

Article

A Fast and Sensitive Enzyme-Mediated Duplex Exponential Amplification Method for Field Detection of *Bursaphelenchus xylophilus*

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Abstract: The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is a pathogenic organism that causes pine wilt disease (PWD). To date, several molecular diagnostic methods have been developed; however, rapid, convenient, and inexpensive field diagnostic tools for detecting PWN are still limited. In this study, an enzyme-mediated duplex exponential amplification (EmDEA) method for detecting PWN from extracted nematodes or pinewood sawdust was developed and tested. This method comprised an EmDEA molecular test kit, which consisted of freeze-dried enzyme pellets that can be stored at room temperature (approximately 20–25 °C) for one year, a dry block heater, and a portable isothermal fluorescence amplification instrument. The whole procedure was completed within 30 min. The EmDEA assay could detect a single PWN at all life stages from a mixture of other nematode species or from pinewood sawdust. The detection limit was 10 copies (plasmid weight 32.66 ag) or 1/500 of that of a single adult PWN per reaction. Therefore, the EmDEA assay has potential applications in PWN detection in the field, as well as quarantine inspection in international trade. Moreover, modification of primers and probes will allow the rapid identification of other nematode species.

Keywords: *Bursaphelenchus xylophilus*; diagnosis; pine wilt disease; isothermal fluorescence amplification



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1. Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) [1], is a destructive pest to pines [2] and causes pine wilt disease (PWD). The nematode is vectored by cerambycid beetles of the genus *Monochamus* from healthy, wilt-killed pine trees [3]. Since its introduction to Japan from the United States of America in the early 1900s, the PWN has caused extensive damage. Currently, PWN has spread to other Eurasian countries, including China, Korea, Portugal, and Spain. Hence, *B. xylophilus* poses a significant threat to pine forests worldwide [4,5]. The economic importance of PWN introduction into new areas has increased the need for its rapid and accurate detection.

Reliable early detection and identification are fundamental for implementing PWN control and management strategies, improving quarantine regulations, and preventing the further spread of PWD.

PWN is traditionally identified based on morphological characteristics using microscopy [6]. However, the coexistence of various nematode species in pine trees makes morphological identification difficult, largely owing to variable female mucron lengths. For populations detected in wood, female *B. xylophilus* tails show a broadly rounded terminus in most cases; however, the tail may occasionally have a terminal nipple-like extension or short mucron of <2 µm. Gu et al. [7] found that these typical bluntly rounded female tails were rare (2–3%), and most females had an obvious mucron (0.2–3.2 µm long).

Therefore, a foundation has been established for the rapid detection of PWN using polymerase chain reaction (PCR), restriction fragment length polymorphism, real-time PCR, loop-mediated isothermal amplification, recombinase polymerase amplification (RPA), RPA combined with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 12a, and enzyme-mediated amplification (EMA) [8–16].

Skilled nematode expertise is required to diagnose PWD; however, this can be time-consuming, and the results can be subjective depending on the expert's capability. Molecular diagnostic methods for the detection of PWN are relatively easy and accurate for non-experts. However, most of these methods are not suitable for field use and are limited to the laboratory. This is mainly because the required instruments are heavy, and *B. xylophilus* genomic DNA extraction has many operational steps and a high risk of aerosol pollution after amplification. To overcome these limitations, Suzhou GeneVide Biotech Co., Ltd. (Suzhou, China) and the Ningbo Customs Technical Center (Ningbo, China) collaboratively developed a portable isothermal fluorescence amplification instrument and kit. Therefore, this study aimed to determine the specificity and sensitivity of the developed instrument and kit and verify their operability.

2. Materials and Methods

2.1. Nematode Populations

Four *B. xylophilus* (Bx), eight *B. spp.*, and other populations (Table 1) from the nematode collection of the Technical Centre of Ningbo Customs, China, were tested. These populations were reared on a *Botrytis cinerea* fungal mat on potato dextrose agar media at 25 °C for several generations and were identified using morphological characteristics and genetic differences.

Table 1. Different nematode species used in this study.

No.	Species	Isolation Number	Source	Host
1	<i>Bursaphelenchus xylophilus</i>	BXNB1	Ningbo, China	<i>Pinus massoniana</i> Lamb.
2	<i>Bursaphelenchus xylophilus</i>	BXUS1	USA	<i>Pinus taeda</i> L.
3	<i>Bursaphelenchus xylophilus</i>	BXWH1	Weihai, China	<i>Pinus massoniana</i>
4	<i>Bursaphelenchus xylophilus</i>	BXBX1	Benxi, China	<i>Pinus koraiensis</i> Siebold et Zuccarini
5	<i>Bursaphelenchus mucronatus kolymensis</i> (Braasch, Gu & Burgermeister, 2011)	BM2	Turkey	<i>Pinus</i> packaging wood

Table 1. Cont.

No.	Species	Isolation Number	Source	Host
6	<i>Bursaphelenchus mucronatus mucronatus</i> (Mamiya & Enda, 1979)	BM3	Huzhou, China	<i>Pinus massoniana</i>
7	<i>Bursaphelenchus doui</i> (Brassch, Gu, Burgermeister & Zhang, 2004)	BKR1	Republic of Korea	<i>Pinus</i> packaging wood
8	<i>Bursaphelenchus fungivorus</i> (Franklin & Hooper, 1962)	BNE	The Netherlands	Peat
9	<i>Bursaphelenchus rainulfi</i> (Brassch & Burgermeister, 2002)	BR1	Ningbo, China	<i>Pinus massoniana</i>
10	<i>Aphelenchoides</i> sp.	NBA1	Ningbo, China	<i>Pinus massoniana</i>
11	<i>Pseudaphelenchus</i> sp.	D23	USA	<i>Pinus taeda</i>
12	<i>Ektaphelenchoides</i> sp.	H46	USA	<i>Pinus taeda</i>

2.2. Wood Samples

Wood samples were collected from PWN-infested forests in China or quarantine samples collected at the port (Table 2). A 4–6 mm diameter, 5–10 cm long drill bit mounted on an electric drill was used. Each wood sawdust sample was drilled from at least five holes, approximately 3–5 cm in depth. Sawdust was collected, each weighing approximately 1 g, mixed well, and approximately 100 mg was used for molecular testing. Two replicates were used for each wood sample. To determine the live PWN number, 50 g of wood from each sample was cut into 0.5–1 cm wide and 5–10 cm long pieces. Nematodes were extracted using a modified Baermann funnel technique for 24 h and counted.

Table 2. Comparison between the EmDEA and Baermann methods.

Region (Country/Province)	Host Species	No. of Samples	EmDEA Results	Baermann Results	Result Consistency
Zhejiang, China	<i>Pinus massoniana</i>	25	15 +, 10 –	13 +, 12 –	Nematodes not isolated by the Baermann method in 2 EmDEA-positive samples
	<i>Pinus tabuliformis</i>	1	1 +	1 –	Detected by EmDEA only
	<i>Pinus sylvestris</i>	2	2 +	2 +	Full agreement
Jilin, China	<i>Pinus tabuliformis</i>	2	2 +	1 +, 1 –	Nematodes not isolated by the Baermann method in 1 EmDEA-positive samples
USA	<i>Pinus taeda</i>	6	6 +	4 +, 2 –	Nematodes not isolated by the Baermann method in 2 EmDEA-positive samples

“+” indicates a positive result using the corresponding method; “–” indicates that the target nematode was not detected by the EmDEA method or not isolated by the Baermann method.

2.3. Portable Enzyme-Mediated Duplex Exponential Amplification (EmDEA) Instrument and Commercial Kit

A portable EmDEA instrument (including a dry block heater and a portable optical isothermal device) and test kit were provided by Suzhou GeneVide Biotech Co., Ltd. (Suzhou, China) (Figure 1). The portable optical isothermal device weighed <1 kg, the dry block heater weighed <0.7 kg, the amplification temperature was 42 °C, and the total reaction time was 20 min. Up to 16 samples could be tested simultaneously.

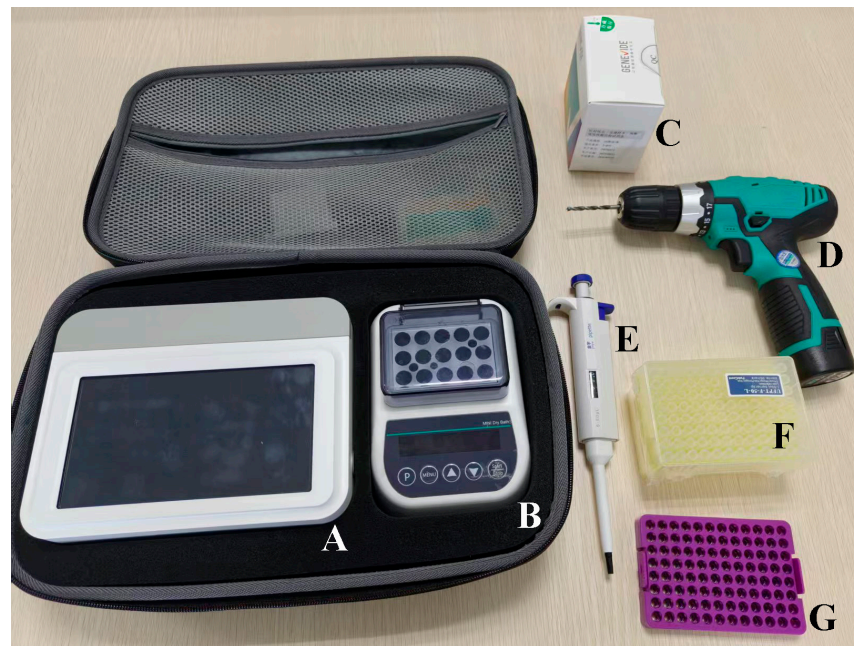


Figure 1. Dry block heater portable enzyme-mediated duplex exponential amplification (EmDEA) instrument and commercial kit. (A) Fluorescence detector or portable optical isothermal device; (B) dry block heater; (C) commercial kit; (D) electric drill; (E) pipette; (F) pipette tip; (G) reaction tube rack.

To drill the sawdust, an electric drill with a 4–6 mm diameter drill bit was used, and a 50 μ L micropipette was also used to transfer the liquid.

2.4. Genomic DNA (gDNA) Extraction from Nematodes

Genomic DNA was extracted from multiple nematodes or a single nematode in water using the same commercial kit. Lysis buffer (80 μ L) was added into 200- μ L tubes, and 10 μ L of nematodes suspended in water or a single nematode was transferred into the tubes. The tubes were heated in the dry block heater instrument at 95 $^{\circ}$ C for 5 min, then 50 μ L of the resultant DNA was used for isothermal fluorescence amplification.

2.5. Genomic DNA Extraction from Pinewood Sawdust

Genomic DNA was extracted from the sawdust using another commercial kit. Lysis buffer (800 μ L) was pre-aliquoted into a 1.5-mL tube, and a flat spoon of sawdust from each wood sample (the spoon was provided with the kit; one flat spoon of sawdust was approximately 100 mg) was added into the 1.5-mL tube. Subsequently, the sample was mixed and heated in the dry block heater instrument at 95 $^{\circ}$ C for 10 min. The solution (50 μ L) was diluted in 200 μ L dilution buffer pre-aliquoted in 500- μ L tubes, and then 50 μ L of the resultant DNA was used for isothermal fluorescence amplification.

2.6. Isothermal Fluorescence Amplification Assay

The primers for the EmDEA test kit were synthesized by Azenta Life Science (Suzhou, China) for the amplification of the internal transcribed spacer 2 (ITS2) and subsequently designed after aligning partial ribosomal RNA sequences from *B. xylophilus* (GenBank accession number: KX856336). The primer sequences Bx_F1 5'-TCGCATTGTTTCGCGCAATGTTAGGC-3' and Bx_R1 5'-AACGGTTTAGCCGCGCAC-GACAATC-3' were designed to amplify a 114-base-pair fragment exclusively from the ITS2 of *B. xylophilus*.

The EmDEA assay was performed using an EmDEA test kit (GeneVide, Suzhou, China) according to the manufacturer's instructions. To ensure field-use suitability, instead of using agarose gel to indicate the target amplicon, fluorescent probes (5'-ctgcccgcacggacaaacag/i6-FAMdT/g/idSp/g/iBHQ1dT/agaagatattggctgcgg-3' [C3 Spacer]) were integrated into the EmDEA reaction system to ensure that the amplification reaction was visualized via fluorescent signals.

Owing to the necessity for on-site rapid testing, the reagents were pre-configured, fractionated, and transformed into lyophilized powders. To achieve this, the freeze-drying methodology was investigated through multidimensional analysis, encompassing key parameters such as pre-freezing duration and concentrations of polyethylene glycol (PEG) and mannitol in the freeze-drying matrix. Following meticulous experimentation, the optimal conditions were a pre-freezing duration of 2 h and a freeze-drying matrix comprising 12% PEG and 0.5% mannitol. Therefore, all the EmDEA reaction components, including primers and probes, were lyophilized with the enzyme pellet into a 50- μ L final volume using the above parameters, with final concentrations of 0.2 μ M and 1.5 μ M, respectively. As shown in Figure 2, the lyophilized pellet remained durable at room temperature (approximately 20–25 °C) for up to 12 months.

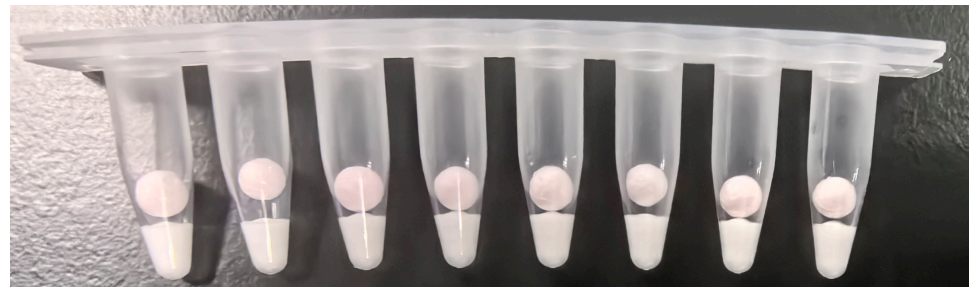


Figure 2. Lyophilized pellets in test tubes including primers and probes that could be kept at room temperature for up to 12 months.

A DNA template (50 μ L) obtained from the nematode solutions or pinewood sawdust during the DNA extraction procedures was placed into the EmDEA enzyme pellet tube, mixed briefly, and then incubated for 20 min at the optimal temperature (42 °C) using the portable optical isothermal device. The fluorescent signal was collected simultaneously, and the test results were directly displayed on the instrument screen.

2.7. Evaluation of EmDEA Assay Specificity

A specificity test of the EmDEA assay was conducted to confirm the ITS primers and probes that could amplify the specific ITS fragment from four *B. xylophilus* populations but not from eight other species (Table 1).

2.8. Evaluation of EmDEA Assay Sensitivity and Stability

Similar to the specificity test of the EmDEA assay, the sensitivity and stability were determined under optimal conditions. First, gradient dilution tests were performed on PWN plasmid DNA solutions of known concentrations. A 10-fold dilution series, starting from 1×10^5 copies of *B. xylophilus* plasmid DNA to 10 copies in each reaction, was used. Second, the stability of a single adult PWN was tested using a five-fold dilution method. Third, the diluted DNA for a single adult PWN was tested. Finally, second- (J2), third- (J3), and fourth-stage (J4) juveniles were tested to ensure that the method was suitable for juvenile detection. In addition, nematode liquids (5 μ L) were tested, including pure PWNs (approximately 1 and 10 individuals), PWNs mixed with other species (1 PWN in 100 other

species and 10 PWNs in 100 other species), and pure sets of other species (approximately 100 individuals). Three replicates were used for each group.

2.9. Comparison of the Baermann Funnel Technique and the Sawdust Method

Pinewood samples (36) stored in the laboratory were inspected for *B. xylophilus* using both the traditional modified funnel method for 24 h and the EmDEA method for 30 min.

3. Results

3.1. EmDEA Assay Specificity

The specificities of the designed primers and probes were assessed using four populations of *B. xylophilus*, five populations (four species) of *Bursaphelenchus* spp., and three other species (Table 1). All four populations of *B. xylophilus* resulted in positive amplification, whereas no amplification was observed in the other populations (Figure 3). These results indicated that the EmDEA assay could distinguish *B. xylophilus* from other closely related nematode species.

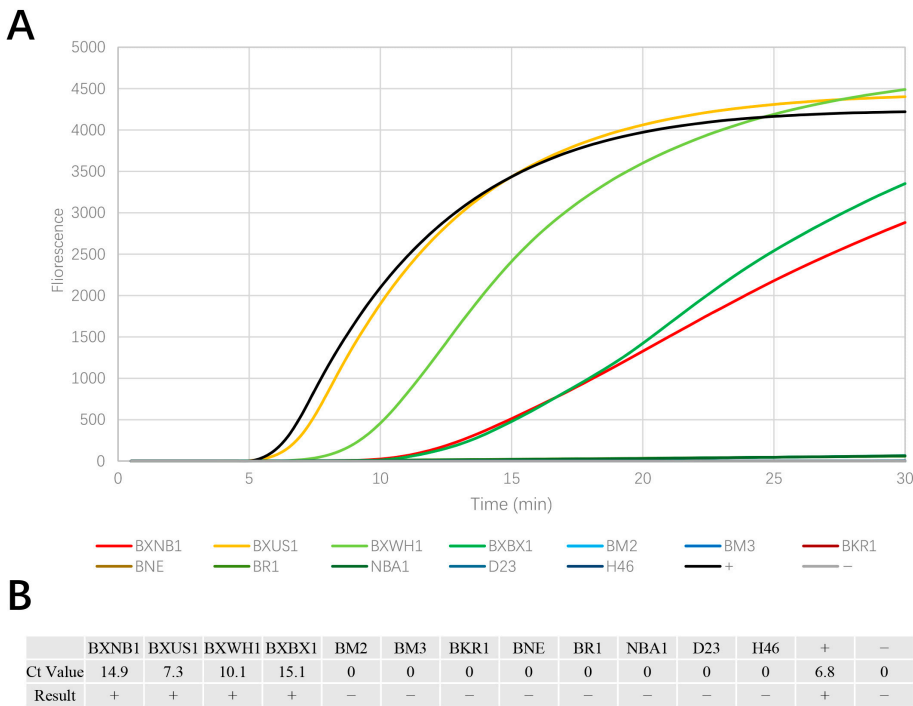


Figure 3. Specificity testing of single adult *Bursaphelenchus xylophilus* and other species. (A) Amplification curves; (B) cycle threshold (Ct) values. BXNB1: *Bursaphelenchus xylophilus* isolate from Ningbo, Zhejiang; BXUS1: *Bursaphelenchus xylophilus* isolate from the USA; BXWH1: *Bursaphelenchus xylophilus* isolate from Weihai, Shandong; BXBX1: *Bursaphelenchus xylophilus* isolate from Benxi, Liaoning; BM2: *Bursaphelenchus mucronatus* kolymensis; BM3: *Bursaphelenchus mucronatus*; BKR1: *Bursaphelenchus doui*; BNE: *Bursaphelenchus fungivorus*; BR1: *Bursaphelenchus rainulfi*; NBA1: *Aphelenchoides* sp.; D23: *Pseudaphelenchus* sp.; H46: *Ektaphelenchoides* sp.; +: Positive control; -:No template control.

3.2. Gradient Dilution Tests on PWN Plasmid DNA

The sensitivity of the EmDEA assay was examined using a 10-fold dilution series and was stable in the detection of *B. xylophilus* at 10 copies (plasmid weight: 32.66 ag). Positive amplifications were determined using amplification curve analysis (Figure 4A) and Ct values (Figure 4B).

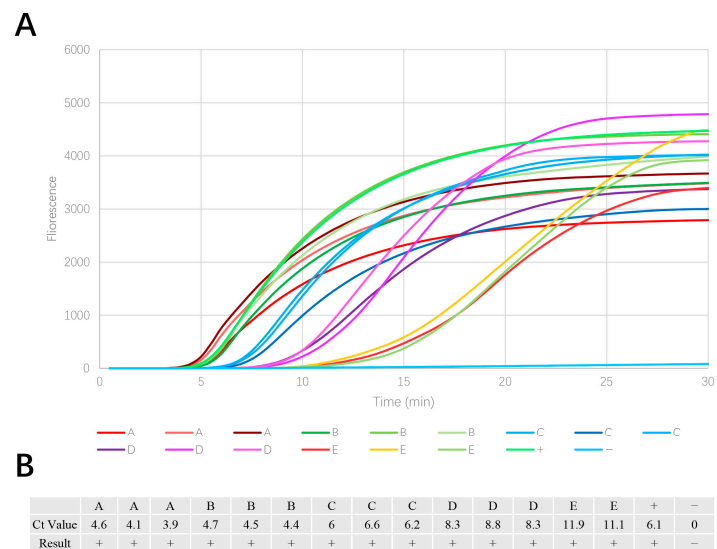


Figure 4. Gradient dilution tests of known solutions of *Bursaphelenchus xylophilus* DNA. **(A)** Amplification curves for enzyme-mediated duplex exponential amplification (EmDEA); **(B)** Table of cycle threshold (Ct) values. A: 100,000 copies total in the system, plasmid weight 326.60 fg; B: 10,000 copies total in the system, plasmid weight 32.66 fg; C: 1000 copies total in the system, plasmid weight 3.27 fg; D: 100 copies total in the system, plasmid weight 0.33 fg; E: 10 copies total in the system, plasmid weight 32.66 ag; +: Positive control; -: No template control.

3.3. Dilution Test for Single Adult PWN

The DNA templates of single nematodes extracted using the *B. xylophilus* extraction kit were tested for sensitivity using a five-fold dilution method. A single adult *B. xylophilus* DNA template extracted using the kit was stably expressed in the five-fold dilution. The test limitation was 1/500 of that of a single PWN for each reaction. Positive amplifications were determined using amplification curve analysis (Figure 5A) and Ct values (Figure 5B).

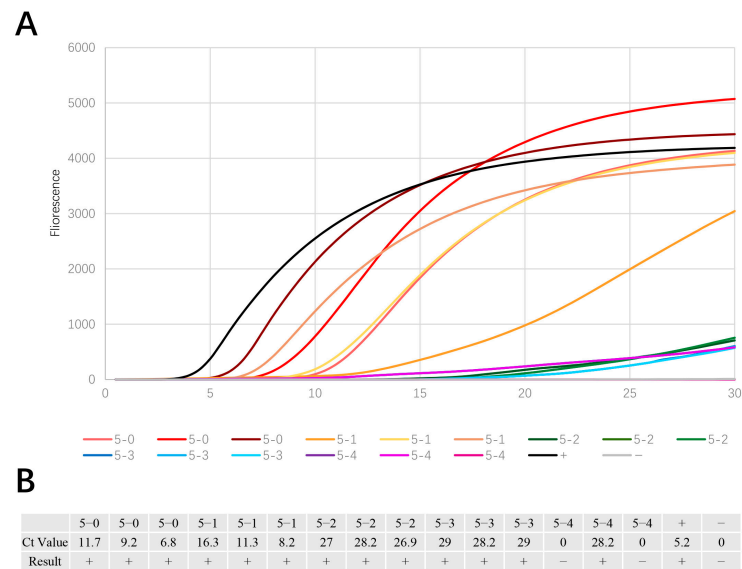


Figure 5. Dilution test for single adult *Bursaphelenchus xylophilus* DNA. **(A)** Amplification curves for enzyme-mediated duplex exponential amplification (EmDEA). **(B)** Table of cycle threshold (Ct) values: 5-0: 1/4 of total single adult PWN DNA in the system; 5-1: 1/20 of total single PWN DNA in the system; 5-2: 1/100 of total single PWN DNA in the system; 5-3: 1/500 of total single PWN DNA in the system; 5-4: 1/2500 of total single PWN DNA in the system; +: Positive control; -: No template control.

3.4. Stability Test of Single PWN in Different Juvenile Stages

Male and female DNA templates of PWN at different juvenile stages (J2, J3, and J4) were tested for stability. Positive amplifications were observed using amplification curve analysis (Figure 6A) and Ct values (Figure 6B). This indicated that any juvenile stage of *B. xylophilus* was detected.

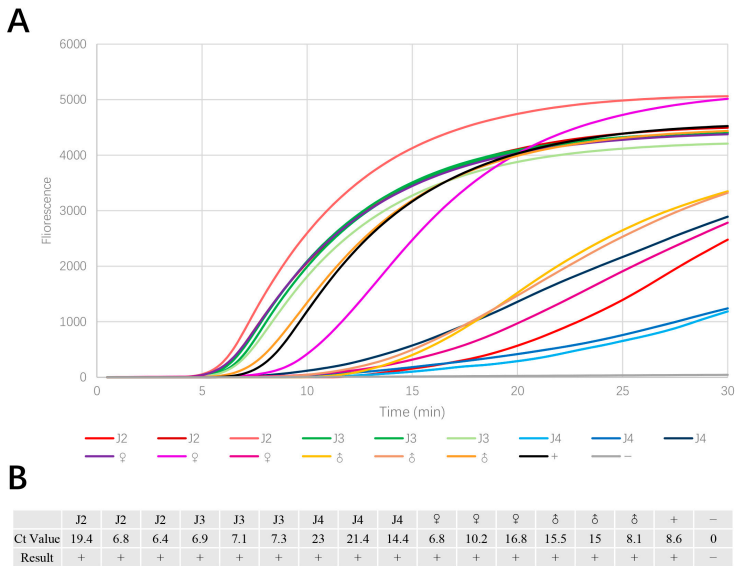


Figure 6. Stability test for a single *Bursaphelenchus xylophilus* at different juvenile stages. (A) Amplification curves. (B) Cycle threshold (Ct) values and results. J2: single second-stage juvenile PWN DNA; J3: single third-stage PWN DNA; J4: single fourth-stage PWN DNA; ♀: single female PWN DNA; ♂: single male PWN DNA; +: Positive control; -: No template control.

3.5. Mixed Nematode Liquid Test

Nematode liquids were tested, including pure PWNs, PWNs mixed with other species, and pure sets of other species. The results revealed that a single PWN was detected in more than 100 other species of mixed nematodes (Figure 7).

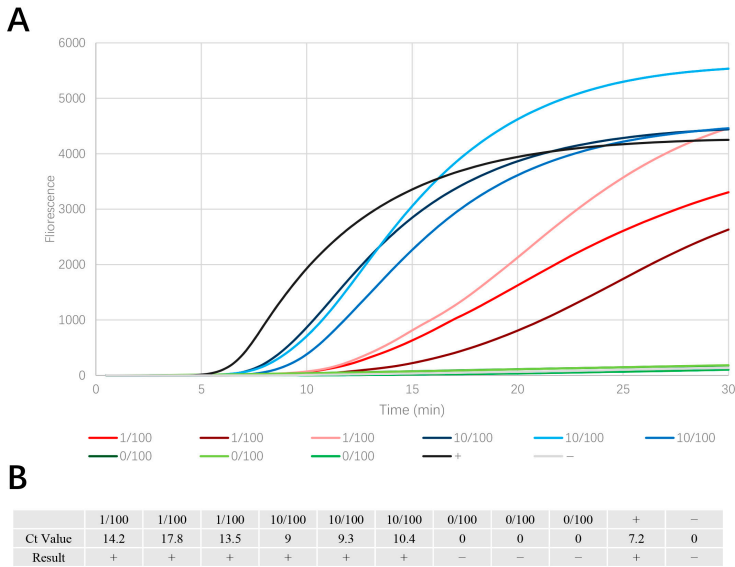


Figure 7. Mixed nematode liquid test. (A) Amplification curves for enzyme-mediated duplex exponential amplification (EmDEA). (B) Table of cycle threshold (Ct) values: 1/100: single PWN in DNA of 100 other species; 10/100: 10 PWN in DNA of 100 other species; 0/100: 0 PWN in DNA of 100 other species; +: Positive control; -: No template control.

3.6. Comparison of the Baermann Funnel Technique and the Sawdust EmDEA Method Based on Real Pinewood Samples

The results are summarized in Table 2. PWNs were detected in 20 out of 36 batches using the Baermann method (1–3000 PWNs/50 g), and all of these batches were also positive by the EmDEA method. A total of 10 batches were negative by both methods, indicating high consistency in non-PWN-infected samples. However, in six batches, PWNs were detected by the EmDEA method but not isolated using the Baermann method. These included two samples from *P. massoniana* in Zhejiang, one sample from *P. tabuliformis* in Zhejiang, one sample from *P. tabuliformis* in Jilin, and two samples from *P. taeda* in the USA. These discrepancies suggest that non-viable or dead PWNs may have been present in these wood samples, which were detected molecularly by EmDEA but could not be recovered by Baermann funnel extraction. Notably, one *P. tabuliformis* sample from Zhejiang was positive only by EmDEA, further supporting its higher sensitivity. In contrast, samples from *P. sylvestris* in Jilin showed full agreement between the two methods.

4. Discussion

A specific and rapid diagnosis of PWN is required for port quarantine, national disease surveys, and control strategy selection. The portable EmDEA assay described herein was reliable for the rapid detection and identification of single nematodes in nematode liquids or pinewood sawdust. This method offers several advantages: it is user-friendly (requiring only two operation steps), portable (<2 kg in total and can be put in a bag), accurate (differentiating PWN from closely related species), and sensitive (detecting as few as 10 copies, equivalent to 32.66 ag of plasmid DNA, or 1/500 of a single adult PWN). It is suitable for different samples, sawdust, nematode liquid samples, and individual nematodes of various juvenile stages. Additionally, it enables rapid detection (<30 min, including the DNA extraction procedure) and does not require specialized equipment, making it more efficient than traditional morphology-based detection techniques and PCR-based molecular methods. The EmDEA assay will be invaluable to those working with PWNs, substantially improve the monitoring and management of PWNs and PWD in forests, and benefit quarantine management.

Compared with other amplification reactions for PWN detection, such as PCR, quantitative real-time PCR, the LAMP method, and the RPA assay, the EmDEA method has the advantages of shorter reaction time and simplicity. First, PCR and quantitative real-time PCR-based PWN detection methods were not suitable for on-site diagnosis since these methods require expensive experimental devices such as PCR machines or electrophoresis devices. Furthermore, the experimental time required to confirm the detection result is more than an hour [10,17–20]. In the LAMP method, four to six primers are used to synthesize target DNA amplicons. If six primers are used, amplification time is reduced, but the probability of cross-contamination by aerosols is increased [21,22]. When four primers are used, however, the amplification time required for detection of the result takes more than an hour [9,11,23]. RPA with the portable optical isothermal device (POID) was developed, and the whole diagnostic procedure was completed within 25 min in the field [14], but after incubation, 25 µL of the SYBR green I dye mixture and 50 µL of the RPA reaction product must be mixed before the UV light detection, which could lead to contamination and make the operation complicated.

Onsite testing requires quick turnaround of results. The EmDEA method achieves this and requires little sophisticated equipment. The EmDEA method accurately detected PWN-infected wood samples, regardless of PWN viability. Therefore, the EmDEA rapid detection method is suitable for initial screening, as shown in Figure 8. If the EmDEA result is negative, the product qualifies for release. If positive, then the product must be further

evaluated using the conventional Baermann funnel technique because quarantine-treated samples with dead PWN may still be positive. Once the nematode is isolated from wood samples, the EmDEA rapid detection system can also help technicians distinguish PWN from other types of nematodes, which increases detection accuracy and efficiency.

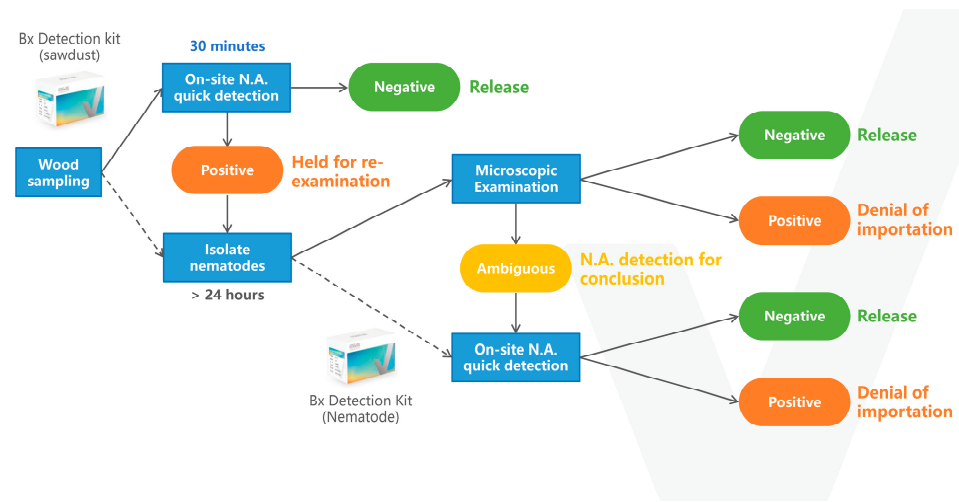


Figure 8. Schematic of the preliminary pine wood nematode screening procedure from wood or packaging wood.

When customs or forestry departments need to detect PWN in pine wood or packaging wood, staff can carry backpacks (containing equipment shown in Figure 1; a portable charger must be carried if no electronics are in the field) to the field. The operational procedures are as follows: Preheat the dry block heater to 95 °C. Drill sawdust from approximately 5 points on the sample wood and collect a mixed sawdust of at least 100 mg. Transfer the sawdust into a tube prefilled with 1 mL lysis buffer and heat at 95 °C for 5 min. Then, pipette 50 µL of the lysate into a tube containing 200 µL dilution buffer and mix thoroughly. Transfer 50 µL of the mixture into the lyophilized pellets (as shown in Figure 2) and shake to dissolve. Put it into the portable optical isothermal device, with results available within 20 min. If negative, no problem. If positive, take the wood sample to the lab, conduct further confirmation using the Baermann funnel method to detect nematodes, perform microscopic identification, or employ the EmDEA method for auxiliary verification.

5. Conclusions

This study described an EmDEA method for detecting PWNs from extracted nematodes or pinewood sawdust. This method comprised an EmDEA molecular test kit and a portable isothermal fluorescence amplification instrument. Moreover, the entire procedure was completed within 30 min. PWNs were detected individually at all life stages, in a mixture of other species, or in pinewood sawdust. The limit was 10 copies (plasmid weight 32.66 ag) or 1/500 of that of a single adult PWN per reaction. This technique can be applied to field-based PWN detection and quarantine inspection for international trade. Future research should focus on primer modifications to allow for the swift identification of other nematode species.

Author Contributions: K.G. and J.G. performed all experiments and wrote the manuscript; X.M., W.D. and Y.F. tested all instruments and reagents; Y.W. and Z.H. designed the primers, probes, and instruments; W.Y. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated and/or analyzed during the current study are available for consultation upon request from the corresponding author.

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Conflicts of Interest: Authors Yao Wu and Zhenxin Hu was employed by the company GeneVide Biotech Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PWN	pinewood nematode
EmDEA	enzyme-mediated duplex exponential amplification

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