A Droplet Digital PCR (ddPCR) Assay to Detect *Phthorimaea absoluta* (Lepidoptera: Gelechiidae) in Bulk Trap Samples

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Abstract

The moth species *Phthorimaea absoluta* (Meyrick) (formerly *Tuta absoluta*) is serious threat to tomato and other Solanaceous crops worldwide and is invasive throughout Europe, Asia, and Africa. While *P. absoluta* has not yet been found in the U.S. recent detections in the Caribbean have raised concerns that the species could be introduced to mainland North America. To improve detection capacity, a droplet digital PCR (ddPCR) assay was developed that employs a nondestructive bulk DNA extraction method able to detect one *P. absoluta* sample among 200 nontargets. Such high-throughput and sensitive molecular assays are essential to preventing introductions through early detection and response. This assay can also be used in areas where *P. absoluta* is established to monitor outbreaks and track migratory patterns.

Key words: South American tomato leaf miner, Solanaceae, tomato, invasive species, agriculture

The South American tomato leaf miner, *Phthorimaea absoluta* (Meyrick), is a pest species that has spread throughout Europe, Africa, Central America, much of Asia, and recently, to several Caribbean islands (Campos et al. 2017, Han et al. 2019, Santana et al. 2019, Verheggen and Fontus 2019, Zhang et al. 2020). It was recently reinstated as *P. absoluta*, as originally described, after having been treated as *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) for many years (Chang and Metz 2021). Its success as an invasive species and the widespread production of its preferred host species, tomato (Tonnang et al. 2015), has raised concerns that it may become established in the United States. The establishment of *P. absoluta* could have a devastating impact on the $1.5 billion U.S. tomato crop (USDA 2021) given that some outbreaks have resulted in near total crop loss (e.g., Desneux et al. 2010, Ghaderi et al. 2019, Mansour et al. 2019). Surveillance for the presence of *P. absoluta* is conducted using pheromone lures affixed to sticky traps (Vick et al. 1990). Its success as an invasive species and the widespread production of its preferred host species, tomato (Tonnang et al. 2015), has raised concerns that it may become established in the United States. The establishment of *P. absoluta* could have a devastating impact on the $1.5 billion U.S. tomato crop (USDA 2021) given that some outbreaks have resulted in near total crop loss (e.g., Desneux et al. 2010, Ghaderi et al. 2019, Mansour et al. 2019). Surveillance for the presence of *P. absoluta* is conducted using pheromone lures affixed to sticky traps which are deployed in tomato growing regions. These traps capture a wide variety of bycatch including other Gelechiidae and a variety of small moths (Vick et al. 1990). Due to its small size, *P. absoluta* is difficult to identify through morphology or dissection, especially when captured on sticky traps. Consequently, several molecular assays have been developed for identification of this species using DNA (Đurić et al. 2014, Sint et al. 2016, Tabuloc et al. 2019, Zink et al. 2020, Butterwort et al. 2022). One such method, a real-time PCR assay, has been shown to be sensitive and specific for detection of *P. absoluta* in the United States (Zink et al. 2020). The assay uses a set of primers and a hydrolysis probe that target a portion of the internal transcribed spacer 1 (ITS1) region of rDNA. The primers and probe were also shown to be suitable for droplet digital PCR (ddPCR) on purified DNA samples. Here we optimize the Zink et al. (2020) ddPCR assay and demonstrate that it can be used for identifying *P. absoluta* in bulk samples using a nondestructive DNA extraction technique. These improvements will allow the assay to be used to screen hundreds of moths at a time to determine the presence of *P. absoluta* on sticky traps. The capability to conduct high-throughput screening of bulk samples is essential to early detection of this species in areas where it is not yet present and to monitor outbreaks of this species where it is already established.

Materials and Methods

Specimen Collection

*P. absoluta* specimens were obtained from a lab colony maintained at Geisenheim University, Germany and stored in 100% ethanol at −20°C until use. Nontargets for bulk samples were collected in Florida in March 2019 and in Colorado, Montana, Ohio, and Utah.
in June and July 2021. All nontargets were collected using light traps. Nontargets were stored in envelopes at 4°C until use.

DNA Extraction

For all bulk and low-quality individual DNA extractions, a single step technique using a modified squish buffer (Gloor et al. 1993) formulation as described by Perera et al. (2015) or Oliveira et al. (2021) was carried out by incubating samples overnight at 56°C in a Thermomixer FP (Eppendorf AG, Hamburg, Germany). For destructive DNA extractions, samples were pulverized using 1.4 mm silica-zirconium beads in a Mini-Beadbeater (Bio-Spec Products, Bartlesville, OK) and 1,000 µL of squish buffer was used for every 0.1g of material; or 30 µL of squish buffer for every individual. Nondestructive extractions were carried out with whole moths and enough squish buffer was used to saturate the moths, approximately 1,000–1,500 µL per 0.1 g of material. Following incubation, samples were centrifuged in an Eppendorf 5424 bench-top microcentrifuge at 16873 × g and the supernatant was retained for use. Both types of extractions were carried out for masses of moths ranging from 0.1 g to 0.5 g of total tissue and for numbers of nontargets from 10 to 200 individuals. All bulk samples were supplemented with a single P. absoluta. When appropriate, the DNA was bead purified using AmpureXP paramagnetic beads (Beckman Coulter Inc., Brea, CA) following Oliveira et al. (2021). Higher-quality individual extractions were carried out using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following manufacturer’s instructions.

Droplet Digital PCR

All ddPCR was carried out following the protocol from Zink et al. (2019) using the primers and probe from Zink et al. (2020) on the Bio-Rad QX200 ddPCR system (Bio-Rad Laboratories Inc., Hercules, CA). All reactions consisted of 10 µL 2x ddPCR Supermix for Probes (no dUTP) (Bio-Rad Laboratories Inc.), 200 nM of each forward (5'-GGCAAGCAGATGCACCATACCG-3') and reverse (5'-AGGGTACATGTCAGCTAATG) primer, 100 nM of probe (5'-56FAM-CTTAAAGAATZEN/CATGTCAGCAGGACG-3IABkFQ), 1 µL of DNA of varying concentration, and water to complete the dilution. Primers and probes were synthesized by IDT (Integrated DNA Technologies Inc., Coralville, IA). The thermocycling protocol used was as follows: denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, then heated to 98°C for 10 min and cooled to 4°C with a ramp rate of 2°C/s between all steps. Following droplet reading, the threshold for calling positive droplets was determined using ‘definetherain’ (Jones et al. 2014). The false positive rate was determined following Zink et al. (2017) using nondestructive DNA extractions of 25 nontargets using high salt squish buffer and bead purification.

Screening Test

One specimen of P. absoluta was combined with 9 nontarget specimens of a similar size in a 1.5 ml tube with 1 ml of Oliveira et al. (2021) squish buffer and incubated overnight as described above for nondestructive DNA extractions. After the incubation, specimens were removed from squish buffer, rinsed briefly in 95% ethanol, and each was placed in a new 1.5 ml tube to dry. The supernatant was used for ddPCR as described above. The dried specimens were used for nondestructive DNA extraction using the Qiagen DNeasy Blood and Tissue kit as outlined above. The DNA from each specimen was then used for real-time PCR as described in Zink et al. (2020).

Results

The lowest concentration of P. absoluta DNA detectable by ddPCR, using a purified squish buffer extraction from a single moth and the optimized reaction conditions, was 0.001 ng/µL (Supp Fig S1 [online only]).

When testing bulk extraction methods initially, a destructive DNA extraction technique was tested in which varying numbers of whole moths, including a single P. absoluta, were ground to powder for use in the single-step extraction protocol, but due to the high level of pigmentation in the supernatant, it was unsuitable for use in ddPCR (Fig. 1A). This may be due to the presence of PCR inhibitors found in pigmented structures like the eyes of insects (Beckmann and Fallon 2014). Even after bead purification, these samples did not reliably reveal the presence of P. absoluta DNA in the extraction when used for ddPCR (Fig. 1B). Conversely, when nondestructive DNA extractions from bulk samples that included P. absoluta were used for ddPCR, P. absoluta was more reliably detected but the amplitude of negative droplets remained inconsistent and positive results were not clear (Fig. 1C). The addition of bead purification to the nondestructive DNA extraction method allowed successful detection of P. absoluta in pools of 100 or 200 nontarget moths (Fig. 1D). Similarly, if moths are combined by mass, P. absoluta is more often detectable when moths are kept whole for DNA extraction up to a total mass of 0.5 g and when using the squish buffer formulation described in Oliveira et al. (2021; Fig. 1D). The best method for extraction was a nondestructive extraction in 5x salt and EDTA squish buffer, followed by bead purification of the samples. When nontargets with wingspans smaller than 1.5 cm were used exclusively, P. absoluta was detected in ratios from 1:10 to 1:200 (P. absoluta to nontarget moths) when using the high salt and bead purification extraction (Fig. 2).

False positive rate (FPR) testing was carried out using a nondestructive extraction of 25 nontarget moths that was bead purified in the same manner as target samples. In total, 43 replicates were run in which there were two false positive events totaling 0.05 events per well (Supp Fig S2 [online only]). In order to call a well positive for P. absoluta with 99% confidence, only two positive droplets need be present, equating to approximately 5 copies of the target gene.

During a P. absoluta survey, specimens would be combined in a bulk extraction for screening by ddPCR. If the assay returned a positive result, individual specimens would require identification using the real-time PCR assay described in Zink et al. (2020). In a test of whether these methods are complementary and whether DNA can be extracted from the same specimen using both relevant techniques, an experimental sample was made containing 1 specimen of P. absoluta along with 9 visually similar specimens collected in Florida, USA. These specimens were extracted in bulk, removed from squish buffer, washed, and then extracted individually, in a nondestructive manner, with the Qiagen DNeasy Blood and Tissue kit. The bulk extraction was used for ddPCR and showed a positive result for the presence of P. absoluta (Fig. 3A). The individual extractions were used for real-time PCR following the protocol described in Zink et al. (2020) resulting in a single sample that was positive for P. absoluta and 9 samples that were negative for P. absoluta but positive for the 18S control (Fig. 3B).

Discussion

The utility of ddPCR for the detection of P. absoluta in bulk samples hinges on the quality of the DNA extraction. Despite the assumption that ddPCR is extraordinarily resistant to PCR inhibitors and
contaminants (e.g., Rački et al. 2014), our results replicate those of Zink et al. (2019) and Oliviera et al. (2021) in showing that the method of DNA extraction is determinate to assay performance for species detection in bulk samples. In this case, the physical disruption of specimens was detrimental to the detection of the target species possibly due to the increased amounts of PCR inhibitors released during pulverization (Beckmann and Fallon 2014). Additionally, use of moths larger than 1.5 cm in wingspan, whether in destructive or nondestructive extractions, greatly decreased the efficiency of the reactions in a manner that is nonlinear to the overall mass or number of nontargets included, possibly due to the presence of too much nontarget tissue or DNA. Fortunately, the nondestructive extraction method is preferred for field detections of P. absoluta because identification of individual moths using real-time PCR or another method would become necessary in such cases.

In addition to the extraction method optimization, the size of the moths included in screening should also be considered. While a sticky trap may collect any size or type of nontargets, only specimens that can reasonably be suspected to be P. absoluta should be included in molecular screening. Because bulk extraction from sticky trap samples necessitates removal of specimens from a trap (Butterwort et al. 2022), it is reasonable to then do a cursory sorting of trapped specimens by order (Lepidoptera) and size. Such efforts will increase the likelihood of successfully detecting P. absoluta if present. While whole-specimen DNA extractions still run the risk of fragmentation of small fragile specimens during removal from sticky traps, many of the morphological features used for diagnosis, such as genitalia, will remain undamaged. It should be noted that the P. absoluta specimens used in this study were collected from a colony and the nontargets were collected by light trap, both of which result in relatively clean well-preserved samples. Specimens that have been collected on sticky traps and exposed to the elements may show some level of DNA degradation and microbial growth potentially affecting the utility of
PCR-based identification, but the Histoclear (citrus oil) used to remove samples from the traps does not affect DNA quality or inhibit PCR (Butterwort et al. 2022). Our recommendation is to use the smallest practical sample size to maintain processing efficiency for bulk samples, which will also reduce the number of post-ddPCR individual identifications.

This ddPCR assay can be used along with the real-time PCR assay from Zink et al. (2020) to identify a specimen of *P. absoluta*. In a mock screening test of 10 specimens, only the *P. absoluta* individual met criteria outlined therein for diagnosis: a FAM Cq of <30 and a ΔCq between FAM and Quasar670 <3.5 cycles. The remaining specimens tested showed some detection of the FAM probe (Cq > 30) that did not meet diagnostic criteria as a detection of *P. absoluta*. Additionally, three had high Cq values for the 18S control probe indicating poor DNA quality (Fig. 3B). Due to the use of bulk extraction, there may be some low-level contamination of the nontarget samples by *P. absoluta* DNA as indicated by late amplification in real-time readouts. Improved external cleaning methods for specimens from bulk samples that require downstream identification of individuals may help reduce this effect. The effect may also be diminished in a larger sample size which would dilute the amount of foreign target DNA on nontarget samples due to the larger volume of extraction buffer used.

If DNA extraction best practices are employed, this ddPCR assay should be sufficient for detection in a typical trap catch as the pheromone lure used for trapping *P. absoluta* has little off-target effect when used in the United States. Like the real-time PCR assay outlined in Zink et al. (2020) this assay is not suited for use outside of the United States without further testing. As increased sampling increases the likelihood that an introduction of *P. absoluta* will be discovered before it becomes established, methods such as the ddPCR assay outlined here should be employed more regularly to help prevent the introduction and spread of *P. absoluta* while reducing the amount of time required for identification of each specimen.

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**Supplementary Data**

Supplementary data are available at *Journal of Economic Entomology* online.

Supplementary Figure S1: sensitivity of the ddPCR assay to detect purified *P. absoluta* DNA. The assay can reliably detect DNA to a concentration of 0.001ng/µL.

Supplementary Figure S2: False positive detection for the ddPCR assay to detect *P. absoluta* in bulk DNA extractions. Negative controls are extracted from 25 non-target moths in a non-destructive manner. Positive controls are purified *P. absoluta* DNA of varying concentrations.

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