid being tested to get the certainty of having only individual predator DNA and no gut-content DNA in the sample. The second negative control was distilled water instead of DNA. A 1:10 (prey: predator) DNA mix was used for one of the positive controls to simulate IG prey in the predator gut and a second of 100% prey DNA was used as well.

Cross-reactivity test
The primers were also tested for cross-reactivity with coccinellid species used in the experiment, including *Hippodamia convergens* Guérin-Meneville, a coccinellid species less common in northern Québec. For all primer pairs, 3 samples of each coccinellid species DNA were tested to assess the primer specificity.

Digestion rate
Furthermore, we conducted an experiment to evaluate digestion rate of each coccinellid species. Thus, we confronted each coccinellid species with another species to get all possible cross-species combinations. The confrontation consisted of a last-larval-instar predator and 5 eggs of the prey species. Last larval instars and eggs were used because they are more probable to engage in, and suffer from IGP, respectively (Cottrell and Yeargan, 1998; Lucas *et al.*, 1998). Before each feeding experiment, individuals were starved after molting for 48h to obtain de same degree of hunger and increase their motivation to forage. Also, the eggs used were less than 5 days old and were kept at 4°C until the experiment. The experimental setup consisted of a 9 cm diameter Petri dish, each containing a filter paper and a piece of moistened cotton. During the experiment, each individual was allowed to feed on 5 eggs of the prey species for a maximum of 30 min and specimens that ate less than 1 egg were discard. After feeding, the number of eggs eaten was recorded and individuals were allowed to digest their meals for time-spans ranging from 0 to 16h, with interval of 4 h. During digestion, individuals were kept at 22°C in growth chambers for their respective time and they were frozen at –80°C until DNA extraction.

Results
All the primers used (Fig.1) in the experiment are of small fragment size, between 100 and 140 bp (*C. septempunctata* = 105 bp, *P. quatuordecimpunctata* = 115 bp, *H. axyridis* = 120 bp and *C. maculata* = 137 bp). For each primer pair, annealing temperature, $MgCl_2$ concentration or primers concentration were optimized to obtain the best degree of specificity. All primers were extremely specific except for *H. axyridis* and *C. maculata.* For the former one, use of higher annealing temperature (55°C) and use of a hot start were necessary to get specific detection. The *C. maculata* primer also amplified *H. convergens* DNA. This primer was retained for our study since this species is present at very low densities in Québec soybean fields. Also, each primer pair was able to detect the prey species as a 10% mixture (represent 50 pmoles) with each coccinellid species (Fig. 2). For all other species, the cross-reactivity test showed that no DNA was amplified with other species of coccinellids than the primer species.

Figure 1. Ethidium-bromide-stained agarose gel of PCR products from 4 coccinellid species. Lane 1 shows primers pair for *C. septempunctata*, lane 2, *P. quatuordecimpunctata*, lane 3, *H. axyridis* and lane 4, *C. maculata.* "M" is the molecular-size marker (25 bp DNA step ladder, Promega).

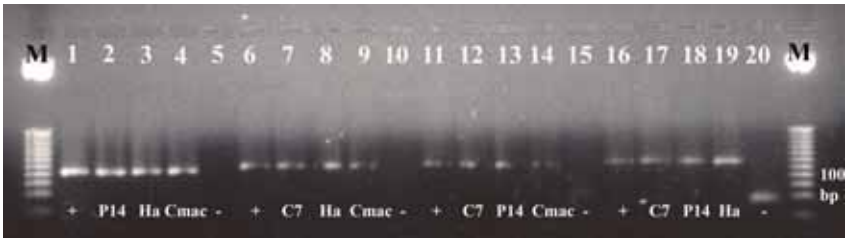

Figure 2. DNA amplification of mixed DNA [1:10 (prey: predator)] using all of the prey: predatory combinations. Lanes 1-5 represent *C. septempunctata* with positive control (+), the 3 IG prey and negative control (-); lanes 6-10 *P. quatuordecimpunctata* with +, the 3 IG prey and -; lanes 11-15 *H. axyridis* with +, the 3 IG prey and -; lanes 16-20 *C. maculata* with +, the 3 IG prey and -. IG prey are C7: *C. septempunctata*, P14: *P. quatuordecimpunctata*, Ha: *H. axyridis* and Cmac: *C. maculata.* "M" is the molecular-size marker (25 bp DNA step ladder, Promega).

The digestion rate experiment was done for each coccinellid species. The results of this experiment will be presented elsewhere (Gagnon *et al*., in prep.), but we summarize them here. The digestion rate seems to vary between species. For example, it was possible to detect prey DNA in the gut of *H. axyridis* after 16 h of digestion, but with other species like *C. maculata*, it was difficult to detect prey DNA after only 4 h or 8 h.

Discussion

PCR markers can be of great use for gut-contents analysis of predators. 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 (Chen *et al*., 2000). Regions of the internal transcribed spacer of the ribosomial gene complex (ITS-1) and the mitochondrial cytochrome oxidase I gene (COI) that we used in this experiment are both present in multiple copies (Hoy, 2003). Since ITS-1 regions possess high genetic variability among coccinellids species (von der Schulenburg *et al*., 2001), it is relatively easy to find specific markers for closely related species (Symondson, 2002).

Many factors can modify the detection period for prey remaining in the gut. The fragment size detected by the pair of primer seems to affect detectability. Designing a pair of primers that amplify a shorter fragment (150 or 100 bp) may increase the detection period (Agustí *et al*., 1999; Hoogendoorn and Heimpel, 2001). The fragment sizes of our primers were all between 100 and 140 bp, thereby, enhancing the probability of detecting prey DNA in the gut content for a longer period of time. The temperature also has an impact on detectability of prey DNA as a higher temperature resulted in an increase in the digestion rate (Hoogendoorn and Heimpel, 2001). Hoogendoorn and Heimpel (2001, 2002) showed that detectability does not seem to be influenced by meal size, coccinellid predator species (*C. maculata* and *H. axyridis*), predator weight, or predator sex and stage. In their experiments, they used *Ostrinia nubilalis* eggs that were fed to larval and adult coccinellids and they found that a fragment of 150 bp remained detectable for up to 12 h in *C. maculata* (Hoogendoorn and Heimpel, 2001).

Finally, this study provides a reliable measure of IGP that can overcome the constraints related to the use of experimental setups. It will now be possible to evaluate the occurrence of IGP under field conditions.

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