Rapid Identification of the Mediterranean Fruit Fly (Diptera: Tephritidae) by Loop-Mediated Isothermal Amplification

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ABSTRACT The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), ranks as one of the world's most destructive agricultural pests. This pest is also widespread and highly invasive; thus, it is a high priority for pest detection and quarantine programs. Although Mediterranean fruit fly adult and third-instar larvae can usually be identified and distinguished from other species by morphological keys, it is often difficult or impossible to identify or distinguish this species from other tephritids by using material from other stages of development. In such situations, use of a molecular technique known as loop-mediated isothermal amplification (LAMP) would be valuable as a rapid and robust alternative species diagnostic tool. This method uses isothermal conditions and requires only relatively inexpensive equipment. In this study we have developed a simple and rapid procedure that combines a Chelex-based DNA extraction procedure with LAMP to rapidly detect the presence of Mediterranean fruit fly DNA and discriminate it from other species, by using material from different stages of development. Amounts of DNA as little as that recovered from a single egg were shown to be adequate for the analysis, and LAMP itself required only 45 min to complete.

KEY WORDS LAMP, *Ceratitis capitata*, molecular identification, quarantine

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), has expanded its host range to >400 plant species (Liquidó et al. 1991, 1998; De Meyer et al. 2002) since it migrated out from its presumed origin in Africa ≈200 yr ago (White and Elson-Harris 1992). It is considered to be one of the world's most widespread and damaging pests, and areas that are currently free of this pest heavily emphasize quarantine procedures designed to keep it from invading new agriculturally sensitive localities (Fimiáni 1989). For traditional detection procedures, Mediterranean fruit fly material from adult or third-instar stages of development can be identified by morphological keys. However, morphological keys either do not exist or are not adequate to identify and/or distinguish different tephritid species by using material from earlier stages of the life cycle (White and Elson-Harris 1992, Frias et al. 2008). This is especially problematic for quarantining protocols because fruit infestations detected at ports-of-entry often include egg- and early larval-stage material. For this reason,

the development of a rapid and robust method for species identification that can be applied to material from these early (or any of the) life stages is necessary.

Molecular tools, in particular those based on the analysis of DNA, have already been used to augment or supplement the use of morphological characters for species identification purposes for many insect pest species. The benefits of the DNA-based tools are enhanced even further when methods using the polymerase chain reaction (PCR) are used, primarily because they can often be used even when only minimal amounts of poorly preserved material are available for analysis. Examples of DNA amplification-based methods applied to the identification of insect pest species include the PCR-restriction fragment length polymorphism (RFLP) (Armstrong et al. 1997, Barr et al. 2006), amplified fragment length polymorphism (Kakouli-Duarte et al. 2001), and oligonucleotide array-based methods (Naeole and Haymer 2003).

One disadvantage of any PCR-based method is the requirement of a relatively costly precision thermal cycler. Other methods, such as the loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000), could provide a valuable alternative molecular diagnostic tool. This method is rapid and robust, and it requires only a simple thermostat-based instrument for operation under isothermal conditions. The amplification reaction uses specifically designed pairs of primers and a DNA polymerase (such as BstDNA polymerase) that has strand displacement activity. Recent work using this technique has also shown that the

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addition of an extra pair of primers can improve the method by reducing by half the time required for amplification (Nagamine et al. 2002).

LAMP has been used previously in a wide range of applications. In disease studies, this method has been used for diagnostic identification of bacterial (Iwamoto et al. 2003, Maruyama et al. 2003), protozoan (Poon et al. 2006), and viral infectious agents (Hong et al. 2004); for quantification of pathogens (Parida et al. 2005, Toriniwa and Komiya 2006); and for the detection of viruses in plants (Fukuta et al. 2003, 2004). It also is highly regarded as a field method for the identification of emerging viruses of biomedical importance (Parida 2008). LAMP also has been used to determine the sex of in vitro-fertilized cow embryos (Hirayama et al. 2004) and as a diagnostic tool to discriminate between termite species (Itakura et al. 2006). Up to now, however, there have been no applications showing how this method might be used for rapid identification of insect pests to augment detections of pests requiring quarantine when detected at ports of entry or other venues.

In this study, we describe a simple and rapid protocol for identification of Mediterranean fruit fly material by using a simple DNA extraction procedure. Specifically, DNA was extracted from material representing various stages of the Mediterranean fruit fly life cycle by using a Chelex-based resin. Using primers designed from the internal transcribed spacer (ITS) 1-5.8S-ITS2 ribosomal DNA (rDNA) sequences, products were then amplified by LAMP to show that Mediterranean fruit fly material could be detected and discriminated from other tephritid species.

Materials and Methods

Samples. Specimens obtained from established laboratory colonies were preserved in 95% ethanol. Mediterranean fruit fly adults, third-instar larvae, and eggs were obtained from J. Vontas (Department of Biology, University of Crete, Greece). Early larval-stage material (first and second instar) as well as pupal and pupal thecal material was obtained from D. McInnis (USDA-ARS, Honolulu, HI). The pupal theca refers to the residual shell left after adult emergence. Material from *Bactrocera tryoni* (Froggatt) was provided by the International Atomic Energy Agency laboratory in Seibersdorf, Austria. Olive fruit fly, *Bactrocera oleae* (Gmelin), material was obtained from J. Vontas's laboratory, and the oriental fruit fly, *Bactrocera dorsalis* (Hendel), material was obtained from a strain maintained in our laboratory in Taiwan.

DNA Extraction Methods. To identify an optimal method for rapid and economical extraction of DNA from Mediterranean fruit fly material that could be used in LAMP, three different conventional DNA extraction methods were compared. For each method, the extraction procedures were repeated three times, and the DNA yields were compared. The methods were as follows.

Proteinase K-spin Column (Tissue & Cell Genomic DNA Purification Kit, Genemark, Taichung, Taiwan).

One adult fly was ground in 200 μ l extraction solution. After centrifuging, the supernatant was treated with 20 μ l of proteinase K (18.18 mg/ml) at 56°C for 1 h. Four microliters of RNase A solution was added after protein digestion. After 5 min of incubation, 200 μ l of binding solution was added, and the DNA was precipitated with ethanol for 10 min at -70°C. The DNA pellet was washed with 70% ethanol, dried, and dissolved in sterile water, pH 8.5.

QuickExtract DNA (BuccalAmp DNA Extraction Kit, QuickExtract DNA Extraction Solution 1.0, Epicenter Technologies, Madison, WI). One adult fly was ground in 100 μ l of QuickExtract Solution and then vortex mix for 15 s. The sample solution was incubated at 65°C for 6 min and then transferred to 98°C for 2 min. After incubation, the sample was centrifuged at 13,000 rpm for 3 min, and the supernatant was used as the DNA source.

Chelex (Chelex 100 Resin, Molecular Biology Grade, Bio-Rad, Hercules, CA). One adult fly was ground in 100 μ l of Chelex solution containing 5% Chelex, 0.2% sodium dodecyl sulfate, 10 mM Tris, pH 9.0, and 0.5 mM EDTA (http://www.uga.edu/srel/DNA_Lab/KariChelex_mod'00.rtf). The sample solution was boiled for 10 min and then centrifuged at 10,000 rpm for 3 min. The supernatant was used as the DNA source.

The concentration and quality of DNA recovered by each method was estimated by spectrophotometer (LAMBDA 45, PerkinElmer Life and Analytical Sciences, Boston, MA) by using optical density (OD)₂₆₀ readings and the OD₂₆₀ and OD₂₈₀ ratios, respectively. Except as indicated for specific experiments, $\approx$ 100 ng of DNA was used for each LAMP reaction.

LAMP Primer Design. To design primers for the LAMP reactions, ribosomal gene sequences representing the ITS1-5.8S-ITS2 region as described in GenBank accession no. DQ490237 for the Mediterranean fruit fly, GenBank accession no. AF276516 for the oriental fruit fly, GenBank accession no. AF121159 for *B. tryoni*, and GenBank accession no. AF189690 for *Ceratitis rosa* Karsch were initially aligned as shown in Fig. 1.

Using the Mediterranean fruit fly sequence, the PrimerExplorer program (a primer designing program developed specifically for LAMP assays available at <http://primerexplorer.jp>) was used to design three pairs of primers. The sequences of these primers are given in Table 1. The pairs of primers used (described below) were combined for use in the LAMP reactions.

LAMP Assays. LAMP reactions were each performed in a total of 25- μ l mixture containing 0.05 μ M each of primers F3 and B3, 0.4 μ M each of primers forward inner primer (FIP) and backward inner primer (BIP), 0.2 μ M each of primers LF and LB along with 20 mM Tris-HCl, pH 8.8, 10 mM KCl, and 8 mM MgSO₄ or MgCl₂, 10 mM (NH₄)₂SO₄, 0.1% each of Tween 20 and Triton X-100, 0.8 M betaine, 1.4 mM dNTPs, and 8 U of the BstDNA polymerase (New England Biolabs, Ipswich, MA). The mixture was first incubated at 65°C for 30, 45, 60, or 120 min, and the

Fig. 1. Ribosomal gene sequences of Mediterranean fruit fly used for designing the primers. Partial nucleotide sequences (ITS1-5.8S-ITS2) of the Mediterranean fruit fly, oriental fruit fly, *B. tryoni*, and *C. rosa* are shown. Number at left end of sequences correspond to the positions in Mediterranean fruit fly (GenBank accession no. DQ490237), oriental fruit fly (GenBank accession no. AF276516), *B. tryoni* (GenBank accession no. AF121159), and *C. rosa* (GenBank accession no. AF189690). Shading with black colors are identical from each other sequences. The lines (without arrowheads) show the location of the primers used. These include the LB (20 bp) and LF (18 bp) primers and the F3 (22 bp) and B3 (20 bp) primers. Additional LAMP primers used were the FIP consisting of the sequences found at the locations shown as F1 (20-bp) and F2 (18-bp) primers and the BIP consisting of the sequences found at the locations of B1 (26-bp) primer and B2 (18-bp) primers.

temperature was raised to 80°C for 20 min to terminate the reaction. A thermal cycler model GeneAmp 2700 (Applied Biosystems, Foster City, California) was used for the controlled temperature reactions.

Restriction digestions were performed to confirm that LAMP products were amplifying products of the expected size (124 and 107 bp) from the target sequences. After digestion with PstI (New England Biolabs) according to conditions recommended by the manufacturer, products were loaded into 1.5% agarose gels for electrophoresis.

Visual confirmations of successful reactions also were obtained by adding 1 µl of a 1,000× concentrate of SYBR Green I in dimethyl sulfoxide (Invitrogen, Carlsbad, CA) to 25 µl of LAMP reaction products corresponding to different incubation times. The florescence intensities produced were compared under visible light and UV light at a wavelength of 365 nm.

Detection of Mediterranean Fruit Fly Material. Using material obtained from the Chelex method, LAMP

assays were performed on Mediterranean fruit fly specimens representing different life cycle stages, including eggs, third-instar larva, pupa, and adult, both with quantitation of the extracted DNA (100 ng) and by adding material from the Chelex extraction directly to the LAMP reaction without quantitation. Water (RNase-free) was used to make all necessary dilutions.

Species Discrimination. Single adult specimens of the Mediterranean fruit fly, oriental fruit fly, *B. tryoni*, and olive fruit fly, were removed from ethanol, blotted dry, and placed in a tube for DNA extraction by using the Chelex method. From each extraction, ≈100 ng of DNA was used in LAMP reactions containing the Mediterranean fruit fly primers. Results obtained from different reaction times (25, 30, 35, and 40 min) were analyzed by gel electrophoresis.

Statistical Analysis. DNA concentration and OD ratio results were analyzed statistically using analysis of variance (ANOVA) tests followed by Duncan tests for comparison of treatment means via SPSS version 16.0 (SPSS Inc., Chicago, IL).

Table 1. Sequences of LAMP primers designed from the Mediterranean fruit fly ribosomal DNA sequence (see text for explanation)

Primer	Sequence (5' → 3')
F3	5'-TCTTGACTCTTCATAAAAAGCA
B3	5'-TCTTACAACCCCTCAACCATA
FIP	5'-ACCATGACGCACAGTTTCTCTCTAAGCG CTGGATCAC
BIP	5'-ACATGAACATCGACATTTTGAACGGTACTC CAAGCAGCACTT
LF	5'-CATCGACCCATGAGCCGA
LB	5'-TGCAGTCCATGCTGTTACGT

Results

DNA Extraction and Specificity of LAMP Reaction. The time spent and quantity and purity of material recovered for the different DNA extraction methods used here are listed in Table 2. Although the proteinase K-spin column method, based on the OD₂₆₀/OD₂₈₀ ratio, yielded the highest purity of DNA among these methods, it also took the longest time to complete. Also, the quantity of material recovered by this method was <1/10 of what was recovered using the other methods. The QuickExtract DNA and Chelex

Table 2. Time required, cost, quantity, and purity of DNA extracted by different methods

Method ^a	Time	Cost per sample ^b (New Taiwan dollar)	dsDNA concn. (ng/μl) ^c	Ratio of OD ₂₆₀ /OD ₂₈₀ ^d
Spin column	1.5 h	23.3	63.6 ± 15.3a	1.79 ± 0.043a
QuickExtract	13 min	20.6	1,420 ± 129b	1.38 ± 0.018b
Chelex	15 min	<1	1,520 ± 355b	1.61 ± 0.003c

Data are expressed as mean ± SD by three replications.
^a The individual methods are described in the text (see Materials and Methods, DNA Extraction Methods section).
^b The cost comparisons of the kits and Chelex reactions did not include cost of additional chemical or solutions required. However, these costs are minimal.
^{c,d} ANOVA ($P < 0.001$). Values in the same column followed by the same lowercase letter were not significantly different from one another ($P < 0.05$).

methods required approximately the same amount time and produced similar yields in term of quantity and purity of the DNA recovered.

To compare the suitability of the material obtained, the same amount of DNA (100 ng) recovered from each method was used in separate LAMP reactions. Figure 2 shows that the LAMP reactions worked successfully for adult DNA extracted via all three methods. As a confirmation that the correct sequences were being amplified, products also were digested using the restriction enzyme PstI. As expected, this digestion collapsed the ladder-like pattern into one main band of the predicted size (lane 13). Some faint higher molecular weight bands, probably representing partial digest products, also are visible here.

Primarily because of the short time required and the relatively low cost, the Chelex DNA extraction method was used in all of the following assays.

LAMP Reactions on Material from Different Stages of the Mediterranean Fruit Fly Life Cycle. The quantities of DNA recovered from different developmental

Table 3. Quantity and purity of Chelex DNA extracted from different developmental stages of Mediterranean fruit fly

Stage	dsDNA concn. (ng/μl) ^a	Ratio of OD ₂₆₀ /OD ₂₈₀ ^b
Egg	4.03 ± 0.49a	1.72 ± 0.092a
First-instar larva	25.7 ± 5.14a	1.32 ± 0.098b
Second-instar larva	87.4 ± 29.2a	1.79 ± 0.016a
Third-instar larva	1475 ± 240b	1.36 ± 0.059b
Pupa	865.7 ± 6.82c	1.29 ± 0.093b
Pupal theca	94.7 ± 6.61a	1.16 ± 0.036c

Data are expressed as mean ± SD by three replications.
^{a,b} ANOVA analysis ($P < 0.001$). Values in a column followed by the same lowercase letter were not significantly different from one another ($P < 0.05$).

stages of Mediterranean fruit fly using the Chelex method are listed in Table 3. The total amount of DNA recovered from single egg was the smallest among the stages tested; however, this material also was among the highest in terms of purity. The quantity of DNA recovered from the pupal theca was similar to that of second-instar larvae but was poorest in terms of purity.

Two experiments were done to demonstrate that the LAMP reaction could be carried out on material from each of these different developmental stages. Figure 3A shows that the LAMP reaction was successful when a standard amount of DNA (100 ng) recovered from egg, third-instar larval-, pupal-, and adult-stage material was used. Figure 3B shows a similar successful outcome when material from these same developmental stages was extracted and added directly to the LAMP reaction without quantification.

Visualization of LAMP Reactions. The products of the LAMP assay also can be directly visualized in reaction tubes by the addition of SYBR Green I as a dye indicator. Figure 4 shows the results for detection of adult Mediterranean fruit fly material obtained at

Fig. 2. Electrophoretic patterns of Mediterranean fruit fly LAMP products by different DNA extraction methods. Lanes 1–4, 5–8, and 9–12 contain material from LAMP carried out using different DNA extraction methods, and the different reaction times used in each case are shown at the bottom of lanes. Lane 13 shows the same LAMP product of lane 12 digested by PstI restriction enzyme. Lane 14 is a negative control in which water was used instead of extracted DNA in the LAMP reaction (for 2 h). Lane M contains 100-bp ladder size markers.

Fig. 3. Electrophoretic patterns of LAMP products from various developmental stages of the Mediterranean fruit fly by using quantified versus unquantified amounts of DNA as templates. (A) Results using quantified extracted DNA (100 ng in each case) for various stages as indicated. Lane A1, control (water instead of extracted DNA); lane A2, adult; lane A3, third-instar larva; lane A4, pupa; and lane A5, eggs. (B) Results using extracted DNA without quantitation for various developmental stages as indicated. Lane B1, control (water instead of extracted DNA); lane B2, pupal theca; lane B3, second-instar larva; lane B4, first-instar larva; and lane B5, egg. Lane M, 100-bp ladder size markers.

time points of 0, 30, 45, 60, and 120 min under visible (A) and UV light (B) conditions. Material from each of these reactions also was examined using agarose gel electrophoresis (Fig. 4C) to confirm the results observed in the reaction tubes.

Specificity of Detection. The results presented in Fig. 5 show that material from the Mediterranean fruit fly can be specifically detected by LAMP. No positive results were obtained using material from any of these species at the 25-min reaction time point. For reaction times of 30, 35, and 40 min, LAMP produced positive results only for the Mediterranean fruit fly material in all cases; the LAMP reactions were negative using

material from other species at the same time points. However, when the experiment was extended to include 90- and 120-min time points, reaction products were seen in the other lanes (data not shown).

Discussion

In an effort to develop a rapid and reliable method for identification of the pest species causing infestations by using material from any stage of the life cycle, a series of experiments have been carried out to 1) compare the efficiency (economic and time costs versus yield) of DNA extraction methods for use in LAMP assays (Table 2; Fig. 2); 2) show that LAMP can be used to detect material from various life stages (Fig. 3); 3) determine the minimum incubation time required to detect LAMP products (Fig. 4); and 4) test the ability of this method to discriminate between material from various fruit fly species that cause similar infestations but that can be difficult to distinguish using traditional taxonomic approaches (Fig. 5).

Of the DNA extraction methods analyzed here, overall the Chelex-based procedure seems to be optimal in the sense that it is a simple procedure requiring only a few steps, takes <45 min to complete DNA, yields reliable material for amplification by LAMP, and is economical in terms of material costs (compared with the other methods). The values for purity of the DNA recovered by the Chelex method were in between those of the spin column and the QuickExtract methods, both of which are more labor-intensive and costly.

To provide template material for LAMP, the material recovered from the different DNA extraction methods examined here did not seem to have a strong influence on the success of the reaction. This may be because in general, LAMP seems to be less sensitive to the presence of nontarget DNA or reaction inhibitors compared with other amplification methods (Notomi et al. 2000, Soliman and El-Matbouli 2005). Regardless, our results support the use of the Chelex method for DNA extraction because it is the least expensive and nearly the fastest. This may be most relevant in situations where the rapid detection of pest infestations is required to implement quarantine procedures in a timely manner. Furthermore, using the Chelex-extracted material, our reactions were successful whether or not the DNA was quantified before use in LAMP.

For the LAMP reactions, we have shown that by using three pairs of primers designed to amplify specific regions of the ribosomal DNA of the Mediterranean fruit fly, we were able to successfully carry out the LAMP reaction in as little as 30 min (Figs. 4 and 5). This is a reduction by approximately one-half of the reaction time reported previously as being required for LAMP (Nagamine et al. 2002).

We have also shown that Mediterranean fruit fly material can be detected by LAMP by using material from virtually any stage of the life cycle. This includes single egg where as little as 4.03 ng of template DNA (the approximate amount of DNA extracted from one

Fig. 4. The fluorescence of DNA-binding SYBR Green I visually detected under visual light (A) and UV light (B). (C) Agarose gel electrophoresis of LAMP products obtained using different reaction times (0, 30, 45, 60, and 120 min) as indicated. DNA was obtained in all cases using the Chelex extraction method.

egg) was sufficient for detection. Other studies have shown that as little as 0.1 pg of DNA from KHV viruses can be detected by LAMP (Soliman and El-Matbouli 2005). However, our level of sensitivity is certainly enough for identification of insect material. Also, even for material where the DNA yield was of relatively low purity, such as pupal thecal material (Table 3), LAMP was still successful (Fig. 3B).

To further demonstrate the utility of this approach, the SYBR Green I dye also was used for detection of LAMP reaction products. Reaction products stained with SYBR Green were clearly detected using gel electrophoresis. The incorporation of this dye also allows for direct visualization of reaction products by eye in the presence of UV light.

Finally, we also have shown that using LAMP, Mediterranean fruit fly material can be discriminated from material derived from other pest species in the family Tephritidae including the oriental fruit fly, *B. tryoni*, and olive fruit fly. Using the Mediterranean fruit fly primers, after a 30-min time interval, LAMP reaction products were clearly observed only when the Mediterranean fruit fly material was used as the template (Fig. 5). Further checking at later time points (35 and 40 min) also confirmed that products were seen only in the Mediterranean fruit fly lane, although when greatly extended reaction times of 90 min or more were checked, products were seen in the other lanes (data not shown). Therefore, for species identification purposes, it will be necessary to limit obtaining results

Fig. 5. Electrophoretic patterns of results of LAMP reactions by using single adult specimens of different tephritid pest species at 25-, 30-, 35-, and 40-min time points. For each time point shown, lane 1, Mediterranean fruit fly; lane 2, oriental fruit fly; lane 3, *B. tryoni*; lane 4, olive fruit fly; and lane 5, water instead of extracted DNA. Lane M, 100-bp ladder size markers.

of the LAMP reactions to within a 30–60-min time window.

Overall, our combined use of a rapid DNA extraction procedure and modified LAMP reaction conditions shows that it is possible to identify Mediterranean fruit fly material and discriminate it from other potentially infesting fruit fly species in <1 h. In other molecular applications, the use of the highly conserved rDNA sequences requires much more detailed analysis to allow for differentiation between species in this family. However, additional testing will be required to see if the Mediterranean fruit fly can be differentiated from other species that are even more closely related species (for example species belonging to the same genus) using this method. To facilitate this, LAMP may be applied in a stepwise manner beginning with conserved sequences from genes such as the ribosomal genes described here to first make genus level identifications. This could then be followed up with a second round of LAMP reactions by using primers derived from other genes with greater power to discriminate between even closely related species, such as those described by Naeole and Haymer (2003) for identification of species with the *B. dorsalis* complex. Alternatively, the LAMP results could be combined with other diagnostic tools, such as those described by Armstrong et al. (1997) to identify critical pest species.

In conclusion, a rapid and simple diagnostic procedure has been successfully developed to identify material from Mediterranean fruit fly specimens by using LAMP. LAMP has the advantage that it requires only relatively simple equipment for isothermal amplification and analysis of the target material. We also show here that using a simple and rapid Chelex-based DNA extraction procedure, minimal amounts of material from the Mediterranean fruit fly, such as single egg or body parts including the pupal theca, can be successfully detected. Results of LAMP also can be observed using either gel electrophoresis or visual detection when SYBR Green I is incorporated. Finally, because the entire process from the extraction of DNA to the identification of the presence of material from a particular species (such as the Mediterranean fruit fly analyzed here) can be completed in a relatively short time (<1 h), this methodology may be extremely valuable for countries or regions subject to infestations of the Mediterranean fruit fly (or other pests) where decisions about implementations of quarantine procedures need to be made rapidly to maintain pest-free status.

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