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RESEARCH ARTICLE

Field friendly identification of the peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae), using loop-mediated isothermal amplification

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The rapid and accurate identification of invasive pest insects at quarantine stations or ports of entry is crucial for preventing their establishment and spread in new territories. Here, we present an effective and rapid genetic identification technique utilising loop-mediated isothermal amplification (LAMP) to detect the presence of the peach fruit fly, *Bactrocera zonata*. The LAMP assay was designed to target the cytochrome oxidase subunit I (COI) gene region of *B. zonata*, ensuring high specificity and sensitivity, and to discriminate it from a range of other economically important *Bactrocera* species. By utilising isothermal conditions, LAMP eliminates the need for lengthy thermal cycling and enables rapid amplification of target DNA within a short period. Additionally, a rapid DNA extraction method combined with the use of an intercalating UV fluorescent dye allows for fast and reliable interpretation of results without the need for specialised equipment. Validation studies were conducted using known samples of *B. zonata*, as well as other *Bactrocera* species commonly encountered in quarantine settings including *B. dorsalis*, *B. correcta*, *B. tryoni*, *B. oleae*, *B. cucurbitae* and *B. tau*. The power of this LAMP assay lies in its specificity to accurately distinguish *B. zonata* from other closely related *Bactrocera* species. Moreover, the assay exhibits a remarkably short turnaround time, with results obtained in under an hour, facilitating timely decision-making at quarantine stations.

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INTRODUCTION

The inadvertent spread of invasive insect species through global trade causes substantial economic losses in agriculture (Bacon et al. 2012; Horton et al. 2013; Szyniszewska et al. 2016). With the continuous growth in global trade, it is expected that the number and impact of successful invasions by plant pests will also increase (Levine & D'Antonio 2003; Essl et al. 2011). The European Union (EU) has established regulations that classify non-EU Tephritidae as quarantine organisms, prohibiting their introduction into the EU (EFSA Panel on Plant Health (PLH) 2020). Consequently, imported produce must undergo inspection by each EU member state to prevent the entry, establishment, and spread of these quarantine organisms. While experts can differentiate between adult tephritid species, identifying the larval stage, which is commonly found in intercepted produce, is challenging, even at the third larval instar (Balmès & Mouttet 2017; EPPO 2011). Larvae could be reared to the adult stage for morphological identification, however this prolongs the identification process, and adult identifications can be complicated by species complexes.

DNA-based molecular tools, particularly those involving DNA sequence or gene region analysis, have been increasingly used alongside morphological characters for species identification in various insect pest species. The advantages of DNA-based tools are further amplified when polymerase chain reaction (PCR) methods are employed, as they can often be applied even with limited quantities of poorly preserved specimens. Several DNA amplification-based techniques have been utilised for identifying insect pest species, including PCR-restriction fragment length polymorphism (RFLP) (Armstrong et al. 1997; Barr et al. 2006), amplified fragment length polymorphism (AFLP) (Kakouli-Duarte et al. 2001), oligonucleotide array-based methods (Naeole & Haymer 2003) and, more recently, DNA barcoding (Van Houdt et al. 2010; Barr et al. 2012; Virgilio et al. 2017) and multiplex PCR assays (Andrews et al. 2022). However, PCR-based methods have certain drawbacks, such as the need for a relatively expensive precision thermal cycler and the time-consuming process of shipping samples from quarantine checkpoints to reference laboratories, leading to significant delays in species identification. This delay poses a challenge, especially for perishable plant imports like fruits, as it can result in substantial economic losses for importers. To overcome this issue, rapid molecular on-site tests conducted directly at the point of entry have shown promise (Blaser et al. 2018; Alon et al. 2023).

One such rapid molecular diagnostic tool is loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000). This method offers rapid and robust amplification and operates under isothermal conditions, requiring only a simple thermostat-based instrument. The LAMP reaction employs specially designed primer pairs and a DNA polymerase, such as *Bst* DNA polymerase, which possesses strand displacement activity. Recent studies have demonstrated that the inclusion of an additional pair of primers (loop primers) can enhance the efficiency of the LAMP method by

reducing the amplification time by half (Nagamine et al. 2002). Overall, LAMP represents a valuable molecular diagnostic tool due to its simplicity, rapidity, and reliable performance.

The loop-mediated isothermal amplification (LAMP) technique has found diverse applications across various fields. LAMP has been utilised for diagnostic identification of bacterial, protozoan and viral infectious agents (Iwamoto et al. 2003; Thai et al. 2004; Poon et al. 2006). Additionally, LAMP has been employed for pathogen quantification (Toriniwa & Komiya 2006), as well as for the detection of plant viruses (Fukuta et al. 2004), as a field method for identifying emerging viruses of biomedical significance (Parida 2008), and as a diagnostic tool for distinguishing between termite species (Itakura et al. 2006). To date, there have been several reported applications demonstrating the potential of LAMP for the rapid identification of insect pests, particularly tephritids, to enhance quarantine interception and identification at ports of entry or other relevant settings. LAMP kits have been developed for rapid identification of the *Bactrocera dorsalis* complex of *Bactrocera cucurbitae*/*Bactrocera latifrons* and of *Bactrocera correcta*/*Bactrocera zonata* (Blaser et al. 2018), as well as for *Bactrocera tryoni* (Blacket et al. 2020). In addition to the *Bactrocera* species, LAMP assays are available for *Dacus ciliatus* (Sabahi et al. 2018) and *Zeugodacus scutellatus* (Kitano & Takakura 2020). The other major tephritid pest species namely *Ceratitis*, have LAMP assays developed for *Ceratitis capitata* (Huang et al. 2009), and Dermauw et al. (2022) developed a user-friendly extraction method and LAMP assay for *C. capitata* and *C. cosyra* group1 (as defined by Virgilio et al. 2017) or *Ceratitis* species belonging to the FARQ complex, which consists of *Ceratitis rosa, Ceratitis quilicii, Ceratitis fasciventris* and *Ceratitis anonae* (Virgilio et al. 2019; Zhang et al. 2021). Most recently, the isothermal amplification technique has been combined with detection through CRISPR-Cas12a (Alon et al. 2023) to provide rapid and easy-to-use DNA-based detection of *C. capitata* and *B. zonata*.

The peach fruit fly, *B. zonata* (Saunders) (Diptera: Tephritidae), is native to South and South-East Asia but it has invaded and become established in a number of countries in the Middle East, the Arabian Peninsula, North Africa and some of the Indian Ocean Islands (i.e. Mauritius and La Réunion) (EPPO 2010; De Meyer et al. 2014; El-Gendy 2017). It is frequently detected in California, USA (Papadopoulos et al. 2013) and every year since 2010 in Vienna, Austria (Egartner et al. 2019). It has also recently established in Israel (EPPO 2002). In tropical and subtropical regions, where its host plants are available year-round, it causes significant economic losses (Stonehouse et al. 1998; OEPP/EPPO 2005). *Bactrocera zonata* is a species of agricultural interest due to its strong flying ability (Qureshi et al. 1975), short generation time, adaptability to different habitats, and wide range of host plants (El-Gendy 2017). The recent expansion of its host range to olives following changes in its microbiome (Awad et al. 2022) highlight the threat posed by *B. zonata* to commercially grown crops.

In this study we describe a novel field-friendly, rapid protocol for the identification of *Bactrocera zonata* from adult and larval tissue. Using the LAMP method, we show that *B. zonata* can be discerned from other *Bactrocera* species in less than 1 hour, without the need for complicated laboratory equipment.

MATERIALS AND METHODS

Samples and sequence data

Physical specimens used for DNA extractions for testing of LAMP assays were obtained from a collection held at Stellenbosch University, Conservation Ecology and Entomology Department, and originating from the International Atomic Energy Agency (IAEA, Austria), and the Royal Museum of Central Africa (RMCA, Tervuren, Belgium). Table 1 provides a summary of the species used in the current study as well as the accession numbers and origins of sequence data used for LAMP primer design and the number of specimens available. Sequence data of the cytochrome oxidase subunit I gene region (*COI*) for LAMP primer design was obtained from GenBank (https://ncbi.nlml.nih.gov) and the Barcode of Life Database (BOLD; https://boldsystems.org/) in fasta format. All sequences were deposited either by the Royal Museum of Central Africa (RMCA, Tervuren, Belgium) or the International Atomic Energy Agency (IAEA, Austria).

LAMP primer design

Sequences were aligned using MAFFT (Katoh et al. 2002). First, the same species' sequences were aligned (if more than one representative sequence was present for a species; Table 1) and consensus sequences obtained. Consensus sequences for all *Bactrocera* species (Table 1) were then aligned. Using the *COI* sequence for *B. zonata* as input sequence, the NEB primer design tool (https://lamp.neb.com/#!/) was used with default settings to design LAMP primers. Primer options given by the NEB primer design tool were placed on the MAFFT sequence alignments of all *Bactrocera* species and primer sets were visually inspected for sequence similarity or dissimilarities. Primers were selected if they showed at least one nucleotide difference per primer between *B. zonata* and another *Bactrocera* species (including the re-named *Zeugodacus cucurbitae* and *Zeugodacus tau*) and *C. capitata*.

Table 1: Species list of samples used in the current study including origin of physical samples used for testing LAMP assays and origin and accession number of sequence data used for LAMP primer design.

Species	GenBank Accession No.†	Specimen origin and number of specimens available (*n*)	Sequence origin
Bactrocera dorsalis	**KM023410.1**	CRI, Nelspruit (*n* = 10)	RMCA (GenBank)
Bactrocera correcta	**FFIPM488-22** & FFIPM489-22	Vietnam (lab strain) (*n* = 2)	IAEA (BOLD)
Bactrocera zonata	**KM023423.1**	Pakistan (lab strain) (*n* = 6)	RMCA (GenBank)
Bactrocera tryoni	AB720890.1, **FFIPM508-22**, FFIPM509-22	Queensland, Brisbane, Australia (lab strain) (*n* = 2)	IAEA (NCBI) and IAEA (BOLD)
Bactrocera oleae	**KM023417.1**	Greece (lab strain) (*n* = 2)	RMCA (GenBank)
Bactrocera cucurbitae (now *Zeugodacus cucurbitae*)	HQ664517.1 to HQ664547.1, GQ154090.1 to GQ154135.1, **KM023407.1** to KM023409.1	Hawaii (lab strain) (*n* = 2)	RMCA (GenBank)
Bactrocera tau (now *Zeugodacus tau*)	GQ154157 to GQ154161, **KM023421**	China (lab strain) (*n* = 2)	GQ154161
Ceratitis capitata	Gene ID: 808516	CRI, Nelspruit (*n* = 6)	GenBank

†Note: Accession numbers in bold were used for alignment depicted in Figure 1.

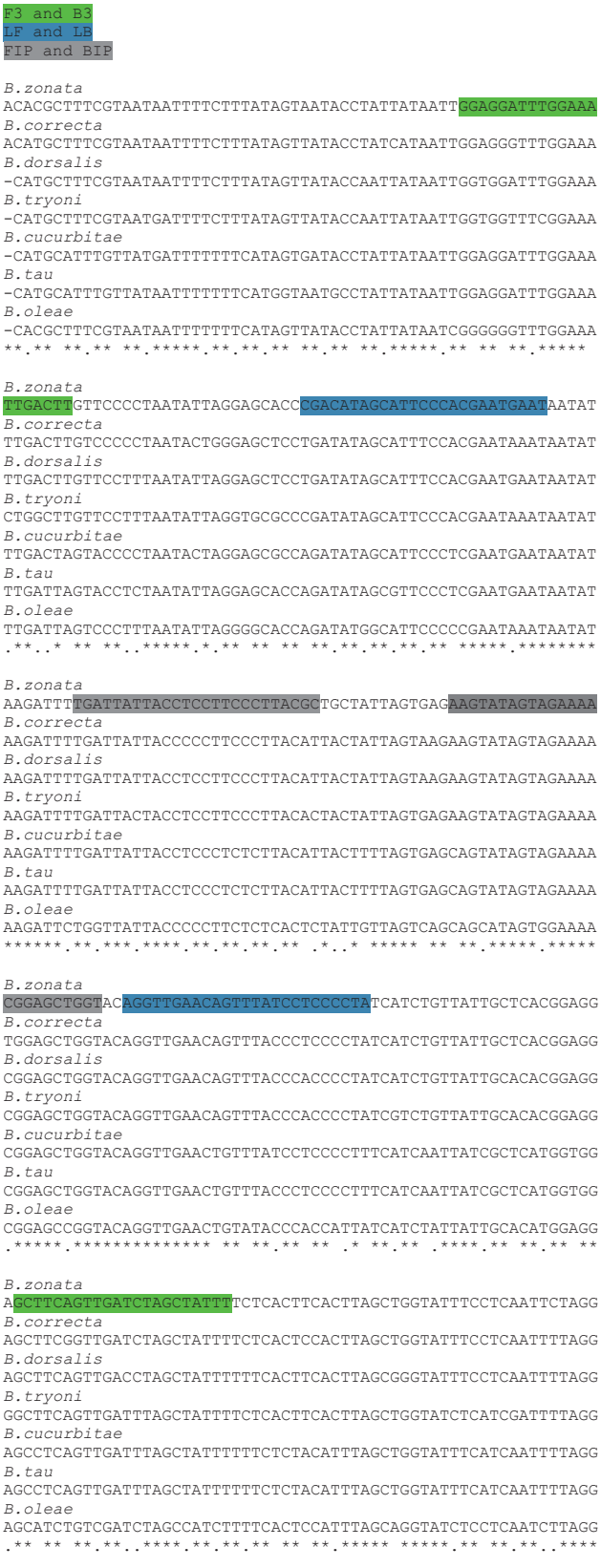

Figure 1: MAFFT sequence alignment of *B. dorsalis, B. zonata, B. latifrons* and *B. oleae* depicting primer locations of F3 and B3 primers (in green), LF and BF primers (in blue) and FIP and BIP primers (in grey). Bold letters specifically highlight sequence differences between *B. zonata* and other *Bactrocera* species. Stars under each block of alignment indicate nucleotide matches while a dot indicate nucleotide differences that are still biochemically similar and a space indicates nucleotide differences that have no biochemical similarity. All *Bactrocera* species used in the LAMP assay displayed in Figure 2 are included here. Sequence accessions used for the alignment are displayed in Table 1.

DNA extraction

For optimisation of LAMP assays, DNA from all physical specimens (Table 1) were extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) using the optimised protocol for insect tissue (Qiagen DNeasy Blood and Tissue Kit supplementary protocol DY14). Only one insect was used per extraction and samples were not pooled. Whole insects were extracted individually, and DNA quality and quantity was assessed using the NanoDrop1000 (Thermo Fisher). Samples were diluted with nuclease free water to 100 ng for use in the LAMP assay.

To assess more field-friendly DNA extraction methods, three alternative extraction protocols were tested on *B. zonata* and *B. dorsalis* third instar larvae. Due to the low number of samples available and because we wished to utilise pooled samples from two different individuals to account for biological variability, two larval halves (randomly selected posterior or anterior) were used per DNA extraction (Dermauw et al. 2022). Larval heads were not included to keep the tissue per extraction consistent. DNA from adult *B. zonata* and *B. dorsalis* samples extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) were included with the field-friendly assays as controls.

Method 1

A method by Kitano and Takakura (2020) was adapted wherein larval posterior and anterior halves were added to 0.2 mL microcentrifuge tubes (Eppendorf) and 30 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) was added (see Supplementary Material for detailed buffer preparation method). The solution was incubated at 95 °C for 5 min on a thermal cycler (Labnet MultiGene™ OptiMax thermal cycler), and used as-is for LAMP assays as the supernatant would contain the roughly extracted DNA.

Method 2

Adapted from Blaser et al. (2018), the posterior and anterior larval halves were added to 600 µM potassium hydroxide (KOH) (see Supplementary Material for detailed buffer preparation method) to a volume of 30 µl in 0.2 mL microcentrifuge tubes (Eppendorf). The solution was heated to 95 °C for 5 min on a thermal cycler (Labnet MultiGene™ OptiMax thermal cycler), and used as-is for LAMP assays as the supernatant would contain the roughly extracted DNA.

Method 3

Lysis buffer from the Qiagen DNeasy Blood and Tissue kit (Qiagen) was added to the posterior and anterior larval halves at 200 µl per sample in 1.5 mL microcentrifuge tubes (Eppendorf) and incubated at 95 °C for 5 min on a heat block (Thermomixer Comfort). Proteinase K, though used as part of the lysis process for the Qiagen DNeasy Blood and Tissue kit, was omitted from the reaction in an attempt to design a cost-effective extraction procedure. The reaction was used as-is for LAMP assays as the supernatant would contain the roughly extracted DNA.

LAMP assay optimisation

LAMP primer specificity was first assessed using DNA extracted from all *Bactrocera* species using the Qiagen DNeasy Blood and Tissue kit (Qiagen) (Table 1). Assay success was evaluated by running products on a 1.5% agarose gel (see Supplementary Material for detailed preparation method) at 90 V for 1 hr and visualising the resultant products under UV light. A negative control was run in every instance as well as a 100 bp ladder and samples were stained using SmartGlow LD (Accuris). The LAMP assay was set up to a total volume of 25.0 µL using the Warm Start NEB LAMP kit (New England Biolabs) as per

manufacturer's instructions. Briefly, a 10× Primer Master Mix was prepared as follows: 16 µM FIP, 16 µM BIP, 2 µM F3, 2 µM B3, 4 µM LF and 4 µM LB to a final volume of 2.5 µL. For the LAMP assay (25.0 µL reaction volume), 12.5 µL Warm Start Master mix, 2.5 µL 10× Primer Master Mix, 0.5 µL fluorescent dye, and 1.0 µL template DNA were added to a total volume of 25.0 µL in 0.2 mL reaction vials. Samples were incubated at 65 °C for 30 min and the reaction was terminated at 81 °C for 5 min on a thermal cycler (Labnet Multigene™ OptiMax thermal cycler). After amplification, 10 µL products were loaded onto the agarose gel for electrophoresis. Primer specificity would be confirmed if a ladder-like product was observed at around 500 bp, only for the *B. zonata* sample, and all other samples resembled the negative control.

To optimise the protocol to be more field-friendly, a more rapid assay confirmation method than the agarose gel was assessed. A LAMP assay was conducted as described above and instead of loading the resultant product on an agarose gel, 10 µL of the product was mixed with 0.5 µL ethidium bromide (Promega) in the 0.2 mL microcentrifuge tube it was amplified in. The tubes were directly visualised under UV light. Primer specificity would be confirmed if fluorescence was observed only for the *B. zonata* sample and all other samples resembled the negative control.

RESULTS

LAMP primer design

LAMP-specific primers for the *B. zonata COI* gene were designed and assessed visually for specificity by placing primers on MAFFT sequence alignments of all *Bactrocera* consensus sequences. Figure 1 shows such an example MAFFT alignment for *B. dorsalis*, *B. latifrons*, *B. oleae* and *B. zonata* to showcase the sequence differences within primer binding sites. Sufficient sequence differences between *B. zonata* and other *Bactrocera* species were needed to ensure primer specificity. Final primer sequences are provided in Table 2.

LAMP assay

Primer specificity was experimentally confirmed by performing the LAMP assay with the selected primers (Table 2) using DNA extracted with the Qiagen DNeasy Blood and Tissue kit (Qiagen) from all *Bactrocera* specimens in this study (Table 1). Negative controls for the assay were run in every instance where all components of the assay were added except for DNA. The specificity was visually confirmed on agarose gel electrophoresis where the ladder-like amplification of *B. zonata* can only be seen around 500 bp, with all other samples resembling the negative control (Figure 2). Table 3 depicts a summary of the detection times for all assays.

Field-friendly optimisation

The assessment of a more field-friendly DNA extraction method proved most successful using Method 1 (Kitano and Takakura 2020). The addition of 30 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and subsequent incubation at 95 °C for 5 min on a thermal cycler (Labnet MultiGene™ OptiMax thermal cycler) yielded DNA of sufficient quality and quantity to be amplified in the LAMP assay (Figure 3). Methods 2 and 3 were not successful in detection of larval samples and only the the adult *B. zonata* sample extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) and serving as positive control was detected (see Supplementary Figure 1).

Visualising LAMP assay products by the addition of ethidium bromide (Promega) to the 0.2 mL vials in which amplification took place proved to be successful given that a negative control is also visualised to calibrate accordingly the fluorescence emitted by the positive sample. Figure 3 shows the successful LAMP assay performed using the field-friendly DNA extraction method with in-tube visualisation by ethidium bromide staining, yielding a fluorescent product only for *B. zonata*. A summary of detection times for all assays is provided in Table 3.

Table 2: Primer sequences of LAMP primers designed for specific amplification of *Bactrocera zonata* DNA.

Primer	Sequence (5'–3')
F3	GGA GGA TTT GGA AAT TGA CTT
B3	AAA TAG CTA GAT CAA CTG AAG C
FIP	GCG TAA GGG AAG GAG GTA ATA ATC AGT TCC CCT AAT ATT AGG AGC AC
BIP	AAG TAT AGT AGA AAA CGG AGC TGG TCC GTG AGC AAT AAC AGA TGA
LF	ATT CAT TCG TGG GAA TGC TAT GTC G
LB	AGG TTG AAC AGT TTA TCC TCC CCT A

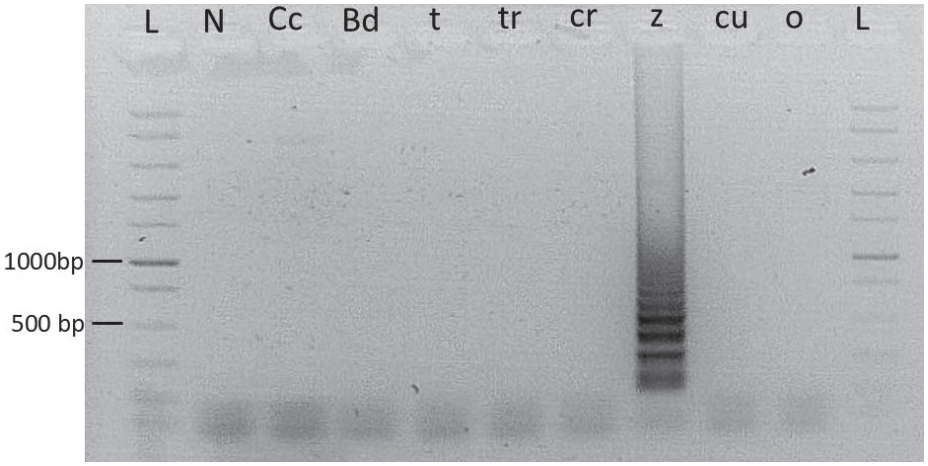

Figure 2: LAMP assay products on 1.5% agarose gel**.** From left to right lanes contain: L - 100 bp ladder; N - negative control; Cc - *Ceratitis capitata*; Bd - *Bactrocera dorsalis*; t - *Bactrocera tau*; tr - *Bactrocera tryoni*; cr - *Bactrocera correcta*; z - *Bactrocera zonata*; cu - *Bactrocera cucurbitae*; o - *Bactrocera oleae*. The ladder-like bands around the 500 bp mark for *B. zonata* indicate the successful amplification of the LAMP primers while all other samples resemble the negative control.

Table 3: All samples and assays run, depicting the number of times a sample was correctly detected.

Sample	Extraction	Number of times correctly detected	Total number of assays
Ceratitis capitata whole adult	Qiagen Blood & Tissue Kit	2	2
Bactrocera dorsalis whole adult	Qiagen Blood & Tissue Kit	4	4
Bactrocera tau whole adult	Qiagen Blood & Tissue Kit	2	2
Bactrocera tryoni whole adult	Qiagen Blood & Tissue Kit	2	2
Bactrocera correcta whole adult	Qiagen Blood & Tissue Kit	2	2
Bactrocera zonata whole adult	Qiagen Blood & Tissue Kit	4	4
Bactrocera cucurbitae whole adult	Qiagen Blood & Tissue Kit	2	2
Bactrocera oleae whole adult	Qiagen Blood & Tissue Kit	2	2
Third instar *Bactrocera zonata* larva in EtOH	Field-friendly extraction	3	3
Third instar *Bactrocera dorsalis* larva in EtOH	Field-friendly extraction	2	2

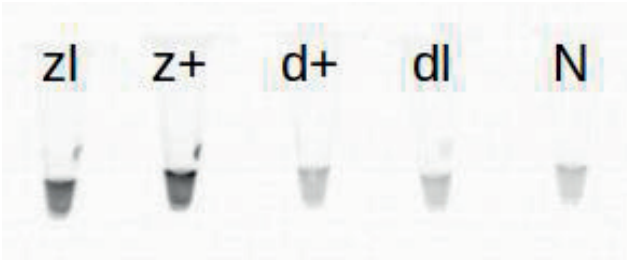

Figure 3: LAMP assay products visualised using the field-friendly in-tube method of adding ethidium bromide as fluorescent, intercalating dye in 0.2 mL tubes. Samples are extracted using the field-friendly DNA extraction method of incubating larvae in TE buffer and compared to positive controls extracted using the Qiagen DNeasy DNA extraction kit. Samples are as follows from left to right: zl - *Bactrocera zonata* larval segments extracted using field-friendly method; z+ - adult *Bactrocera zonata* extracted using the Qiagen DNeasy kit; d+ - adult *Bactrocera dorsalis* extracted using the Qiagen DNeasy kit; dl - *Bactrocera dorsalis* larval segments extracted using field-friendly method; N - negative control. Only samples containing *B. zonata* show fluorescence under UV light.

DISCUSSION

The LAMP technique has been applied to other tephritids; however, this study and the CRISPR-based assay of Alon et al. (2023) are the first assays specifically targeting the accurate and rapid identification of *B. zonata*. The current study, in particular, discerns *B. zonata* from among a series of other agriculturally important *Bactrocera* and *Zeugodacus* species as well as *C. capitata*. Through a combination of a more rapid DNA extraction and visualisation method, the target species can be identified in under one hour, providing a field-friendly detection method for *B. zonata*. Figure 4 depicts the workflow for the LAMP assay and indicates the time taken at each step of the workflow. In terms of visual detection, previous studies have used agarose gel electrophoresis (Huang et al. 2009) and speciality fluorescence-based isothermal amplification devices (Huang et al. 2009; Blaser et al. 2018; Blacket et al. 2020; Dermauw et al. 2022). This study provides a methodology for detecting LAMP products in-tube only by adding an intercalating UV fluorescent dye, which can be visualised immediately under any UV light source.

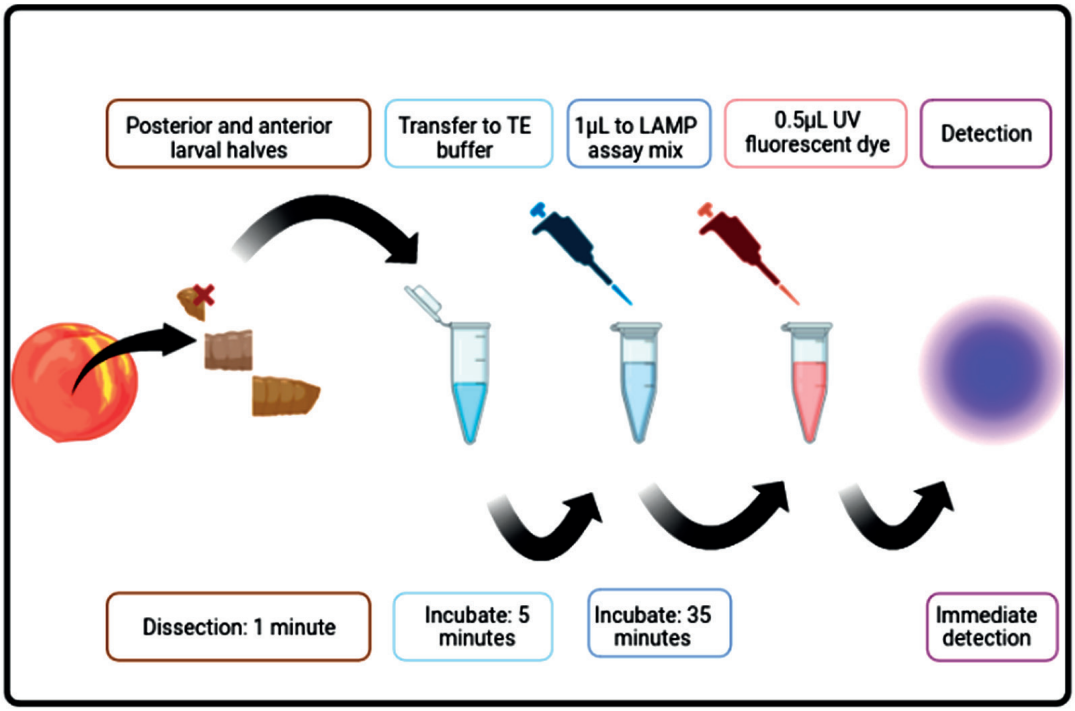

Figure 4: LAMP assay workflow depicting the steps and duration in time for each part of the workflow from dissection of larval samples on the left, through to the DNA extraction in TE buffer and transfer of 1 µL crude extract to the LAMP assay mixture followed by addition of 0.5 µL of a UV-fluorescent dye such as Ethidium Bromide and immediate visualisation under UV light. The complete workflow can be accomplished in under 1 hour if sample material is at hand.

Alon et al. (2023) recently presented a protocol for detecting *B. zonata* and *C. capitata* using a CRISPR-Cas12a detection assay. Several notable differences exist between our study and the study conducted by Alon et al. (2023). First, while Alon et al. (2023) solely focused on *C. capitata* and *B. zonata*, our study included a greater diversity of closely related species. Though the assay of Alon et al. (2023) aimed to detect either *B. zonata* or *C. capitata*, the authors did not test the assay for specificity on any other species as our study did. Second, the field-friendly DNA extraction method presented in our study does not require manual grinding of samples or ultra-pure water, unlike the Chelex-based protocol used by Alon et al. (2023). The completion time for the protocol by Alon et al. (2023) is approximately 1.5 hours.

In contrast, our protocol can be completed in under one hour and does not require a benchtop centrifuge that might be cumbersome or unavailable to field-based applications. Lastly, the protocol by Alon et al. (2023) utilises a specialised plate reader for visualisation, mentioning that hand-held fluorometers could be used. However, this assessment was not conducted. In contrast, our protocol only requires a UV light source, which is less expensive and more readily available than fluorometers.

LAMP is an isothermal process and does not require temperature ramping up and down as in conventional PCR reactions (Notomi et al. 2000). This shortens the reaction time and simplifies the equipment (i.e. a thermal cycler might not always be necessary). In addition to the low complexity equipment required to perform LAMP assays, the lack of background interference due to the high specificity of LAMP primers to the target sequence makes LAMP particularly suited to field-based applications (Notomi et al. 2000). The advantages of LAMP including its low cost, rapidity, sensitivity and amenability to field applications make it ideally suited to species confirmation of economically important insects such as Tephritids.

The advantages also far outweigh the disadvantages, including the slightly complicated (but not insurmountable) primer design and challenges in compiling multiplex reactions and quantifying target DNA. However, care should be taken as cross-contamination by material present in aerosols is still a risk and could lead to false positive results and the difficulty in detecting inhibitors in the reaction could lead to false negatives (Soroka et al. 2021). Further limitations of this study include the narrow scope of its tests as no larval instars other than the third instar were used in assays and no larvae collected from varying conditions were utilised for any of the assays.

Most DNA-based species diagnostic tools utilise the *COI* gene or the 16S ribosomal RNA gene. Though these regions are often sufficient, instances do occur where species cannot be discriminated based on these gene regions alone. As discussed by Alon et al. (2023), the increased availability of genomic data for fruit flies of economic importance will be a significant benefit to the future development of DNA-based assays for species identification allowing researchers to widen their searches for unique DNA regions outside of the traditional *COI* gene region.

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DATA AVAILABILITY

All data are available from the corresponding author upon reasonable request.

DECLARATION OF COMPETING INTEREST

The authors have not disclosed any competing interests.

AUTHORSHIP CONTRIBUTION STATEMENT

All authors contributed to the study conception and design. Specimen collection, sample preparation and experimental analysis were conducted by Anandi Bierman, Annelie Smit and Minette Karsten and Marc de Meyer (sample collection). Data analysis were performed by Anandi Bierman. Anandi Bierman, Minette Karsten, and John S. Terblanche contributed to the first draft of the manuscript and all authors commented on subsequent versions of the manuscript, revisions and responses to referees. All authors read and approved the final manuscript.

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