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Research Article

Rapid Identification of the Spruce Bark Beetle *Ips typographus* (Linnaeus) Basing on a New Amplification and Analysis Platform

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Abstract

Insects, one of the major disturbance agents, are regarded as a big challenge to forests. Bark beetles (Coleoptera: Curculionidae: Scolytinae) are among the most destructive pests around the world. The European spruce bark beetle *I. typographus* (Linnaeus) is considered the most dangerous species to mature spruce forests throughout Eurasia. In order to improve efficiency, accuracy, and operability of identification, a rapid, simple, highly sensitive and specific screening method is in urgent need. In this study, a rapid classification approach for *I. typographus* was established based on the enzyme-mediated duplex exponential amplification (EmDEA) amplification and analysis platform. The method development process consists of target gene selection, primer design, primer screening, and method validation. Parameter analysis demonstrated that this new method has a detection limit of 1.96×10^3 copies/μL, which is comparable to conventional molecular tools such as PCR. Stable repeatability and high specificity were confirmed by testing 5 samples of *I. typographus* and 4 related beetles. Besides, this screening protocol was easy to use, and could be completed in 30 min. With the advantage of isothermal amplification, this method could be further applied in non-laboratory scenarios such as port rapid screening and wild survey. This rapid screening method for *I. typographus* is believed to assist precise prediction and efficient prevention of exotic insect species.

Keywords

Bark Beetle, *Ips Typographus*, Enzyme-Mediated Duplex Exponential Amplification, Invasive Insect

1. Introduction

Insects, one of the major disturbance agents, are regarded as a grand challenge to forests [1]. Bark beetles (Coleoptera: Curculionidae: Scolytinae) are among the most destructive pests, especially in coniferous stands around the world [2]. Researchers have given an estimation that 8% of the tree loss was due to bark beetles from 1850 to 2000 in Europe [3].

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Meanwhile, they are ecologically significant as early decomposers when they attack dead or dying trees in the forest ecosystem [4]. Normally, bark beetles live in close association with many arthropods, as well as filamentous fungi and yeasts [5].

The European spruce bark beetle *Ips typographus* (Linnaeus) is considered the most dangerous species to mature spruce forests throughout Eurasia [6]. Its main host is the Norway spruce, *Picea abies*. It also live on trees from *Pinus*, *Larix*, *Abies* or *Pseudotsuga* [7]. As a true tree-killer, *I. typographus* lives in symbiosis with the fungus *Endoconidiophora polonica* which can aid the insect by weakening tree defense system through over-stimulation [8, 9]. Dead or dying spruce trees are preferred breeding sites for this bark beetle. Outbreak of the population of *I. typographus* after windfalls and droughts pose a great crisis to mature spruce stands [10]. In addition, climate change, like temperature in most cases, could influence the dynamic of insect population [11]. In a nutshell, *I. typographus* ranks among the primary destructive pests to coniferous forests.

By contrast, other projects have also described the ecological importance of *I. typographus* as a keystone species, aiding forest regeneration and acting as an ecosystem engineer [7]. Despite being an early decomposer, this beetle lives in diverse relationships with numerous arthropods and microbes, thus contributing to diversity of forest ecosystems [12].

Nowadays, the problem of pest transmission along with international trade is gaining more and more attention. For the safety of local ecosystem, exotic species, especially invasive species and pests, must be stopped at the border [13]. It is highly important to take effective and efficient measures for port inspection [14]. For insect inspection, commonly used methods are morphological identification, and molecular assays such as PCR. The morphological method relies heavily on the professional taxonomic background. Molecular methods need to be conducted in labs, and often take a long time. Developing a rapid, easy-to-use, sensitive, and specific approach for the identification of *I. typographus* is essential.

In this survey, an easy-to-handle method was established for rapid screening of *I. typographus* based on a new amplification and analysis platform, enzyme-mediated duplex exponential amplification (EmDEA). This method would be suitable for application in ports and wild surveys.

2. Materials & Methods

2.1. Sample Preparation & DNA Extraction

Five samples of *I. typographus* and 4 other related bark beetles (Table 1) were collected and tested using the newly established assay. The recombinant plasmid containing the selected target sequence was synthesized by BGI Co., Ltd (Guangdong) and used accordingly in method establishment. DNA extraction from beetle samples was performed using a rapid protocol [15].

2.2. Target Region Selection & Primer Design

Based on these genetic data of *I. typographus* in GenBank, the ITGT1B6 microsatellite sequence (accession no. AY243329.1) was selected for primer design which was carried out by GeneVide Biotech Co., Ltd.. Initial primers consisted of 6 RNA primers, 6 upstream DNA primers, and 6 downstream DNA primers (Table 2). These RNA primers were all modified with FAM and BHQ1 at both ends, respectively.

2.3. Establishment of EmDEA Assay

The reaction mixture consisted of 0.2 μL of RNA primer (250 ng/μL), 0.5 μL of each DNA primer (20 μM), 1.8 μL ddH_2O (RNase free), 5 μL template and 12 μL activation buffer (from Fluorescent isothermal amplification assay blank kit, GeneVide Biotech Co., Ltd. Jiangsu). Add all these reagents into a PCR tube containing dried enzyme powder (from Fluorescent isothermal amplification assay blank kit, GeneVide Biotech Co., Ltd. Jiangsu). Amplification and analysis were performed on a Roche LightCycler 480 II (Switzerland). A total of 30 cycles were performed, each at 42 ℃ for 1 min. The fluorescence signal was collected at the end of each cycle.

Initial primers went through 3 steps of selection with the recombinant plasmid as a template to achieve the final primer set for amplification. First step was to select the most efficient RNA primer according to the primer combination series 1 (Table 3). Second and third steps were for the most suitable downstream and upstream DNA primers based on primer combination series 2&3 (Table 4&5), respectively.

2.4. Method Validation, Specificity and Sensitivity Analysis

Five samples of *I. typographus* were used for method validation and specificity analysis with 4 other bark beetles (Table 1). For sensitivity test, the recombinant plasmid was employed accordingly.

The ddH_2O (RNase free) was used as a negative control. Each treatment had three repetitions.

3. Result & Analysis

3.1. Primer Selection

After first round of primer selection, RNA primer 3 was chosen based on the Ct and final fluorescence value. Primer combination of RNA3F3R4 had the lowest Ct and the highest final fluorescence value. Fix RNA primer 3 and upstream DNA primer 3 (F3), the downstream DNA primer 4 (R4) gave the best result. Fix RNA primer 3 and downstream DNA primer 4 (R4), the upstream DNA primer 6 (F6) had the lowest Ct and highest final fluorescence value.

3.2. Method Parameter Analysis

Method validation and specificity analysis were conducted in one survey using 5 *I. typographus* samples and 4 related beetles. The amplification result showed that all *I. typographus* samples expressed positive curves, while other insects had negative ones (Figure 1), demonstrating the high feasibility and specificity of this new method.

Sensitivity test was carried out using the recombinant plasmid which was diluted into concentration series from 1.96×10^5 copies/μL to 1.96×10^0 copies/μL, respectively. Altogether, 6 solutions of recombinant plasmid were used, and only the concentrations of 1.96×10^5 copies/μL, 1.96×10^4 copies/μL, and 1.96×10^3 copies/μL had positive amplification curves (Figure 2). Thus, the detection limit of this new method was 1.96×10^3 copies/μL.

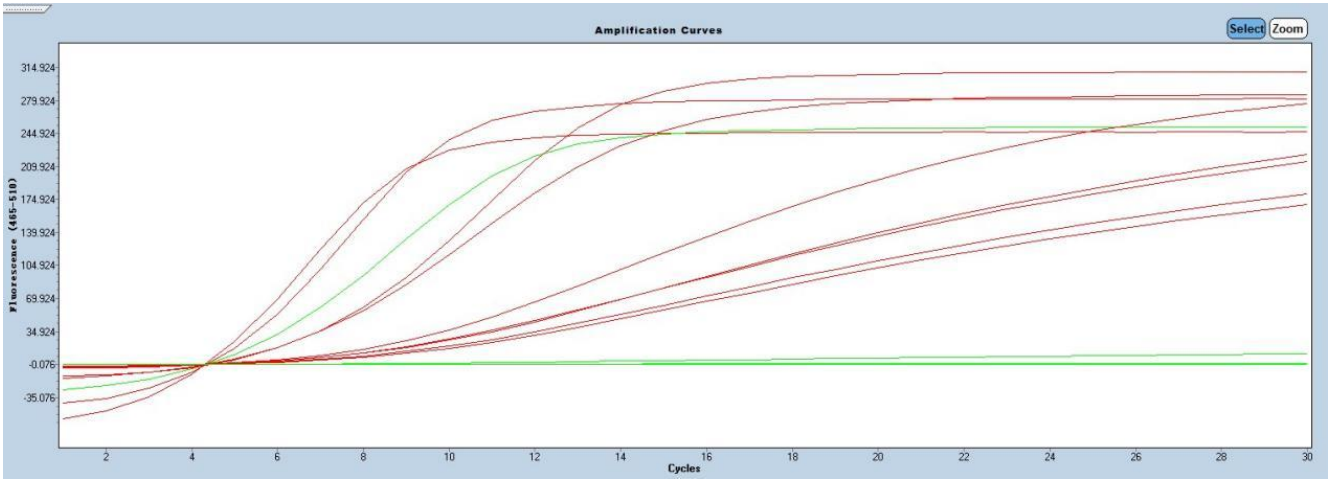

Figure 1. *Method validation and specificity analysis result.*

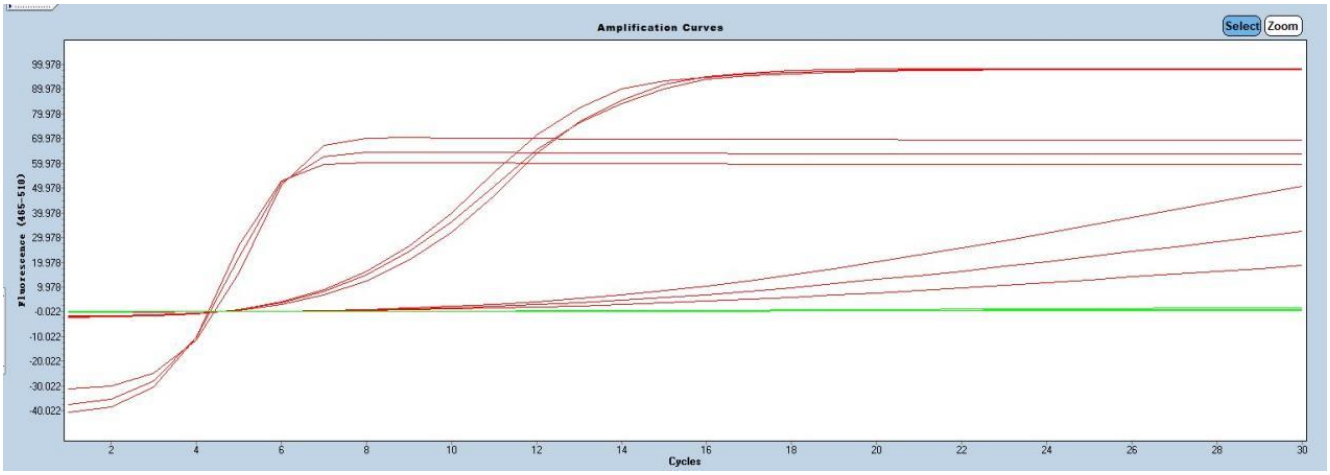

Figure 2. *Sensitivity test result.*

4. Discussion

Exotic species, of which a subset are invasive, form a grand challenge to local ecosystems globally [16]. The accelerated speed of commercial and social globalization provides numerous opportunities for non-native species to move into new parts of the world [17]. They could spread, get established and exert an influence on native biodiversity. Alien species could influence local biodiversity through both direct and indirect impacts. The former includes food web interactions, and hybridization with native species, while the latter is related to pathogen vectors, and resource competition [18]. Once an alien species becomes invasive, it could lead to environmental, economic, as well as human health problems. Meanwhile, their interactions with both native and other exotic species make them increasingly difficult to control. As they move outside the native range, they get rid of population-controlling predators, parasitoids, and pathogens and come into contact with local plants and animals. Exotic insects cause serious issues in ecosystem balancing, industrial development, food

supply, and human health. Researchers have predicted that invasive insects would be the most widespread and underestimated factor of great challenges to sustainable development and resilient economy, both locally and globally [19].

A true invasion usually consists of several steps: occasional arrival, establishment, and further spread. Responses range from prediction and prevention, to early detection and rapid response, to mitigation and management [20]. Obviously, efforts towards earlier stage of invasion are more cost-effective, if they work. To assist precise prediction and efficient prevention, more sophisticated methods are required to detect insects *en route*.

In this study, a rapid screening approach for *I. typographus* was established based on the EmDEA amplification and analysis platform. Parameter analysis demonstrated that this new method has a detection limit of 1.96×10^3 copies/μL, which is comparable to conventional molecular tools such as PCR. Stable repeatability and high specificity were confirmed by testing 5 samples of *I. typographus* and 4 related beetles. Besides, this screening protocol was easy to use, and could be completed in 30 min. With the advantage of isothermal amplification, this method could be further applied in non-laboratory scenarios such as port rapid screening and wild survey. To improve identification efficiency, a fast, simple DNA extraction protocol for insect samples, especially larvae and debris, is needed. Traditional identification methods based on morphological characteristics for insect samples could not handle debris, and lose accuracy for larvae.

5. Conclusion

Insects, one of the major disturbance agents, are regarded as a big challenge to forests. Bark beetles (Coleoptera: Curculionidae: Scolytinae) are among the most destructive pests around the world. The European spruce bark beetle *I. typographus* is considered the most dangerous species to mature spruce forests throughout Eurasia. In order to improve efficiency, accuracy, and operability of identification, a rapid, simple, highly sensitive and specific screening method is in urgent need. In this study, a rapid screening approach for *I. typographus* was established based on the EmDEA amplification and analysis platform. The method development process consists of target gene selection, primer design, primer screening, and method validation. Parameter analysis demonstrated that this new method has a detection limit of 1.96×10^3 copies/μL, which is comparable to conventional molecular tools such as PCR. Stable repeatability and high specificity were confirmed by testing 5 samples of *I. typographus* and 4 related beetles. Besides, this screening protocol was easy to use, and could be completed in 30 min. Non-professionals without molecular background can operate with simple training. With the advantage of isothermal amplification, this method could be further applied in non-laboratory scenarios such as port rapid screening and wild survey. This rapid screening method for *I. typographus* is believed to assist precise prediction and efficient prevention of exotic insect species. Moreover, accurate species identification could also aid in monitoring insect population dynamics and evolutionary researches.

6. Recommendations

This study provides useful data and methodology for further researches on quarantine and detection of specific plant pests in non-lab settings. Based on this new method, a corresponding amplification and analysis machine can be designed, which should be small in size, and light in weight.

Abbreviations

EmDEA	Enzyme-Mediated Duplex Exponential Amplification
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction

Author Contributions

Wang Jiaying: Funding acquisition, Investigation, Writing – original draft

Cui Junxia: Project administration

Yan Shuyi: Investigation

Liu Li: Data curation, Investigation, Resources

Chen Xianfeng: Conceptualization, Supervision

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Conflicts of Interest

The authors declare no conflicts of interest.

Appendix

Table 1. *Sample list.*

No.	Sample name	Source
1	*Ips typographus*	reserved sample
2	*Ips typographus*	donated by Nanjing Customs
3	*Ips typographus*	reserved sample
4	*Ips typographus*	reserved sample
5	*Ips typographus*	reserved sample
6	*Ips sexdentatus*	donated by Nanjing Customs
7	*Ips grandicollis*	donated by Nanjing Customs
8	*Xylosandrus germanus*	donated by Guangzhou Customs
9	*Dendroctonus terebrans*	reserved sample

Table 2. *Primer list.*

Primer Name	Seq (5'-3')
1-Ips-F1	AAGCTAATACGACTCACTATAGGGTTAGATCTCGTTAAAACAATATTGCATA
1-Ips-F2	AAGCTAATACGACTCACTATAGGGCTCGTTAAAACAATATTGCATAATGATC
1-Ips-F3	AAGCTAATACGACTCACTATAGGGAAAACAATATTGCATAATGATCTTAAAG
1-Ips-F4	AAGCTAATACGACTCACTATAGGGATATTGCATAATGATCTTAAAGTGCTAA
1-Ips-F5	AAGCTAATACGACTCACTATAGGGCATAATGATCTTAAAGTGCTAATAATAA
1-Ips-F6	AAGCTAATACGACTCACTATAGGGGATCTTAAAGTGCTAATAATAACACACA
1-Ips-RNA1	AAAGAGGUGAGUCGAGUGUGUGUGUG
1-Ips-RNA2	AAACUAAAAGAGGUGAGUCGAGUGUGUG
1-Ips-RNA3	AUCGGAAAACUAAAAGAGGUGAGUCGAG
1-Ips-RNA4	UGUUAGAUCGGAAAACUAAAAGAGGUGA
1-Ips-RNA5	CGUUGCUGUUAGAUCGGAAAACUAAAAG
1-Ips-RNA6	AAUUUUCGUUGCUGUUAGAUCGGAAAAC
1-Ips-R1	TCCGTCGATTTTCCTGGGGCTGAAAACA
1-Ips-R2	CTTTTTTCCGTCGATTTTCCTGGGGCTG
1-Ips-R3	GAGACTCTTTTTTCCGTCGATTTTCCTG
1-Ips-R4	GCGGTGGAGACTCTTTTTTCCGTCGATT
1-Ips-R5	AGTGGTGCGGTGGAGACTCTTTTTTCCG
1-Ips-R6	AGGTCCAGTGGTGCGGTGGAGACTCTTT

Table 3. RNA primer selection series.

No.	Primer combination	RNA primer	Upstream DNA primer	Downstream DNA primer
1	RNA1F3R3	1-WA1-RNA1	1-WA1-F3	1-WA1-R3
2	RNA1F3R4	1-WA1-RNA1	1-WA1-F3	1-WA1-R4
3	RNA1F4R3	1-WA1-RNA1	1-WA1-F4	1-WA1-R3
4	RNA1F4R4	1-WA1-RNA1	1-WA1-F4	1-WA1-R4
5	RNA2F3R3	1-WA1-RNA2	1-WA1-F3	1-WA1-R3
6	RNA2F3R4	1-WA1-RNA2	1-WA1-F3	1-WA1-R4
7	RNA2F4R3	1-WA1-RNA2	1-WA1-F4	1-WA1-R3
8	RNA2F4R4	1-WA1-RNA2	1-WA1-F4	1-WA1-R4
9	RNA3F3R3	1-WA1-RNA3	1-WA1-F3	1-WA1-R3
10	RNA3F3R4	1-WA1-RNA3	1-WA1-F3	1-WA1-R4
11	RNA3F4R3	1-WA1-RNA3	1-WA1-F4	1-WA1-R3
12	RNA3F4R4	1-WA1-RNA3	1-WA1-F4	1-WA1-R4
13	RNA4F3R3	1-WA1-RNA4	1-WA1-F3	1-WA1-R3
14	RNA4F3R4	1-WA1-RNA4	1-WA1-F3	1-WA1-R4
15	RNA4F4R3	1-WA1-RNA4	1-WA1-F4	1-WA1-R3
16	RNA4F4R4	1-WA1-RNA4	1-WA1-F4	1-WA1-R4
17	RNA5F3R3	1-WA1-RNA5	1-WA1-F3	1-WA1-R3
18	RNA5F3R4	1-WA1-RNA5	1-WA1-F3	1-WA1-R4
19	RNA5F4R3	1-WA1-RNA5	1-WA1-F4	1-WA1-R3
20	RNA5F4R4	1-WA1-RNA5	1-WA1-F4	1-WA1-R4
21	RNA6F3R3	1-WA1-RNA6	1-WA1-F3	1-WA1-R3
22	RNA6F3R4	1-WA1-RNA6	1-WA1-F3	1-WA1-R4
23	RNA6F4R3	1-WA1-RNA6	1-WA1-F4	1-WA1-R3
24	RNA6F4R4	1-WA1-RNA6	1-WA1-F4	1-WA1-R4

Table 4. Downstream DNA primer selection series.

No.	Primer combination	RNA primer	Upstream DNA primer	Downstream DNA primer
1	RNA3F3R1	1-WA1-RNA3	1-WA1-F3	1-WA1-R1
2	RNA3F3R2	1-WA1-RNA3	1-WA1-F3	1-WA1-R2
3	RNA3F3R3	1-WA1-RNA3	1-WA1-F3	1-WA1-R3
4	RNA3F3R4	1-WA1-RNA3	1-WA1-F3	1-WA1-R4
5	RNA3F3R5	1-WA1-RNA3	1-WA1-F3	1-WA1-R5
6	RNA3F3R6	1-WA1-RNA3	1-WA1-F3	1-WA1-R6

Table 5. Upstream DNA primer selection series.

No.	Primer combination	RNA primer	Upstream DNA primer	Downstream DNA primer
1	RNA3F1R4	1-WA1-RNA3	1-WA1-F1	1-WA1-R4
2	RNA3F2R4	1-WA1-RNA3	1-WA1-F2	1-WA1-R4
3	RNA3F3R4	1-WA1-RNA3	1-WA1-F3	1-WA1-R4
4	RNA3F4R4	1-WA1-RNA3	1-WA1-F4	1-WA1-R4
5	RNA3F5R4	1-WA1-RNA3	1-WA1-F5	1-WA1-R4
6	RNA3F6R4	1-WA1-RNA3	1-WA1-F6	1-WA1-R4

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