Rapid Detection of the *Bursaphelenchus Xylophilus* by Denaturation Bubble-mediated Strand Exchange Amplification

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*Bursaphelenchus xylophilus* (*B. xylophilus*) is one of the most important causal agents of infectious diseases in forest pathology. Obviously, the rapid detection of *B. xylophilus* is an urgent need for its prevention and cure. We have developed a detection method of *B. xylophilus* by strand exchange amplification (SEA). This method could detect $10^5$ copies of genomic DNA of *B. xylophilus*, and it was sufficiently sensitive to detect a single nematode as short as 40 min. Moreover, because the amplification result could be visualized by the naked eyes, the only equipment required throughout the process was a simple isothermal block. Therefore, our method would be a potential for developing on-site detection of *B. xylophilus* to prevent and control its spread.

**Keywords** Isothermal amplification, strand exchange amplification, *Bursaphelenchus xylophilus*, visual detection, on-site detection

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Introduction

*Bursaphelenchus xylophilus* (*B. xylophilus*) is the causal agent of pine wilt disease (PWD),¹ known as pine cancer, which is difficult to prevent and cure. At the beginning of the 20th century, PWN was introduced into Japan from the United States, and became responsible for a massive mortality of native pine trees (*Pinus densiflora*, *P. thunbergii* and *P. luchuensis*) and then spread to China,² Korea³ and Taiwan,⁴ Spain⁵ and many other countries. In recent years, with the rapid development of international trade and the growing amount of communication among countries, the economic damage caused by *B. xylophilus* is becoming increasingly serious.⁶ In order to control further spread of PWD, there is an urgent need for a simple and rapid detection of *B. xylophilus*-infected materials from quarantine to field management.

Different detection methods of *B. xylophilus* have been developed, including morphological observation by microscopic as well as cellular and molecular biomarkers detection.⁶ The traditional morphological observation is to isolate nematodes from wood samples using the Baermann funnel technique⁷ followed by microscopic observation.⁸ However, considering that the pathogenic species *B. xylophilus* and non-pathogenic related species *B. mucronatus* have very similar morphologies,⁹ the operation staff has required specialized knowledge about the nematode morphology, and it is extremely difficult to distinguish the pathogenic species from non-pathogenic related species. In recent years, polymerase chain reaction (PCR)-based detection methods for nematodes have been developed, such as conventional¹⁰ or real-time PCR.¹¹ Real-time PCR can offer an accurate diagnosis of *B. xylophilus*. However, complicated operation and relatively expensive equipment required during the PCR reaction limit it within a laboratory area.¹²,¹³ Thus, a simple and rapid detection method is a very important research goal for the on-site detection of *B. xylophilus*.

Herein, we proposed a simple and robust detection strategy of *B. xylophilus* using the strand exchange amplification (SEA) technique.¹⁴ The SEA technique is identified as isothermal PCR, which only requires a pair of specific primers, and then utilizes successive natural strand “breathing” to unwind double-stranded DNA, rather than heat denaturation.¹⁵ Thus, our strategy was expected to provide a novel nucleic acid amplification method for detecting pinewood nematode, which can be further applied to other target specific detection and electrochemical signal amplification strategy research.¹⁶-¹⁸
Experimental

Materials and reagents

All oligonucleotides used (Table S1 in Supporting Information) in this work were designed by NUPACK software (http://www.nupack.org/) and synthesized by Sangon Biotech (Shanghai, China), respectively. *B. xylophilus* was provided by Qingdao University. The SEA detection kit and colorimetric kit were purchased from Qingdao Navid Biotechnology Co., Ltd. (China). Liquid RNase Erasol was purchased from Solarbio (Beijing, China). All chemical agents were purchased from Sangon Biotech (Shanghai, China).

Nucleic acid extraction from *B. xylophilus*

According to the reference, 19 genomic DNA of *B. xylophilus* were extracted from more number of specimens. Additionally, a simple treatment of *B. xylophilus* sample was performed to be heated at 95°C for 5 min in a lysis buffer and then cooled to room temperature, and used as a template for the SEA reaction.

Primers for SEA reaction

A specific sequence of 28s rDNA was chosen as the target, aligned by using BioEdit software. Finally, the corresponding primers of the SEA reaction were designed (Table S1). The expected size of the amplification products was 40 bp.

SEA reaction

The SEA reaction was performed according to the manufacturer’s instructions. Briefly, the reaction was performed in 10 μL of reaction mixture containing 1 μL of the target, 1.5 × 10^-6 M of each primer of BXF and BXR, and 5.0 μL 2× reaction mix. The reaction mixture was incubated at 61°C, and SEA amplifications were monitored by an isothermal amplification instrument at 1-min intervals or by color changes.

Results and Discussion

The design of SEA to detect *B. xylophilus*

The SEA is a new isothermal nucleic acid amplification method presented recently. To develop a simple and rapid nucleic acid detection method for *B. xylophilus*, herein SEA was used because of its advantages. A pair of specific primers targeting the region of 28S rDNA were designed with an amplification product of 40 bp. The workflow for detecting *B. xylophilus* was shown in Fig. 1. To develop a technique release target using simple methodology compatible with on-site and rapid detection, a *B. xylophilus* sample was firstly heated to 95°C for 5 min to release nucleic acid, and the resulted lysates were directly used as input to initiate the SEA reaction. Single-stranded denaturation bubbles of double-stranded DNA (dsDNA) could be produced at the reaction temperature, which presented a great opportunity for primers to invade and bind to the targets for amplification by DNA polymerase. According to the *Tm* values of primers, the reaction temperature was set at 61°C. To realize the detection requirement of an on-site setting, colorimetric detection was performed using a SEA colorimetric kit. The color of positive reactions changed from light-yellow to red, while the color of a negative reaction remained unchanged. Thus, the SEA method would show great advantages compared with previous methods for detecting *B. xylophilus*, when they were used for on-site and rapid detection. Especially, SEA has been proved to be able to directly detect RNA without any extra transcription step (Fig. S1, Supporting Information), so both DNA and RNA were together detected when a cell was lysed, which might greatly improve the sensitivity of this method for the detection of real samples.

Sensitivity of SEA to detect *B. xylophilus*

The sensitivity of the SEA detection method for *B. xylophilus* was evaluated in Fig. 2. The sensitivity of the SEA assay for the detection of *B. xylophilus* was firstly determined by testing serial 10-fold dilutions of the target DNA fragment, which was obtained by PCR, as described in Materials and Methods. As shown in Fig. 2A, the fluorescence signal showed good regularity with an increase of the target DNA copy number. The fluorescence signals significantly increased with the targets DNA, ranging from 10^5 to 10^7 copies compared with that of NTC. SEA was then carried out using a 10-fold serial dilution of genomic DNA extracted from *B. xylophilus*. The results showed that SEA was able to detect specific genomic DNA at 40 pg in the reaction solution (Fig. 2B). Compared with the target DNA fragment, the fluorescence signal of genomic DNA

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**Fig. 1** Schematic illustration of detecting *B. xylophilus* by the isothermal SEA method.
was late, which could be explained why the denaturation bubble was more difficult to be formed than a short target DNA fragment due to the complicated structure of genomic DNA.

To further validate the feasibility of our method in real sample, a

\textit{B. xylophilus} sample was simply heated to assess for sensitivity of our method. Ten adult \textit{B. xylophilus} were lysed in 10μL of lysis buffer and 2.0, 1.0, and 0.5 μL of the lysate were directly used as templates, respectively. We could see from Fig. 2C that all targets could induce an increase of the fluorescence signal within 40 min, but NTC could not; 0.5 μL of the lysate amounting to 1/2 of one \textit{B. xylophilus} also could be well distinguished from NTC. The good sensitivity of this method for real samples was likely to strongly depend on that the SEA method could detect both DNA and RNA from the lysate. Although existing nucleic acid-based method including our method could detect a single nematode, our method was more advantageous due to the simple sample treatment and a rapid amplification as well as being cost-effective, because of the simple design with a pair of primers and expensive instrument-free (Table 1).

\textbf{Specificity of strand exchange amplification (SEA) for the detection of \textit{B. xylophilus}}

The pathogenic species \textit{B. xylophilus} could induce pine wilt disease to bring great threat to the forest health, but related species \textit{B. mucronatus} with a similar morphology is non-pathogenic. Thus, it is very difficult to distinguish the pathogenic species from a non-pathogenic related species of pinewood nematodes. To examine the specificity of SEA method to detect \textit{B. xylophilus}, 28S rDNA fragments of \textit{B. xylophilus} and \textit{B. mucronatus} were used as targets, respectively. As can be seen in Fig. 3, the fluorescence signal of \textit{B. xylophilus} was significantly increased, and no fluorescence signal was observed from \textit{B. mucronatus} and NTC. These results indicated that the SEA method to detect \textit{B. xylophilus} had good specificity, and could well distinguish \textit{B. xylophilus} from a closely related species, \textit{B. mucronatus}.

\textbf{SEA for RNA of \textit{B. xylophilus}}

SEA reactions were performed with primer BXF and BXR, and monitored by fluorescence and polyacrylamide gel electrophoresis (PAGE). The RNA of \textit{B. xylophilus} was extracted and then amplified by the SEA isothermal amplification and colorimetric reaction, respectively. As can be seen in Fig. 4A, the fluorescence signal of \textit{B. xylophilus} was
significantly increased, and no fluorescence signal was observed from NTC. Correspondingly, as can be seen in Fig. 4B, the expected 40 bp amplification product from *B. xylophilus* appeared in native PAGE (Fig. 4B. Lane 1), while there were no amplification products from NTC (Fig. 4B. Lane 2). The ability of SEA to detect RNA from Fig. S1, these results indicated that the SEA method could one-step detection of RNA.

Our challenge was to realize rapid and low-resource setting detection. In order to achieve this, a visual detection method based on SEA was established by using a SEA colorimetric detection kit. The colorimetric result was shown in the inset Fig. 4A, the color of positive reactions changed from light yellow to red, while the color of a negative reaction was still light yellow. The success of visual colorimetric detection would greatly contribute the application of the SEA method in on-site and inconvenient situations.

**Conclusions**

In this work, a strand exchange amplification method to detect the pine wood nematode *B. xylophilus* was developed. At present, the detection of nematode *B. xylophilus* based on nucleic acid has been reported, some of which are PCR-derived alternating temperature amplification. As a result, PCR-derived methods require expensive instruments, and are too expensive, not portable, or inconvenient in the harsh conditions of remote regions. The others are isothermal amplification methods, such as the loop-mediated isothermal amplification (LAMP), which has been used to detect *B. xylophilus* with high sensitivity and specificity. The drawback of this system is that it requires two pairs of primers, and is very difficult to design for ordinary staff members, which limits widespread use. Overall, the outbreak of PWD highlights the need of a simple and rapid detection method of RNA.20

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**Supporting Information**

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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