A loop-mediated isothermal amplification (LAMP) assay for rapid identification of *Ceratitis capitata* and related species

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**Abstract**

True fruit flies (Tephritidae) are among the most destructive agricultural pests in the world, attacking a wide range of fruits and vegetables. The Mediterranean fruit fly *Ceratitis capitata* is a highly polyphagous species but, being widely established in the Mediterranean region, is not considered as a EU quarantine pest. Hence, it is important to discriminate *Ceratitis capitata* from non-EU tephritid species, present in imported fruit and vegetables, as non-EU species have a quarantine status. However, morphological identification of tephritid larvae, the most frequently intercepted stage in non-EU produce, is difficult and an easy-to-use molecular diagnostic tool would be helpful for rapid species identification. Therefore, a loop-mediated isothermal amplification (LAMP) method was developed for *C. capitata* and non-EU tephritids *Ceratitis cosyra* group I and *Ceratitis* species from the FARQ complex, *C. fasciventris*, *C. anona*, *C. rosa* and *C. quillicii*. LAMP assays were run with DNA from ILVO collected specimens and DNA samples collected during previous research surveys. LAMP primers were species-specific, with LAMP amplification occurring within 45 minutes for the targeted species. In addition, LAMP assays were successful for all *C. capitata* life stages or a limited amount of tissue. To conclude, the LAMP assays developed in this study were able to distinguish *C. capitata* from non-EU Tephritidae species and could be a useful tool for the rapid identification of *C. capitata*.

1. Introduction

The Tephritidae is a group of colourful fruit flies and includes approximately 5000 species from 500 genera (Brown et al., 2018; EFSA Panel on Plant Health, 2020). Currently, around 35% of the Tephritidae are recognized as pests of commercial fruit and vegetables. According to the European Commission (EU) 2016/2031 regulation, the so-called non-EU Tephritidae have a quarantine status and may not be introduced into the EU. Hence, imported produce needs to be examined by each EU member state to prevent the entry, establishment and spread of these quarantine organisms. In Belgium, these controls are performed by the Federal Agency for the Safety of the Food Chain (FASFC) which also acts as the National Plant Protection Organisation (NPPO). Suspected products are further analyzed by the National Reference Laboratory (NRL) for Entomology (at the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)) for conformation of non-EU fruit flies. During the identification of the fruit fly, the suspected cargo is halted until further notice. The longer the cargo - chilled, perishable products - is blocked, the higher the financial consequences may be for the importer, putting great pressure on all parties involved, including operators, importers, NPPO and NRL.

The Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), is one of the most destructive pests in the world. It occurs in more than 130 countries and has a very wide host range, including important commercial fruits and vegetables such as citrus, apple, peach, and mango (White & Elson-Harris, 1992). Although *C. capitata* originates from Sub-Saharan Africa (De Meyer et al., 2002), it is now widespread in Southern Europe, including Spain, Italy, Greece, Malta, Cyprus and Portugal (EPPO, 2021) and therefore, not considered as a EU quarantine pest *stricto sensu* (Regulation (EU) 2016/2031). Consequently, it is important to discriminate *C. capitata* from non-EU tephritids listed in the new directive for reasons described above. Experts are able to discriminate between adults of different tephritid species, but morphological identification of larva - the most frequently found stage in intercepted fruit and vegetables - is difficult even when dealing with the third larval instar (Balmès & Mouttet, 2017; EPPO, 2011). Further rearing to the adult stage prolongs the identification procedure considerably and, in most cases, the success rate is low. Moreover, *C. capitata* feeds on...
the same hosts as many non-EU Tephritidae, including Ceratitis cosyra, Ceratitis rosa and Bactrocera dorsalis, further complicating correct identification. As a consequence, molecular diagnostic assays have been recommended to assist in the identification of tephritid larvae. Different molecular methods for identification of C. capitata have been described (PCR-RFLP method based on ITS1 primers (Douglas & Haymer, 2001), RFLP-PCR identification method based on the cytochrome oxidase subunit 1 gene (Barr et al., 2006) and DNA barcoding (Barr et al., 2012; Van Houdt et al., 2010)) to distinguish C. capitata from other Ceratitid species. These techniques require a well-equipped laboratory with expensive equipment and well-trained staff. Moreover, these tests significantly increase the analysis cost and, in general, take a considerable amount of time, especially when some steps of the protocol need to be outsourced (e.g. sequencing). Therefore, a fast, reliable and inexpensive identification technique to discriminate C. capitata from non-EU Tephritidae would be very useful and accelerate the release of suspected shipments.

The loop-mediated isothermal amplification (LAMP) method, developed by Notomi et al. (2000), is an appropriate alternative for the rapid molecular identification of quarantine insects. Currently, LAMP assays already exist for certain quarantine insects such as Thrips palmi, Bemisia tabaci and Liriomyza sp.. These were commercialized by Optigene (http://www.optigene.co.uk/plant-health-pests-diagnostics) and are used for species identification at two ports of entry in Switzerland (von Felten, 2017). For a similar purpose, a LAMP kit was developed for rapid identification of Bactrocera flies (Bactrocera dorsalis complex, Bactrocera cucurbitae/Bactrocera latifrons and Bactrocera correcta/Bactrocera zonantis) (Blaser et al., 2018), while recently a LAMP identification method was reported for B. tryoni (Blacket et al., 2020), Dacus ciliatus (Sabañé et al., 2018) and Zeugodacus scutellatus (Kitano & Takakura, 2020). In 2009, Huang et al. developed a LAMP assay for C. capitata but species-specificity of these primers was not assessed (Huang et al., 2009). In this study, we describe a user-friendly DNA extraction method for tephritid species and developed LAMP- assays that could aid in the rapid identification of C. capitata, C. cosyra group1 (according to Virgilio et al. (2017)) or Ceratitis species belonging to the FARQ complex (Virgilio et al., 2019).

2. Materials and Methods

2.1. Collection of tephritid samples and DNA extraction

Fruit and vegetable samples that arrived at the Diagnostic Centre for Plants (DCP) of the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO, Merelbeke, Belgium) were inspected for the presence of tephritid larvae. Detected larvae (either L2 or L3 instars) were stored in 90% ethanol until DNA extraction or lysis solution preparation. Before DNA extraction or lysis preparation, larvae were allowed to dry on filter paper to remove excess of ethanol. The NucleoSpin Plant II Kit (Machery-Nagel, Germany) with lysis buffer PL1, which is based on the CTAB lysis method (a method that has been previously used for DNA extraction of tephritid specimens, see e.g. Asokan et al. (2011)), and an elution volume of 50 μL, was used to extract DNA of larvae. Alternatively, some fresh intercepted larvae were cut into two pieces (separating the anterior part from the rest of the body) and the rest of the body was again divided into two pieces. These two posterior pieces were transferred to a 1.5 mL Eppendorf tube containing 200 μL plant lysis buffer (EXT-001 Buffer; Optigene, UK) (Witters et al., 2018). Subsequently, the larval lysis solution was vortexed for 1 min, incubated for 5 min at room temperature, and 1/10th diluted in water. Lastly, some intercepted tephritid larvae were grown on an artificial diet at 22°C. The artificial diet was based on those of Lebretón et al. (2014) and Enriquez et al. (2019) and consisted of 1 L water, 15 g agar, 50 g sucrose, 30 g inactive dry brewer’s yeast, 20 g commel, 50 g carrot powder, 1.2 g methyl 4-hydroxybenzoate (Nipagin) dissolved in 12 mL ethanol, 2.22 g tartaric acid, 0.89 g ammonium sulphate, 0.22 g magnesium sulphate and 0.67 g potassium phosphate. Once larvae had developed into adults, an adult was transferred to a 1.5 mL Eppendorf tube, subsequently held in liquid nitrogen for five minutes and homogenized using a pestle. Next, DNA was extracted using the NucleoSpin Plant II Kit (Machery-Nagel, Germany), in an elution volume of 50 μL in addition to DNA from fresh specimens, DNA from tephritid species (adult males or females), kindly provided by Dr. Marc De Meyer and Dr. Massimiliano Virgilio (Royal Museum of Central Africa (RMCA), Brussels, Belgium), was also used. The Qiagen Blood and Tissue kit (Qiagen, Belgium) was used to extract DNA from these RMCA samples, using an AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0) elution volume of 70 μL. All adult specimens were morphologically identified using taxonomic keys of White and Elson-Harris (1992), Virgilio et al. (2014) and/or Carroll et al. (2019).

2.2. PCR of Ceratitis COI region and phylogenetic analysis

PCRs were performed to amplify a 710 bp region of the cytochrome oxidase subunit 1 (COI) gene using the primers of Folmer et al. (1994) and MyF/M-bar (Meridian Bioscience, Belgium). The PCR mix consisted of 12.5 μL MyFi-mix, 1 μL HCO2198 (10 μM), 1 μL LCO1490 (10 μM), 2 μL of kit extracted DNA (see above) or 5 μL of a 1/10th dilution of larva lysis solution (larva homogenized in plant lysis buffer) and 8.5 or 5 μL of ultrapure water, respectively. PCR conditions were as follows: 95°C for 1 min followed by 35 cycles of 95°C for 1 min, 51°C for 30 sec and 72°C for 15 sec. PCR amplicons were purified using the Smartpure PCR Kit (Eurugenetics, Belgium) and sent for sequencing to Macrogen (The Netherlands), GeneWiz (Germany) or Eurofins Scientific (Luxembourg) using both the forward (HCO2198) and reverse primer (LCO1490). COI sequences were aligned with those of a selection of tephritid species available in the NCBI/EPPO-Q-BANK database using MAFFT version 7 (Katoh & Standley, 2013) and default settings. A phylogenetic analysis was subsequently performed at the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) with sequence type set to “codon”, genetic code set to “Invertebrate mitochondrial”, model set to “AUTO” and with 1000 ultrafast (UF) bootstraps (Nguyen et al., 2014; Trifinopoulos et al., 2016).

2.3. LAMP assays

LAMP primers were designed for C. capitata (Ccap), C. cosyra group1 (Ccos g1) and Ceratitis species belonging to the FARQ complex (C. fasciventris, C. ananoe, C. rosaee and C. quilicii) (CFARQ) (Virgilio et al., 2019). For all species, the COI gene was used as template for primer design. Primers were designed to be as specific as possible using the Primer Premier Software (PREMIER Biosoft, USA) or Primer Explorer V5 (Eiken Chemical Company, Japan) and taken into account COI sequences of the most closely related species available in either the NCBI or EPPO-Q-BANK databases. LAMP assays were performed using the GpSSD Isothermal Mastermix (ISO-001; Optigene, UK) and a Quantstudio 5 Real-Time PCR system (Qiagen, Belgium) with the fluorescence reporter set to TAM. The LAMP reaction volume was 20 μL consisting of 10 μL ISO-001, 0.25 μM of F3/B3, 1.25 μM of BIP/FIP and 2 μL of sample: 1/10th dilution of larva homogenized in plant lysis buffer or kit extracted DNA (see Table S1). A 1/10th dilution of the larva lysis solution was used as the anneal derivative melting temperature (Ta, see below) of LAMP products in LAMP assays using this dilution was similar to the Ta of LAMP products in LAMP assays using kit extracted (pure) DNA as input, while for LAMP products in LAMP assays using undiluted larva lysis solution the Ta was 3-4°C lower (data not shown). For each LAMP run, a positive control (LAMP primers with DNA from targeted species) and a negative control (LAMP primers with water or a 1/10th dilution of plant lysis buffer) were included. Each LAMP primer set was also tested on Nucleospin Plant II kit extracted DNA (5-10 ng/μL of Za-priornus ornatus (Diptera: Drosophilidae)). LAMP assays were run at 65°C for 1 hour, followed by a melt curve analysis from 95°C to 60°C and
Figure 1. Cladogram of tephritid samples used in LAMP assays. COI sequences of tephritid species were aligned (File S1) using MAFFT (Katoh et al. 2013) and used in a maximum likelihood phylogenetic analysis. Tephritid samples of the Flanders research institute for Agriculture, Fisheries and Food (ILVO) are indicated in red, while those of the Royal Museum of Central Africa (RMCA) are indicated in blue font and those of NCBI/QBANK in yellow font (see Table S1 for sample details). "-" indicates that the country of origin was not known. Only UF bootstrap values above 90 and at phylogenetically important nodes are shown. For the Ceratitis FARQ complex a letter between brackets indicates to which Ceratitis species of the FARQ complex the specimen was morphologically assigned (A: C. anonae, R: C. rosa, F: C. fasciventris, Q: C. quinaria).

Table 1

| stage         | Lysis buffer | Lysis buffer + pestle |
|---------------|--------------|----------------------|
|               | Tp¹ | Ta²  | Tp¹ | Ta²     |
| egg           | "   | "    | 19:10 (02:03)** | 81.12 (0.18)** |
| L1            | "   | "    | 19:31 (00:24)  | 80.82 (0.06)   |
| L2            | 19:39 (1:42) | 81.13 (0.14) | nd     | nd      |
| L3            | 19:17 (1:41) | 81.07 (0.07) | nd     | nd      |
| adult leg     | 25:21 (04:11)** | 81.19 (0.06)** | 20:54 (0:55) | 81.10 (0.13) |
| pupa          | 18:54 (0:36) | 81.00 (0.01) | nd     | nd      |
| empty pupa    | 25:52 (02:15) | 81.25 (0.12) | nd     | nd      |

¹ time in mm:ss (standard deviation)  
² anneal derivative temperature in°C (standard deviation)  
* no LAMP amplification  
** LAMP amplification in only three out of four biological replicates  
*** nd, not determined,
analyzed using the Quantstudio Design and Analysis software v 1.4.3 (Qiagen, Belgium). Time to positivity (Tp, expressed in mm:ss) reported in this study was determined using a deltaRn threshold of 75,000 and “Automatic baseline” in the “Ct settings” of the “Analysis settings” of the Quantstudio Design and Analysis software, while the Ta (expressed in°C) was determined by a melt-curve analysis. A heatmap of LAMP assays Tps was generated using ComplexHeatmap package in R (Gu et al., 2016). In addition to LAMP experiments with the Quantstudio 5 Real-Time PCR system, we also ran LAMP assays with each LAMP primer set and a 1/10th dilution of larva lysis solution of C. capitata (ILVO-10), C. cosyra g1 (ILVO-21) and kit extracted DNA of C. quilicii (RMCA-56, with a concentration of 5 ng/μl) on a Genie II instrument (Optigene, UK), with default settings for Tp and Ta assessment (melt curve analysis from 70°C to 98°C) and with “Amplification height” set at 5000. A 1/10th dilution of B. dorsalis lysis solution (ILVO-26) and a 1/10th dilution of plant lysis buffer were used as a negative control. Last, we also performed LAMP assays with the C. capitata primers described in Huang et al. (2009) on the Genie II device but using the LAMP reaction specifications described in this study.

### 2.4. Sensitivity of Ceratitis LAMP primers

The sensitivity of our LAMP assays was assessed by testing serial dilutions of DNA with the Ccap, Ccos g1 and CFARQ primers. Around 50 ng/μl DNA from a C. capitata larva (ILVO-09), C. cosyra g1 larva (ILVO-17), and a Ceratitis quilicii adult (RMCA-56) was diluted 10-fold, 100-fold and 1000-fold and LAMP assays were performed on a Quantstudio 5 Real-Time PCR system as described in section 2.3. Four technical replicates were performed for each dilution. An average Tp was calculated when a Tp value could be determined for three out of four replicates.

### 2.5. LAMP assays with different stages of Ceratitis capitata

*Ceratitis capitata* stages - egg, 1st instar (L1), 2nd instar (L2), 3rd instar (L3), pupa, empty pupa and adults - were provided by the International Atomic Energy Agency (Vienna) and stored in 90% ethanol at 4°C (one stage/10 mL Falcon tube) until use. Single specimens were allowed to dry on filter paper before being transferred to plant lysis buffer (Optigene, UK). Egg and L1 stages were not manipulated and directly transferred to 30 µl lysis buffer while L2 and L3 stages were decapitated and the posterior region (cut in two) was transferred to 200 µl lysis buffer (see also section 2.1). Pupa and empty pupa were cut into two pieces and both pieces were transferred to 200 µl lysis buffer. For adults, a leg was detached using a forceps and subsequently transferred to 200 µl lysis buffer. Next, tubes were vortexed for 1 min, incubated for 5 min at room temperature, and 2 µl of a one-tenth dilution (in water) of the lysis solution was used in LAMP assays on a Quantstudio 5 instrument as described above (see section 2.3). Last, another batch of LAMP assays with eggs, L1s and adult legs were performed but differing from the assays described above by microspore homogenisation of tissue before vortexing the sample for 1 min.

### Table: LAMP assays with different stages of Ceratitis capitata

| Stage       | Ccap | Ccap g1 | CFARQ |
|-------------|------|---------|-------|
| Egg         |      |         |       |
| 1st instar  |      |         |       |
| 2nd instar  |      |         |       |
| 3rd instar  |      |         |       |
| Pupa        |      |         |       |
| Empty pupa  |      |         |       |
| Adult       |      |         |       |

### Figure 2: Heatmap of Tp values for LAMP assays with Ccap, Ccos g1 and CFARQ primers and ILVO and RMCA collected tephritid DNA samples. Each sample name (ILVO or RMCA) is followed by the country of origin of the sample. See Table S1 for detailed Tp values. All LAMP assays were run on a Quantstudio 5 system. An asterisk indicates that an atypical amplification curve (with Tp values of 49 and 47 minutes) was observed for these samples (ILVO-14 and ILVO-17, respectively) with Ccap primers.

**Table 1:** Sensitivity of LAMP assays with different stages of Ceratitis capitata.
3. Results and Discussion

*Ceratitis capitata* (Diptera: Tephritidae) is not considered a EU quarantine pest and non-EU fruit and vegetables infested with this species pass import control at the port of entry. The larval stage is the most frequently intercepted stage of Tephritidae in imported produce. However, tephritid larvae are difficult to identify using morphological characteristics only and a molecular diagnostic tool might aid in species identification. In 2009, a LAMP assay, targeting the ITS1-5.8S-ITS2 region, was developed for *C. capitata* (Huang et al., 2009) but was not highly specific as these LAMP primers also amplify *C. cosyra* and *B. dorsalis* DNA (Figure S1). Hence, the main goal of this study was to develop a new LAMP assay that is more specific for *C. capitata*. Therefore, we first collected DNA samples from a selection of tephritid species, originating from different countries, and subsequently PCR-amplified a COI fragment of each sample. A maximum likelihood phylogenetic analysis showed that COI sequences form ILVO or RMCA collected samples clustered with either *Ceratitis sp.*, *Bactrocera sp.* or *Dacus sp.* COI sequences from the NCBI database. Of particular note, sequences of *C. cosyra* group2 (as defined by Virgilio et al. (2017)) clustered as a sister group of *C. quinaria* COI sequences. RMCA or ILVO COI sequences from adult specimens also clustered according to their morphological identification (Figure 1, Table S1, File S1). Next, LAMP primers were designed for *C. capitata* (Ccap, Table S2, Figure S2) and tested on all tephritid samples for which a COI fragment was obtained (Figure 2). Tp values with Ccap primers were below 30 minutes for all *C. capitata* samples (Figure 2, Table S1) and the melting temperature (Ta) was 81.3°C (SD, 0.2). Ccap primers did not result in LAMP amplification of *Ceratitis* FARQ complex species, *B. dorsalis, Dacus* sp. samples, while for two out of 17 *C. cosyra* samples (ILVO-14 and ILVO-17) an atypical amplification curve was observed, with a Tp values of 49 and 47 minutes (Figure 2, Figure S3). To complement the Ccap primers, we also developed LAMP primers for *C. cosyra* species (Ccos g1) and *Ceratitis* species from the FARQ complex (CFARQ), which could be run in parallel with the *C. capitata* LAMP assays (Table S2, Figure S2). We also attempted to design LAMP primers for *C. cosyra* group2 species but were not successful (data not shown). Tp values for Ccos g1 LAMP primers with *C. cosyra* group1 DNA samples varied between 31 and 41 minutes with a Ta of 84.4°C (SD, 0.2), while LAMP amplification did not occur with other tephritid samples. CFARQ primers with *C. rosa*, *C. ananassae* or *C. quilonii* samples resulted in Tp values between 29 and 38 minutes with a Ta of 80.7°C (SD, 0.2) and LAMP amplification with CFARQ primers did not occur with other tephritid samples tested (Figure 2, Table S1). LAMP amplification was also not observed with *Zaprionus ornatue*, an unrelated fruitfly that feeds on plant hosts on which *Ceratitis capitata* also occurs (Poligui et al., 2014) (Johan Witters, pers. communicaton) (Figure S4). LAMP assays are prone to false positives, and two false positives (with atypical amplification curves) were observed in our study (Figure 2, Figure S3). Time-gating has been suggested as a valuable tool to reduce the probability of false positive results. In addition, the Ta might be used to discern specific from non-specific amplification (Moeling et al., 2021; Rolando et al., 2020). Therefore, we suggest to use the following Tp and Ta thresholds to assess whether the result of our LAMP assays can be considered as a true positive: Tp < 35 min and Ta of 81±1°C for Ccap LAMP primers, Tp < 45 min and Ta of 84.5±1°C for Ccos g1 primers and Tp < 40 min and Ta of 80.5±1 for CFARQ primers. Lastly, our *C. capitata* LAMP assay might not be able to discriminate between *C. capitata* and the closely related *Ceratitis caetrita*, as the COI sequence diversity (“bar-code gap”) is too low between these species (Barr et al., 2012; 99% identity between the LAMP primer region (273 bp) of *C. capitata* (GenBank accession AJ242872.1) and *C. caetrita* (GenBank accession AV788414)). However, as *C. caetrita* has not been reported for the last ten years (http://projects.bebif.be/fruityfly_taxonomo.html?id=2) and only occurs in Kenya on hosts that are not commercially exported (De Meyer et al., 2002), it is highly unlikely that this species would be present in produce imported in the EU.

LAMP assays with serial dilutions of *Ceratitis* sp. DNA revealed that reliable detection (Tp SD less than 5 min, amplification in all four technical replicates) of *C. capitata* and *Ceratitis* species from the FARQ complex was possible at 1 ng of input DNA while *C. cosyra* group1 DNA could be reliably detected up to 10 pg (Table S3). The sensitivity of the *C. capitata* LAMP assay is lower than the sensitivity of the recently developed *Bactrocera tryoni* LAMP assay (Blacket et al., 2020) but seems more than sufficient to reliably identify L3 larvae and most other stages
of C. capitata. LAMP amplification with Ccap primers occurred using L2, L3, pupa as template, but did not occur for egg and L1s (Table 1) or only for three out of four replicates in case of adult legs. We speculated that this difference in amplification success could be related to the limited amount of DNA that is released during vortexting of eggs, L1s or adult legs. Hence, we performed an alternative extraction protocol (addition of a “pestle homogenisation” step, see section 2.5 in Materials and Methods) for these stages/samples. LAMP assays using this alternative extraction method resulted in successful LAMP amplification for eggs, L1s and adult legs (Table 1).

A set of LAMP assays was also run with larva lysis solution and the Genie II (Figure 3, Figure 4, File S2), which is a portable device and can easily be used for in-field diagnostics (see e.g. Blacket et al. (2020)). Noteworthy, for the samples tested on the Genie II, Ta values were 0.5°C to 1°C less than those obtained using the QuantStudio 5 system, indicating that Ta values can slightly deviate when running LAMP assays on different devices. The LAMP assays developed in this study and those designed for B. tryoni (Blacket et al., 2020), B. dorsalis complex/B. latifrons (Blaser et al., 2018) and Z. scutellatus (Kitano & Takakura, 2020) might be run simultaneously, allowing rapid screening of tephritid pests. Such screening might also be achieved by PCR amplification and sequencing of a fragment of the COI gene but, to our knowledge, on-site sequencing facilities at ports of entry are currently rather exceptional and PCR requires more trained personnel and special equipment compared to LAMP assays (Hsieh et al., 2012). To conclude, in this study, we developed LAMP assays for C. capitata and related species that could aid in the rapid identification of these pests in imported produce.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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