Gut-content analysis of predatory phytoseiid mites using fluorescent-labeled polymerase chain reaction: age of spider-mite eggs influences detection rates

Norihide HINOMOTO¹*

¹Biological Control Research Group, Division of Applied Entomology and Zoology, Central Region Agricultural Research Center, National Agriculture and Food Research Organization (NARO), Kannondai 2-1-18, Tsukuba, Ibaraki 305-8666, Japan
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ABSTRACT

As spider mites become increasing resistant to chemical acaricides, interest in establishing biological control programs using natural enemies has also risen, requiring verification of spider-mite predators. Although some phytoseiid mites are considered effective predators in agro-ecosystems, their small size makes it difficult to confirm predation through field observation. Polymerase chain reaction (PCR) can be an effective detection technique and has been used commonly in studies on microfaunal predator-prey interactions. However, preliminary data revealed that ordinary agarose gel electrophoresis cannot detect PCR products from phytoseiid mites that consumed spider-mite eggs. In this study, I used fluorescent-labeled primers and genetic analyzers to successfully amplify the gut contents of phytoseiid mites and confirmed they were derived from spider-mite eggs based on fragment analysis. The results indicated that spider-mite eggs can be detected ≥24 h post-oviposition, but not within 3 h. Thus, fluorescent PCR is an effective tool for elucidating predator-prey interactions among microfaunal food chains.

Key words: Phytoseiid mite, spider mite, biological control, predator-prey interaction, gut content analysis, fluorescent primers

INTRODUCTION

Spider mites (Acari: Tetranychidae) are major pests of many agricultural crops. The historically heavy usage of acaricides has caused spider mites to develop resistance against chemical pesticides (e.g., Dennehuy et al., 1987; Nauen et al., 2001; Osakabe et al., 2009; Sato et al., 2004; Stumpf and Nauen, 2001, 2002; Van Leeuwen et al., 2004), leading to increasing interest in biological control methods. Several phytoseiid mite species (Acari: Phytoseiidae) are candidates for natural enemies of spider mites. Studies have attempted to identify the most
effective phytoseiid predators and increase their populations for spider-mite control (e.g., Abad-Moyano et al., 2009; Funayama et al., 2015; Hoddle et al., 2000; Katayama et al., 2006; Kishimoto, 2002; Oliveira et al., 2007; Toyoshima, 2003). However, with reduced acaricide use, many phytoseiid species are present in the field, and their small size causes difficulties in determining true spider-mite predators through field observations.

Recently, gut content analysis using molecular markers have been developed and used for many predatory species (e.g., Agustí et al., 2003; Greenstone, 2003; Hoogendoorn and Heimpel, 2001; Itou et al., 2013; Kamikawa et al., 2016; Symondson, 2002; Wari et al., 2014; Zhang et al., 2007). Phytoseiid mites—especially specialist species—tend to consume spider-mite eggs (Blackwood et al., 2001; Furuichi et al., 2005), and the small size of this preferred prey item hampers detection in the gut. The establishment of an effective analytical method that can identify egg remains from the phytoseiid gut will allow us to better evaluate predator-prey interactions between phytoseiid mites and spider mites, while also identifying the best predator. However, few such studies have examined the gut contents of phytoseiid mites (Melo et al., 2015; Rivera-Rivera et al., 2012; Wari et al., 2014). Therefore, in this study, I conducted laboratory feeding experiments in conjunction with fluorescent-labeled PCR to analyze the gut contents of phytoseiid mites.

MATERIALS AND METHODS

Mites

The phytoseiid mite Neoseiulus californicus (McGregor), obtained from Arysta LifeScience Corporation, was selected as the potential predator. Laboratory strains of two spider mite species (Tetranychus urticae Koch and T. kanzawai Kishida) were used as prey. Phytoseiid mites were provided with a diet of T. urticae prior to the experiment. Likewise, spider mites were maintained on leaf discs of the kidney bean (Phaseolus vulgaris L.). Both phytoseiid and spider mites were maintained in the laboratory under a 16L:8D photoperiod at 25°C.

Feeding experiments

Twenty females of spider mites were placed on a leaf disc (1.5cm × 1.5cm) of the kidney bean and allowed to oviposit for 2 h. After removing them, discs with spider-mite eggs were either used immediately (0 h), or placed in an incubator at 25°C for the following periods: 3, 6, 12, 24, 36, and 60 h.

Adult females of N. californicus were food-deprived for over 6 h in 1.5-mL microtubes supplemented with small water drops. They became slightly thin, but their behavior seemed normal. Subsequently, a single female was placed on the leaf disc and observed under a microscope. After the female consumed a spider-mite egg completely, it was transferred to a 0.5-mL microtube with small water drops and maintained in an incubator at 25°C. Females were excluded from subsequent analysis if they did not feed on any eggs within 5 min; most females ate an egg immediately upon introduction. Within 1 h (typically between 15 min and 46 min), microtubes were dipped in liquid nitrogen and preserved in a freezer at −80°C until DNA analysis.
DNA extraction, amplification, and fragment analysis

Phytoseiid mites were ground at 1,500 rpm for 5 min in a bead mill (Shakemaster® ver 1.2; Bio Medical Science) containing zirconium dioxide beads (1.0 mm in diameter) and 20 µL of PrepMan Ultra Reagent (Applied Biosystems). Crushed mites were heated at 100°C for 2 min and centrifuged at 16,000 × g for 3 min. The supernatant was then used for polymerase chain reaction (PCR). Control DNA was extracted in the same manner from phytoseiid eggs, and adult spider mite females.

The marker for spider-mite DNA detection was the internal transcribed spacer 1 (ITS1) region of nuclear ribosomal RNA gene, amplified with primers rD02 (5′-GTCGTAACAAGGTTTCCGTAGG-3′) and rD03 (5′-TGGCTGCCTTCTTCATCG-3′) (Hinomoto and Takafuji, 2001). The forward primer rD02 was labeled with fluorescent dye (Beckman Dye D4). Successful DNA extraction was checked with primers rD01 (5′-TCCAGCTCCAATAGCGTA-3′) and rD05 (5′-GCAGGTTCACCTACGGAAC-3′), designed based on the conserved region of the gene encoding acarine 28S ribosomal RNA gene (Crampton et al., 1996). The PCR mix (10 µL) contained 0.2 mM dNTPs, 0.2 µM of each primer, 1 µL of DNA, and 0.2 U of Ex Taq DNA polymerase (Takara). The mixture was added to 0.2-mL thin-walled PCR tubes and subjected to the following reaction profile in a thermocycler (iCycler; Bio-Rad Laboratories): 94°C for 3 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; and 72°C for 10 min. Resultant PCR products were visualized with agarose gel electrophoresis, and non-amplified samples were eliminated from the further analysis. A restriction reaction was conducted on some amplified fragments with the following mixture: 0.8 µL of 10× restriction buffer, 2.2 µL of distilled water, 1 µL of restriction enzyme Mun I (Promega), and 4 µL of PCR products. Next, the mixture was incubated in a thermal cycler at 37°C for two to three hours.

Fragment analysis was then performed on the PCR products of rD02 and rD03. Mixtures of 2.0 µL of PCR products, 0.5 µL of 600-bp size standard (Beckman Coulter), and 40 µL of Hi-Di Formamide (Applied Biosystems) were applied to a CEQ 8000 genetic analysis system (Beckman Coulter). Fragment lengths were estimated with the included software.

RESULTS AND DISCUSSION

In this study, PCR with fluorescent-labeled primers successfully detected DNA fragments derived from spider-mite eggs in whole-body extracts of phytoseiid mites. A preliminary study using ordinary agarose gel electrophoresis failed to amplify any DNA fragments (data not shown), indicating that fluorescent primers must increase detection sensitivity. These results are extremely promising for future gut-content analysis of microfaunal predators.

Fragment analysis comparing data from adult spider mite females, phytoseiid eggs, and adult phytoseiid mite females revealed that DNA of the two mite groups are clearly distinguishable based on bp differences (Fig. 1). Spider mite-derived fragments were approximately 580 bp (n = 9), consistent with predictions based on DNA sequences (Fig. 1). Phytoseiid mites that consumed a spider-mite egg exhibited a 580-bp fragment and ca. 270-bp fragments. Phytoseiid mite females that did not feed on spider mites, along with phytoseiid eggs, both possessed only 270-bp fragments (n = 23 and 3 respectively) or no fragments (n = 2 and 2). These results indicated the
580-bp fragments were derived from spider mites, whereas the 270-bp fragments were nonspecific. Hereafter, I refer to the 580-bp fragment as the “spider-mite fragment.”

The lengths of amplified ITS1 were estimated as 578 bp and 580 bp for *T. urticae* and *T. kanzawai*, respectively, based on sequences deposited with the International Nucleotide Sequence Database (INSD; accession numbers AB076370 and AB076369; Osakabe et al., 2002). The estimated length of the entire amplified fragment was 2 bp longer in *T. kanzawai* than in *T. urticae* (Fig. 2 [1] and [3]). *Tetranychus kanzawai* and *T. urticae* are predicted to have four and three restriction sites for *Mun* I, respectively, leading to 163-bp and 135-bp fragment lengths at the fluorescent-labeled 5′ end. Electrophoresis results show that the restriction fragments of *T. kanzawai* and *T. urticae* differed by about 30 bp (Fig. 2 [2] and [4]). Thus, I confirmed that the amplified fragments were derived from the spider-mite eggs consumed by phytoseiid mites.

Detection rates of spider-mite fragments in phytoseiid mites were separated into blocks based on time after oviposition (i.e., egg age) to understand the point at which egg-derived DNA becomes measurable. For example, 0-h indicates 0–2-h-old eggs, because spider mites oviposited for two hours before incubation began. Spider-mite fragments were not detected in phytoseiid mites fed on 0-h and 3-h eggs, but were detected in all females fed on ≥24-h eggs (Fig. 3). Spider-mite eggs enter the blastula stage after 22 hours post-deposition at 24–25°C (Gotoh et al., 1994), when they appear to have enough genome copies for detection. Furthermore, the egg stage...
of *T. urticae* is about 4.5 days at 25°C (Saito, 1979). Thus, over 70% of consumed eggs can be detected using the method employed here if phytoseiid mites do not prefer younger eggs over older eggs.

In conclusion, this study demonstrated that fluorescent primers and genetic analyzers allow the amplification of trace PCR products. Because prey DNA degrades in the predator’s gut and detection is therefore time-dependent, future studies need to determine the maximum duration before ingested spider-mite DNA becomes undetectable. Nonetheless, the fluorescent-labeling method contributes to our understanding of predator-prey food chains by increasing the applicability of gut content analysis to more organisms.

**Fig. 2.** Representative results of fragment analysis on fluorescent-labeled PCR-amplified DNA. Templates are as follows: (1) *Neoseiulus californicus* female that consumed a 24-h-old egg of *Tetranychus kanzawai*, (2) restriction fragments of (1), (3) *N. californicus* female that consumed a 24-h-old egg of *T. urticae*, and (4) restriction fragments of (3). Arrows A, B, and C indicate spider mite-derived PCR fragments, the *T. kanzawai*-specific restriction fragment, and the *T. urticae*-specific restriction fragment, respectively.
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**Fig. 3.** Detection rates of spider-mite DNA fragments from adult females of *Neoseiulus californicus*. Rates are grouped by blocks of time post-oviposition, during which *N. californicus* females (number indicated with N above bars) consumed *T. urticae* eggs.
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摘要
カブリダニに捕食されたハダニ卵齢が蛍光プライマーを用いた検出率に及ぼす影響
日本 典秀（農研機構 中央農業研究センター）

ハダニ類は様々な作物の重要害虫であるが、薬剤抵抗性の発達が著しく、近年は天敵を利用した生物的防除への関心が高まっている。カブリダニ類は有力な天敵候補の一つであるが、減農薬体系下では多様なカブリダニ類が出現するため、どの種が有望であるか特定する必要がある。しかし、体サイズが小さなカブリダニ類の捕食行動を野外で観察することは困難である。近年、PCRを利用した被食者検出技術が様々な捕食者─被食者系の研究で用いられるようになってきた。本研究では、カブリダニ類─ハダニ類の系においてこの方法を用いて、ハダニ卵を捕食させたカブリダニからハダニ由来 DNA を検出できるか試みた。ハダニ特異的 PCR 産物は、通常のアガロースゲル電気泳動では検出できなかったが、蛍光プライマーによる PCR 産物のジェネティックアナライザーによるフラグメント解析によって、効率的に検出可能であった。産下後 3 時間以内のハダニ卵を捕食した場合は全く検出できなかったが、24 時間以上経過したハダニ卵の場合は 100% 検出可能であった。本方法は、カブリダニ類─ハダニ類の捕食─被食関係を明らかにするために利用可能と期待できた。