A LAMP Assay for the Detection of Thecodiplosis japonensis, an Alien Gall Midge Species Pest of Pine Trees

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Simple Summary: Thecodiplosis japonensis is considered the most harmful pest to pines in South Korea. T. japonensis is a native species of Japan. Recently, T. japonensis was discovered in China and has caused serious damage to local pine trees. Due to the small size and little morphological difference with its related species, it is difficult to accurately identify T. japonensis by morphological methods. Accurate and efficient molecular identification methods are urgently needed to detect this invasive gall midge pest, yet there was no molecular identification method for T. japonensis. In this study, we developed a LAMP assay to detect T. japonensis based on the COI gene sequence. The LAMP assay could detect as little as 300 fg of gDNA. Using colorimetric amplification and a crude gDNA extraction method, the total procedure could be processed in 75 min. The method established in the study can be easily used in both laboratory and field conditions, enabling rapid molecular identification of T. japonensis.

Abstract: Pine needle gall midge (T. japonensis), native to Japan, has become a serious invasive pest in South Korea and, more recently in 2006, in China. It was first discovered in Qingdao, Shandong Province, and has caused serious damage to local Pinus thunbergii. The insect’s small size makes morphological-based identification difficult; therefore, molecular detection techniques are urgently needed for monitoring and preventing its further spread. At present, there is no simple and accurate field molecular identification tool. To solve this problem, a LAMP-based molecular diagnosis technology of T. japonensis was developed. Four LAMP primers were designed to specifically amplify T. japonensis DNA. Positive LAMP reactions usually produce amplification in one hour. The optimal incubation conditions for LAMP detection were determined with 4 LAMP primers for 60 min at 61 °C. The LAMP detection range of gDNA concentrations is wide, with a minimum detectable gDNA concentration of 300 fg. A non-destructive DNA-releasing procedure, HotSHOT “HS6”, which could extract “crude DNA” for LAMP assay in 10 min, was used for larval and adult samples. Therefore, we established a LAMP-based rapid molecular identification method that can be applied in the monitoring and management of T. japonensis.

Keywords: pine needle gall midge; Thecodiplosis japonensis; COI; loop-mediated isothermal amplification (LAMP); field diagnostics

1. Introduction

Pine needle gall midge, T. japonensis (Uchida and Inouye) (Diptera: Cecidomyiidae), is a native pest of Japan. T. japonensis belongs to the Cecidomyiidae family, which contains 6651 known species and 832 genera [1]. The family is divided into six subfamilies. The largest subfamily, the Cecidiomyiinae, best known as herbivores and plant gall makers, such as pine needle gall midge, includes some of the most destructive pests of grains, fruits
and vegetables and also many fungivores and predators of plant-feeding arthropods [1–3]. In addition to T. japonensis, there are another five species in the genus Thecodiplosis recorded: Thecodiplosis brachyntera in Europe; Thecodiplosis brachynteroides, Thecodiplosis pinirgidae, Thecodiplosis piniradiatae, and Thecodiplosis piniresinosae in North America. These gall midges broke out locally from the 1930s to the 1990s [4–6]. However, there have been no reports of spread and harm caused by them in the past two decades.

First discovered in South Korea in 1924, T. japonensis has widely spread throughout the country South Korea [7]. Discovered in China in 2006, the tiny invasive pest has seriously endangered P. thunbergii Parlatore, Pinus densiflora Sieb. et Zucc. and Pinus tabuliformis Carriere [8]. It is considered the most harmful pest to pines in South Korea, causing great damage to the South Korean ecological environment and economic development [9,10]. In Japan, T. japonensis could attack Pinus massonian Lambert [11]. T. japonensis is mainly spread by humans over long distances with the transfer of host plants or soil-carrying plants, and natural dispersion mainly depends on the flight of adults [12]. T. japonensis is an univoltine species in Shandong Province. Larvae hibernate in the soil where they pupate in late May. Adults emerge from the soil from the end of late May until July. After mating, females search for a suitable host plant and lay eggs on needle pairs of current-year shoots in the vicinity of where they emerged from the soil. Larvae develop inside galls at the base of needle pairs. In the autumn, they leave the galls and drop to the soil where they pupate and remain until the following year [8,13]. Galls are formed when larvae feed on the needles, stopping needle growth and causing them to become shorter than healthy needles, which then gradually wither and die [14,15].

In China, there is a pest of the same genus that infests P. massoniana, mainly in Fujian Province. Since its larval and adult morphological characteristics are almost the same as T. japonensis, it has long been misidentified as T. japonensis [16] until Jiao separated the two by larval morphology and mitochondrial gene differences [13]. These two gall midges cause similar damage to their host plants [8,16]. Specialized entomological knowledge is needed to accurately distinguish them [13,16]. Therefore, to improve monitoring, early detection and pest management of T. japonensis, an effective identification method is urgently needed. However, there is no molecular detection method for T. japonensis so far. In addition to the morphological method for T. japonensis identification, there is an urgent need for rapid detection methods that can be used for field detection.

At present, the molecular methods for identifying similar pests include DNA barcoding based on COI gene, real-time qPCR and so on. Loop-mediated isothermal amplification (LAMP) is a one-step nucleic acid amplification technology based on auto-cycling strand-displacement DNA synthesis and could be undertaken in the field without high equipment and personnel [17–22]. In general, the LAMP method only requires a hot block to amplify DNA, and the result is detectable by the naked eye. This method has been widely used in the detection of many kinds of pests, such as Myzus persicae, Spodoptera frugiperda, etc. [23–28].

In our study, a sensitive, specific LAMP assay for the rapid identification of T. japonensis was developed. A rapid DNA extraction method that can be used in the field is applied to save time. Colorimetric LAMP assay requires only a portable heating block for clear visual detection with the naked eye. We also designed a pair of T. japonensis species-specific primers based on the COI gene sequences and compared the results of two diagnostic methods.

2. Materials and Methods

2.1. Samples Examined

During the adult emergence period, a net was used to catch the mating adults of T. japonensis and Thecodiplosis sp. on the grass under the infested pine tree. The infected pine trees were identified by searching the galls formed on shortened needles of the previous year. Individual larvae of T. japonensis and Thecodiplosis sp. were obtained in galls formed on current-year needles before they left the galls and dropped to the soil.
Other non-target gall midge specimens collected are listed in Table 1. Specimens were confirmed by DNA barcoding of COI using a pair of universal primers (C1-J1709 and C1-N2353) [29] and a morphological method. Molecular phylogenetic analysis was carried out using the neighbor-joining method by MEGA 7. The COI sequences of other gall midges were downloaded from NCBI (see Supplementary File S1) and compared to the reference sequence of *Drosophila melanogaster* and *Aedes aegypti*.

Table 1. Specimens used in LAMP assay and SS-COI PCR to identify *T. japonensis* from other gall midges.

| Genus     | Species            | Stage and Amount | Collection Date | Location Information             |
|-----------|--------------------|------------------|-----------------|-----------------------------------|
| Thecodiplosis | *T. japonensis*   | Adult/Larvae (58/52) | June 2020      | Shandong China (33°23’18” N, 126°37’40” E) |
|           | *Thecodiplosis sp.*| Adult/Larvae (24/98) | March 2020   | Fujian China (24°32’29” N, 116°54’40” E) |
| Contarinia | *Contarinia sp.*   | Larvae (21)       | April 2018     | Xinjiang China (41°9’34” N, 80°10’14” E) |
|           | *Contarini caryafloralis* | Larvae (85)   | August 2018    | Anhui China (30°26’23” N, 118°44’23” E) |

Genomic DNA (gDNA) was extracted from adults and larvae using the TIANamp Micro DNA Kit (TIANGEN, Beijing, China), following the manufacturer’s instructions. The extracted DNA was quantified by a spectrophotometer (Thermo Fisher, Waltham, MA, USA) and stored at −20 °C. Another “crude” DNA extraction method that is non-destructive to specimens was tested in the LAMP assay. An individual gall midge larva or adult was processed according to a modified HotSHOT protocol [30]. Briefly, a single larval or adult was placed in 20 µL of premixed TE buffer, pH 8.0 (Invitrogen, Waltham, MA, USA) and 25 mmol L-1 NaOH (1:1), and denatured at 95 °C for 10 min, followed by > 1 min incubation on ice. DNA was stored at −20 °C.

2.2. LAMP Primer Design

Based on the analysis of mitochondrial genome sequences (unpublished data) from *T. japonensis*, *Thecodiplosis sp.* and mitochondrial genome sequences in NCBI from three other gall midges (*Mayetiola destructor*, *Orseolia oryzae* and *Rhopalomyia pomum*), a partial COI sequence region was chosen as a target region to design LAMP primers. Nine COI sequences containing the target region were downloaded from GenBank and NCBI (see Supplementary File S2) from the closely related gall midges. For comparative analysis, COI sequences were aligned using MEGA 7. Six LAMP primers were manually designed targeting eight DNA regions and synthesized by a commercial company (SinoGenoMax, Beijing, China). Potential primer–dimer interactions of LAMP primers were analyzed.

2.3. LAMP Assay

A WarmStart® LAMP Kit (New England Biolabs, Ipswich, MA, USA) was used for the LAMP assay. The general LAMP protocol followed the manufacturer’s guidelines using a 25 µL reaction mixture. Each reaction contains 4 primers (outer primer F3 and B3, and inner primer FIP and BIP) and 30 ng of gDNA. Two additional loop primers (Floop and Bloop) were tested to reduce the incubation time. Different reaction temperatures (range 59 to 63 °C) were tested to determine the optimum working conditions. LAMP assays were performed on a CFX96 thermocycler (BIO-RAD, Hercules, CA, USA), and the amplification curves could be visualized on the thermocycler screen. The results could also be checked under UV light with GelRed (BIOTIUM, Fremont, CA, USA), visible light or upon gel electrophoresis.
2.4. Analytical Detection Limit of the LAMP Assay

DNA extract from *T. japonensis* was ten-fold serial diluted using ultrapure water (Invitrogen, Waltham, MA, USA). Starting DNA concentration was quantified by a spectrophotometer (Thermo Fisher, Waltham, MA, USA). The DNA concentration was diluted from 30 ng/µL to 3 fg/µL serially. Detection limit of the LAMP assay was tested following the same assay conditions as mentioned above using the serially diluted DNA. The results could be checked under UV light with GelRed (BIOTIUM, Fremont, CA, USA), visible light or upon gel electrophoresis.

2.5. Species-Specific PCR Assay

A pair of *T. japonensis*-specific primers, named COI-F and COI-R, were designed by Primer 5.0 according to the difference in COI gene sequence between *T. japonensis* and related gall midges. The Primer-BLAST found in the NCBI database showed that the primer set only matched the COI gene fragment of *T. japonensis*. The primers were synthesized by a commercial company (SinoGenoMax, Beijing, China). For SS-COI PCR, Prime Star Mix (Tsing Ke, cat#R045) was used together with two SS-COI primers (COI-F and COI-R) following the protocol: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min. The serial dilution of gDNA extracts was the same as the LAMP assay. PCR fragments were separated by 1.0% agarose gel and checked under UV light with GelRed.

3. Results

3.1. Specimens Examined

All specimens examined were confirmed by DNA barcoding of COI and the morphological method in the current study (Table 1). The *Thecodiplosis* sp. was the most homologous species of *T. japonensis* in NCBI, which showed high similarity by MegaBLAST. The phylogenetic relationship between COI genomes of 18 species was examined (Figure 1), and the result of the phylogenetic relationship was similar to the megaBLAST result.

![Figure 1. Phylogenetic tree reconstructed using NJ based on COI sequence.](image)

3.2. Development of the LAMP Assay

Four LAMP primers (Table 2), including two outer primers of Tjap_F3 and Tjap_B3, and two inner primers of Tjap_FIP and Tjap_BIP (Figure 2), were designed manually by aligning COI gene sequences of *T. japonensis* and related gall midges. The primer ratio of outer primers and inner primers was tested, and the optimal primer ratio was determined to be 1:8, with final concentrations of 0.2 µM and 1.6 µM, respectively (Table 3). LAMP
assay was tested in a CFX96 thermocycler at various incubation temperatures of 59–63 °C. The optimal reaction condition was incubated at 61 °C for 60 min. Two loop primers of Tjap_FL/Tjap_BL were tested in a CFX96 thermocycler (BIO-RAD, Hercules, CA, USA) for acceleration of the reaction [31]. However, there was no significant increase in amplification efficiency by adding Tjap_FL and Tjap_BL.

**Table 2. Primers for LAMP and SS-COI PCR.**

| Assay     | Primer Name | Sequence (5'-3')                                                                 |
|-----------|-------------|---------------------------------------------------------------------------------|
| LAMP      | Tjap_F3     | CGTTTGAATTAAGTCTGTAATCTAAATTGATAG                                              |
|           | Tjap_B3     | CTGCTTAATACGGTAAGAAAGTAAAGTGAG                                                    |
|           | Tjap_FIP    | CCTGTTCCTCCGTCTCTTCACATTTCTCAGATTCTCCTCTCCTATTTCTT                              |
|           | Tjap_BIP    | GAACGTGTTATCCCTCCTCTCTTCAATTATTGCTAGAGAAAAATTGAAATGTGTTCTAG                     |
|           | Tjap_LF     | CCTGCTTCGTTCTCAGATTTCTCCTCAGATTCTCCTCTCCTATTTCTT                              |
|           | Tjap_LB     | GTTATTCCCTCTCCTCTCAGATTCTCCTCTCCTATTTCTT                                      |
| SS-COI PCR| COI-F       | CAGGTAAAGAAGATAGAATGTAATGTTAG                                               |
|           | COI-R       | GATTGTGATTACCTCCCCCTCTAATTC                                                     |

**Figure 2.** Alignment of COI gene sequences of T. japonensis with related gall midges. Primer regions are marked with boxes. FIP is composed of the reverse complement of F1 and F2; BIP is composed of B1 and the reverse complement of B2.
Table 3. Optimized LAMP components for the detection of *T. japonensis*.

| LAMP Components | Concentrations Used for Optimization | Final Concentrations Used in LAMP |
|-----------------|-------------------------------------|----------------------------------|
| WarmStart®LAMP Master Mix | 2× | 1× |
| F3 | 10 µM | 0.2 µM |
| B3 | 10 µM | 0.2 µM |
| FIP | 10 µM | 1.6 µM |
| BIP | 10 µM | 1.6 µM |
| DNA sample | Variable | Variable |

3.3. Performance of the LAMP Assay

Usually, the LAMP assay amplifies the gDNA of *T. japonensis* within 60 min, and the anneal derivative temperature is approximately 79 °C (Figure 3). All non-target gall midge species, including the most closely related *Thecodiplosis* sp., did not amplify. The positive reaction can be checked under visible light with the color change from red to yellow, confirmed by gel electrophoresis or under UV light with GelRed (Figure 4).

![Figure 3](image_url)

Figure 3. Different reaction temperatures (range: 59 to 63 °C) were tested to determine the optimal reaction temperature of LAMP. The optimal incubate condition was 61 °C for 60 min with an anneal derivative temperature of 79 °C.

![Figure 4](image_url)

Figure 4. Specificity of the LAMP assay with four LAMP primers. (A) Checking results under visible light, (B) checking results under ultraviolet light with GelRed and (C) checking results by gel electrophoresis. NC: negative control, +: *T. japonensis*, TSP: *Thecodiplosis* sp., CSP: *Contarinia* sp., CCA: C. caryafloralis.
The detection limits of the LAMP assay were tested using the “clean” DNA (from a standard DNA isolation protocol). Whether under visible light, using ultraviolet light or gel electrophoresis, the four LAMP primers could detect as little as 300 fg of gDNA (Figure 5).

Figure 5. Identification of the detection limit of gDNA in the LAMP assay from 30 ng to 3 fg. (A) Checking results under visible light, (B) checking results under ultraviolet light with GelRed and (C) checking results by gel electrophoresis. NC, non-template control.

The non-destructive DNA extraction method from whole specimens was applied to obtain *T. japonensis* gDNA from single adult and larva, suitable for the LAMP assay. A colorimetric LAMP assay was conducted to test this “crude” gDNA extraction method in a heating block. The result was also checked by gel electrophoresis. Both adult and larval samples of *T. japonensis* generated positive results within 60 min (Figure 6), with 100% success rates when 10 samples per adult and larva were tested. This allows the preservation of complete morphological voucher specimens for further species identification by molecular or morphological identification method.
Figure 6. Identification of the “crude” gDNA extract using a non-destructive DNA extraction method with four LAMP primers. (A) Individual larva and adult, (B) under visible light, (C) under gel electrophoresis.

3.4. Performance of Species-Specific PCR Assay

The specificity of the species-specific primer pair COI-F and COI-R was tested, resulting in a 299-bp fragment obtained from the gDNA of *T. japonensis*, with no amplification detected for the three other gall midges. Sensitivity tests were performed on larvae and adults. The detection limit of the species-specific PCR assay was 3 pg of *T. japonensis* gDNA (Figure 7).

Figure 7. Species-specific PCR assay results with two SS-COI primers. (A) Species-specificity test of *T. japonensis* SS-COI primers. (B) Identification of the detection limit of gDNA in the species-specific PCR assay from 30 ng to 30 fg. (C) Species-specific PCR assay results with genomic DNA extracted from larvae and adults. NC: negative control, +: *T. japonensis*, TSP: *Thecodiplosis* sp., CSP: *Contarinia* sp., CCA: *C. caryafloralis*. 
4. Discussion

In recent years, *T. japonensis* have caused serious damage to *P. thunbergii* in Shandong, China. In South Korea, *T. japonensis* have been reported for nearly 100 years and have caused serious ecological and economic losses [9,10]. Inoculation tests showed that *T. japonensis* could infest a variety of pine trees, including *P. massoniana*, *P. resinosa*, *P. thunbergii*, *Pinus sylvestris*, *Pinus mugo*, *Pinus luchuensis*, *Pinus tabulaeformis*, *Pinus radiata*, *Pinus coulteri*, *Pinus nigra*, *P. densiflora*, *Pinus taiwanaensis* and *Pinus insularis* [15].

The potential geographical distribution of *T. japonensis* in China was predicted by a MaxEnt niche model and CLIMEX model (unpublished data). The results showed that most areas of southern and central China were highly suitable areas for *T. japonensis* establishment and outbreak, coinciding with a wide distribution of pine trees that inoculation tests showed could be infested by *T. japonensis*. Thus, the risk of *T. japonensis* spreading in China, and even globally, is high.

Early detection and management of invasive pests are crucial. It is difficult to accurately identify *T. japonensis* by traditional morphological methods due to the small size and little morphological difference with its related species. The small shape difference of sternal spatula (width no more than 50 microns) on the pronotum of mature larvae and the wing vein of adults are key identification plural of genus *Thecodiplosis*. Jiao used the geometric morphology method to identify *T. japonensis* by analyzing the shape of the sternal spatula of larvae [13]. This morphological identification method requires a series of operations such as making slide specimens, micrographs and picture analysis with professional software, which is extremely time-consuming and requires trained professionals. Accurate and efficient molecular identification methods are urgently needed to detect this invasive gall midge pest, yet there was no molecular identification method for *T. japonensis*.

LAMP has higher sensitivity and amplification efficiency than other molecular identification methods, and its results can be visually monitored either through a color change of fluorescent intercalated dye or turbidity. Recently, LAMP has been widely used in many fields, such as ecology, medical research and also to diagnose mutations associated with resistance in insects [23,32]. The accuracy, simplicity and high-throughput adaptability of LAMP analysis are advantageous [33–35]. In this study, a LAMP method for on-site identification of *T. japonensis* was developed. We designed a new set of specific LAMP primers based on the COI sequence. In the LAMP assay, the detection limit of four primers was 300 fg gDNA within 60 min. The optimized method is fast, stable and specific, and even the most closely related *Thecodiplosis* sp. did not produce positive amplification. Two loop primers (Tjap_FL and Tjap_BL) were used to reduce the incubation time. However, there was no significant increase in amplification efficiency by adding both Tjap_FL and Tjap_BL or adding them separately.

It is essential that the field detection of LAMP obtains a fast, reliable and simple DNA extraction method. Nowadays, some non-destructive DNA extraction methods are used to obtain insect DNA for molecular experiments [36]. The published HotSHOT protocol provides a simple and rapid method for obtaining genomic DNA for LAMP assay. Genomic DNA can be successfully obtained by simple incubation of specimens in NaOH and Tris mixed solution by HotSHOT protocol. The specimens extracted by this non-destructive method still retain the morphological characteristics, which can be used for morphological identification for further confirmation. Thus, in our study, even without special equipment such as a PCR machine or electrophoresis equipment, DNA can be detected from adult and larvae specimens within 75 min using only hot blocks. Besides LAMP, we also developed a PCR method based on a mitochondrial DNA COI marker to detect *T. japonensis*. According to the difference in COI gene sequence between *T. japonensis* and related gall midges, a pair of *T. japonensis*-specific primers were designed. The specificity of the primers was tested on related gall midges. The detection limit of this method was 3 pg gDNA of *T. japonensis*. Thus, the two molecular detection methods can be applied to the identification of *T. japonensis*. Each method provides a different advantage depending on the situation. The LAMP assay is appropriate in field situations or for urgent samples, while the SS-COI
PCR method is more suitable for a large number of sample extracts, without a time limit, in a laboratory setting with experienced personnel.

In this study, it was the first time that LAMP was used for molecular detection of cecidomyiid insects. It is worth further investigation to determine whether other congeners such as *T. brachyntera* and *T. brachynteroides*, which have not been tested in this study due to the unavailability of DNA samples, could amplify their gDNA using the LAMP assay.

5. Conclusions

In this study, a LAMP assay was successfully developed to detect the invasive pest *T. japonensis* based on the *COI* gene sequence. Using colorimetric amplification and crude gDNA extraction method, the total procedure could be processed in 75 min. Specific primers, optimized LAMP reaction temperatures and easily timed steps make the method specific, stable and reproducible. Therefore, the LAMP assay could be used to detect *T. japonensis* in field-collected populations. The new method established in the study is a portable, rapid molecular identification tool for *T. japonensis*, which can be easily used in both laboratory and field conditions, enabling rapid molecular identification of *T. japonensis*.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/insects13060540/s1](https://www.mdpi.com/article/10.3390/insects13060540/s1), File S1: *COI* sequences used in molecular phylogenetic analysis; File S2: *COI* sequences used in LAMP primers design.

**Author Contributions:** Y.L., J.T. and L.R. conceived the study. J.J. and R.C. prepared samples. J.J. performed the bioassay and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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