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ORIGINAL CONTRIBUTION

Real-time PCR for identification of the soybean aphid, *Aphis glycines* Matsumura

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Abstract

The soybean aphid (*Aphis glycines* Matsumura) is an economically significant pest in North America, causing extensive damage to soybean crops through direct feeding damage and disease transmission. If unchecked, this pest could cause billions of dollars of damage to soybean crops. Identification of the soybean aphid can be difficult due to its small size, complex life cycle and morphological plasticity. Generally, an expert is required to identify a specimen. Additionally, identification of some life stages, such as eggs, is impossible. DNA barcoding has been successfully used to differentiate aphid species, including *A. glycines,* based on sequencing of a standardized gene region. Although this method represents an important step towards accurate identification, samples must still be sent to specialized facilities for analysis. Using existing DNA barcode sequences in the publically accessible Barcode of Life Data System (BOLD; www.boldsystems.org), species-specific differences were identified and used to develop a real-time PCR assay to identify soybean aphids. This assay can be run on portable systems for rapid, accurate and simple identification in the field. The use of a non-destructive DNA extraction protocol allows the original insect to be vouchered and therefore available for further study if necessary. This work represents an important step in soybean aphid management.

Introduction

The soybean aphid, *Aphis glycines* Matsumura, is an invasive pest of soybean crops. From its arrival in North America in 2000–2003, *A. glycines* had spread to 20 states and three provinces (Ragsdale et al. 2004), and by 2009, it was found in 30 states (Ragsdale et al. 2011). There is a risk of further spread through the United States and South America (Venette and Ragsdale 2004; Ragsdale et al. 2011).

Populations can change quickly, doubling in <2 days under ideal conditions (McCornack et al. 2004). Economic loss from this pest can occur due to direct feeding damage and disease transmission (Blackman and Eastop 2000). Evidence suggests that effects on photosynthesis in soybean can occur even at low densities of *A. glycines* (Macedo et al. 2003).

To mitigate the potential economic impact of this pest, including continued spread, early identification and management is a key step. However, morphological identification can be complicated by complex life cycles, morphological plasticity and similarity to other species that share the same hosts. In North America, *A. glycines* and *Aphis nasturtii* Kaltenbach are difficult to distinguish on the shared primary (overwintering) hosts, *Rhamnus* species (Voegtlin et al. 2004). A polyphagous aphid, *Aphis gossypii* Glover, is morphologically similar to *A. glycines* and may also occur on both soybean and *Rhamnus*. Other species related to *A. gossypii* occur on *Rhamnus* in Asia and Europe (Blackman and Eastop 1994).

Molecular techniques, such as DNA barcoding, may be more accurate than visual identification in cases where species are morphologically similar. DNA barcoding of the 5′ end of the mitochondrial cytochrome

c oxidase I (COI) gene for species identification (Hebert et al. 2003) has been shown to differentiate species of Aphididae (Foottit et al. 2008; Lee et al. 2011), and sequence data has been used to distinguish soybean aphid from related aphids on Rhamnus (Kim et al. 2010). This method also allows for identification of immature individuals, including eggs (Shufran and Puterka 2011). This is especially important for soybean aphids, as adults are not always found upon initial colonization of a soybean field (Ragsdale et al. 2004), and allows detection of eggs on overwintering hosts.

Although DNA barcoding represents an important step towards improved pest identification, the lack of portability can still present an obstacle to rapid implementation of pest management. A simpler, field-ready form of molecular identification is becoming increasingly desirable.

Real-time PCR has been used for pest insect identification (Walsh et al. 2005; Yu et al. 2005; Huang et al. 2010), including aphids (Naaum et al. 2012). This method is quicker and yields results that are easier to interpret than DNA barcoding. An additional benefit is that portable platforms exist, allowing these assays to be run in the field. Paired with simple extraction techniques, it has been demonstrated that the entire process of molecular identification, including DNA extraction, can be performed in the field (Tomlinson et al. 2005). Rapid, non-destructive DNA extraction techniques for arthropods (Castalanelli et al. 2010) allow for complete analysis from sample to identification in the field for insect pests, while preserving the sample as a voucher for further morphological analysis.

We describe here a real-time PCR assay, combined with the ANDE DNA extraction, for the portable, rapid and accurate identification of the soybean aphid. This assay was designed using existing high-quality DNA barcode sequences derived from vouchered specimens representing over three hundred species of aphid, including those morphologically similar to *A. glycines*, from the Barcode of Life Data Systems (BOLD; www.boldsystems.org; Ratnasingham and Hebert 2007).

Methods

Real-time PCR primer and probe design

Species-specific primers and a TaqMan probe were developed for *A. glycines* using sequences from the publically available BOLD project 'Barcoding the Aphididae'. This project contains 690 sequences from 338 species, including *A. gossypii* and *Aphis nasturtii*, which share hosts with *A. glycines*. These sequences were derived from individuals covering a wide geographical range. Two hundred and nine unique haplotypes, representing all sequences in this project, were identified using the haplotype identification tool at ibarcode.org (Singer and Hajibabaei 2009). This included 2 haplotypes of *A. glycines* from 5 individuals collected in Japan, Quebec, Kentucky, Ontario and Australia. Primers and probes (Table 1) were designed using AlleleID (version 7.7, Premier Biosoft International, Palo Alto, CA, USA). The TaqMan probe was tagged at the 5′ end with the fluorescent reporter 6-carboxyfluoroscein (FAM) and at the 3′ end with BHQ-1. Optimization of probe and primer concentrations and cycling conditions was carried out according to the guidelines set by Cepheid for the SmartCycler II System (SmartNote 6.2; http://www.cepheid.com/systems-and-software/smartcycler-system).

Species selection and sample collection

A hierarchy of different species related to *A. glycines* were selected for specificity testing as in Naaum et al. 2012, with the following differences: *Aphis rubicola* Oestlund, *Aphis farinosa* J.F. Gmelin and *Aphis hyperici* Monell were not included in this study, and *Aphis sedi* Kalthenbach was included. Table S1 contains the detailed collection information for aphids used in this study. Voucher specimens for these collections were deposited in the Canadian National Collection of Insects (Agriculture and Agri-Food Canada, Ottawa).

DNA extraction

DNA extraction was carried out on individual aphids using the XytXtract Insect (ANDE) extraction kit (Xytogen, Perth, Australia). Aphids were placed in 0.2-µl tubes. 18.5 µl of solution 1 and 5.5 µl of solution 2 were added. Tubes were then incubated at 95°C for 20 min. Following this incubation, 6.5 µl of

Table 1 Sequences of species-specific oligonucleotides used in this study and amplicon length. All primers and probes target portions of the *Aphis glycines* cytochrome C oxidase I (COI) DNA barcode region

Forward primer sequence (5′–3′)	GGATGTCCAGATATATCTTTTC
Reverse primer sequence (5′–3′)	CCTGTTCCTGTTCCATTA
Probe sequence (5′–3′)	6FAM-AGATTCTGATTATTGCCACCTTCATT-BHQ1
Amplicon length	113 base pairs

solution 3 was added. After gentle mixing, the specimen was removed and DNA was stored until use in real-time PCR. DNA concentration was determined using a Nanodrop 8000 (Thermo Scientific, Wilmington, DE, USA) and ranged from 65 to 475 ng/μl.

Real-time PCR

Reactions were carried out in 25 μl volumes containing sterile water, 5 μl template DNA directly from extraction without dilution, forward and reverse primers at a concentration of 0.3 μM and 0.5 μM, respectively, probe at a concentration of 0.1 μM and OmniMix HS lyophilized master mix (Cepheid, Sunnyvale, CA, USA) according to manufacturer's guidelines. PCR cycling conditions were as follows: an initial step of 95°C for 120 s, followed by 40 cycles of 94°C for 11 s, 60°C for 40 s and 72°C for 25 s. Fluorescence readings were taken at the annealing step of each cycle. All reactions were carried out on the SmartCyclerII (Cepheid). The number of cycles required for fluorescence to exceed the threshold (cycle threshold, Ct) was recorded for each sample. The default threshold of 30 was used. If no signal was observed, a Ct of 40 was recorded. Ten individuals from the target species were tested. Three individuals from each of the other species selected were also tested.

Sensitivity and linearity

A standard curve was generated from 10-fold serial dilutions of *A. glycines* DNA from 120 to 0.12 ng/μl to determine the range, detection limits and efficiency of the assay.

Results

Signals were observed in the FAM fluorescence channel for *A. glycines* between Ct = 19.81–25.72 and for *Aphis oestlundi* between Ct = 30.75–32.51. None of the other non-target species produced a signal. No false negatives were observed in this study, and no signal was observed for any no-template 'blank' controls. The standard curve (r^2 = 0.994) showed a linear range of 120–0.12 ng/μl, a detection limit of 0.012 ng/μl and efficiency of 110% (fig. 1).

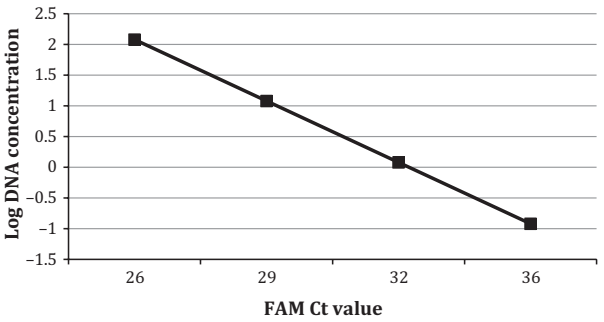

Fig. 1 Standard curve for *Aphis glycines* (r^2 = 0.994, slope = −0.325), generated from 10-fold serial dilutions of target DNA from 120 to 0.12 ng/μl.

Discussion

The availability of existing sequence data from a large number of aphids and related groups for a standard gene region makes the DNA barcode library ideally suited for real-time PCR assay development. Therefore, a real-time PCR assay to identify the soybean aphid was developed using the extensive DNA barcode library housed on BOLD. Vouchered DNA barcode sequences representing over 300 species of aphid were used in probe and primer design. The assay proved to be an accurate method of identifying *A. glycines*, correlating with the morphological identification in 100% of trials. This indicates that this method could be used to augment morphological identification of this species. Cross-amplification was only observed for one non-target species, always over 30 cycles. The latest Ct for any *A. glycines* individual was 25.72. Therefore, the use of a Ct = 30 cut-off for positive identification of *A. glycines* can be used to avoid incidence of false positives. Given that no DNA replication was observed for any of the other 26 non-target species, we also conclude that the assay is specific enough to differentiate *A. glycines* from closely related species, most significantly two species with which it shares hosts: *A. gossypii* and *A. nasturtii*. Therefore, rapid identification of the soybean aphid is possible on either its primary or secondary host.

The ANDE DNA extraction method takes just 30 min to complete and requires only that the solution be heated to 95°C to obtain DNA (Castalanelli et al. 2010). This simple, rapid method can therefore be carried out in the field using a heating block of any kind, including the real-time PCR platform itself. This improves on previous studies where DNA extraction proved a practical limitation to field identification of pests with real-time PCR (e.g. Naaum et al. 2012). Additionally, the non-destructive nature of the extraction provides a means for adult specimens to be recovered for independent morphological analysis and/or archival. All DNA concentrations obtained from the target species after extraction were successfully amplified, indicating that DNA extract can be

used directly in this assay without dilution and therefore need not be measured in the field.

The use of real-time PCR on a portable platform, such as the SmartCycler II used here, combined with the ANDE extraction, allows for identification in field settings in approximately 90 min for <$5.00 per sample. Field identification can result in more rapid implementation of pest control. This represents an improvement over earlier molecular methods proposed for aphid identification, which require a two-step PCR-gel electrophoresis procedure (e.g. Rebijeth et al. 2012) or DNA barcoding as discussed previously. These two methods both require non-portable equipment, and results can require more advanced interpretation than with real-time PCR, which can be run and interpreted by a non-specialist with minimal training. Lyophilized reagents in commercial kits, along with positive control DNA to eliminate false negatives occurring from failed PCR, could help extend the application of this method and may further reduce the likelihood of human error.

These results are encouraging and represent a successful preliminary optimization of this assay. Further validation by multiple laboratories and field testing should be undertaken in the future to confirm the efficacy of this assay in identification of *A. glycines*.

This assay has great potential as an important tool to aid in rapid soybean aphid identification and management because traditional morphological identification requires an adult specimen to be cleared, stained and slide-mounted for microscopic examination by an expert taxonomist. Our method can be used for identification in the field or at border crossings, where rapid identification at any life stage is a key to improve the accuracy of soybean aphid identification and to help limit the continued spread of this pest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Collection information for aphid specimens used in this study.