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Development of a Rapid Isothermal Amplification Assay for the Fall Armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), Using Species-Specific Genomic Sequences

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Abstract: The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is native to tropical and subtropical regions of the Western Hemisphere, but is now regularly appearing in crop fields across South Korea, particularly in corn fields. Therefore, it is crucial to promptly and accurately identify the presence of FAW in crop fields to effectively eradicate it as a regulated quarantine species. We developed a loop-mediated isothermal amplification (LAMP) assay, which allows for rapid in-filed identification. To develop the LAMP assay, we selected FAW-specific genomic regions from the whole-genome sequences of one FAW and 13 other lepidopteran species and validated five primer sets that consistently produced positive reactions in ten FAW samples collected from eight different locations in four countries. The assay successfully identified FAW in a maximum of 45 min, starting from crude DNA extraction (~15 min) to diagnosis (30 min) from the following samples, which were deposited outdoors for 30 days: a 1st-instar larva, an adult leg, an adult antenna, and 1/16 and 1/8 of an adult thorax. The five assays can be used selectively or in combination to cross-check and provide further confidence in the in-field diagnosis of FAW.

Keywords: fall armyworm; *Spodoptera frugiperda*; loop-mediated isothermal amplification; FAW-specific primers

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1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is a highly polyphagous insect that consumes at least 353 species of plants. It is a significant pest of corn, rice, and forage grasses [1,2]. Originating in the tropical regions of the western hemisphere from USA to Argentina, the species began spreading in 2016 and has now been detected in over 70 countries in Africa, Asia, and Oceania as of 2021 [3–12]. In South Korea, after FAW was first detected in 2009, the number of places where it has been detected is expanding annually [13,14]. It is believed that the possible source of the infestation was FAW populations residing in eastern part of southern China, aided by seasonal winds [13]. Unlike other invasive species introduced by human activity, the arrival and spread of FAW in South Korea will continue as long as the dispersal pressure in the source populations remains. Consequently, all crop fields in rural areas across the country are potential sites

for quarantine measures against the entry of FAW as a regulated quarantine species. It may be difficult to confine the infestation to a specific region based solely on the previous year's records.

The species identification of FAW is typically performed using the adults captured in FAW-specific pheromone traps. However, this method may not be sufficient to determine the absence of FAW, as leftover egg masses on corn leaves and various stages of FAW larvae inside the whorl of corn leaves are often found. Additionally, several lepidopteran moths, the larvae of which damage corns, can be also detected. Moreover, dead, distorted, and partial bodies of FAW larvae and other lepidopteran species, which are not suitable for morphological inspection, are occasionally found inside the whorl of corn leaves and on the soil. It is possible that these specimens were left undisturbed for a while after being killed by pesticides before FAW's presence was recognized in the area.

Under such circumstances, accurate and rapid diagnosis is essential for making prompt control decisions to minimize potential settlement. To fulfill this purpose, the loop-mediated isothermal amplification (LAMP) assay has effectively been applied to diagnose the speculated samples outdoors [15–19]. LAMP assay for species identification has several merits over conventional PCR, in that thermos-cycler is not required, quick extracted DNA can be used, and the time required for diagnosis is shorter [20–22]. For FAW, a few studies have already developed the LAMP assay, targeting the mitochondrial region spanning from $tRNA^{Arg}$-$tRNA^{Asn}$ to *ND5* genes, the mitochondrial *COI* gene, or the FAW-specific nuclear gene, Sf00067 [23–26]. These studies successfully demonstrated the usefulness and superiority of in-field application in various aspects including the time required for the completion of the assay, diagnosis power in various types of tissues, and sensitivity in DNA concentrations.

Nevertheless, the limited availability of trained local specialists in molecular diagnosis, in contrast to the ubiquitous occurrence of FAW in nationwide crop fields, necessitates a LAMP assay, which includes in-field referable tests to determine the sensitivity of the developed markers, thereby increasing the confidence in the diagnosis results. This is particularly important considering that local inspectors are responsible for control decisions once the LAMP results are positive. Moreover, the improvement in any in-field processes for diagnosis would be helpful for a more accurate LAMP assay. On the day of field diagnosis, inspectors should prepare the chemical mixture, spend a significant amount time focusing on the trace of the FAW remnant, and perform actual in-field diagnosis under diverse conditions and using various forms of FAW samples that may be encountered in the field.

In this study, we present five primer sets for LAMP assays that can be used either independently or in combination for cross-checking. These five loci were selected from FAW-specific genomic regions through whole-genome sequencing of one FAW and 13 lepidopteran species, consisting of seven corn-damaging species, three *Spodoptera* species found in South Korea, and three *Spodoptera* species found in the USA, which are also regulated for quarantine in South Korea. After validating the markers, the five loci were tested using crude DNA obtained using samples from all life stages, including 1–3 eggs, a 1st-instar larva, partial pupa, and partial adult tissues, which were exposed to outdoor ambient temperatures for up to 30 days to confirm the applicability of the assay in the field. Moreover, the stability of the pre-made LAMP mixture, which was stored at −70 °C for up to ten days, was tested.

2. Materials and Methods

2.1. Specimen Collection

The FAW larvae were hand-collected form the whorl of corn leaves in eight different locations across four countries: three in South Korea, two in Vietnam, two in Zimbabwe, and one in the United States of America (Table 1). They were initially identified based on their morphological characteristics and later confirmed through sequencing of the 3′-end of the mitochondrial cytochrome oxidase subunit I (*COI*) and the nuclear Z-chromosome-

linked Triose phosphate isomerase (*Tpi*), which are used to determine the host type of FAW [27–29]. To collect non-target, corn-damaging species found in South Korea, species-specific sex pheromone traps (GreenAgrotech, Gyeongsan, Korea) were installed in various agricultural fields. However, the false army worm, *Mythimna loreyi*, was collected in a FAW-specific pheromone trap. Three *Spodoptera* species found in the USA were collected as adults in the fields during 2019–2020. These 13 non-target species were sequenced for DNA barcoding for species identification (Table 1).

Table 1. Collection information of the samples.

No.	Species	Specimen No.	Locality	Coordinates	Date	Stage
1	*Spodoptera frugiperda*	CNU15404	Gyeongsan-si, Gyeongsangbukdo, Republic of Korea	35°50′47″ N 128°48′05″ E	12 June 2019	Larva
2	〃	† CNU13926	Hallim-eup, Jejudo, Republic of Korea	33°25′51″ N 126°16′05″ E	6 June 2020	Larva
3	〃	CNU13911	Hallim-eup, Jejudo, Republic of Korea	33°25′51″ N 126°16′05″ E	6 June 2020	Larva
4	〃	CNU13942	Unnam-myeon, Muan-gun, Jeollanamdo, Republic of Korea	34°57′29″ N 126°20′46″ E	15 June 2020	Larva
5	〃	CNU13959	Unnam-myeon, Muan-gun, Jeollanamdo, Republic of Korea	34°57′29″ N 126°20′46″ E	15 June 2020	Larva
6	〃	CNU13946	Newark, DE, USA	39°46′25″ N 75°44′37″ W	5 July 2020	Larva
7	〃	CNU13969	Ninh Binh, Vietnam	20°14′10″ N 105°57′44″ E	30 September 2019	Larva
8	〃	CNU13972	Hanoi, Vietnam	21°02′39″ N 105°51′07″ E	30 September 2019	Larva
9	〃	CNU13975	Harare, Zimbabwe	17°45′22″ S 30°57′40″ E	10 January 2019	Larva
10	〃	CNU13978	Manicaland, Zimbabwe	18°40′57″ S 32°43′30″ E	27 January 2019	Larva
11	*S. praefica*	CNU13987	MPG Ranch, Missoula Co, Montana, USA	46°40′23″ N 114°00′56″ W	20 April 2020	Adult
12	〃	† CNU13988	Yolo Co., Rumsey Cyn., CA, USA	38°53′45″ N 122°14′58″ W	9 July 2019	Adult
13	*S. eridania*	CNU13981	Santa Rosa Co., FL, USA	30°48′29″ N 87°02′37″ W	26 June 2019	Adult
14	〃	† CNU13982	Wilson Co., NC, USA	35°41′18″ N 77°51′59″ W	10 October 2019	Adult
15	*S. ornithogalli*	CNU13984	Wayne Co., NC, USA	35°41′18″ N 77°51′59″ W	4 October 2019	Adult
16	〃	† CNU13986	Manatee Co., FL, USA	27°27′43″ N 82°19′58″ W	24 September 2019	Adult
17	*S. depravata*	CNU14444	Samseo-myeon, Jangseong-gun, Jeollanamdo, Republic of Korea	35°13′15″ N 126°39′25″ E	11 May 2020	Adult
18	〃	† CNU13996	Samseo-myeon, Jangseong-gun, Jeollanamdo, Republic of Korea	35°13′15″ N 126°39′25″ E	11 May 2020	Adult
19	*S. exigua*	CNU14456	Samseo-myeon, Jangseong-gun, Jeollanamdo, Republic of Korea	35°13′15″ N 126°39′25″ E	14 May 2020	Adult
20	〃	† CNU13997	Nampyeong-eup, Naju-si, Jeollanam-do, Republic of Korea	35°25′27″ N 126°51′28″ E	18 May 2020	Adult
21	*S. litura*	CNU14541	Unnam-myeon, Muan-gun, Jeollanamdo, Republic of Korea	34°57′29″ N 126°20′46″ E	26 June 2020	Adult
22	〃	† CNU14000	Yongbong-dong, Buk-gu, Gwangju, Republic of Korea	35°10′25″ N 126°53′54″ E	21 May 2020	Adult
23	*Helicoverpa armigera*	CNU14457	Nampyeong-eup, Naju-si, Jeollanam-do, Republic of Korea	35°25′27″ N 126°51′28″ E	18 May 2020	Adult

Table 1. *Cont.*

No.	Species	Specimen No.	Locality	Coordinates	Date	Stage
24	〃	† CNU13990	Yongbong-dong, Buk-gu, Gwangju, Republic of Korea	35°10′25″ N 126°53′54″ E	19 May 2020	Adult
25	*H. assulta*	CNU14492	Nampyeong-eup, Naju-si, Jeollanam-do, Republic of Korea	35°25′27″ N 126°51′28″ E	3 June 2020	Adult
26	〃	† CNU13999	Yongbong-dong, Buk-gu, Gwangju, Republic of Korea	35°10′25″ N 126°53′54″ E	22 May 2020	Adult
27	*Agrotis ipsilon*	CNU14489	Samseo-myeon, Jangseong-gun, Jeollanamdo, Republic of Korea	35°13′15″ N 126°39′25″ E	3 June 2020	Adult
28	〃	† CNU14001	Yongbong-dong, Buk-gu, Gwangju, Republic of Korea	35°10′25″ N 126°53′54″ E	26 May 2020	Adult
29	*Mythimna loreyi*	CNU14475	Unnam-myeon, Muan-gun, Jeollanamdo, Republic of Korea	34°57′29″ N 126°20′46″ E	28 May 2020	Adult
30	〃	† CNU13989	Hallim-eup, Jeju-do, Republic of Korea	33°25′49″ N 126°15′57″ E	7 June 2020	Adult
31	*M. separata*	CNU13880	Buan-gun, Jeollabukdo, Republic of Korea	35°43′33″ N 126°42′26″ E	1 July 2020	Adult
32	〃	† CNU13880	Buan-gun, Jeollabukdo, Republic of Korea	35°43′33″ N 126°42′26″ E	1 July 2020	Adult
33	*Cnaphalocrocis medinalis*	CNU13894	Gogeum-myeon, Wando-gun, Jeollanamdo, Republic of Korea	34°23′10″ N 126°48′42″ E	14 July 2020	Adult
34	〃	† CNU13889	Gogeum-myeon, Wando-gun, Jeollanamdo, Republic of Korea	34°23′10″ N 126°48′42″ E	14 July 2020	Adult
35	*Ostrinia furnacalis*	CNU14465	Daedeok-myeon, Damyang-gun, Jeollanamdo, Republic of Korea	35°15′04″ N 127°02′12″ E	21 May 2020	Adult
36	〃	† CNU13998	Daedeok-myeon, Damyang-gun, Jeollanamdo, Republic of Korea	35°15′04″ N 127°02′12″ E	3 June 2020	Adult

† Used for whole-genome resequencing.

2.2. Preparation of Libraries for Next-Generation Sequencing

Whole-genome sequencing was performed from on one individual FAW collected from a Korean locality (specimen no. CNU13926) and 13 non-target species (Table 1). Total DNA was extracted from the thoracic muscles of larvae and hind legs of adults using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), Proteinase K (Thermo Fisher Scientific, Rockford, IL, USA), isopropyl alcohol (Amresco, Solon, OH, USA), and 70% ethanol, following the manufacturer's instructions, and stored at –70 °C until use. The purity and concentration of the extracted DNA were estimated using the standard procedure of the Quant-iT PicoGreen dsDNA Assay kit (Molecular Probes, Eugene, OR, USA) with a Synergy HTX Multi-Mode Reader (Biotek, Winooski, VT, USA), and normalized to a concentration of 12.5 ng/μL.

Sequencing libraries were prepared using the Illumina TruSeq Nano DNA kit (Illumina, SD, USA), and the quality of the constructed library was assessed using a DNA High-Sensitivity LabChip kit on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Paired-end sequencing with 151-bp reads was conducted on the Illumina Nextseq500 platform. A summary of the sequencing data can be found in Table S1.

2.3. Draft Genome Assembly and Isolation of FAW-Specific Regions

Sequencing artifacts and low-quality bases in the generated reads were removed using the PE mode of Trimmomatic v0.39 [30], with TruSeq 3 adapter sequences included in Trimmomatic and the following parameters: LEADING, 3; TRAILING, 20; and MINLEN, 125. Before conducting genome assembly, sequencing errors in the generated reads were corrected using the error correction module included in Allpaths-LG r52488 [31]. The error-corrected paired-end reads were then merged into an interleaved fasta file using fq2fa of IDBA_UD, and assembly was conducted using IDBA_UD [32] with the pre_correction option. To identify the species-specific region of each genome, the merged reads were

mapped to assembled genomes of 13 remaining lepidopteran species using bwa-mem2 [33], and the unaligned reads were isolated using SAMtools [34] with Flag 0 × 04, iteratively. Unaligned reads of each species to the remaining 13 species are each unique to each genome. These isolated reads were aligned to each genome to identify species-specific regions and read mapping coverage of mapping regions was calculated using genomecov of bedtools [35]. Minimum read mapping coverage over 20 were identified and the sequences (>1000 bp) of the species-specific regions in each genome were isolated. The specificity of these regions was confirmed using iterative mapping with minimap2 [36], which is the same method used for short reads.

2.4. Conventional PCR

Conventional PCR was performed to determine the host type of FAWs, to identify 13 non-target species, and to assess sequence divergence among FAWs in the five loci, based on which the LAMP primers were designed (Table S2). To accomplish this, total DNA was extracted from an additional set of nine FAWs obtained from four countries, including South Korea, as well as one individual from each of the 13 non-target species (Table 1) using the same method employed for whole-genome sequencing and stored at −20 °C until further use.

To amplify the 3′-end of the *COI* gene and a fragment of the *Tpi* gene for the host-type identification of FAWs, primers were adapted from Nagoshi et al. [37] (Table S2). The inner primer pair, which amplifies a 360-bp fragment (excluding primer sites), was primarily used to amplify the 3′-end of *COI*. However, in cases where amplification using the inner primer set was insufficient, the amplicon acquired using the outer primer set (560 bp) was used as a template to amplify the target region. Similarly, the target *Tpi*-gene fragment was mostly amplified using the inner primer set, resulting in a 444-bp product. Occasionally, the amplicon acquired using the outer primer set (492 bp) was used as a template for amplification of the target amplicon. For amplification of the 658-bp fragment of *COI*, corresponding to the DNA barcoding region for species identification of 13 non-target species, a pair of primers was adapted from Folmer et al. [38] (Table S2). In order to assess sequence variation among ten FAWs within each locus of the five LAMP loci, which were eventually selected for LAMP assay, PCR was conducted using a pair of primers designed for the beginning and end regions of each locus (Table S2, Figure 1).

Amplification was conducted using AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) under the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 50–53 °C, and 1 min at 72 °C, and a final extension for 7 min at 72 °C. Electrophoresis was conducted to confirm successful DNA amplification using 0.5× TAE buffer on a 0.5% agarose gel. PCR products were purified using a PCR Purification kit (Qiagen, Hilden, Germany). DNA sequencing was conducted using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit with an ABI PRISMTM 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). All products were sequenced from both strands.

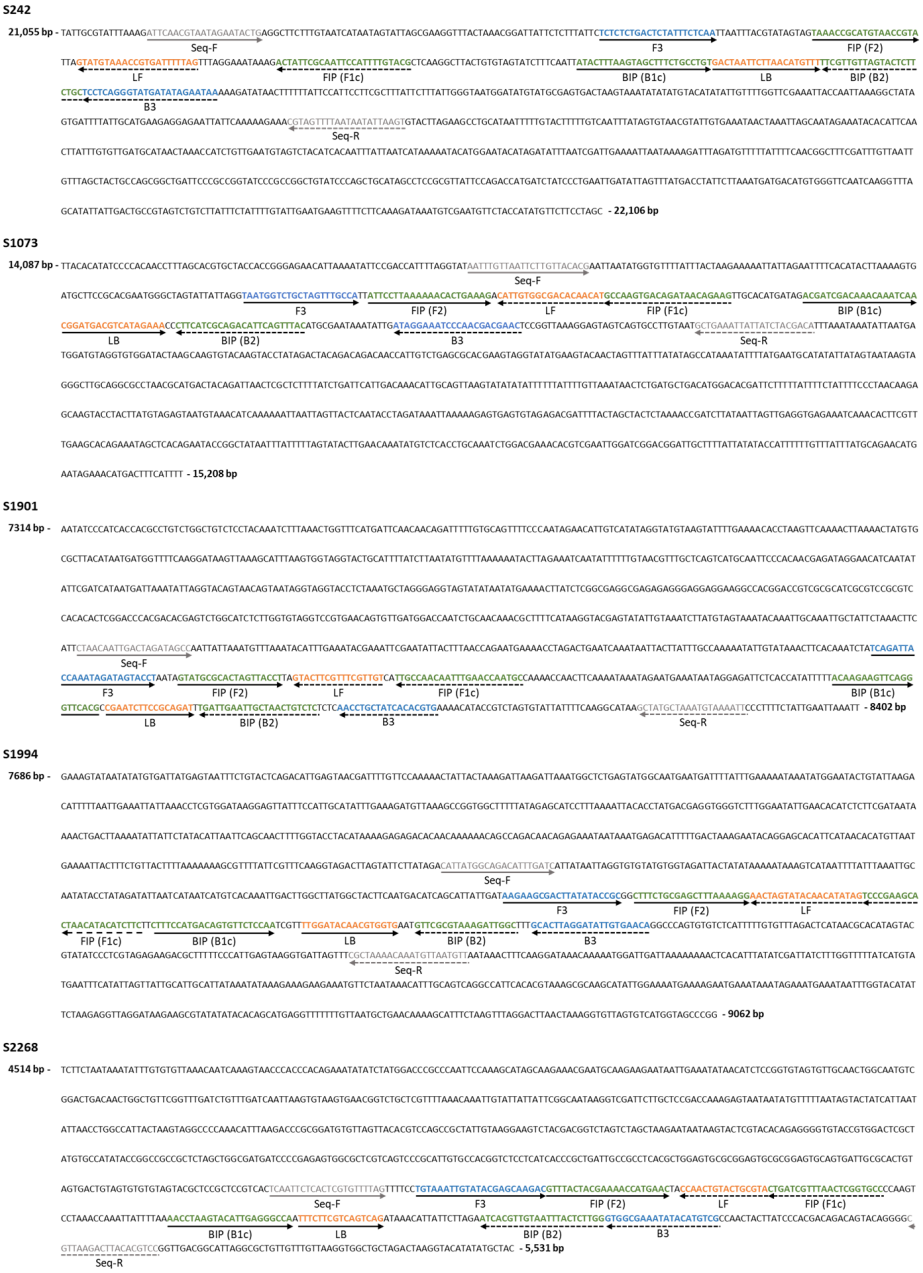


Figure 1. Sequences of five FAW-specific scaffolds used for designing six primer sets for the LAMP assay. The nucleotide positions indicated at the beginning and end of each sequence represent the start and end positions of regions that are unique to FAW among 14 lepidopteran species, including one FAW. The forward primers are marked with arrows, while the reverse primers are marked with dotted arrows. FIP (5′ to 3′) was designed by combining the F2 of FIP with the reverse complementary sequences of F1c of FIP. Similarly, BIP (5′ to 3′) was designed by combining the B2 of BIP with the reverse complementary sequences of B1c of BIP. Seq-F and Seq-R are the primers designed to sequence each locus to check for sequence conservation among FAW samples.

2.5. LAMP Primer Design from FAW-Specific Regions

Three pairs of primers were designed for each of the five loci using PrimerExplorer ver. 5 software (available at http://primerexplorer.jp/lampv5e/index.html; accessed on 18 February 2022). The length of the primers was specified to be 18–25 nucleotides, with an annealing temperature of 55–63 °C, and a GC ratio of 30–65% (Figure 1). The primers were synthesized by Macrogen (Daejeon, Republic of Korea). FIP and BIP were purified through

HPLC, while other primers were purified using the Macrogen Oligonucleotide Purification Cartridge method.

2.6. Validity Test for the Five Loci

The validity of the five loci for FAW detection was tested using ten FAW samples from four countries (including both corn and rice types) and 13 non-target species (Table 1). In the negative control, an additional 1 µL of Isothermal Master Mix was added instead of DNA. Each experiment was repeated at least three times.

Each LAMP reaction mix was prepared in a 200 µL thin-wall, plat-cap, portable micro-centrifuge tube (Corning Life Science, Painted Post, NY, USA). The mix included 2.5 µL of primer master mix (0.05 µL each of F3 and B3; 0.2 µL each of FIP and BIP; 0.1 µL each of LoopF and LoopB; and 1.8 µL of ultrapure water), 15 µL of Isothermal Master Mix (ISO-004, OptiGene, Horsham, UK), 1 µL of template DNA, and 6.5 µL of ultrapure water, as recommended in the LAMP user guide (OptiGene, Horsham, UK). The DNA extracted for conventional PCR was used for this validation test. The purity and concentration of the extracted DNA were determined using a BioDrop spectrophotometer (Biochrom, Ltd., Cambridge, UK) and normalized to a concentration of 10 ng/µL. LAMP reactions were performed for 40 min at 60 °C using an incubator (MiniT-100, Hangzhou Allsheng Instruments Co., Hangzhou, China). After the reaction, 2 µL of SYBR (Life Technologies, Eugene, OR, USA) was added to each tube for visualization under daylight and ultraviolet (UV) light. Positive reactions were further confirmed through electrophoresis on a 2% agarose gel using 1× TAE buffer at 100 V for 25 min.

2.7. Sensitivity Test for Tissue Type, Reaction Time, and DNA Concentration

The sensitivity of the five loci was tested using tissues from each life stage, which presumably can be encountered outdoors: an egg, 1st-instar larva, 1/8 of a pupa, and adult antenna. An egg mass from a female FAW, which was collected originally in Korea (Gyeongsan, specimen no. CNU15404), was reared on a general-purpose artificial diet for Lepidoptera (Bio-Serv, Frenchtown, NJ, USA) to produce all the necessary life-stage samples. Egg sampling was conducted from an egg mass using a brush under general-purpose microscope. The pupa was sampled by cutting approximately 1/8 segment of the middle part of a pupa with bare eyes. An antenna was detached from an adult body using forceps. For 2.8. Sensitivity test for outdoor deposited FAW each adult leg and adult thorax was additionally obtained by detaching a leg from an adult body using forceps and cutting each 1/16 and 1/8 segment of an adult thorax with bare eyes, respectively. DNA extraction was performed using a quick DNA extraction method. Each specimen was crushed approximately ten times using a micro-pestle in a 1.5-mL micro-centrifuge tube. Then, 250 µL of Lucigen Quick Extract DNA extraction solution (BioSearch, Middleton, WI, USA) was added, and the mixture was incubated for 6 min at 65 °C and then for 2 min at 98 °C using an incubator (MiniT-100). The extracted DNA was stored at −20 °C until further use. LAMP reactions were performed under the same conditions described in the Section 2.6. "Validity test for the five loci" but with varying reaction times (10, 20, 30, and 40 min) and using crude stock DNA. Once a consistent positive reaction time was obtained for all loci, that particular reaction time was used for the sensitivity test with diluted DNA (stock, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions from the crude stock DNA). Negative controls included *S. exigua* (specimen no. CNU14456) and a no-DNA LAMP mixture. DNA from *S. exigua* was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and normalized to a concentration of 10 ng/µL. Each experiment was repeated at least three times.

2.8. Sensitivity Test for Outdoor Deposited FAW

To further manage outdoor situations, where dead and distorted FAW specimens at varying stages are used for species identification, all stages of FAW (1–3 eggs, 1st-instar larva, 1/8 of a pupa, adult leg, adult antenna, 1/8 thorax of an adult, and 1/16 thorax

of an adult) exposed to ambient temperatures during June–July for 10, 20, and 30 days were tested using crude stock DNA. LAMP reactions were performed for 30 min under the same conditions as previously described. The methods for detecting positive reactions, the negative controls, and the number of experimental repeats were the same as described earlier. The approximate size, weight, DNA concentration (ng/μL), and purity (at $A_{260/280}$) of each tissue after being exposed for varying periods were measured for in-field reference. The concentration of DNA (ng/μL) and purity ($A_{260/280}$ ratios) were evaluated using a BioDrop spectrophotometer (Biochrom, Ltd., Cambridge, UK).

2.9. Stability of Pre-Made Chemical Mixture

One of the omissible procedures in the field for the LAMP assay is the preparation of chemical mixtures, such as the primer master mix and Isothermal Master Mix. Preparing these mixtures beforehand can save time and prevent contamination in the field, particularly for unskilled testers. Thus, the stability of the pre-made total mixture, consisting of the primer master mix and Isothermal Master Mix, was tested by incubating at −20 °C for different periods (zero, one, three, five, seven, and ten days). The crude stock DNA from one egg, one 1st-instar larva, 1/8 of a pupa, and one adult antenna were used for testing. LAMP reactions were performed for 30 min under the same conditions as described earlier. A positive reaction was visualized under daylight after adding 2 μL of SYBR. The negative controls and number of experimental repeats were the same as described earlier.

3. Results

3.1. Host Type of FAWs and Sequence Identity of 13 Non-Target Species

Host-type identification of the ten FAWs, according to Nagoshi et al. [37], revealed that two Korean local samples and one USA sample were corn-type in both *COI* and *Tpi*, while the remaining eight samples were rice-type in *COI* and corn-type in *Tpi* (Table S3). Previous studies have shown that *Tpi* can be reliably used for host plant identification, rather than *COI* [39,40]. The DNA barcoding sequences of the 13 non-target species were analyzed using The Basic Local Alignment Search Tool (BLAST), which showed 99.70% to 100% identity to the corresponding species in the databases of the National Center for Biotechnology Information (NCBI) and Bold Systems (available at http://www.boldsystems.org/; accessed on 9 October 2024) (Table S4). These sequences were deposited in GenBank (Table S4).

3.2. Genome Assembly and Selection of Species-Specific Regions

Draft genomes of one FAW and 13 non-target species were assembled using the generated whole-genome sequencing data (Figure S1). The genome size of the 14 species ranged from approximately 428 Mbp in *S. praefica* to approximately 1 Gbp in *Mythimna separata*, resulting in a difference of more than two-fold and an average genome size of approximately 731 Mbp (Figure S1A). The N50 length was the shortest (281 bp) in *Cnaphalocrocis medinalis* and the longest at (2329 bp) in *S. praefica*, with an average of about 10 kbp (Figure S1B). The difference in N50 length was significant across species, but there was no discernible trend based on genome size or depth coverage of the generated data. By performing short-read iterative mapping using draft genomes with trimmed reads, a unique genomic region was identified for each species (Figure S2). The number of species-specific regions in FAW was 158 scaffolds, but it varied greatly among species, ranging from 32 to 3663 regions (Figure S2A). The total length of the species-specific regions also exhibited high variability, ranging from about 49 kbp in *Helicoverpa armigera* to about 5 Mbp in *Ostrinia furnacalis* (Figure S2B).

3.3. Sequence Homology of the FAW-Specific Loci to Public Data

A total of 11 out of 158 loci (1005–1554 bp) were randomly selected as the initial set of target loci for LAMP primer design. The primer-targeted regions, spanning from F3 to B3 (146–307 bp), were then searched using BLAST with the option "somewhat similar

sequences (blastn)" (Table 2). Four loci did not yield any matching sequences, while the remaining seven loci produced a variable number of matching sequences (2–18 sequences). These matches were found in different categories: FAW only (S1073), non-insects (S96 and S590), non-lepidopteran insects (S2268 and S590), lepidopteran Noctuidae (which include FAW; S96, S590, S1910, S1992, S2268, and S2326), and non-Noctuidae lepidopterans (S590 and S2268). Most of the matched sequences had a very low query cover, accounting for less than 40% of the total length, excluding those matched to FAW sequences in the public data. However, there were also sequences with longer query cover that could potentially nullify FAW. In the case of S1992, two sequences matched to *Spodoptera* sequences with sequence identities of 69.14% and 78.49%, covering 97% (*S. exigua*) and 100% (*S. littoralis*) of S1992, respectively. Similarly, S96 also detected two matched sequences that covered most of the sequences of S96. Therefore, these two loci were abandoned due to the risk of false-positive amplification, particularly when encountering geographic variation. Excluding these two loci, the remaining matched sequences in the five loci had a query cover of less than 50% to the given loci, covering only a limited length of the primer sequences.

Table 2. Matching sequences for each locus obtained from Blast search.

Locus	Size (Bp)	Taxonomy	Description	Query Cover (%)	Identity (%)	Overlapping Region to Primer Sequences	Accession No.
S96	240	Lepidoptera: Noctuidae	*Spodoptera exigua* genome assembly, chromosome: 11	97	80.43	Partial F3–Partial B3	LR824612
		Lepidoptera: Noctuidae	*Spodoptera littoralis* genome assembly, chromosome: 11	97	78.72	Partial F3–Partial B3	LR824542
S242	245	-	-	-	-	-	-
S590	249	Lepidoptera: Sesiidae	*Synanthedon tipuliformis* genome assembly, chromosome: 18	21	87.04	Partial B1c	OX392425
		Non-insect	*Marchantia polymorpha* subsp. ruderalis Tak-1 DNA, chromosome: 5	23	82.26	Partial B1c	AP019870
		Non-insect	*Ailanthus altissima* genome assembly, chromosome: 19	16	92.68	Partial B1c	OX327701
		Coleoptera: Nitidulidae	*Brassicogethes aeneus* genome assembly, chromosome: 5	20	88.00	Partial B1c	OV121136
		Diptera: Pediciidae	*Pedicia rivosa* genome assembly, chromosome: 4	33	77.65	Partial LB	OY720154
		Diptera: Bibionidae	*Dilophus febrilis* genome assembly, chromosome: 1	36	76.34	Partial LB	OY284468
		Non-insect	*Musa balbisiana* cv. Pisang Klutuk Wulung BAC MBP-17D14c, complete sequence	19	86.00	Partial F3–Partial F2	HE983609
		Non-insect	*Musa balbisiana* genomic DNA, BAC clone:MBP_31O07, complete sequence	19	86.00	Partial F3–Partial F2	AP009334
		Lepidoptera: Crambidae	*Elophila nymphaeata* genome assembly, chromosome: 23	20	84.62	Partial B1c	OY039089
		Lepidoptera: Nymphalidae	*Melitaea cinxia* chromosome 23	17	90.91	Partial F3–Partial F2	CP049670
		Non-insect	*Buxus sempervirens* genome assembly, chromosome: 2	12	96.88	Partial F2	OX387183

Table 2. *Cont.*

Locus	Size (Bp)	Taxonomy	Description	Query Cover (%)	Identity (%)	Overlapping Region to Primer Sequences	Accession No.
		Lepidoptera: Erebidae	*Catocala fraxini* genome assembly, chromosome: 7	21	83.93	Partial F1c	OV884031
		Non-insect	*Neostethus bicornis* genome assembly, chromosome: 1	20	84.31	Partial B1c	LR738542
		Non-insect	*Gossypium raimondii* isolate D5-4 chromosome D5_11	17	86.36	Partial B1c	CP032563
		Non-insect	*Gossypium hirsutum* cultivar TM1 chromosome D11	17	86.36	Partial B1c	CP023744
		Lepidoptera: Geometridae	*Eupithecia subfuscata* genome assembly, chromosome: 19	22	82.14	Partial B1c	OY751115
		Lepidoptera: Coleophoridae	*Coleophora deauratella* genome assembly, chromosome: Z	22	83.05	Partial B1c	OY282387
		Lepidoptera: Sesiidae	*Synanthedon andrenaeformis* genome assembly, chromosome: 15	18	86.96	Partial B1c	OW387791
S1073	202	Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* uncharacterized LOC126911878 (LOC126911878), ncRNA	73	81.88	Partial LF–Partial B3	XR_007706379
		Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* major facilitator superfamily domain-containing protein 6 (LOC118278837), mRNA	73	82.43	Partial LF–Partial B3	XM_050703552
		Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* peroxisomal membrane protein PEX16 (LOC118268658), mRNA	73	81.05	Partial LF–Partial B3	XM_035583230
		Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* uncharacterized LOC126911752 (LOC126911752), mRNA	73	81.76	Partial LF–Partial B3	XM_050700507
S1205	185	-	-	-	-	-	-
S1901	232	Lepidoptera: Noctuidae	*Spodoptera frugiperda* sequence from BAC clone 72B06	100	99.57	F3–B3	FO681373
		Lepidoptera: Noctuidae	*Spodoptera exigua* genome assembly, chromosome: 8	24	80.70	Partial B1c–Partial B2	LR824609
S1992	247	Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* histone-lysine N-methyltransferase PRDM9-like (LOC126911818), mRNA	100	100.00	F3–B3	XM_050700757
		Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* zinc finger protein 62-like (LOC118281175), mRNA	100	91.60	F3–B3	XM_035601678
		Lepidoptera: Noctuidae	*Spodoptera littoralis* genome assembly, chromosome: 28	100	78.49	F3–B3	LR824559
		Lepidoptera: Noctuidae	*Spodoptera exigua* genome assembly, chromosome: 28	97	69.14	Partial F3–B3	LR824629
		Lepidoptera: Noctuidae	*Thalpophila matura* genome assembly, chromosome: 30	27	79.71	Partial LB–Partial B3	OX419206
S1994	181	-	-	-	-	-	-

Table 2. *Cont.*

Locus	Size (Bp)	Taxonomy	Description	Query Cover (%)	Identity (%)	Overlapping Region to Primer Sequences	Accession No.
S2088	218	-	-	-	-	-	-
S2268	210	Lepidoptera: Noctuidae	*Spodoptera littoralis* genome assembly, chromosome: 9	29	88.71	Partial LF–Partial B1c	LR824540
		Lepidoptera: Noctuidae	*Spodoptera littoralis* genome assembly, chromosome: 23	29	85.71	Partial LF–Partial B1c	LR824554
		Lepidoptera: Noctuidae	*Tholera decimalis* genome assembly, chromosome: 3	42	77.78	Partial B1c–Partial B3	OW964551
		Coleoptera: Lucanidae	*Dorcus parallelipipedus* genome assembly, chromosome: 1	28	81.36	Partial B1c–Partial B2	OY284475
		Lepidoptera: Tortricidae	*Pammene fasciana* genome assembly, chromosome: 14	48	72.28	B1c–Partial B3	OU452286
S2326	230	Lepidoptera: Noctuidae	PREDICTED: *Spodoptera frugiperda* cytochrome P450 9e2-like (LOC126911294), mRNA	64	100.00	F3–Partial B1c	XM_050697701
		Lepidoptera: Noctuidae	*Spodoptera frugiperda* clone U7451 cytochrome P450 (CYP9A10) mRNA, complete cds	39	100.00	F3–Partial LF	MZ945701
		Lepidoptera: Noctuidae	*Spodoptera frugiperda* clone U7158 cytochrome P450 (CYP9A10) mRNA, complete cds	33	100.00	F3–Partial LF	MZ945698
		Lepidoptera: Noctuidae	*Spodoptera frugiperda* clone CL147C34 cytochrome P450 (CYP9e2) mRNA, complete cds	33	100.00	F3–Partial LF	MZ945545
		Lepidoptera: Noctuidae	*Spodoptera frugiperda* clone U5644 cytochrome P450 (CYP9A10) mRNA, complete cds	31	100.00	F3–Partial LF	MZ945683
		Lepidoptera: Noctuidae	*Spodoptera littoralis* genome assembly, chromosome: 3	50	77.24	Partial LF–Partial B2	LR824534
		Lepidoptera: Noctuidae	*Spodoptera exigua* genome assembly, chromosome: 3	45	73.04	F1c–B2	LR824604

The locus with bold-faced letters are those eventually developed for LAMP assay.

3.4. Initial LAMP Reaction Using Nine FAW-Specific Loci

Even with prior genomic analysis to select FAW-specific loci, the LAMP assay using the first nine loci under the initial reaction conditions (e.g., reaction at 60 °C for 40 min, with 10 ng/µL of DNA and four primer sets, excluding loop primers) failed in four loci, resulting in no consistent amplification reaction among ten FAW samples (e.g., weak or no reaction), even after repeated experiments. As a result, these four loci were discarded without further inspection. However, the remaining five loci, which were consistently amplified in the ten FAWs and showed no amplification in 13 non-target species, were further examined.

3.5. Sequence Divergence of the Five Loci among Ten FAW Individuals

To determine the sequence divergence and positions of variable sites among the ten FAW individuals, the five loci were PCR-amplified and sequenced. All ten FAWs were successfully amplified in all five loci, while the 13 non-target species did not produce any equivalent PCR product, confirming that these loci are specific to FAW. The percentage of

sequence divergence and number of substitutions (including insertions/deletions) among the 10 FAWs varied among loci, ranging from zero to 4.86% (12 bp) in the S242 locus, zero to 5.94% (12 bp) in the S1073 locus, zero to 4.18% (10 bp) in S1901, zero to 1.66% (3 bp) in S1994, and 0.48% (1 bp) to 2.38% (5 bp) in the S2268 locus (Table 3). The majority of substitutions (and insertions/deletions) were found at the 5′-end of each primer, with a smaller number of substitutions detected at the non-primer sites (Figure S3). However, a few substitutions were located within 4–8 nucleotides at the 3′-end of the primers, including one substitution at the first nucleotide at the 3′-end of FIP (F2) in S1994, one at the 3′-end of BIP (B1c) in S1994, one at the second nucleotide at the 3′-end of F3 in S2268, and one at the third nucleotide at the 3′-end of BIP (B1c) in the S242 locus (Figure S3). Therefore, the negative effect of template mismatches was expected to be minimal for the LAMP assay.

Table 3. Pairwise comparisons among ten FAWs for each locus used in the LAMP assay.

	S242	1	2	3	4	5	6	7	8	9	10
1.	CNU13926	-	0.00	0.00	0.00	0.00	4.86	0.00	0.00	0.00	0.00
2.	CNU15404	0	-	0.00	0.00	0.00	4.86	0.00	0.00	0.00	0.00
3.	CNU13911	0	0	-	0.00	0.00	4.86	0.00	0.00	0.00	0.00
4.	CNU13942	0	0	0	-	0.00	4.86	0.00	0.00	0.00	0.00
5.	CNU13959	0	0	0	0	-	4.86	0.00	0.00	0.00	0.00
6.	CNU13946	12	12	12	12	12	-	4.86	4.86	4.86	4.86
7.	CNU13969	0	0	0	0	0	12	-	0.00	0.00	0.00
8.	CNU13972	0	0	0	0	0	12	0	-	0.00	0.00
9.	CNU13975	0	0	0	0	0	12	0	0	-	0.00
10.	CNU13978	0	0	0	0	0	12	0	0	0	-
	S1073	1	2	3	4	5	6	7	8	9	10
1.	CNU13926	-	3.47	2.97	1.98	0.00	3.96	4.95	3.47	4.46	3.47
2.	CNU15404	7	-	1.49	4.46	3.47	2.48	3.47	1.98	2.48	1.98
3.	CNU13911	6	3	-	3.96	2.97	1.98	2.97	1.49	2.48	1.49
4.	CNU13942	4	9	8	-	1.98	4.95	5.94	4.46	5.45	4.46
5.	CNU13959	0	7	6	4	-	3.96	4.95	3.47	4.46	3.47
6.	CNU13946	8	5	4	10	8	-	3.96	1.49	1.49	1.49
7.	CNU13969	10	7	6	12	10	8	-	3.47	4.46	3.47
8.	CNU13972	7	4	3	9	7	3	7	-	2.97	0.00
9.	CNU13975	9	5	5	11	9	3	9	6	-	2.97
10.	CNU13978	7	4	3	9	7	3	7	0	6	-
	S1901	1	2	3	4	5	6	7	8	9	10
1.	CNU13926	-	0.00	0.00	3.35	2.93	0.00	0.00	4.18	4.18	3.35
2.	CNU15404	0	-	0.00	3.35	2.93	0.00	0.00	4.18	4.18	3.35
3.	CNU13911	0	0	-	3.35	2.93	0.00	0.00	4.18	4.18	3.35
4.	CNU13942	8	8	8	-	0.42	3.35	3.35	0.84	1.67	0.00
5.	CNU13959	7	7	7	1	-	2.93	2.93	1.26	1.26	0.42
6.	CNU13946	0	0	0	8	7	-	0.00	4.18	4.18	3.35
7.	CNU13969	0	0	0	8	7	0	-	4.18	4.18	3.35
8.	CNU13972	10	10	10	2	3	10	10	-	2.51	0.84
9.	CNU13975	10	10	10	4	3	10	10	6	-	1.67
10.	CNU13978	8	8	8	0	1	8	8	2	4	-
	S1994	1	2	3	4	5	6	7	8	9	10
1.	CNU13926	-	0.00	0.00	1.11	0.55	1.11	0.00	0.00	0.55	1.11
2.	CNU15404	0	-	0.00	1.11	0.55	1.11	0.00	0.00	0.55	1.11
3.	CNU13911	0	0	-	1.11	0.55	1.11	0.00	0.00	0.55	1.11
4.	CNU13942	2	2	2	-	1.66	1.11	1.11	1.11	1.66	2.21
5.	CNU13959	1	1	1	3	-	1.66	0.55	0.55	1.11	1.11
6.	CNU13946	2	2	2	2	3	-	1.11	1.11	1.66	2.21
7.	CNU13969	0	0	0	2	1	2	-	0.00	0.55	1.11
8.	CNU13972	0	0	0	2	1	2	0	-	0.55	1.11
9.	CNU13975	1	1	1	3	2	3	1	1	-	0.55
10.	CNU13978	2	2	2	4	2	4	2	2	1	-

Table 3. *Cont.*

S2268	1	2	3	4	5	6	7	8	9	10
1. CNU13926	-	0.95	1.43	1.91	1.43	1.43	1.43	1.91	1.91	1.91
2. CNU15404	2	-	0.48	1.91	1.43	1.43	1.91	1.91	1.91	1.91
3. CNU13911	3	1	-	2.38	1.91	1.91	2.38	1.43	2.38	1.91
4. CNU13942	4	4	5	-	0.95	1.91	1.91	1.91	0.00	1.43
5. CNU13959	3	3	4	2	-	0.95	0.95	1.43	0.95	1.43
6. CNU13946	3	3	4	4	2	-	1.43	1.43	1.91	2.38
7. CNU13969	3	4	5	4	2	3	-	2.38	1.91	2.38
8. CNU13972	4	4	3	4	3	3	5	-	1.91	1.43
9. CNU13975	4	4	5	0	2	4	4	4	-	1.43
10. CNU13978	4	4	4	3	3	5	5	3	3	-

Numbers above the diagonal are percent distance values; numbers below the diagonal are absolute distance values.

3.6. Validity of the Five Loci

The amplification results of the LAMP assay in the five loci, under the initial reaction condition (60 °C for 40 min with 10 ng/μL of genomic DNA), but with six primer sets, including loop primers showed a strong positive reaction only in FAWs (Figure 2). In all loci, amplification of the ladder-shaped DNA fragments in gel electrophoresis, the color changed from orange to green after adding SYBR green, and fluorescence after adding SYBR green under UV light were detected in all 10 FAWs collected from different localities and countries (Figure 2). On the other hand, no reaction was detected in the 13 non-target species in any loci (Figure 2). These results indicate that the five LAMP loci are capable of diagnosing FAWs and provide a consistent reaction, at least for the FAW samples used in this study.

3.7. Sensitivity Test for Reaction Time, DNA Concentration, and Tissue Types

To test the sensitivity of the five loci, LAMP reactions were performed using various reaction times (10–40 min) and DNA concentrations (up to 10^5-fold dilution from the stock) with different types of FAW tissues (an egg, one 1st-instar larva, 1/8 length of a pupa, and one adult antenna), using crude DNA (Figures S4 and S5). Amplification failed at the 10 min reaction time in all tissues and loci. All tissues, except for one egg, exhibited successful amplification at a 20 min reaction time in all loci; the one egg was successful only in S242 and S1091. All tissues exhibited successful amplification at the 30 min reaction time in all loci (Figure S4). In the DNA concentration test, stock DNA from all FAW tissues successfully provided a positive reaction in all loci within a 30 min reaction time. However, as DNA was diluted, the overall amplification success decreased (Figure S5). For the one egg, only S242 and S2268 were successful with the 10-fold diluted DNA, whereas other loci were only successful with stock DNA. For the one 1st-instar larva, all loci were successful with up to a 100-fold dilution, but S242 was successful with up to 10^3-fold dilution. For 1/8 of a pupa, all loci were successful up to a 10-fold dilution, but S1073 and S2268 loci were successful with up to a 100-fold dilution. For one adult antenna, two loci (S1073 and S1901) were successful with up to a 100-fold dilution, but the remaining three loci were successful with up to 10^3-fold dilution. In conclusion, a single egg is the most challenging tissue to work with when using diluted crude DNA, while the other tissues are more tolerant. However, stock DNA provided the most consistent and reliable results in all tissues and loci.

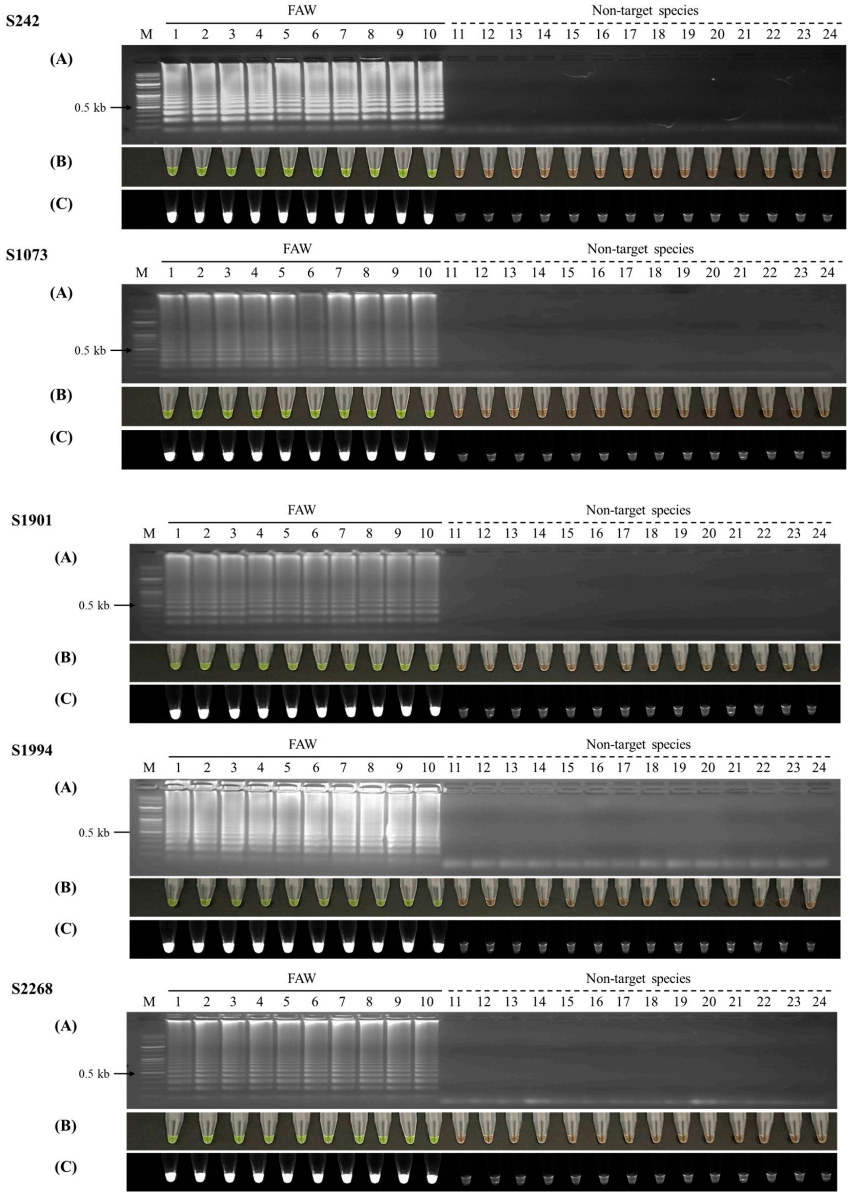


Figure 2. Specificity test of the five loci conducted on ten geographic samples of *Spodoptera frugiperda* (FAW) and 13 non-target species. The LAMP reaction was performed at 60 °C for 40 min using 10 ng/μL of genomic DNA. (**A**) Electrophoresis on a 2% agarose gel, with 5 μL of the LAMP reaction product. (**B**) Color change (from orange to green) under daylight after adding SYBR green. (**C**) Fluorescence under UV light after adding SYBR green. M, 100-bp plus molecular marker. 1–10, individuals of FAW (1, CNU15404; 2, CNU13926; 3, CNU13911; 4, CNU13942; 5, CNU13959; 6, CNU13946; 7, CNU13969; 8, CNU13972; 9, CNU13975; and 10, CNU13978); 11–23, non-target species (11, *S. praefica*; 12, *S. eridania*; 13, *S. ornithogalli*; 14, *S. depravata*; 15, *S. exigua*; 16, *S. litura*; 17, *Helicoverpa armigera*; 18, *H. assulta*; 19, *Agrotis ipsilon*; 20, *Mythimna loreyi*; 21, *M. separata*; 22, *Cnaphalocrocis medinalis*; and 23, *Ostrinia furnacalis*); and 24, negative control (no DNA). Detailed sample information is provided in Table 1.

3.8. Sensitivity Test for Outdoor Deposited FAWs

To test the sensitivity of the five loci for outdoor deposited samples (for 10, 20, and 30 days), a LAMP reaction was performed at 60 °C for 30 min using crude DNA from various FAW tissues (one egg, two eggs, three eggs, one 1st-instar larva, 1/8 of a pupa, one adult antenna, one adult leg, 1/16 of an adult thorax, and 1/8 of an adult thorax), which could be encountered in the field (Figure 3). In the case of the pupa, outdoor deposited

samples did not respond at all regardless of the deposit period in any loci, whereas the zero-day deposited pupa reacted well in all loci. Excluding the pupa, all 10-day-old samples showed a positive reaction, regardless of tissue type, in all loci. In the case of 20-day-old samples, all tissues, except for the pupa, reacted positively only in S242. In the other four loci, one egg did not yield a positive reaction in S1994 and S2268, and one to three eggs did not react in S1073 and S1901, whereas the remaining tissues exhibited successful amplification. The 30-day-old samples reacted identically to 20-day-old samples, but one egg did not show a positive reaction in any loci. In summary, 1/8 of a pupa, when deposited outdoors, did not yield a positive reaction in any loci; one egg reacted positively only in the 10-day-old sample, and the eggs deposited for more than 10 days showed variable responses depending on the deposit period, number of eggs, and loci. On the other hand, the remaining tissues, except for the pupa and eggs, were always successful in producing a reaction, regardless of the deposit period, in all loci.

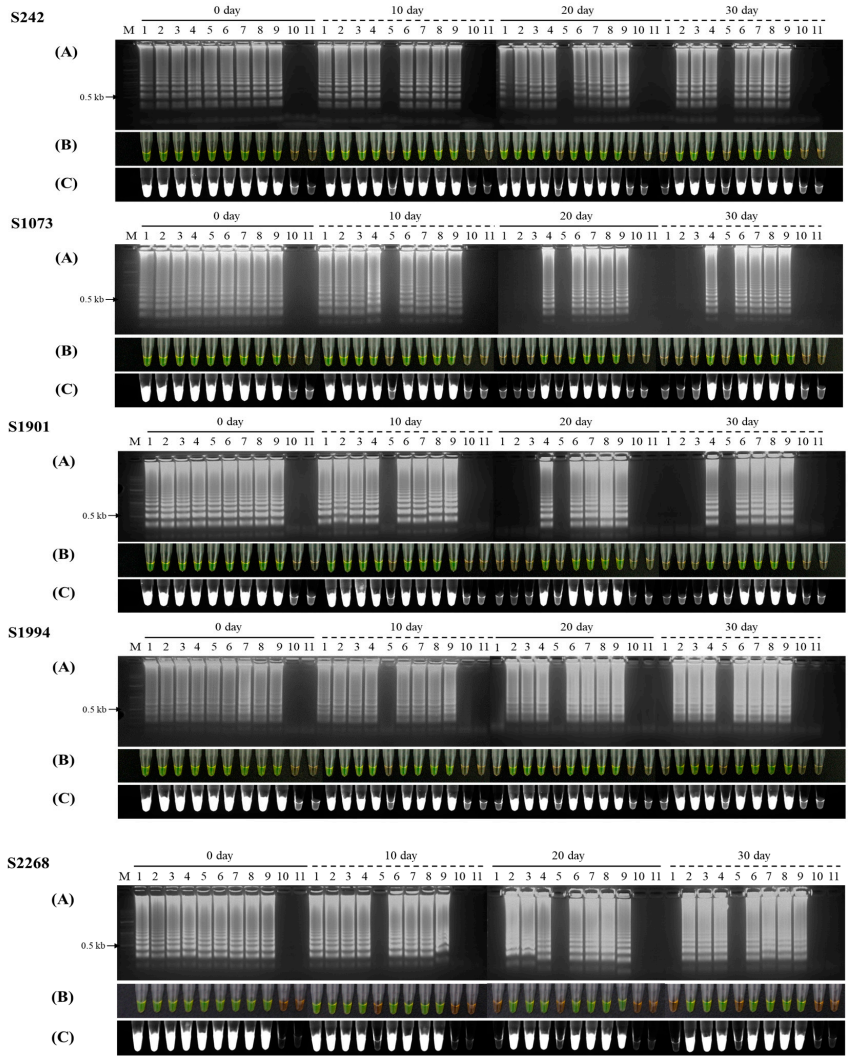


Figure 3. Sensitivity test of the five loci based on tissue types and the duration of field deposition. The LAMP reaction was conducted for 30 min using crude stock DNA. (**A**) Electrophoresis on a 2% agarose gel, with 5 µL of LAMP reaction product. (**B**) Color change (from orange to green) under daylight after adding SYBR green. (**C**) Fluorescence under UV light after adding SYBR green. M, 100-bp plus molecular marker; 1, one egg; 2, two eggs; 3, three eggs; 4, 1st-instar larva; 5, 1/8 of a pupa; 6, one adult antenna; 7, one adult leg; 8, 1/16 of an adult thorax; 9, 1/8 of an adult thorax; 10, *Spodoptera exigua* (non-target species); and 11, negative control (no DNA).

As a reference for in-field diagnosis, the tissue weight, length, DNA concentration, and purity of the outdoor deposited samples were measured (Figure 4). All tissues deposited from day zero to day 30 weighed less than 1 mg. The change in tissue length (the longest axis) varied among tissues, resulting in similar sizes (e.g., pupa) or substantial reductions (e.g., 50% in 1st-instar larva) between zero and 30 days of deposition. There was no clear pattern of reduction, which could be attributed to smaller tissue size (less than 10 mm), shape changes during drying, and tissue-specific characteristics. A gradual reduction in DNA concentration was generally observed as the deposition period increased in most tissues, but this trend was not consistently dependent on the length of this period (Figure 4). The DNA concentration of the samples deposited for 30 days was approximately 70% lower in the pupa, antenna, and leg; ~60% lower in 1st-instar larva; ~50% lower in two eggs; ~40% lower in 1/16 of an adult thorax; and ~30% lower in one egg, three eggs, and 1/8 of an adult thorax, compared to the zero-day deposited samples. In terms of DNA purity, the overall purity was poor even in the zero-day deposited samples (0.841 in 1/8 of a pupa to 1.438 in 1st-instar larva) compared to the optimal purity of 1.8–1.9. No clear pattern of reduction was detected according to the deposit period, but the zero-day deposited samples generally had higher purity than the outdoor deposited samples. Among the tissues, the segment of a pupa always had the lowest purity at 0.707 or below, while other samples, regardless of the deposition period, had purities higher than 1.

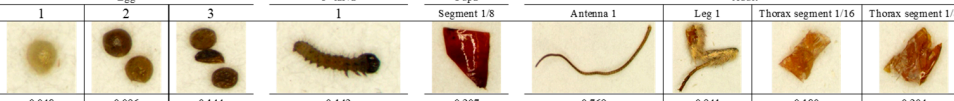
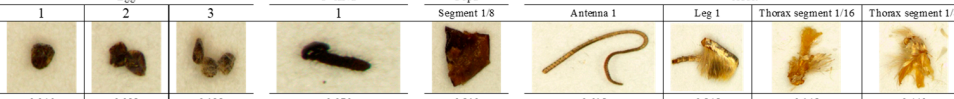
0 day									
Stage	Egg			1st larva	Pupa	Adult			
Number/Tissue	1	2	3	1	Segment 1/8	Antenna 1	Leg 1	Thorax segment 1/16	Thorax segment 1/8
Picture									
Length (cm)	0.048	0.096	0.144	0.142	0.207	0.769	0.941	0.180	0.204
DNA concentration (ng/μL)	10.665	23.323	32.485	12.866	380.513	13.611	97.158	107.180	226.705
Purity (260/280)	1.262	1.244	1.191	1.439	0.841	1.187	1.216	1.259	1.214
10 day									
Stage	Egg			1st larva	Pupa	Adult			
Number/Tissue	1	2	3	1	Segment 1/8	Antenna 1	Leg 1	Thorax segment 1/16	Thorax segment 1/8
Picture									
Length (cm)	0.049	0.098	0.147	0.074	0.192	0.600	0.653	0.155-0.190	0.295
DNA concentration (ng/μL)	8.285	14.853	31.662	12.761	215.776	13.560	61.639	105.649	199.258
Purity (260/280)	1.117	1.165	1.336	1.284	0.577	1.051	1.450	1.067	1.256
20 day									
Stage	Egg			1st larva	Pupa	Adult			
Number/Tissue	1	2	3	1	Segment 1/8	Antenna 1	Leg 1	Thorax segment 1/16	Thorax segment 1/8
Picture									
Length (cm)	0.046	0.092	0.138	0.076	0.210	0.615	0.845	0.145	0.440
DNA concentration (ng/μL)	6.986	14.885	21.223	9.163	192.232	6.720	35.439	75.242	158.523
Purity (260/280)	1.092	1.241	1.110	1.260	0.707	1.195	1.300	1.204	1.163
30 day									
Stage	Egg			1st larva	Pupa	Adult			
Number/Tissue	1	2	3	1	Segment 1/8	Antenna 1	Leg 1	Thorax segment 1/16	Thorax segment 1/8
Picture									
Length (cm)	0.043	0.086	0.129	0.069	0.235	0.682	0.759	0.233	0.325
DNA concentration (ng/μL)	7.473	12.278	22.380	5.589	113.252	3.914	36.133	65.918	151.699
Purity (260/280)	1.476	1.379	1.427	1.254	0.682	1.187	1.487	1.240	1.164

Figure 4. Length, DNA concentration and purity of FAW tissues deposited to outdoors. The weight of all tissues was less than 1 mg. The lengths for two and three eggs were determined by multiplying the length of a single egg. Antenna and leg lengths were determined by combining each straight line.

3.9. Stability of Chemical Mixture

To test the stability of the pre-made chemical mixture, the total mixture, which consisted of primer master mix and Isothermal Master Mix, was prepared and incubated at –20 °C for different periods (0, 1, 2, 5, 7, and 10 days) (Figure 5). The LAMP reaction was performed at 60 °C for 30 min for one egg, a 1st-instar larva, 1/8 of a pupa, and one adult antenna, all of which provided a positive reaction in all five loci (Figure 5).

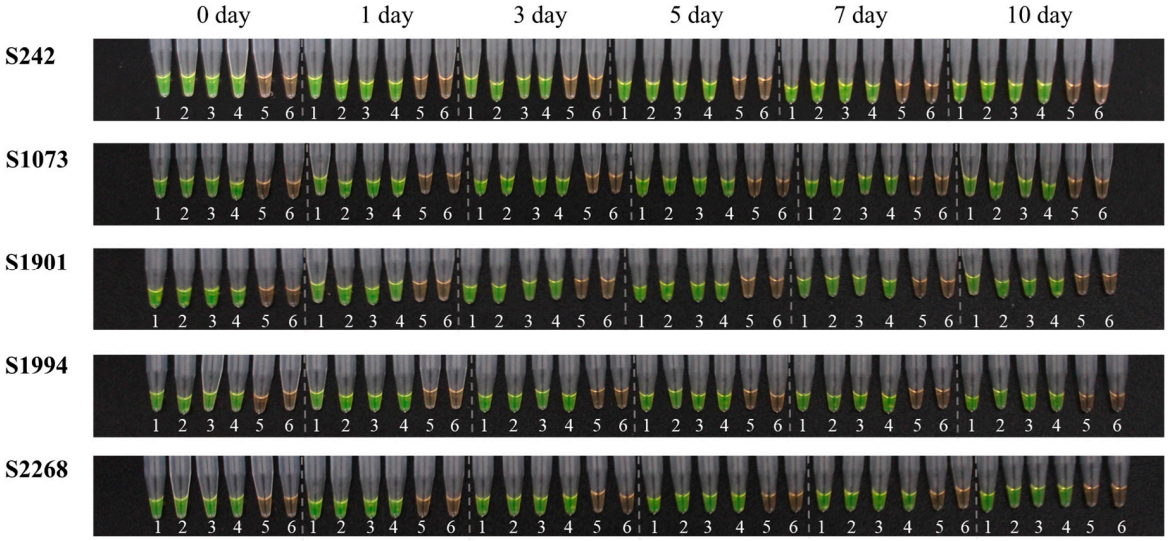


Figure 5. Stability test of pre-made chemical mixtures incubated at −20 °C for indicated time periods. The LAMP reaction was performed for 30 min. 1, one egg; 2, one 1st-instar larva; 3, 1/8 of a pupa; 4, one adult antenna; 5, *Spodoptera exigua* (non-target species); and 6, negative control (no DNA). Color change (from orange to green) was observed under daylight after adding SYBR green.

4. Discussion

FAW is found in various areas in South Korea [13,41]. It is difficult to predict its arrival location solely based on previous years' records. Moreover, dead bodies of insects premature stages, which indicate their development in corn fields, have been detected, along with adults trapped in pheromone traps. A field experiment conducted in Jeju, where FAW was first detected in South Korea (33°N), showed successful pupation of the early migrant generation in June [41], suggesting that premature stages may occur either as live or dead in corn fields. In addition, the limited availability of local specialists for molecular diagnosis necessitates a suitable molecular assay that can be supplemented with in-field reference data on its sensitivity, which would aid in making confident decisions regarding control practices. To meet these requirements, we selected five FAW-specific loci from whole-genome sequences that can be used individually or in combination to cross-check a diverse range of dead or live tissues that can be encountered during the in-field monitoring of FAW.

4.1. Specificity and Validity of Markers

In order to develop the LAMP assay, previous studies have selected suitable markers with high specificity to FAWs, often from mitochondrial DNA [23,24,26]. On the other hand, Osabutey et al. [25] searched for RNA-seq data from a public database and selected an FAW-specific nuclear gene through a bioinformatics process. Parallel to the concept of Osabutey et al. [25], we attempted to select sequence segments of FAW that lack or nearly lack equivalent regions in non-target species to increase specificity to FAW and minimize the potential for false-positive reactions, which is one of the central concerns of the LAMP assay [42–46]. By generating whole-genome sequences of an FAW and 13 non-target species, which consisted of seven corn-damaging lepidopteran species and three species of *Spodoptera* occurring and not occurring in South Korea, a high number of FAW-specific loci were obtained (Figure S2, Table 2).

Among the initial nine loci tested, only five were ultimately selected as the final markers as they consistently produced results among the ten FAW samples collected from four countries, including both corn and rice types (Figure 2). In contrast, the remaining four loci showed either a weak or no reaction in some FAW samples, even after repeated experiments. We speculated that such inconsistent reactions among FAW samples were mainly due to population genetic differences, although not all of the population genomic

variation could be accounted for during marker development. This inference is supported by the sequence analyses of the five successful loci, which indirectly indicated a significant sequence variation among samples in certain loci (e.g., 5.94% in S1073; Table 3). Fortunately, the majority of substitutions and insertions/deletions detected in the primer sequences of the five successful loci were located closer to the 5′-end of each primer, with only a few mismatches near the 3′-end of the primers (Figure S3). While the specific impact of mismatched primers on templates cannot be estimated, it appears that a consistent LAMP reaction is closely related to the conservation of primer sites on the template.

4.2. Sensitivity of Markers and Stability of Pre-Mixed Chemicals

FAWs, as either whole individuals or in parts, can be found inside the whorl of corn, on the ground, and in pheromone traps. They can remain in the corn fields for varying periods of time. However, when encountering samples that have been deposited outdoors, it is important to note that the quality and quantity of DNA extracted from these samples may be lower compared to fresh samples, particularly when using crude DNA. To address this issue, the sensitivity of the five loci was tested using crude DNA extracted from outdoor deposited samples (10, 20, and 30 days) from various FAW stages (Figure 3). Most of the 30-day deposited samples showed a positive reaction, but there were some exceptions. A single egg was only detectable in samples up to 10 days old; the detection of 2–3 eggs varied depending on the loci and deposit period; and 1/8 of a pupa only yielded a reaction if it had not been deposited at all (Figure 3). Based on these findings, we recommend avoiding the use of field-deposited pupae and eggs that are more than 10 days old. However, if necessary, two or more outdoor deposited eggs, up to 30 days old, can be diagnosed using the S242, S1994, and S2268 loci.

The measurement of the weight, length, DNA concentration, and purity of the outdoor deposited samples provides valuable in-field information in connection with the results of the sensitivity test (Figures 3 and 4). However, the measured data may have limited significance as they were obtained through a one-shot experiment, although they were measured several times mostly from identical samples. First, the measurements suggest that a higher DNA concentration is not the sole decisive factor affecting a positive reaction. For example, eggs deposited for 30 days provided a higher DNA concentration (one egg, 7.473 ng/μL; 2–3 eggs, 12.278–22.380 ng/μL) compared to one antenna and one 1st-instar larva (3.914 and 5.589 ng/μL; Figure 4). However, a single egg did not yield a positive reaction in any loci, and 2–3 eggs failed in two loci (S1073 and S1901), unlike one antenna and one 1st-instar larva (Figure 3). Second, a certain level of DNA purity also appears to be crucial for a proper LAMP reaction. Despite having the highest DNA concentration, the pupa had the lowest DNA purity compared to other outdoor deposited samples (Figure 4) and did not yield a positive reaction when deposited outdoors (Figure 3). Typically, an $A_{260/280}$ ratio between 1.8 and 2.0 indicates pure DNA without contamination [47,48]. Lower purity values suggest the potential presence of protein contamination [49]. Actually, pupa tissue demonstrated a purity below 1.0, and crude DNA extraction from pupa tissue resulted in the highest production of residues. Also, insect pupae are known to contain a high concentration of potential enzyme inhibitors [50], which can limit diagnostic sensitivity. Therefore, the LAMP assay using outdoor deposited tissues collectively suggests that both DNA concentration and purity are important factors that can affect false-negative reactions, although further tests on each factor should be conducted.

The molecular experiment conducted in the field requires more attention compared to experiments performed in an indoor laboratory, particularly for those with limited experience. Therefore, any process that can simplify the outdoor experimental procedure would be beneficial for achieving accurate results. The chemical mixture, which consists of a primer master mix (six primers), Isothermal Master Mix, and water, were prepared in advance and stored at −20 °C for up to ten days. On the day of the LAMP reaction, 1 μL of template DNA was added to the mixture after crude DNA extraction. All tested tissues, such as one egg, one 1st-instar larva, a 1/8 length of one pupa, and one adult antenna,

successfully reacted even after being stored for up to ten days, effectively distinguishing negative controls (Figure 5). Moreover, to account for potential temperature variations in the field, an additional stability test was conducted using the pre-made chemical mixture deposited at room temperature for 24 h after being stored for ten days at −20 °C. This test also yielded positive reactions in all tested samples. Therefore, the pre-made chemical mixture remains stable for a certain period, even with minor disruptions, as long as the chemicals are properly stored in a cold block in the field.

5. Conclusions

The five loci developed in this study allows for distinguishing FAWs from non-target species in a maximum of 45 min, starting from crude DNA extraction (~15 min) to diagnosis (30 min). As long as the FAW samples are fresh, 1–3 eggs, one 1st-instar larva, 1/8 length of a pupa, one adult antenna, one adult leg, and 1/16 and 1/8 of an adult thorax can be diagnosed in 30 min in all loci. Moreover, for fresh samples, one 1st-instar larva, 1/8 length of one pupa, and one antenna of an adult can be diagnosed in 20 min in all loci. For outdoor deposited tissues, one 1st-instar larva, one adult antenna, one adult leg, and 1/16 and 1/8 of an adult thorax can be diagnosed using samples deposited for up to 30 days in 30 min in all five loci. However, 1/8 of a pupa can only be diagnosed from fresh samples in all loci, and one-to-three eggs can only be diagnosed using samples deposited for up to 10 days in the five loci. However, two or more eggs can be diagnosed using samples deposited for 30 days in the S242, S1994, and S2268 loci. The use of pre-mixed primer mix and Isothermal Master Mix, which were stored at −20 °C for up to 10 days and then left at room temperature for 24 h, did not affect the reaction. The five loci developed for FAW diagnosis can be used for various types of tissues encountered in the field, whether fresh or left as dead for variable time periods. They can be used selectively or in combination for cross-checking, if necessary, to increase the reliability of the result in the field.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010219/s1, Figure S1: Bar chart illustrating the summary statistics of 14 assembled genomes.; Figure S2: Bar chart illustrating species-specific regions; Figure S3: Sequence alignments of the ten FAWs for each locus.; Figure S4: Sensitivity test of the five loci based on tissue types and reaction time.; Figure S5: Sensitivity test of the five loci based on tissue types and their DNA concentration.; Table S1: Summary statistics of the whole-genome sequencing data generated using the Nextseq500 platform.; Table S2: List of primers used for conventional PCR.; Table S3: GenBank accession numbers for the specimens used in this study.; Table S4: Blast search result for the DNA barcoding sequences of 13 non-target species.

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