Communication

Monochamus Saltuarius Endangers Pinus tabuliformis Carr. and Carries Bursaphelenchus xylophilus (Steiner and Buhrer) in China

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Abstract: The pinewood nematode (Bursaphelenchus xylophilus) can cause fatal damage to trees and is transmitted by the vector species of the Monochamus genus. In December 2017, pinewood nematodes were found to be harming a large plot of pine trees, Pinus tabuliformis, in a new region with an average annual temperature of 6.7 °C in China. However, the vector insects were unknown and urgently needed to be identified. Hence, in April 2018, we collected wood sections of P. tabuliformis trees that had died from pine wilt disease. All 127 longhorn beetles that emerged from the P. tabuliformis samples were identified as Monochamus saltuarius, and the nematodes they carried were fourth-stage dispersal juveniles of the pinewood nematode. The carrier rate of pinewood nematodes in the M. saltuarius population was 58.3%, and the average carrying capacity was 642.4 ± 89.3. In the same P. tabuliformis plot, 742 M. saltuarius beetles were collected from hormone traps. Our results revealed that M. saltuarius can damage P. tabuliformis and is a new vector species of the pinewood nematode in China. These findings can inform the prevention and control of pinewood nematode damage to pine forests.

Keywords: vector insect; pinewood nematode; dispersal juveniles

1. Introduction

Pine wilt is a complex disease caused by the pinewood nematode, Bursaphelenchus xylophilus (Steiner and Buhrer) Nickle. Distribution of the pinewood nematode requires vector insects, which are typically Monochamus spp. [1,2]. When the external environment of the pinewood nematode changes unfavorably, it will transform from a propagative juvenile to a dispersal juvenile. The dispersal-stage juveniles are able to endure lower temperatures and gather around the pupal chamber of Monochamus beetles [3,4]. Monochamus beetles can carry a large number of four-stage dispersal juveniles in their surface respiratory trachea and reproductive system after eclosion from pines killed by pine wilt disease. These Monochamus migrate to healthy pine trees, thereby spreading the pine wilt disease when feeding on the tender branches [5–7].

In China, pinewood nematodes are distributed in areas where the average annual temperature is above 10 °C [8,9] by the vector insect, Monochamus alternatus [10,11]. However, the pinewood nematode has continuously adapted to low-temperature environments and has expanded its distribution from South to North. In the process, it has also adapted to new insect vectors. Fushun City, Liaoning Province is located in the middle of the temperate zone influenced by China’s monsoons. Its average annual temperature is 6.7 °C with a minimum temperature of around −30 °C in winter [12]. In 2018, the pinewood nematode was found to have severely damaged Pinus tabuliformis in Fushun City,
and the vector insect urgently needed to be identified to provide a theoretical basis for the prevention and control of pine wilt disease in this district. Hence, we collected wood sections of *P. tabuliformis* that were dying from pine wilt disease to identify the vector species of the pinewood nematode. Our investigation identified the new vector of the pinewood nematode, and our findings can be used to inform efforts to prevent and control pinewood nematode infestations.

2. Materials and Methods

2.1. Collection and Morphological Identification of *M. saltuarius*

In February 2018, in the surrounding area of Dahuofang Reservoir and Nanzamu Town in Fushun City, Liaoning Province, three sample plots of *P. tabuliformis* that were seriously damaged by pinewood nematodes were selected. In each sample plot, five wood sections were collected from randomly selected dead *P. tabuliformis* that had entering holes made by longhorn beetles. The diameter at breast height of the wood sections was 20–30 cm and the length 50–80 cm. The cross-sections of the collected wood were sealed with sealing wax and placed in a rearing cage at room temperature. The longhorn beetles that emerged from the blocks of *P. tabuliformis* were collected and counted. Emerged adults were observed under binocular continuous zoom stereo microscopes, and identified using a catalogue of longhorn beetle species [13]. Additionally, ten *Monochamus* sex pheromone traps (Lisen, China) were set in each of the above three sample plots in July 2018. The traps were suspended at a height of 1.5 m and were spaced 200 m apart. The longhorn beetles in the trap were collected and counted every seven days for one month. The *M. saltuarius* specimens were identified and counted.

2.2. Extraction and Morphological Identification of the Pinewood Nematode

The emerged adult longhorn beetles were cut into pieces and soaked in a water-containing petri dish (d = 12 cm) for 12 h. Then, the carrier rate and carrying capacity of the longhorn beetles for nematodes were determined using a microscope. This confirmed the source of the pinewood nematodes [14]. The nematodes in the bodies of longhorn beetles were picked up using a 10 mL pipette and dropped onto a glass slide. The slide was placed over the flame of an alcohol lamp for 3–5 s. After the pinewood nematodes were heat-killed, they presented a “(" shape. A temporary slide of the pinewood nematode was made for observing and measuring its morphology. According to the findings of previous studies, all the nematodes in the longhorn beetles were fourth-stage dispersal juveniles [15].

2.3. Molecular Identification of the Pinewood Nematode

Fifty nematodes were selected under a microscope using a 10 mL pipette. Their DNA was extracted according to the manufacturer’s instructions of the DNA preparation kit, AP-MN-MS-GDNA-50, purchased from Axygen, USA. The PCR (Polymerase Chain Reaction) reaction volume was 25 µL in total, which included 12.5 µL PCR Mix; 1 µL each of upstream and downstream primers; 1 µL DNA template; and 9.5 µL of ddH2O [16]. The upstream primer used was 5′-CGTAACAAGGTAGCTGTA-3′ and downstream was 5′-TTTCATCGCCGTTACTAAGG-3′. The PCR procedure was performed as follows: 94 °C initial denaturation for 2 min; 35 cycles of 94 °C denaturation for 1 min, 55 °C annealing for 1 min and 72 °C extension for 1 min; followed by the final extension at 72 °C for 5 min [17].

3. Results

3.1. Morphological Identification and Emergence Number of *M. saltuarius*

In April 2018, 127 longhorn beetles emerged from the collected wood sections of *P. tabuliformis*. After morphological evaluation, the body surface of the longhorn beetles was found to be black or brown with a tint of bronze luster and two small yellow spots in front of the pronotum. The yellow-brown villi on the elytral surface were relatively dense with many light yellow or white spots that formed
three bands. The antennae of males were black, while the bases of the females’ antennae had light grey rings (Figure 1C–F). The 71 male and 56 female adult longhorn beetles were identified as *M. saltuarius* (Gebler, 1830). In July 2018, a total of 1663 longhorn beetles were collected in 30 traps in the same sample plots (Figure 1A,B). Of those, 742 were *M. saltuarius*.

**Figure 1.** Morphological characteristics of *M. saltuarius* and affected *P. tabuliformis* in the field. (A, B) *P. tabuliformis* affected by *M. saltuarius* and *Bursaphelenchus xylophilus* in the field; (C) female *M. saltuarius* and emergence hole on *P. tabuliformis;* (D) the reverse side of male *M. saltuarius;* (E) the right side of male *M. saltuarius;* (F) the side face of male *M. saltuarius.*
3.2. Morphological Identification, Carrier Rate and Carrying Capacity of the Pine Wood Nematode

The nematodes were isolated from *M. saltuarius* and were found to have no stylet or median esophageal bulb (Figure 2B). The nematode bodies (excluding the heads) were filled with a large number of fat granules (Figure 2C). Their tails were tapered (Figure 2D). The observed characteristics are consistent with the features of fourth-stage dispersal juveniles. Of the 127 *Monochamus saltuarius*, 74 carried the pinewood nematode (carrier rate of 58.3%). The average carrying capacity of *M. saltuarius* of pinewood nematodes was found to be 642.4 ± 89.3. Under normal temperature conditions, after 48 h, most of the fourth-stage dispersal juveniles turned into adults in the distilled water. Figure 2E (male) and Figure 2G (female) show the adult form of the pinewood nematodes that had transformed from fourth-stage dispersal juveniles. The observed characteristics (Figure 2F,H,I) are consistent with the features of the pinewood nematode.

Figure 2. Morphological characteristics of *Bursaphelenchus xylophilus* isolated from *M. saltuarius*. (A–D) fourth-stage dispersal juveniles of *B. xylophilus*; (E–I) adult *B. xylophilus*. 
3.3. Molecular Identification of the Pinewood Nematode

PCR amplification and sequencing were conducted with the sequence-specific primers of ITS (Internal Transcribed Spacer) and a template of nematode DNA. The length of the fragment was 815bp. The sequence was blasted with sequences from the NCBI, and the results indicated that its similarity and coverage were 100% with an E-value of 0 when aligned with KF025323.1, EF446943.1 and AB294736.1. The above molecular sequencing results indicated that the nematodes in M. saltuarius were the Bursaphelenchus xylophilus.

4. Discussion and Conclusions

A total of 127 M. saltuarius emerged from the collected wood sections of dead P. tabuliformis. Combined with the damage characteristics (emergence holes, frass holes and tree entering holes) and a large number of M. saltuarius found in the traps, comprehensive assessment proved that M. saltuarius was the vector species of the nematodes that harmed P. tabuliformis. There are no existing reports on the damage of P. tabuliformis by M. saltuarius under natural conditions, so our study is the first to prove that P. tabuliformis was the host of M. saltuarius [18].

There are 13 species of vector insects of the pinewood nematode, all of which belong to Monochamus [19]. In this study, we isolated the nematodes from collected M. saltuarius and comprehensively determined that the pinewood nematodes were fourth-stage dispersal juveniles through morphological and molecular biological methods [20]. The above results proved that M. saltuarius was the vector insect transmitting the pinewood nematode in the P. tabuliformis forest in China that had experienced large-scale outbreaks of pine wilt disease [21].

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