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TECHNICAL NOTE

Rapid detection of the red fire ant *Solenopsis invicta* (Hymenoptera: Formicidae) by loop-mediated isothermal amplification

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Abstract
We developed a loop-mediated isothermal amplification (LAMP) assay to detect the red imported fire ant, *Solenopsis invicta* Buren, an invasive species in Japan. We designed species-specific LAMP primers on the basis of the nucleotide sequence of the *S. invicta* mitochondrial cytochrome *c* oxidase subunit 1 region. The system we developed has a sensitivity and specificity enabling detection of one *S. invicta* in a mixed sample including nine individuals of a non-target ant species. We were easily able to detect the *S. invicta* DNA visually based on the turbidity of the reaction mixture. This rapid and easy LAMP assay system provides a powerful tool for the early detection and identification of *S. invicta* in invaded regions.

Keywords Invasive alien species · *Solenopsis invicta* · Loop-mediated isothermal amplification · *COX1*

Introduction

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), has been documented as entering Japan within ship cargo since June 2017. *S. invicta* queens and workers have been captured around international port areas, as well as in inland areas, and have even been seen in premises (Ministry of the Environment 2017; http://www.env.go.jp/nature/dobutsu/fireant.html#ABOUT). Although no established *S. invicta* nests have been found, it is believed to be only a matter of time before this species will naturalize in Japan.

Early detection is an important strategy to prevent *S. invicta* expansion. Because *S. invicta* may be artificially introduced across broad geographic regions via commercial transport, wider monitoring by officials and citizens is needed. However, non-experts cannot easily identify *S. invicta* based on morphological characteristics alone. In fact, we ourselves have needed specialists to confirm our identification of *S. invicta*, a process requiring at least several days. Therefore, it is crucial to develop a rapid and easy procedure to identify *S. invicta*.

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Recently, Valles et al. (2016) developed a lateral-flow immunoassay detection system for *S. invicta* identification by raising monoclonal antibodies against *S. invicta*-specific venom proteins. However, that system requires protein from at least five workers to identify the species. Because the number of *S. invicta* individuals captured in the field during the early stages of invasion will be very small, the sensitivity of that immunoassay system is likely insufficient.

We considered that the loop-mediated isothermal amplification (LAMP) assay (Notomi et al. 2000) may enable easy and rapid detection of *S. invicta*. The assay is a single-tube isothermal technique for DNA amplification. Because the assay procedure is simple and rapid, LAMP has proven to be a powerful tool for monitoring invasive species. For example, Ide et al. (2018) developed a LAMP assay protocol to detect another invasive ant, *Linepithema humile* (Mayr).

In the present study, we designed *S. invicta*-specific LAMP primers and evaluated their specificity by performing LAMP assay amplification of DNA extracted from whole or partial individuals of *S. invicta* and several other ant species.

Materials and methods

Ant samples

Solenopsis invicta workers were collected from the Tokyo, Nagoya, and Kobe Ports in June and July 2017. They were

stored individually at − 20 °C in a microtube until DNA extraction. Workers of *Cardiocondyla* sp., *Crematogaster osakensis*, *Formica japonica*, *Monomorium chinense*, *Pheidole noda*, *Solenopsis japonica*, *Temnothorax* sp., and *Tetramorium tsushimae* were collected from Tokyo and Tsukuba City, Japan in July 2017. These eight ant species are commonly found in port areas and are likely to be mistakenly identified as *S. invicta*. These samples were immersed in 99.5% ethanol and stored at − 20 °C until DNA extraction.

Primer design

In this study, we designed *S. invicta*-specific LAMP primers based on several genetically distinct populations collected at Japanese ports by the Ministry of the Environment. We selected the mitochondrial cytochrome *c* oxidase subunit 1 (*COX1*) gene as a target sequence. In arthropods, the nucleotide sequence of the *COX1* region is highly divergent, and therefore is useful for species identification (Valles and Porter 2003). The nucleotide sequence of mitochondrial *COX1* was obtained from the mitochondrial genome data of *S. invicta* registered in NCBI (https://www.ncbi.nlm.nih.gov/nuccore/NC_014672.1; accession no. NC_014672). Based on the sequence data, we designed seven sets of LAMP primer candidates using Primer Explorer Ver. 5.0 (https://primerexplorer.jp/v5_manual/index.html) under default conditions.

Evaluation of species specificity using purified DNA

DNA was isolated through SDS–phenol extraction with a modified RNA isolation method described by Nakajima et al. (1988). The whole body of each ant was separately homogenized with a mortar and pestle in 200 µL of extraction buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 1% SDS) and an equivalent amount of water-saturated phenol. The extracts were incubated at 60 °C for 10 min. After centrifugation at 20,000×*g*, the upper phases were transferred to fresh microcentrifuge tubes and sequentially extracted in phenol:chloroform (1:1) and then chloroform. DNA was recovered by ethanol precipitation and washed with 70% ethanol. The air-dried DNA was dissolved in 100 µL of Tris–EDTA (TE) buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA). DNA concentrations were determined with a TapeStation 2200 system (Agilent, Santa Clara, CA, USA).

DNA amplification via the LAMP assay was performed according to the protocol described by Notomi et al. (2000, 2015) using a Loopamp DNA Amplification Kit (Eiken Chemical, Tokyo, Japan). Each reaction contained 12.5 µL of reaction mix provided in the kit, 2 µL of FIP3 primer (20 µM), 2 µL of BIP3 primer (20 µM), 1 µL of F3_3 primer (5 µM), 1 µL of B3_3 primer (5 µM), 3.5 µL of nuclease-free water, 1 µL of Bst polymerase, and 2 µL of DNA. The reactants were placed in a dedicated clean PCR tube, and reactions were performed at 60 °C for 90 min. Turbidity indicative of the reaction progress was recorded with an LA-320C turbidimeter (Eiken Chemical).

Simplification of sample treatment for the LAMP assay

To simplify the DNA extraction processes, we also evaluated DNA samples obtained by the following methods. When an excised leg was used, the sample was homogenized in 40 µL of TE. Nucleases were inactivated by heating at 90 °C for 10 min. Then, 2 µL of the supernatant was used as a template for the LAMP reaction.

When a whole body was used, the sample was homogenized in 40 µL of TE. It was allowed to settle for 1 min, and 20 µL of the supernatant was transferred to another tube and mixed with 200 µL of InstaGene matrix (Bio-Rad, Hercules, CA, USA). The mixture was incubated at 56 °C for 30 min, vortexed for 10 s, incubated at 100 °C for 8 min, and then vortexed for 10 s. After the insoluble material was precipitated for 5 min, 2 µL of the supernatant was used as a LAMP reaction template.

Analysis of mixed samples

We also tested an extract from a mixed sample comprising one *S. invicta* worker and nine *F. japonica* workers. This native ant species is easily obtained and has a large amount of DNA per individual because of its large body size; so it is considered as an ideal sample for verifying the specificity of the LAMP method in the case of amplification using a contaminated DNA sample. In addition, about 10 ants can be homogenized in a microtube; so we chose to use one *S. invicta* and nine *F. japonica* workers in each microtube. The sample was homogenized in 400 µL of TE, and the extract was allowed to settle for 1 min for precipitation. Then, 20 µL of the supernatant was transferred to another tube and mixed with 200 µL of InstaGene. The mixture was treated as described above, and 2 µL of the supernatant was used as a template for the LAMP reaction.

Results and discussion

Species specificity of the LAMP primers

Among the seven primer sets designed, the FIP3 primer set showed the best amplification of *S. invicta* DNA. This primer set could amplify all DNA templates extracted from the *S. invicta* samples. The primer sequences are listed in Table 1, and their locations in the *S. invicta COX1* gene are

Table 1 The FIP3 primer set used for the LAMP reaction to detect *Solenopsis invicta* DNA

Primer	Sequence (5′→3′)
FIP3	AGGCTCGTGTATCAACGTCAA GGTTTTATTGTTTGGGCACA
BIP3	TGAATTTCCACTCTCCACGGA CCTATCGATCATCAGAGTGTG
F3_3	TGCTATAATCGCTATCGGAT
B3_3	CCTGTTAATCCTCCTATAGTGAAT

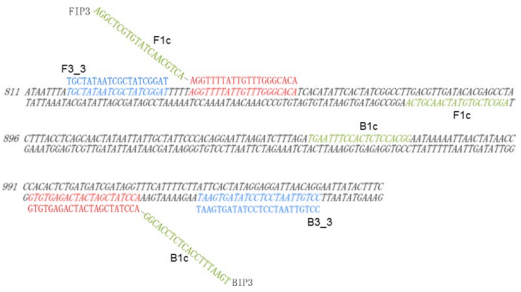

Fig. 1 Nucleotide sequences of the FIP3 primer set for the LAMP reaction and their locations in the *S. invicta* mitochondrial *COX1* gene. Italic indicates the nucleotide sequence of *S. invicta* mitochondrial *COX1* DNA (NC_014672.1). The bases that correspond to *COX1* DNA are colored as follows: red, F2 and B2 regions of FIP3 and BIP3; green: F1c and B1c regions of FIP3 and BIP3; and blue: F3_3 and B3_3

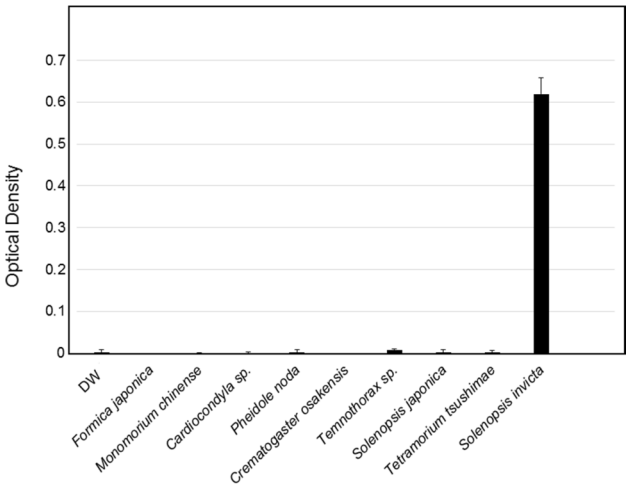

Fig. 2 Specificity of the LAMP reaction with the FIP3 primer set, as measured by the optical density of LAMP reactions containing RNase-free water as a negative control (DW), DNA of eight Japanese native ant species, and DNA of *S. invicta*. Error bars represent the standard deviation of three independent reactions

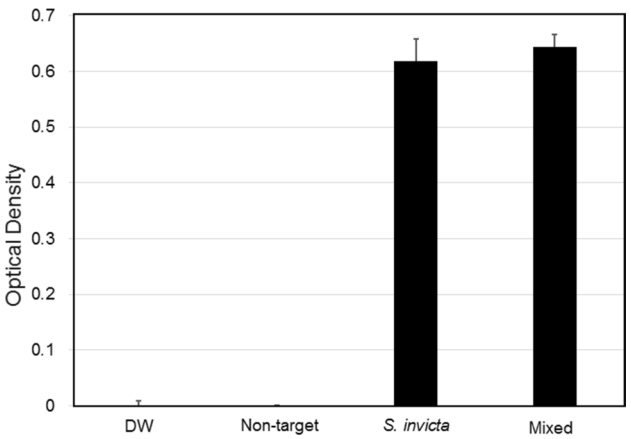

Fig. 3 Detection specificity of the LAMP reaction with the FIP3 primer set. The optical densities of LAMP reactions containing RNase-free water as a negative control (DW), whole-body extract of *F. japonica* (Non-target), whole-body extract of *S. invicta* (*S. invicta*), or mixed extracts of one *S. invicta* worker and nine *F. japonica* workers (Mixed) are shown. Error bars represent the standard deviation of three independent reactions

shown in Fig. 1. On the other hand, the other six primer sets were unable to detect some of the DNA templates.

We then performed LAMP assays with the FIP3 primer set for the eight Japanese native ant species (Fig. 2). Whereas marked turbidity was observed in the assay using *S. invicta* DNA, the diagnostic turbidity did not rise to the threshold level (OD = 0.1) in the reactions with native species' DNA, indicating the specificity of the FIP3 set for *S. invicta*. Therefore, we selected the FIP3 set as the diagnostic primers.

Utility of the LAMP assay for field samples

To check for *S. invicta* at monitoring sites where their presence is assumed to be possible (e.g., port areas, green spaces, and playgrounds), we will occasionally need to use mixed samples of various ant species collected by traps. In some cases, we must identify *S. invicta* only from body parts, such as a leg. In addition, these DNA extracts should be obtained as easily as possible. Therefore, we tested the mixed DNA sample and single-leg DNA sample extracted by our rough protocol.

The assay using an extract from a mixed sample comprising one *S. invicta* worker and nine native ant workers clearly showed a positive turbidity signal for *S. invicta* (Fig. 3). Furthermore, our LAMP assay exhibited the same positive signal for whole-body extract and leg extract of *S. invicta* (Fig. 4a). After completion of the LAMP reaction, the turbidity of the solution in the tube containing the DNA of *S. invicta* could be confirmed visually (Fig. 4b). These results indicate that our LAMP system with the FIP3 primer set does not need highly purified DNA and has sufficient specificity to detect one *S. invicta* individual out of 10 ants.

Of note, given the very high intraspecific variation within ant species distributed worldwide (Ascunce et al. 2011), our

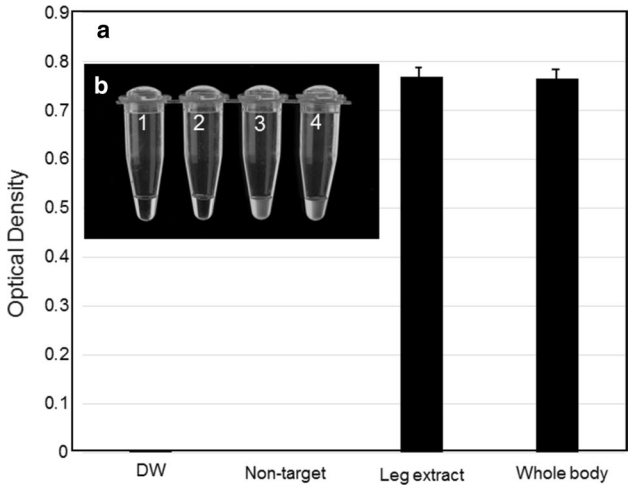

Fig. 4 Detection of *S. invicta* DNA in leg and whole-body extracts via the LAMP reaction with the FIP3 primer set. **a** Optical density of LAMP reactions containing RNase-free water as a negative control (DW), whole-body extract of *Formica japonica* (Non-target), leg extract of *S. invicta* (Leg extract), and whole-body extract of *S. invicta* (Whole body) are shown. Error bars represent the standard deviation of three independent reactions. **b** Turbidity after LAMP reactions in microtubes containing (1) RNase-free water as a negative control, (2) whole-body extract of *F. japonica*, (3) leg extract of *S. invicta*, and (4) whole-body extract of *S. invicta*

LAMP primers might be unable to amplify some *S. invicta* haplotypes that have never been detected in Japan. However, because all *S. invicta* individuals that have been collected so far were detectable, our LAMP primers are currently the most effective tool for the rapid detection of *S. invicta* in Japan. To improve the sensitivity and reliability of the LAMP assay system, it is necessary to monitor the DNA variation of *S. invicta* in Japan.

This method can be easily used by non-scientists. Thus, it can be used not only for quarantine in port areas but also for the detection of *S. invicta* in public spaces such as schoolyards and parks. We are preparing to distribute our *S. invicta* detection system to various organizations throughout Japan to construct a national fire ant monitoring network.

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