

A loop-mediated isothermal amplification assay for the plant-parasitic nematode *Aphelenchoides besseyi* in rice seedlings

Jiue-in Yang* and Guan-yi Yu

Department of Plant Pathology and Microbiology, National Taiwan University, No. 1 Roosevelt Rd., Sec. 4, Da-an Dist., Taipei City, 10617, Taiwan.

*E-mail: jiueinyang@ntu.edu.tw

This paper was edited by Raquel Campos-Herrera.

Received for publication June 22, 2019.

Abstract

Aphelenchoides besseyi is a seed-borne plant-parasitic nematode pathogen that causes rice white-tip disease worldwide. For quarantine purposes at custom border inspection and in field surveillance, a rapid, highly specific, and sensitive loop-mediated isothermal amplification (LAMP) assay for the nematode detection was developed. The assay targets the sequence variable region of the mitochondrial cytochrome oxidase subunit I (COI) gene of the nematode. It is capable of distinguishing the *A. besseyi* rice pathotype from the closely related *A. besseyi* nest fern pathotype. The assay is highly sensitive, with the detection minimum limit at 10^3 copy numbers/ μ l cloned target plasmid or a single nematode crude DNA. A minimum of 35 min is needed for the assay to complete, and the detection results can be rapidly determined by the color change of hydroxynaphthol blue dye or by the indication bar on a specific lateral flow dipstick. The application potential of this assay on rice seedling was demonstrated at the sensitivity level of five nematodes in single rice seedling. This is the first LAMP assay for *A. besseyi* rice race detection using a mitochondria gene as marker. This assay is well-suited for phytosanitary examination and seedling screening purposes in nurseries.

Keywords

Aphelenchoides besseyi, LAMP PCR assay, Leaf and bud nematode, Molecular diagnostic technique, Quarantine detection, Rice.

The leaf and bud nematode, *Aphelenchoides besseyi*, is the pathogen that causes the rice white-tip disease in rice growing regions worldwide. The nematode is widely distributed in Taiwan. Depends on the rice variety, it generally causes 7.3 to 29% disease rate in the field, and resulted in up to 39.3% yield loss (Wu, 2011). Leaf tips of the *A. besseyi*-infected plant turned white, and usually extended 3 to 5 cm long. While the nematodes gradually took the majority space within the seed, the infected plant lost vigor, became stunted and produced lighter grains that flow in water (Togashi and Hoshino, 2003). The disease symptoms on rice seedlings are easily confused with calcium and magnesium deficiency of the crop. Most temperate and tropical countries consider

A. besseyi as important plant pest. The European and Mediterranean Plant Protection Organization had listed *A. besseyi* as an A2 quarantine pest since 1981, and the Caribbean Plant Protection Commission, the Junta del Acuerdo de Cartagena and the Inter-African Phytosanitary Council also considered it of quarantine significance (OEPP/EPPO, 1992).

Currently, the World Trade Organization members follow the International Standards For Phytosanitary Measures (ISPMs) for nematode isolation from symptomatic plant or suspected plant materials. In ISPM, the identification of *A. besseyi* is based on morphology characteristics and PCR assays (IPPC, 2016). Compared to common plant-parasitic nematodes in the same genus, such as *A. blastophthorus*,

A. fragariae, *A. subtenuis* and *A. ritzemabosi*, *A. besseyi* differs from them by only a few morphological characteristics (Franklin and Siddiqi, 1972; Ferris, 2018). *A. besseyi* have a star-shaped mucro, a postvulval sac that is always less than one-third of the distance from the vulva to the anus, its excretory pore is usually positioned near the anterior edge of the nerve ring and the proximal ends have an indistinct dorsal process but the ventral apex is only moderately developed. Observation and measurements of the transparent, slender and microscopic nematode require well-trained nematologists working with skillful techniques under microscopic visions. Therefore, morphological identification can be very time consuming. Several PCR-based assays are available to identify *A. besseyi* from other *Aphelenchoides* spp., using multiple regions of the rDNA genes and mtDNA genes as molecular makers (Rybarczyk-Mydlowska et al., 2012; Youssef, 2014; Devran et al., 2017; Sánchez-Monge et al., 2017). Although experts generally agree that a combination of morphological and molecular methods is required for the current most reliable identification of pathogens in the *Aphelenchoides* genus (IPPC, 2016), the paucity of informative morphological characters for many species and that using only partial ribosomal gene sequences for interspecies identification still might not be sufficient (Rybarczyk-Mydlowska et al., 2012).

The *Aphelenchoides* genus contains over 150 nematode species, and the majority are fungal feeders (Rybarczyk-Mydlowska et al., 2012). To accurately distinguish the quarantine pest from others, only species-specific methods that are highly accurate and discerning would serve well for the quarantine inspection purposes. Lately, the loop-mediated isothermal amplification method (LAMP) has shown its potential as a useful tool for plant pathogen detection (Notomi et al., 2000). Using a set of four to six specific primers, the assay provides a higher specificity than other one-step PCR assays. In addition, without temperature changing steps throughout the amplification process, LAMP assay is a faster detection system with lower cost. To date, a few studies have demonstrated the application of LAMP assays for important plant-parasitic nematode species identification, and even detection directly from plant and soil samples. Successful detection reports include *Radopholus similis* in banana, *Anthurium* and citrus roots (Peng et al., 2012) and *Tylenchulus semipenetrans* in citrus rhizosphere soil (Lin et al., 2016), *Meloidogyne enterolobii* (Niu et al., 2012), *M. incognita* (Niu et al., 2011), *M. mali* (Zhou et al., 2017) and *M. hapla* (Peng et al., 2017)

in root and soil samples. Furthermore, evaluation by Canadian Food Inspection Agency and Canadian Forest Service on the RT-LAMP assay, which allows detection of living *Bursaphelenchus xylophilus* in pine wood samples, had revealed its value at boarder control (Leal et al., 2015).

Due to the mass amount and variety of plant samples that are subjected for nematode pest inspection at custom border and field surveillance, we aimed to develop a sensitive and species-specific LAMP assay for *A. besseyi* detection to support the quarantine inspection measures. In this paper, we describe a LAMP assay that can be applied directly on seedlings, planting materials and nematodes that have been extracted. Furthermore, the result interpretation method of the assay was also optimized to be efficient and user friendly.

Materials and methods

Nematodes samples

Besides the target species *A. besseyi*, nematode species that are commonly encountered at the Taiwan boarder quarantine inspections were also included in this study as none targets. A total of 18 nematode samples were used in this study. All nematodes were identified morphologically with microscopes and molecularly confirmed by analyzing the ribosomal DNA sequences (Powers, 2004). Among them, four were intercepted at the custom border, extracted from chopped Black pine (*Pinus thunbergii*), pine (*Pinus* spp.) and peat moss. The samples were extracted by using modified Baermann funnel method for 24hr and were stored in dH₂O at 4°C prior to DNA extraction. The other 14 samples were collected from seven locations in Taiwan, originally extracted from leaf of rice (*Oryza sativa*), nest fern (*Asplenium* spp.), or strawberry (*Fragaria × ananassa* Duch) through modified Baermann funnel method. The sample names, code, origin of the sample and the plant or materials that it was associated with were listed in Table 1. The field-collected samples were sub-cultured every 14 days on slant cultures in test tubes. *Aphelenchoides* species and *B. xylophilus* were maintained on *Alternaria citri* that had been growing on potato dextrose agar (PDA) containing 500 ppm chloramphenicol at 24°C for 5~8 days (Hsieh et al., 2012). Prior to inoculation or DNA extraction, nematodes were harvested from the slant tube by rinsing the agar surface twice with sterilized ddH₂O.

Table 1. Nematode samples used in this research.

Nematode species	Code	Geographical origin ^a	Origin	Accession number	AB-ID37 LAMP ^b	AbF5/AbR5 PCR ^b
<i>Aphelenchoides besseyi</i>	HW	Yunlin, TW	Rice (<i>Oryza sativa</i>)	MF669509	+	+
<i>Aphelenchoides besseyi</i>	GD	Taipei, TW	Rice (<i>Oryza sativa</i>)	MF669510	+	+
<i>Aphelenchoides besseyi</i>	TG9	Taoyuan, TW	Rice (<i>Oryza sativa</i>)	MF669506	+	+
<i>Aphelenchoides besseyi</i>	YL	Changhua, TW	Rice (<i>Oryza sativa</i>)	MF669504	+	+
<i>Aphelenchoides besseyi</i>	YG	Changhua, TW	Rice (<i>Oryza sativa</i>)	MF669505	+	+
<i>Aphelenchoides besseyi</i>	YLI	Hualien, TW	Rice (<i>Oryza sativa</i>)	MF669503	+	+
<i>Aphelenchoides besseyi</i>	GK	Yunlin, TW	Nest fern (<i>Asplenium</i> spp.)	MF669514	–	+
<i>Aphelenchoides besseyi</i>	HL1	Hualien, TW	Nest fern (<i>Asplenium</i> spp.)	MF669515	–	+
<i>Aphelenchoides besseyi</i>	HL2	Hualien, TW	Nest fern (<i>Asplenium</i> spp.)	MF668516	–	+
<i>Aphelenchoides besseyi</i>	HL3	Hualien, TW	Nest fern (<i>Asplenium</i> spp.)	MF669517	–	+
<i>Aphelenchoides bicaudatus</i>	ABC	Taichung, TW	Peat moss	MF669511	–	–
<i>Aphelenchoides fragariae</i>	SP	Taichung, TW	Nest fern (<i>Asplenium</i> spp.)	MF669507	–	–
<i>Aphelenchoides fujianensis</i>	AS	Taichung, TW	Strawberry (<i>Fragaria</i> × <i>ananassa</i> Duch)	MF669502	–	–
<i>Aphelenchoides</i> spp.	630–2	Germany	Peat Moss	MF669501	–	–
<i>Bursaphelenchus xylophilus</i>	B2	Kinmen, TW	Pine (<i>Pinus</i> spp.)	MF669500	–	–
<i>Ditylenchus</i> spp.	JB2	Japan	Black pine (<i>Pinus thunbergii</i>)	MF669508	–	–
<i>Ditylenchus</i> spp.	68–3	Germany	Peat moss	MF669512	–	–
<i>Ditylenchus</i> spp.	3010	Lithuania	Peat moss	MF669513	–	–

^aTW, Taiwan; ^bthe symbols indicate if the sample was included (+) or was not included (–) in the specific experiment.

Rice seedlings inoculation

Rice seedlings for nematode inoculation were prepared following the standard producing procedure of seedlings producers in Taiwan. Seeds of Taoyuan No. 3 rice (*Oryza sativa*) were first sterilized in 55°C

hot water for 10min, then soaked in dH₂O at 37°C for two days, and finally placed in autoclave-sterilized soil for three days in the dark to induce germination. Later on, the seedlings were maintained under the day/night regime of 12 hr/12 hr at 28°C for seven days. *A. besseyi* was directly inoculated onto the seedlings by placing

the adult individuals on seedlings with picking needles. For each seedling, 0, 1, 5, 10, 20 or 40 nematodes were inoculated. Total DNA of the inoculated rice were extracted on the same day of inoculation. Three repeats were conducted for each treatment.

DNA extraction

Single nematode crude total DNA was prepared after surface-sterilizing the target nematode in 1% NaClO for 1 min, and then rinsed with dH₂O for 1 min. The nematode was then transferred into 20µl 0.25M NaOH in 0.2ml tubes, and incubated for 5hr at room temperature. The lysate was then heated at 95°C for 3min, and then 10µl 0.5M Tris-HCl buffer, 4µl 1.25M HCl and 5µl 2% Triton X-100 were added in a sequential order into the lysate before the secondary heating at 95°C for 3min (Floyd et al., 2002). For rice seedlings, plant tissues were first crushed and homogenized by pestle in mortar with liquid nitrogen, and the total DNA was extracted using PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., CA, USA) following the manufacturer's recommendation. Final DNA products were all stored at -20°C prior to use.

LAMP primers design and reaction preparation

The target gene for LAMP primers was chosen after analyzing all the available sequences of mitochondria and ribosome genes of *A. besseyi* and its closely related nematode species in the GeneBank database. Multiple sequences alignments were performed using Geneious 9.1.6 software (Biomatters Inc., CA, USA)

and the mitochondria COI gene was finally selected as primer target due to its relatively high sequence variation among the *Aphelenchoides* species. The LAMP primers were designed using PrimerExplorer v.5 software (Eiken chemical Co., Ltd, Tokyo, Japan). Four sets of primers, AB-ID14, AB-ID30, AB-ID37 and AB-ID40, were used for further testing. Among them, the AB-ID37 set contains four primers that attached to multiple sites of the mitochondria COI gene accordingly (Table 2), and all primers in the set had low sequence similarity to other closely related *Aphelenchoides* species (Fig. 1). All LAMP PCR reactions were conducted in 25-µl reactions. LAMP reaction mixes were prepared using Loopamp DNA Amplification Kit (Eiken chemical Co. Ltd, Tokyo, Japan) as suggested by the manufacturer. The LAMP mix contained distilled water 7µl, 2x reaction mix 12.5µl, Primer Mix (FIP 40pmol, BIP 40pmol, LF 20pmol, LB20pmol, F3 5pmol, and B3 5pmol) 2.5µl, *Bst* DNA Polymerase 1µl and 2µl template. Reactions were set for incubation at 63°C and for the time required for each examination.

PCR assay comparison

To further understand the developed LAMP assay application potential, a comparison to the prior published detection method was conducted, using the *A. besseyi* specific PCR primer pair AbF5/AbR5 (Devran et al., 2017) (Table 2). The PCR was conducted in 10-µl reactions. The PCR mix contained 5.1µl ddH₂O, 1µl 0.5M Tris buffer (pH 8.3), 1µl 25mM MgCl₂, 0.5µl 10g/L BSA, 0.5µl 2.5mM dNTP, 0.4µl 10mM primer pair, 0.1µl *Taq* polymerase

Table 2. List of primers and probe for LAMP assay and conventional PCR assay for *A. besseyi* detection in this study.

Set/target	Primer/probe	Sequence	Reference
AB-ID37	F3	5'-CGTCTAAATAATTTGAGGTTTTGGT-3'	This study
	B3	5'-ACCTAAAATAGAACTAATCCCAG-3'	
	Biotin-FIP	5'-TCTTGTTCTGCACCCATATCT-TGCCTGTAGCTTTATTTTGC-3'	
	BIP	5'-ATCCTCCTTTAAGAACTCTTGGTCA-CACAATGAAGCCTAAAAATGG-3'	
	Probe 17	5'-AACTCTTGGTCATCCTGGAAGAAG-3'	
AbF5/R5	AbF5	5'-ATGTGTAAGTAGAGCGTTATA-3'	Devran et al. (2017)
	AbR5	5'-ATTCGCCGTTTTTAAGGCG-3'	

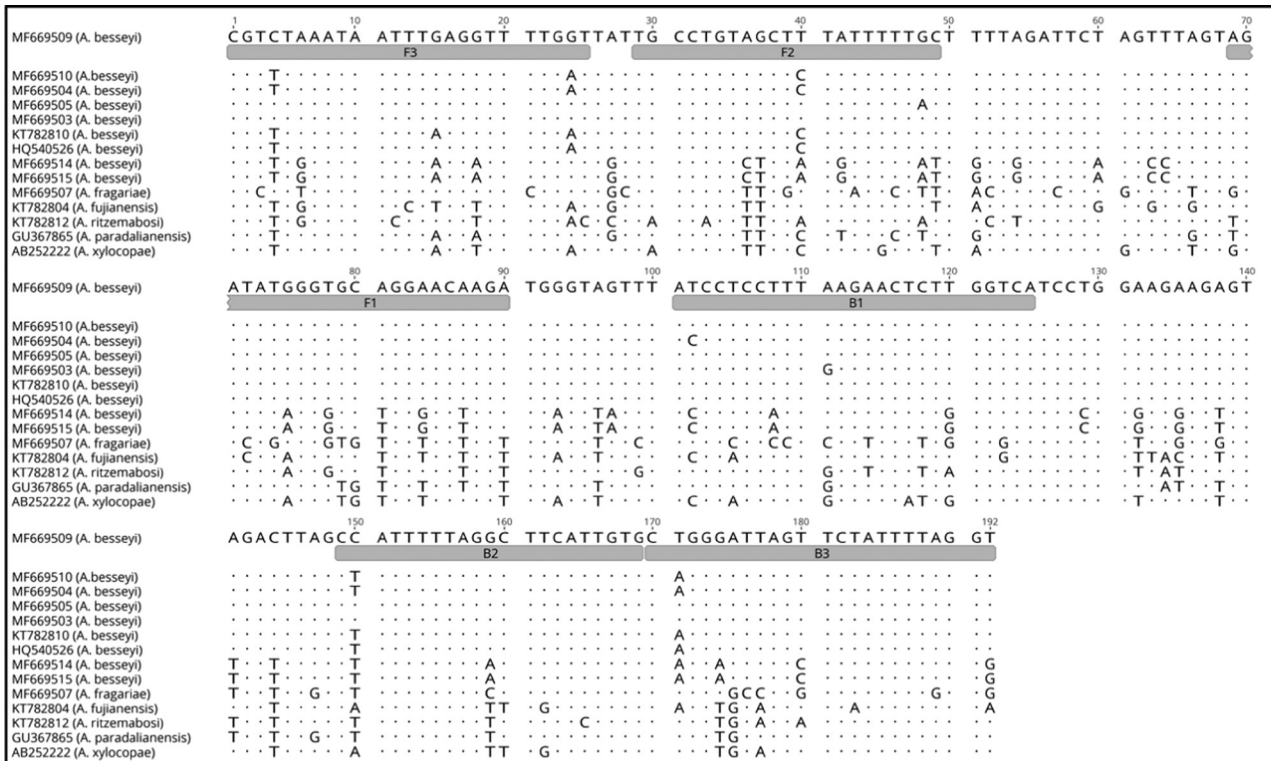


Figure 1: Multiple sequence alignment of partial mitochondria COI gene of *A. besseyi* and selected closely related species. The isolate HW was chosen as the *A. besseyi* reference sequence. In the aligned sequences, a dot indicates a match, a letter indicates a mismatch compared to the reference sequence. The gray arrows under the reference sequence indicate the binding locations of the designed LAMP primer set AB-ID37.

(Biomax Scientific Co. Ltd., Taiwan) and 1 µl template DNA. PCR reactions were carried with the following conditions: 94°C 3min for initial denaturation, 35 cycles with denaturation at 94°C for 30sec, annealing at 56°C 30sec, extension at 72°C for 60sec and final extension at 72°C for 7 min (Devran et al., 2017). Subsequently, 5 µl PCR products were analyzed through electrophoresis in 1.5% agarose, stained with EtBr and imaged with UV light.

Sensitivity, specificity and application test

To determine the sensitivity of the LAMP assay, serial dilutions of single nematode crude total DNA and the target gene cloned plasmids in sterile ddH₂O were used. The cloned plasmids were prepared using pGEM-T Easy Vector System (Promega, Madison, WI, US). LAMP reaction mixes were prepared using Loopamp DNA Amplification Kit (Eiken chemical Co. Ltd, Tokyo, Japan) as

suggested by the manufacturer. The LAMP reaction was conducted in the RealTime Turbidimeter LA-500 (Eiken chemical Co. Ltd, Tokyo, Japan), and the turbidity of each reaction was recorded after each cycle. The specificity of the LAMP assay was tested using crude total DNA of nine above-ground plant-parasitic nematodes species (Table 1). In order to evaluate the application potential of the assay, detection ability of nematode-inoculated rice seedlings prepared as previously described were also examined. Amplification results were analyzed through multiple visualization methods.

Visualization method

Three visualization methods were used and compared for the LAMP assay: gel electrophoresis, hydroxy naphthol blue (HNB) staining and LFD assay. For gel electrophoresis analysis, 30min were run at 110V on 2% agarose gels (Biomax Scientific Co. LTD., Taiwan) in an Owl separation

system (Thermo Fisher Scientific Inc., USA), then stained in EtBr (Bioshop Canada Inc., Canada) for 10min, and the electrophoresis result was analyzed in the Gel Doc EZ imager (Bio-rad Laboratories, Hercules, CA, USA) under UV light. HNB (Dojindo Laboratories Co. Ltd, Japan) was added at the concentration of 120 μ M in each LAMP reaction prior to each reaction. The positive reactions are indicated by a color change from violet to sky-blue (Goto et al., 2009). Custom lateral flow dipstick (LFD) assay was performed using DNA hybridization and immunoprecipitation to specifically detect LAMP reaction products. An oligonucleotide probe labeled with FITC at the 5' end (Table 2) was designed on COI gene of mtDNA between the F2 and B2 region (Fig. 1). After biotin labeled LAMP reaction, 20pmol of the oligonucleotide probe was added to the amplification products and kept at 63°C for 10min for hybridization. Then, 10 μ l of the hybridized products was transferred into 150 μ l assay buffer (Milenia Biotec GmbH, Giessen, Germany), and the reaction results was analyzed from a customized dipstick after a 5min reaction time in the mixture.

Results

The AB-ID37 LAMP primer set performance

The AB-ID37 set performed best in the 90min long efficiency tests; Only 30min was needed to raise its amplicon mix turbidity above the detection level; whereas 42, 47 and 53min were needed for AB-ID40, AB-ID30 and AB-ID14, respectively (Fig. 2). Therefore, AB-ID37 was chosen for further exam and optimization.

Specificity and sensitivity of the LAMP assay

All six *A. besseyi* rice isolates from different geographic locations in Taiwan were successfully detected (Fig. 3A). By contrast, no PCR amplification was observed from nest fern isolates of *A. besseyi* or other common above-ground plant-parasitic species (Fig. 3A, B). The template with 10⁶ copies/ μ l had sufficient amplification for turbidity detection after 35min of reaction. When the reaction time was extended to 90min, similar to the reaction time of a traditional PCR assay, even the plasmids concentration as low as 10³ copies/ μ l could be detected (Fig. 4A). The 10⁻¹ dilution of the crude DNA of single adult nematode was also successfully detected with the assay (Fig. 4B).

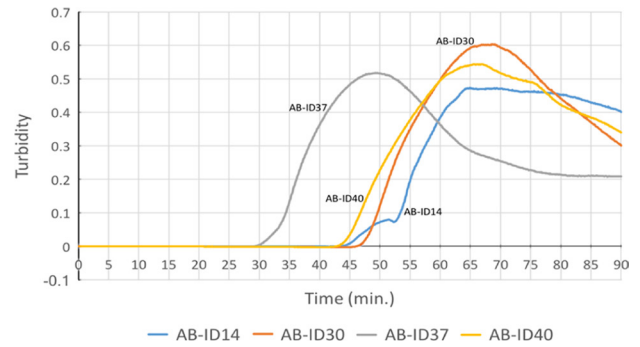


Figure 2: The efficiency test result of four LAMP primer sets. The colorful curves represent different LAMP primer sets. 10⁶ copies/ μ l *A. besseyi* cloned plasmids were used as template.

Comparison of LAMP with other PCR assay

In the specificity test of primer pair AbF5/AbR5, among the 14 *Aphelenchoides* samples, all rice and nest fern *A. besseyi* isolates were detected (Fig. 5A). The sensitivity of the PCR assay is 10³ copies/ μ l. It was capable of detecting the single adult nematode crude total DNA or its 1/10 dilution (Fig. 5B).

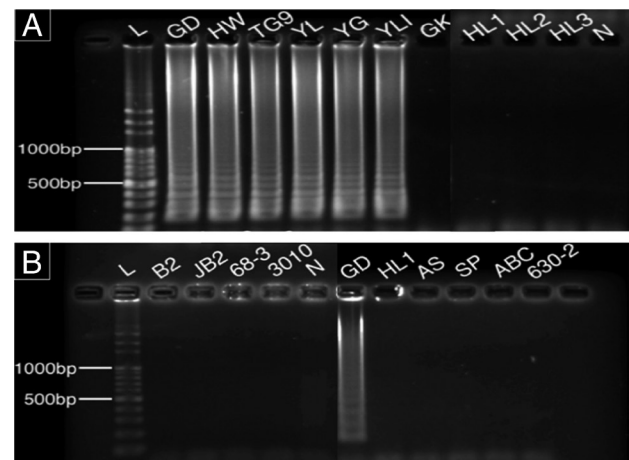


Figure 3: The *A. besseyi* specific AB-ID37 LAMP assay specificity on aboveground plant-parasitic nematodes with gel electrophoresis analysis. L: ladder, N: negative control. (A) Examine with *A. besseyi* isolates from rice (GD, HW, TG9, YL, YG and YL1) and nest fern (GK, HL1-3). (B) Examine with different species of aboveground plant-parasitic nematodes.

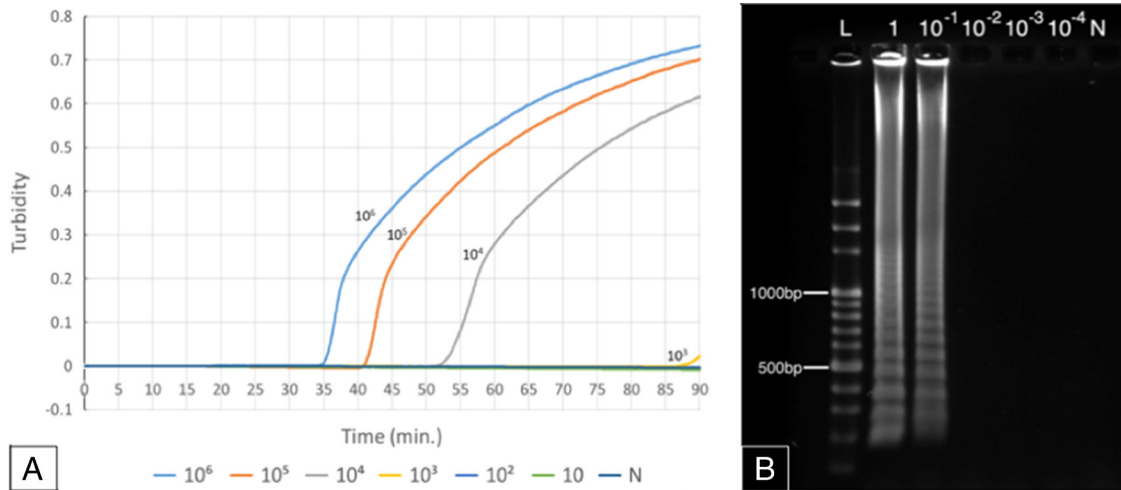


Figure 4: Sensitivity examination of the *A. besseyi* specific AB-ID37 LAMP assay. (A) The turbidity detection using 10⁶~10¹ copies/ μ l target sequence cloned plasmids as template. (B) The electrophoresis gel result when used 10-fold dilutions of single nematode crude DNA. L: ladder, N: negative control.

Application with rice seedling samples

From a 120-minutes long turbidity recording of AB-ID37 assay reaction, 65min was shown to be required for the detection power of 10 nematodes per seedling (Fig. 6), and the detection power was extended to 5 nematode per seedling when the reaction time

was increased to 85min (Fig. 6). Gel electrophoresis images, HNB staining results and LFD results from 90 min of AB-ID37 assay reaction were consistent with the turbidity observation (Fig. 7). The detection result is reliable when there are more than 5 nematode per seedling. Compare to electrophoresis, the visualization with LFD saved 15min of the result analysis time.

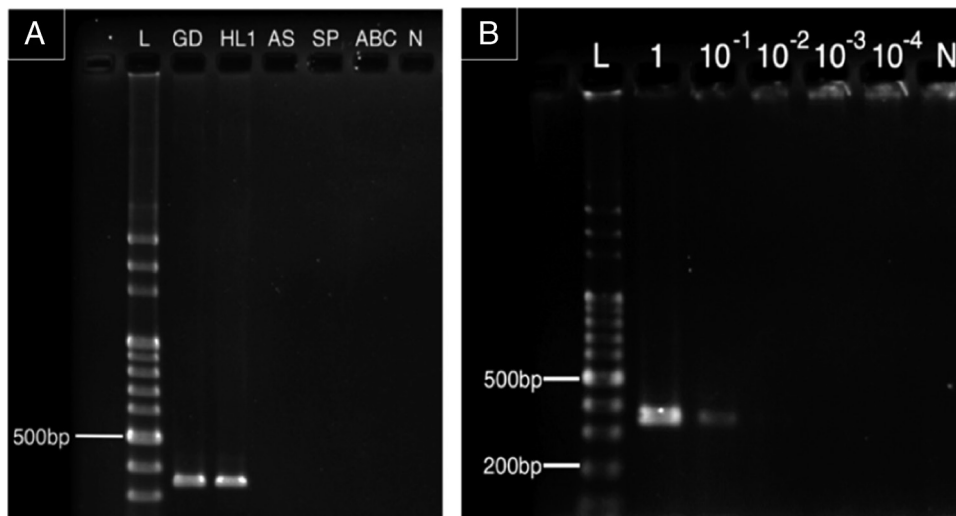


Figure 5: The specificity and sensitivity test result of PCR primer pair AbF5/AbR5. L: ladder, N: negative control. (A) *Aphelenchoides* spp. were examined. GD: *A. besseyi* isolate from rice, HL1: *A. besseyi* isolate from nest fern, AS: *A. fujianensis*, SP: *A. fragariae* and ABC: *A. bicaudatus*. (B) Using serial 10-fold dilutions of single nematode crude DNA as template.

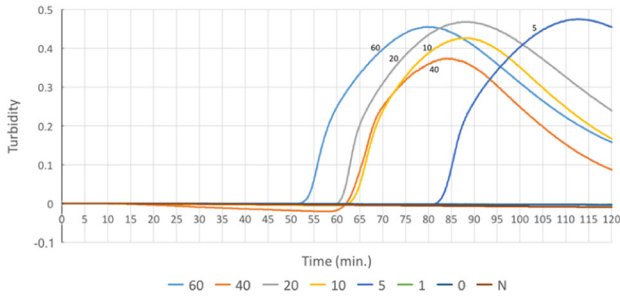


Figure 6: The application sensitivity exam on total DNA of *A. besseyi*-inoculated rice seedlings with the *A. besseyi* specific AB-ID37 LAMP assay. The colorful curves indicate different amount of nematode inoculated on a single rice seedling, ranging from 60, 40, 20, 10, 5, 1 to 0. N: negative control.

Discussion

A. besseyi is a threat economical important crops, including rice, strawberry, nest fern and maize (Wang et al., 1993; Yu and Tsay, 2004; Youssef, 2014), and pathotypes have been described (Wu et al., 2016). For the quarantine detection, a quick and accurate assay that is applicable even with very few nematode samples is very important. In this study, we developed an *A. besseyi* specific LAMP assay with the HNB coloration and lateral flow stick options. The assay provided the disease diagnosis without expensive or sophisticated equipment within 35min from crude DNA of one single nematode. Furthermore, this *A. besseyi* LAMP assay is more specific than the previously reported PCR assay and targets only the *A. besseyi* rice isolates. Lastly, the assay does not require complicated procedures or long training hours for the operation personnel; the result analysis

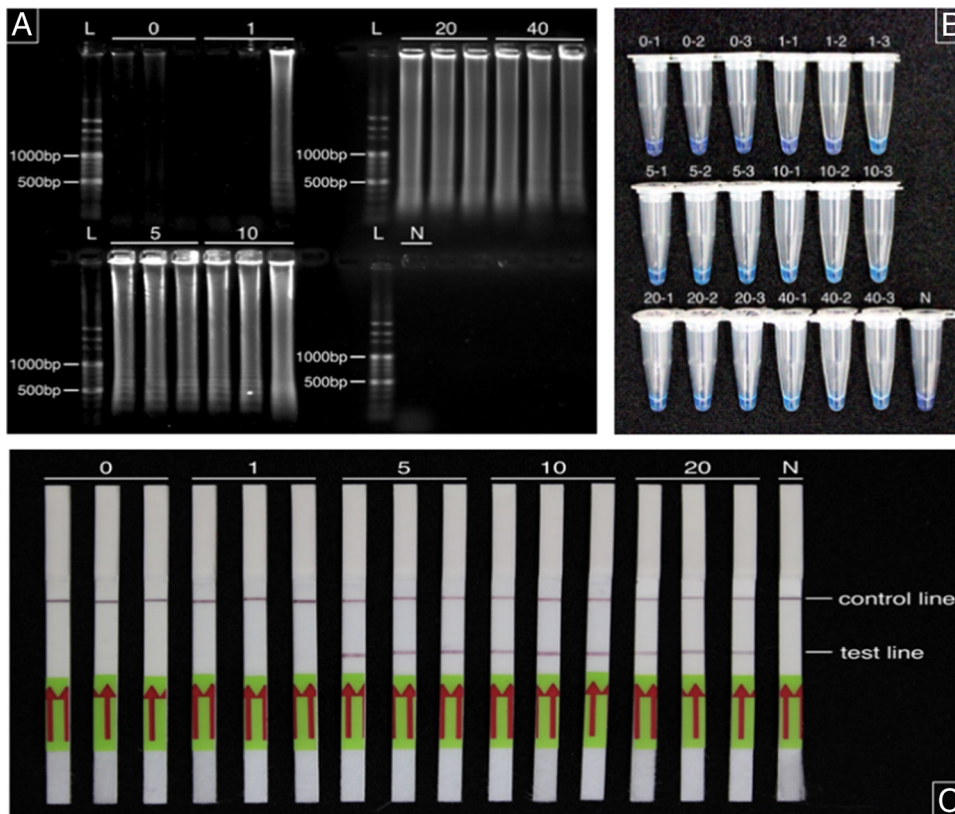


Figure 7: Different visualization options for the application of the *A. besseyi* specific AB-ID37 LAMP assay on total DNA of *A. besseyi*-inoculated rice seedlings. Triplicate of samples inoculation nematode numbers 40, 20, 10, 5, 1, 0 per seedling were tested. N: negative control. (A) Gel electrophoresis. L: ladder, N: negative control. (B) HNB staining. The sky-blue color indicates positive amplification. (C) Lateral flow dipstick (LFD). The purple color appearance at both control line and test line indicates a successful detection of target from LAMP PCR reaction. The test results with 40 nematodes are not shown.

with the LFD or HNB is user friendly. This assay is highly specific, affordable and efficient for quarantine pest identification for boarder quarantine inspections on seeds and seedlings.

Given the fact that rice white-tip disease caused by the nematode is seed-borne, the primary inoculum is the nematode-infected rice seedlings planted at the beginning of each rice production season. In the current rice production system in Taiwan, the rice seedlings are produced by the nursery industry. If a detection assay can identify the infection at an early stage before transplanting it would be very useful for preventing primary inoculum entering the field. *A. besseyi* is heterophytic and the amounts of female are usually much higher than males in the population. Prior studies reported male ratio of the nematode species from field studies was 3 to 21.7% (Fukano, 1962; Huang et al., 1979). In other words, when there are more than five nematodes, the chance of one of them being male is higher. Prior studies have shown that the symptoms on rice were only observed five weeks after 500 nematodes were inoculated onto one seedling (Tzeng and Lin, 2005). The *A. besseyi* LAMP assay developed in this study had the minimum detectable power of five nematode individuals on a seven-days old seedling. Our testing results on the inoculated seedlings suggest our assay meet both criteria and is very sufficient for the application for *A. besseyi* infection diagnosis in rice seedling production systems.

The regions of the rDNA gene is highly divergent region among different nematode species and frequently chose as target sequence in PCR-based diagnosis techniques (McCuiston et al., 2007; Kikuchi et al., 2009; Zhou et al., 2017). It has also showed higher sequence divergences in *Aphelenchoides* spp. than mtDNA COI gene (Yu, 2018). However, in our study, the rDNA LAMP primer sets did not show better specificity in the bioinformatic analysis (data not shown). It is likely due to the characteristics of the LAMP assay, which requires four to six primers that should be carefully designed to not only selectively bind to the target sequence but also would form proper loop structures instead of primer dimers during the amplification process. The related mtDNA COII gene had also been chosen as the target of LAMP assay for *Radopholus similis* diagnosis (Peng et al., 2012), instead of rDNA genes. Mitochondrial DNA sequences often provide evidence of nematode evolutionary horizontal gene transfer events (Scholl et al., 2003; Husnik and McCutcheon, 2018); these could be for population maintenance or involved in innovation purposes such as detoxification, protection and nutrition metabolism (Craig et al., 2009; Danchin

et al., 2010; Paganini et al., 2012). For example, Sánchez-Monge et al. (2017) analyzed the mtDNA COI gene sequences for phylogenetic studies among *Aphelenchoides* species and found the support of multiple origin of plant-parasitism. The collection of more mtDNA information of all plant-parasitic nematodes in the future would benefit all researches in the nematology society, no matter the interests lie in genetics, host-pathogen biology, or pathogen identification and disease diagnostics.

Acknowledgments

The authors thank Dr Meng-Ling Wu (Deputy Director, Taiwan Forestry Research Institute, Council of Agriculture, Executive Yuan, Taiwan) for kindly allowing the authors to use the machinery. This research was funded by the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan, Taiwan, Grant Number 108AS-8.4.3-BQ-B1. The authors declare no conflict of interest.

References

- Craig, J. P., Bekal, S., Niblack, T., Domier, L. and Lambert, K. N. 2009. Evidence for horizontally transferred genes involved in the biosynthesis of vitamin B₁, B₅, and B₇ in *Heterodera glycines*. *Journal of Nematology* 41:281–90.
- Danchin, E. G. J., Rosso, M.-N., Vieira, P., de Almeida-Engler, J., Coutinho, P. M., Henrissat, B. and Abad, P. 2010. Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)* 107:17651–6.
- Devran, Z., Tülek, A., Mistanoglu, İ., Çiftçiçil, T. H. and Özalp, T. 2017. A rapid molecular detection method for *Aphelenchoides besseyi* from rice tissues. *Australasian Plant Pathology* 46(1):43–8.
- Ferris, H. 2018. *Aphelenchoides besseyi*. Nemaplex, University of California, Davis, CA. available at: <http://plpnemweb.ucdavis.edu/nemaplex> (accessed March 23, 2018).
- Floyd, R., Abebe, E., Papert, A. and Blaxter, M. 2002. Molecular barcodes for soil nematode identification. *Molecular Ecology* 11(4):839–50.
- Franklin, M. and Siddiqi, M. 1972. *Aphelenchoides besseyi* Set 1, No. 4. in descriptions of plant-parasitic nematodes. Commonwealth Institute of Helminthology: St. Albans.
- Fukano, H. 1962. Ecological studies on white-tip disease of rice plant caused by *Aphelenchoides besseyi* Christie and its control. *Bulletin Fukuoka of Agricultural Experimental Station* 18:1–108.

- Goto, M., Honda, E., Ogura, A., Nomoto, A. and Hanaki, K.-I. 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* 46:167–72.
- Hsieh, S. H., Lin, C. J. and Chen, P. 2012. Sexual compatibility among different host-originated isolates of *Aphelenchoides besseyi* and the inheritance of the parasitism. *PLoS one* 7(7):e40886.
- Huang, C., Huang, S. and Chiang, Y. 1979. Mode of reproduction and sex ratio of rice white-tip nematode, *Aphelenchoides besseyi*. *Nematologica* 25(2):255–60.
- Husnik, F. and McCutcheon, J. P. 2018. Functional horizontal gene transfer from bacteria to eukaryotes. *Nature Reviews Microbiology* 16(2):67.
- IPPC. 2016. *Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi*. in *Diagnostic Protocols: International Standard for Phytosanitary Measures (ISPM) 27, ANNEX 17*, Food and Agriculture Organization of the United Nations (FAO), Rome.
- Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N. and Kanzaki, N. 2009. A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. *Phytopathology* 99(12):1365–9.
- Leal, I., Allen, E., Foord, B., Anema, J., Reisle, C., Uzunovic, A., Varga, A. and James, D. 2015. Detection of living *Bursaphelenchus xylophilus* in wood, using reverse transcriptase loop-mediated isothermal amplification (RT-LAMP). *Forest Pathology* 45(2):134–48.
- Lin, B., Wang, H., Zhuo, K. and Liao, J. 2016. Loop-mediated isothermal amplification for the detection of *Tylenchulus semipenetrans* in soil. *Plant Disease* 100(5):877–83.
- McCuston, J. L., Hudson, L. C., Subbotin, S. A., Davis, E. L. and Warfield, C. Y. 2007. Conventional and PCR detection of *Aphelenchoides fragariae* in diverse ornamental host plant species. *Journal of Nematology* 39(4):343–55.
- Niu, J., Guo, Q., Jian, H., Chen, C., Yang, D., Liu, Q. and Guo, Y. 2011. Rapid detection of *Meloidogyne* spp. by LAMP assay in soil and roots. *Crop Protection* 30(8):1063–9.
- Niu, J., Jian, H., Guo, Q., Chen, C., Wang, X., Liu, Q. and Guo, Y. 2012. Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. *Plant Pathology* 61(4):809–19.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 28(12):e63.
- OEPP/EPPO. 1992. Quarantine procedure méthode de quarantaine. *EPPO Bulliten* 22(2):217–8.
- Paganini, J., Campan-Fournier, A., Da Rocha, M., Gouret, P., Pontarotti, P., Wajnberg, E., Abad, P. and Danchin, E. G. J. 2012. Contribution of lateral gene transfers to the genome composition and parasitic ability of root-knot nematodes. *PLoS One* 7(11):e50875.
- Peng, H., Peng, D., Hu, X., He, X., Wang, Q., Huang, W. and He, W. 2012. Loop-mediated isothermal amplification for rapid and precise detection of the burrowing nematode, *Radopholus similis*, directly from diseased plant tissues. *Nematology* 14(8):977–86.
- Peng, H., Long, H., Huang, W., Liu, J., Cui, J., Kong, L., Hu, X., Gu, J. and Peng, D. 2017. Rapid, simple and direct detection of *Meloidogyne hapla* from infected root galls using loop-mediated isothermal amplification combined with FTA technology. *Scientific reports* 7:44853.
- Powers, T. 2004. Nematode molecular diagnostics: from bands to barcodes. *Annual Review of Phytopathology* 42:367–83.
- Rybarczyk-Mydlowska, K., Mooyman, P., van Meegen, H., van den Elsen, S., Vervoort, M., Veenhuizen, P., van Doorn, J., Dees, R., Karssen, G. and Bakker, J. 2012. Small subunit ribosomal DNA-based phylogenetic analysis of foliar nematodes (*Aphelenchoides* spp.) and their quantitative detection in complex DNA backgrounds. *Phytopathology* 102(12):1153–60.
- Sánchez-Monge, A., Janssen, T., Fang, Y., Couvreur, M., Karssen, G. and Bert, W. 2017. mtCOI successfully diagnoses the four main plant-parasitic *Aphelenchoides* species (Nematoda: Aphelenchoididae) and supports a multiple origin of plant-parasitism in this paraphyletic genus. *European Journal of Plant Pathology* 148(4):853–66.
- Scholl, E. H., Thorne, J. L., McCarter, J. P. and Bird, D. McK. 2003. Horizontally transferred genes in plant parasitic nematodes: a high-throughput genomic approach. *Genome Biology* 4:R39.1–R39.12.
- Togashi, K. and Hoshino, S. 2003. Trade-off between dispersal and reproduction of a seed-borne nematode, *Aphelenchoides besseyi*, parasitic on rice plants. *Nematology* 5(6):821–9.
- Tzeng, C. and Lin, Y. 2005. The intraspecific variation of *Aphelenchoides besseyi* populations in Taiwan. *Plant Pathology Bulletin* 14(1):67–75.
- Wang, K., Tsay, T. and Lin, Y. 1993. The occurrence of *Aphelenchoides besseyi* on strawberry and its ecology in Taiwan. *Plant Protection Bulliten* 35(1):14–29.
- Wu, G.-L., Kuo, T.-H., Tsay, T.-T., Tsai, I. J. and Chen, P. J. 2016. Glycoside hydrolase (GH) 45 and 5 candidate cellulases in *Aphelenchoides besseyi* isolated from bird's-nest fern. *PLoS One* 11(7):e0158663.
- Wu, H.-Y. 2011. The occurrence of white tip of rice in the northern part of Taiwan and improvement of rice seed treatment method. *Bulletin of Taoyuan District Agricultural Research and Extension Station* 70:51–60.

Youssef, M. M. A. 2014. The leaf and bud nematode, *Aphelenchoides besseyi*, its identification, economic importance and control measures. A review. Middle East Journal of Agriculture Research 3(3):461–4.

Yu, G. Y. 2018. Development and application of a rapid detection assay of white-tip disease and bakanae disease on rice. master thesis, National Taiwan University, Taipei City, Taiwan.

Yu, P. and Tsay, T. 2004. Occurrence of a foliar nematode disease of fern in Taiwan. Plant Protection Bulliten 13(1):35–44.

Zhou, Q., Cai, Y., Gu, J., Wang, X. and Chen, J. 2017. Rapid and sensitive detection of *Meloidogyne mali* by loop-mediated isothermal amplification combined with a lateral flow dipstick. European Journal of Plant Pathology 148(4):755–69.