Rapid and highly accurate detection of *Drosophila*
suzukii, Spotted Wing Drosophila (Diptera:
Drosophilidae) by loop-mediated isothermal
amplification assays

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Abstract

Drosophila suzukii, the spotted wing drosophila (SWD), is currently a major pest that

causes severe economic losses to thin-skinned, small fruit growers in North America and Europe. The monitoring and early detection of SWD in the field is of the utmost importance for its proper management. Although SWD traps using various attractants have been developed and used for SWD monitoring in the field, they are not specific to SWD; consequently, other insects, including *Drosophilidae* flies that share high morphological similarities with SWD, are also easily trapped. Thus, all captured flies need to be identified under microscopes by an expert in fly morphological characteristics. In this study, we developed a simple molecular detection tool that rapidly and accurately identifies SWD without sophisticated instruments or expertise. We first identified a gene, Ds10_00012111, that is present in the SWD genome but not in that of any other insect species. Then, we developed the loop-mediated isothermal amplification (LAMP) assay, which was designed based on genomic DNA of the Ds10_00012111 gene. This LAMP assay detected only genomic DNA from SWD—not from *Drosophila melanogaster*. The minimum amount of genomic DNA required for the LAMP assay was 1.0 picogram, and the duration of this assay at temperatures ranging from 58 °C to 62 °C was 30 min. In addition, this assay could detect genomic DNA in SWD geographical strains collected from 8 different locations in Asia, Europe, Hawaii, and the USA. Our LAMP assay could be a useful detection tool for identifying SWD rapidly in the field.

Key words: spotted wing drosophila, monitoring, identification, loop-mediated isothermal amplification, *Drosophilidae*

Introduction

Spotted wing drosophila (SWD), *Drosophila suzukii* (Matsumura) is an endemic vinegar fly widely distributed in temperate and subtropical regions of South Asia (Kanzawa, 1939). Although SWD shares several biological characteristics with other *Drosophilidae* species, it also has some unique morphological and physiological characteristics. For example, SWD females have unique serrated ovipositors that they use to make a hole in fruits to lay eggs inside them (Kanzawa, 1939; Lee et al., 2011). In addition, adult males have a spot on the outside edge of each wing (Kanzawa, 1939; Lee et al., 2011). The most important physiological characteristic of SWD that differs from other *Drosophilidae* flies is its spawning preference for fresh fruits, although SWD are also known to oviposit on fermented, rotten fruits (Lee et al., 2011). The wide range of feeding preferences gives SWD a strong advantage and makes them more competitive than any other *Drosophilidae* flies (Rota-Stabelli et al., 2013). Compared with *D. melanogaster*, which prefers rotten, fermented foods, SWD showed increased susceptibility to oxidative stressors together with reduced glutathione S-transferase activity and gene numbers in its genome (Nguyen et al., 2016).

Recent invasions of SWD in North America and Europe have had a large impact on industries that produce thin-skinned, small fruits. The economic damage from crop production reductions, market value losses, and the increased pest management costs caused by SWD reaches into the hundreds of million dollars every year in the USA alone (Burrack et al., 2013; Goodhue et al., 2011). The wide range of host fruits infested by SWD makes their management more difficult (Harris et al., 2014). To

properly control SWD, it is important to monitor their occurrence when fruits start to ripen (Lee et al., 2012).

Currently, various types of SWD traps have been developed, and these are used to monitor seasonal populations of SWD in the field. (Basoalto et al., 2013; Landolt et al., 2011; Lee et al., 2013; Mazzetto et al., 2015). However, because most of the attractants used in SWD traps are not specific to them, other insect species are also captured, including *Drosophilidae* flies (Isaacs et al., 2010). Thus, the flies captured in these traps must be identified by an expert to validate the presence of SWD. Several morphological characteristics of SWD have been used to differentiate them from other *Drosophilidae* flies during identification. The wing spots of adult male SWD are easily identifiable by the naked eye. However, other identifying morphological characteristics, such as the serrated ovipositors of adult females and the two dark bands on the forelegs of adult males, must be identified under a stereomicroscope (Isaacs et al., 2010). Thus, developing molecular tools that could be used to identify SWD rapidly without using sophisticated instruments or requiring morphological expertise can be important for the rapid and accurate monitoring of SWD in the field.

In this study, we found an SWD-specific gene that does not occur in other insects, including other species in the genus *Drosophilidae*. Using this SWD-specific gene, we developed a loop-mediated isothermal amplification (LAMP) assay. This newly developed tool is able to detect as little as 1 picogram (pg) of SWD genomic DNA. In addition, this assay detected all SWD-specific genomic DNA samples obtained from 8 geographical regions from Asia, Europe, Hawaii, and the USA. Because this LAMP assay requires only maintaining a constant temperature of between 58 ~ 62°C, it could be used as an on-site, rapid, and accurate detection tool for SWD in the field.

Materials and Methods

***Drosophila* species**

Previously published *Drosophila suzukii* (spotted wing drosophila (SWD)) strains, including strains from Korea (KR), Japan (JP), Italy, (ITY) Hawaii (HI), Oregon (OR), California (CA), Georgia (GA), and New York (NY), were used in this study (Nguyen et al., 2016). The *Drosophila melanogaster* (*Dm*) wild-type control strain Canton-S was obtained from Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA) and used as a negative control. The *Dm* and SWD were raised on a standard *Drosophila* diet or SWD diet, respectively, as reported by Phuong et al. (Nguyen et al., 2016).

Genomic DNA purification

Genomic DNA from adults of the *D. melanogaster* and various SWD strains were purified using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The concentrations of extracted genomic DNA were measured using an Epoch Microplate Spectrometer (BioTek Instruments, Inc., Winooski, VT, USA).

Identification of SWD specific gene

Specific SWD genes were screened from the SWD identified by unpublished SWD transcriptomic analysis data (M.Y.C. & Y.H.K.). One gene had no homologue in *Drosophila*, and the other insect genome was identified by blasting its DNA sequences

in FlyBase blast server (<http://flybase.org/blast>; cut-off e-value < 0.1). The identified gene was then blasted again using SpottedWingFlyBase (<http://spottedwingflybase.oregonstate.edu/blast>). It was annotated to Ds10_00012111 in the SpottedWingFlyBase, and its genomic DNA sequences were obtained.

Design of primers for the loop-mediated isothermal amplification assay

The genomic DNA sequence of Ds10_00012111 was used as a template for designing the loop-mediated isothermal amplification (LAMP) assay. Four primers for Ds10_00012111 were designed using guidelines reported in Tomita et al. (Tomita et al., 2008). The detailed locations and DNA sequences of 4 primers are listed in Table 1. The predicted size of the amplified DNA fragments by the forward primer 3 (F3) and the backward primer 3 (B3) was 206 nucleotides.

PCR, cloning and sequence verification of Ds10_00012111

Genomic DNA of *D. melanogaster* and SWD was amplified with 10 pmol F3 and B3 in 1 x PCR master mix (Elpis Bio, Daejeon, Korea). After 5 min of denaturing at 95 °C, a PCR amplification cycle, consisting of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s and extension at 72 °C for 30 s, was repeated 35 times. The amplified DNA fragments were confirmed using 2.0% agarose gels (0.5 x Tris/Borate/EDTA buffer) and visualized with SYBR green (Life Technologies, Grand Island, NY, USA). Bands were excised and cloned into a pGEM®-T easy vector according to the manufacturer's protocol (Promega, Madison, WI, USA). Cloned amplified DNA fragments were sequenced and compared with those of Ds10_00012111 using Geneious® DNA software

(ver. 8.1.5) (<http://www.geneious.com>) and CLC Sequence Viewer (ver. 7.0) (CLC bio. Aarhus, Denmark).

LAMP reaction conditions

A twenty-five- μ l LAMP reaction mixture consisted of 1.6 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 2 mM of each dNTP (Life Technologies), 8 units of large-fragment GspSSD DNA polymerase (Optigene, Horsham, UK), 1 x buffer (Optigene), 2 mM MgSO_4 (Optigene), 0.2 pmol F3 primer, 0.2 pmol B3 primer, 1.6 pmol forward internal primer (FIP) and 1.6 pmol backward internal primer. LAMP reactions were performed at 58 °C, 60 °C, or 62 °C for 1 hr using an Applied Biosystems 2720 thermal cycler (Life Technologies). To determine the optimal LAMP reaction time, LAMP reactions were incubated at 60 °C for 5, 10, 15, 20, 25, or 30 min. After the LAMP reactions were complete, SYBR green (Life Technologies) was added to each tube for visualization. Every experiment was repeated a minimum of three times.

Results

Ds10_00012111 gene in the SWD genome as a LAMP target

DNA fragments of approximately 200 base pairs (bp) were amplified from PCR mixtures containing 1 or 2 ng of SWD genomic DNA, but not from 2 ng of *Dm* genomic DNA (Figure 1). The sequences of amplified DNA fragments were the same as those of Ds10_00012111 obtained from SpottedWingFlyBase (data not shown). This result suggests that Ds10_00012111 is present only in the SWD genome, not in the *Dm* genome.

Next, we tested whether the Ds10_00012111 gene could be amplified by four LAMP primer sets. When LAMP reactions were performed at 60°C for 1 hr with 1 ng of SWD or *Dm* genomic DNA, only the tube that contained SWD genomic DNA showed fluorescence under daylight (Figure 2A). No fluorescence was detected from either *Dm* genomic DNA or from tubes containing no DNA (negative controls). The differences were even clearer when the tubes were examined under UV illumination (Figure 2B). When the LAMP reaction products were separated by 2.0% agarose gels, the SWD genomic DNA reaction products contained greater quantities of double-stranded DNA (ds-DNA) smeared along the lanes (Figure 2C). To determine the optimal temperature range for the LAMP reaction, we tested the reaction at both 58°C and 62°C. Both temperatures yielded results similar to those obtained at 60°C, suggesting that the LAMP reaction could be performed at temperatures between 58°C and 62°C.

Optimization of LAMP reaction

To determine the minimum duration of the LAMP reaction, 6 LAMP reactions performed at durations that varied by 5 min were examined (Figure 3). When the 6 LAMP reactions were examined, weak fluorescence signals were detected after 25 min of incubation and strong signals were detected after 30 min of incubation under both daylight (Figure 3A) and UV illumination (Figure 3B), respectively. When those reaction products were separated by 2.0% agarose gels, amplified, ladder-like ds-DNA fragments began to be detected after 25 min (Figure 3C). After 30 min of incubation, greater amounts of amplified ds-DNA with typical ladder shapes were present. These data suggest that incubation for 30 min may be sufficient to detect the presence of SWD genomic DNA.

Sensitivity of LAMP

Because LAMP reactions are known to be highly sensitive compared with conventional PCR (Kang et al., 2014; Tomita et al., 2008), the minimum amount of SWD genomic DNAs that could be detected by this LAMP protocol was determined. When 1.0 ng to 1.0 femtogram (fg) of SWD genomic DNAs were used for the LAMP reactions, strong fluorescence signals were detected under daylight (Figure 4A) and UV illumination (Figure 4B) from 1 ng and 1.0 pg LAMP reaction tubes, respectively. Consistently, LAMP reactions without or with less than 0.1 pg of SWD genomic DNA input did not show any amplification of DNA fragments when separated by 2.0 % agarose gels (Figure 4C). These data suggest that the minimum amount of SWD genomic DNA confidently detected by LAMP PCR was 1.0 pg.

This LAMP assay was able to detect 8 geographically different SWD strains

Because it has been reported that SWD could rapidly change their genomic information after invading new territories to adapt new environments (Nguyen et al., 2016; Ometto et al., 2013), we further tested whether this LAMP assay could be applied for detecting SWD strains collected from different geographical locations in Asia, Europe, and the USA. When 1.0 nanogram (ng) of genomic DNA from 8 geographically different SWD strains were used, strong fluorescence signals were detected under daylight (Figure 5A) and UV illumination (Figure 5B) from all strains. In addition, when those LAMP reaction products were separated by 2.0 % agarose gels, greater quantities of ds-DNA ladder-like shapes were detected from all strains (Figure 5C). This result suggests that the LAMP assay designed using the Ds10_00012111 gene as a target can be used to detect SWD from different geographical locations.

Discussion

The most significant contribution of this study is the development of a rapid diagnostic tool requiring no sophisticated instruments or expertise that can be used to differentiate SWD from other *Drosophilidae* flies that share similar morphological characteristics. To accomplish this, we looked for a gene that was present only in SWD and not in other *Drosophila* species or other insect species. Because the reference genome sequence of SWD contains approximately 220 million nucleotides—80% more than that of *Dm*—and because among the 13,583 predicted protein-coding genes, only 12,389 have homologs in the *Dm* genome (Adams et al., 2000; Chiu et al., 2013), we expected to find several SWD specific genes or genomics regions by comparing the transcriptome analysis results of SWD with the genomic DNA or the transcript database of *Dm*. Indeed, we found an SWD-specific gene. Several blast analysis results using the FlyBase and SpottedWingFlyBase blast servers suggest that Ds10_00012111 may be present only in SWD (M.Y.C. and Y.H.K.). Because genomic DNA is usually much more stable than RNA, we identified genomic DNA sequences of Ds10_00012111 from the SpottedWingFlyBase blast server. Then, the LAMP primer sets were designed based on the genomic DNA sequences of Ds10_00012111, which contained an intronic region. More interestingly, this gene has no homologue not only in the genus *Drosophila* but also in the orders Diptera, Lepidoptera, Hymenoptera, Hemiptera, Phthiraptera, and Ixodida at a cut-off e-value of 0.1. Thus, in addition to being a biomarker specific to SWD, this gene may be a possible target for developing species-specific pesticides in the future.

The SWD genome is known to change rapidly because the flies can complete 7 to 13

generations per year depending on the climate, and a single female can lay several hundred eggs (Lee et al., 2011). In a previous study, we also found that an internal transcribed spacer 1 (ITS1) of genomic DNA obtained from geographically different SWD strains showed very high variations (Nguyen et al., 2016). Even within the same geographical strains, many different variations in ITS1 were present. LAMP assays have been widely used to detect a number of pathogens from insects, nematodes, bacteria, and viruses. For example, an allele-specific LAMP assay could be applied to detect single nucleotide polymorphisms in alleles in the malaria vector *Anopheles gambiae* (Badolo et al., 2012). Sometimes, the specificity of LAMP assays can cause problems when detecting pathogens. For example, a LAMP method designed to detect the ITS1 regions of *Bursaphelenchus xylophilus* in Japan failed to detect *B. xylophilus* from some tree discs where infections were confirmed because of possible sequence variations within the ITS1 region (Kanetani et al., 2010; Kang et al., 2014). Thus, we further confirmed that SWD collected from other geographical regions in Asia, Europe, Hawaii, and USA could be detected by our LAMP protocol (Figure 5). Our LAMP assay was able to amplify ladder-like dsDNA fragments from all 8 SWD strains, indicating that it can be used to detect all SWD strains worldwide.

Conclusions

To properly manage SWD on farms growing thin skinned, small fruits, monitoring the occurrence of SWD is of the utmost importance (Goodhue et al., 2011; Lee et al., 2011). Although there are several recommendations for SWD monitoring traps (Landolt et al., 2011; Lee et al., 2013), those are not yet specific to SWD; consequently, they often capture other insects—including other *Drosophilidae* flies—meaning that SWD must

be confirmed under a stereomicroscope using their specific morphological characteristics (Quarles, 2015). Because our LAMP assay requires only limited instruments and short incubation times, it could be used as an on-site rapid diagnostic tool for detecting SWD in the field.

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DNA sequence	+	CATTTGT <u>TAA AATCCGACGT CCCGT</u> TCAAA AGTTATTCCG <u>AAAACAAGAT TTACGCGGTC</u>
No. in a contig		22332
Primer	Name	Forward 3 (F3) Forward 2 (F2)

Table 1. Locations and sequences of LAMP primers in gene coding sequences of SWD

DNA sequence	+	GAACCGTAAG TCTTGTGGTG ACCAAAATCC ATATA <u>AAATT CTCTGTGTAA CTGGGAGATC</u>
No. in a contig		22392
Primer	Name	Forward 1c (F1c)

DNA sequence	+	GATCCAGGTT <u>CCGCAGGTGG TAAGCGAAAAG</u> ATTCTAGTAC TGAATCCGTC ACTAATATG
No. in a contig		22452
Primer	Name	Backward 1 (B1)

DNA sequence	+	<u>GCTCAATAGA ATTCCACAGT CTTCGCTTGT GACG</u> TATTCT TGGTGTATT
No. in a contig	22512	
Primer	Name	Backward 2C (B2) Backward 3c (B3c)

No. of a contig, Numbers in a contig of genomic DNA sequences; Seq, sequence.

Primer sequences were colored, bolded, and underlined. Forward inner primer (FIP) made with F1complemenatry (F1c) and F2. Backward inner primer (BIP) made with B1 and B2complementary (B2c). Backward 3c; Backward 3 complementary

Figure Legends

Figure 1. F3 and B3 primer sets amplified approximately 200-bp DNA fragments from SWD genomic DNA but not from *Dm* genomic DNA. The black arrow indicates amplified DNA fragments just above a 200-bp marker.

Figure 2. Visual inspection and electrophoretic separation of LAMP reaction products

(A) Amplified DNA could be detected under daylight when the LAMP reaction mixtures with genomic DNA of SWD and *Dm* or with no DNA (No) were incubated at 60°C for 1 hr. SYBR green was added to the tubes after incubation was complete.

(B) Strong fluorescence signals were detected only from the SWD genomic DNA-containing tubes, when the same tubes were inspected under UV light,

(C) Enormously amplified double-stranded DNA was detected when LAMP reaction products were separated using a 2.0% agarose gel. The reaction temperature was 60°C; however, LAMP reactions incubated at 58°C or 62°C produced similar amplifications.

Figure 3. Optimization of LAMP reaction incubation time

(A-B) Six LAMP reactions of durations differing by 5 min were examined under daylight (A) and UV illumination (B). Weak fluorescence signals were detected from the 25-min incubation. After 30 minutes, strong signals were detected. (C) Consistent with the fluorescence signals, typical ladder-shaped amplified ds-DNA fragments were detected from the 25-min incubation.

Figure 4. Sensitivity of LAMP reaction for SWD genomic DNA

(A & B) When 1 ng to 1 fg of SWD genomic DNA was used as a template for the LAMP assay, strong fluorescence signals were detected from 1.0 ng to 1.0 pg under daylight (A) and UV illumination (B). (C) Similarly, when LAMP reaction products were separated using 2.0 % agarose gels, amplified ds-DNA ladders were detected only from 1.0 ng to 1 pg.

Figure 5. The LAMP assay detected 8 geographically different SWD strains

(A & B) When 1 ng of genomic DNA from 8 geographically different SWD strains was used as a template, strong fluorescence signals were detected under daylight (A) or UV illumination (B) from all strains. (C) All LAMP reactions had a greater quantity of ds-DNA ladders when separated using 2.0 % agarose gels.

Figure 1.

Figure 2

Figure 3.

Figure 4.

Figure 5

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