Newly Discovered Transmission Pathway of *Bursaphelenchus xylophilus* from Males of the Beetle *Monochamus alternatus* to *Pinus densiflora* Trees via Oviposition Wounds

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Abstract: The transmission of *Bursaphelenchus xylophilus* from *Monochamus alternatus* males to *Pinus densiflora* trees via oviposition wounds has been determined. Nematode-infested males, with mandibles fixed experimentally to prevent feeding, were placed for 48 hours with pine bolts containing oviposition wounds that had been made by nematode-free females. After removal of the nematode-infested males, the pine bolts were held for 1 month and then examined for the presence of nematodes. Reproducing nematode populations were recovered from pine bolts that were exposed to male beetles carrying a high number of nematodes. No reproducing nematode population could be recovered from pine bolts exposed to beetles with a small number of nematodes. Nematode reproduction in the pine bolts was not related to the number of oviposition wounds per bolt. Fourth-stage dispersal *B. xylophilus* juveniles, collected from beetle body surfaces, were inoculated on pine bolt bark 0, 5, 10, and 15 cm away from a single artificial, small hole. These dauer juveniles successfully entered some bolts. The probability of successful nematode reproduction decreased with increased distance between inoculation point and artificial hole. The results indicated that *B. xylophilus* can move a significant distance to oviposition wounds along the bark surface and enter a tree via the wounds. The new transmission pathway is considered important for the nematode to persist in pine forests such as in North America where pine wilt disease does not occur.

Key words: *Bursaphelenchus xylophilus, Monochamus alternatus,* multiple infection, nematode movement, oviposition wound, *Pinus densiflora,* transmission.

The pinewood nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle is distributed in East Asia and North America (Mamiya, 1987) and recently has been introduced into Portugal (Mota et al., 1999). The nematode causes pine wilt disease in stressed or susceptible tree species (Kiyohara and Tokushige, 1971). Pine wilt disease has been an epidemic in East Asia but is not symptomatic in pine forests native to North America, where pines are in their native range (Rutherford and Webster, 1987). In North America, pine species susceptible to pine wilt disease experience a relatively short and cool summer climate, perhaps engendering a level of resistance relative to pine species growing in a hot summer climate (Rutherford and Webster, 1987).

The nematode is transported as fourth-stage dispersal juveniles to new host trees by cerambycid beetles of the genus *Monochamus. Monochamus alternatus* Hope carries pinewood nematode in Japan (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972; Togashi, 1985), whereas *M. carolinensis* (Olivier), *M. mutator* LeConte, *M. scutellatus* (Say), and *M. titillator* (Fabricius) are the vector insects in North America (Edwards and Linit, 1992; Linit, 1990; Luzzi et al., 1984; Wingfield, 1983; Wingfield and Blanchette, 1983). When young adult beetles emerge from dead pine trees, they begin feeding on the twig bark of healthy pine trees. It is during this maturation feeding that the pinewood nematode is transmitted to healthy trees through the feeding wounds (Linit, 1988). Rapid wilting is induced following infection of susceptible pines as the nematode begins to reproduce rapidly in the tree (Mamiya, 1983). Reproductively mature beetles are attracted to dying or recently killed trees, and walk about on the tree trunk surface to copulate and oviposit (Fauziah et al., 1987; Togashi, 1989). The female beetles use their mandibles to disrupt the bark surface so that they can insert their ovipositors under the bark. The female beetles transmit the nematode into the trees via oviposition wounds they incur (Edwards and Linit, 1992; Wingfield, 1983). Progeny beetles carrying the nematodes emerge the following early summer.

Transmission of *B. xylophilus* via oviposition wounds on dying or freshly dead trees incapable of mounting a resistance response is necessary for the nematode to persist in a pine forest ecosystem where it does not cause pine wilt disease (Togashi and Jikumaru, 1996; Wingfield and Blanchette, 1983). In this case, only the nematodes carried by female beetles contribute to the population persistence. Horizontal transmission of nematodes from male to female beetles during mating (Edwards and Linit, 1992; Togashi and Jikumaru, 1996) contributes partly to the persistence of the nematode population because some horizontally transmitted nematodes can enter the tree body via oviposition wounds made by the female beetles (Edwards and Linit, 1992; Togashi and Arakawa, unpubl. data). Thus, this transmission pathway starting from male beetles is suspected to be a relatively minor pathway. In other words, the transmission by female beetles via oviposition wounds is considered to give individual nematodes initially carried by female beetles a greater opportunity of entering pine trees—a component of nematode fitness—than those carried by male beetles because they need to move to female beetles. Interestingly, there is no difference in the initial nematode load between

male and female beetles (Linit et al., 1983; Wingfield and Blanchette, 1983), suggesting that the sexes of beetle vectors do not affect the fitness of individual transported nematodes. However, the possibility of a transmission pathway of nematodes directly from male beetles to dying trees has not been studied.

This research was designed to test the hypothesis that male beetles transmit the nematode to dying trees via conspecific oviposition wounds. It also was designed to observe the nematode movement to artificial wounds from a distance. Finally, a new transmission pathway is discussed as it relates to the spread of pine wilt disease range and the role of multiple infection by a specific parasite in the evolution of virulence (Baalen and Sabelis, 1995; Bonhoeffer and Nowak, 1994; Leung and Forbes, 1998; May and Nowak, 1995; Nowak and May, 1994).

Materials and Methods

Insects: Monochamus alternatus adults infested with *B. xylophilus* were obtained from *Pinus densiflora* recently killed as a result of infection with *B. xylophilus*. More than 80 dead trees were collected between October and December in 1995 to 1999 in Tokuyama City, Yamaguchi Prefecture. They were cut into 70-cm-long logs and were hauled to a *P. densiflora* stand in Higashi-Hiroshima City, Hiroshima Prefecture. The logs were placed in cases in the pine stand in late April to early May. The next year *M. alternatus* adults emerging from the logs were collected daily between May and August and were placed individually in small plastic cases (17.3 cm × 8.5 cm × 4.1 cm tall). The beetles were fed on *P. densiflora* and *P. thunbergii* twigs. The twigs were renewed in the cages at 3 to 4-day intervals. The number of *B. xylophilus* carried by individual beetles varied greatly, and some beetles were contaminated with other parasitic nematodes. To select beetles infested with many *B. xylophilus* but free of other parasitic nematodes, pine twigs on which 10-day or older beetles had fed were chipped and the nematodes extracted and identified. The chipped twig was suspended at 25 ℃ for 2 to 3 days in a Baermann funnel.

Nematode-free female beetles used in experiment 1 were progeny of laboratory populations originating from Tokuyama City, Yamaguchi Prefecture, and Shika Town, Ishikawa Prefecture. These beetles were reared in vitro from eggs collected from *P. densiflora* branches. Newly hatched larvae were inoculated onto 70-cm-long, nematode-free *P. densiflora* logs: The larvae were kept at 25 ℃ for 4 to 5 months under a fixed 12 hours light-12 hours dark photoperiod. They were then stored for 4 to 5 months at 10 ℃ to terminate the larval diapause. The logs containing the post-diapause larvae were returned to the original conditions of 25 ℃. The beetles emerging from the inoculated logs were then individually reared for about 1 week on excised twigs of *P. densiflora* and *P. thunbergii* in small plastic cases. Next, the beetles were paired and supplied with *P. densiflora* branch sections (about 5 cm long and 1.5 to 3.0-cm diam.) as oviposition substrate. The twigs and branch sections were renewed every 3 to 4 days. Pine branch sections were examined for the presence of oviposition wounds as an indication of the reproductive maturation of females. Reproductively mature females were isolated from pine branch sections for several days before use to increase oviposition-driving forces. The Baermann funnel technique was used to examine a subsample of female beetles ($n = 9$). None of the female beetles examined carried nematodes.

Pine bolts: Thin trees and boughs of *P. densiflora* were collected in Higashi-Hiroshima City, Hiroshima Prefecture. Those showing no wounds on the bark surface were cut into 7-cm and 17-cm-long sections without nodes for experiments 1 and 2, respectively. These bolts were soaked in a sufficient amount of hot water at a temperature of about 70 ℃ to kill the plant cells and then placed at room temperature. Five to 12 hours later, the bolts were washed with running tap water and air dried for 0.5 to 1 hour. The cut ends of bolts were sealed with paraffin (melting point, 56 to 58 ℃) to prevent nematodes from entering bolts via cut ends and to limit desiccation. The bolts were kept for short-term storage at 25 ℃ or were preserved for longer times at 5 ℃. Those bolts kept in long-term storage were transferred to 25 ℃ 1 day before use in each test. Mean diameter of bolts was 3.40 cm (SD = 0.33 cm, $n = 20$) and 2.68 cm (SD = 0.40 cm, $n = 40$) for experiments 1 and 2, respectively. In experiment 2, the bolts were separated into three classifications based on the texture of bark surface (Fig. 1). Random sampling of the bolts ($n = 15$) and extraction in a Baermann funnel showed the bolts to be free of *B. xylophilus*.

Containers for experiments: Small transparent plastic containers (16.0 cm × 9.0 cm at the top, 14.5 cm × 7.5 cm at the bottom, × 10.5 cm deep) were used for experiment 1 (Togashi and Jikumaru, 1996). A shallow
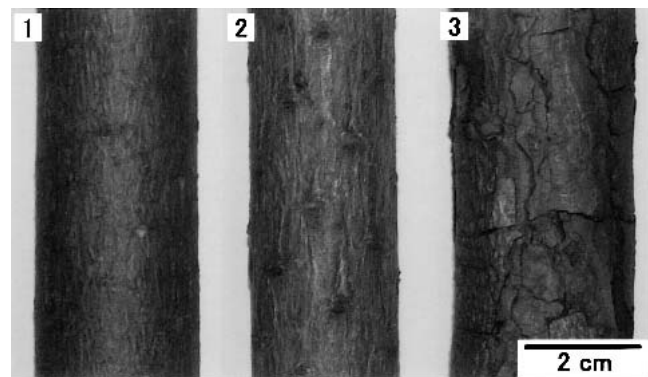

FIG. 1. Different bark surface textures of *Pinus densiflora* bolts. Bark surface was divided into smooth, intermediate, and coarse and scored as 1, 2, and 3, respectively.

layer of distilled water (3 to 5 mm deep) was added to each container to maintain humidity. A stainless steel net was placed over the water layer to hold beetles and pine bolts above the water. The containers were sealed with a polyethylene sheet to maintain high humidity throughout the test.

Medium-sized, polypropylene containers (20.5 cm × 27.0 cm × 9.2 cm-deep) were used for experiment 2. Four plastic slide projector racks (18.4 cm × 5.5 cm × 5.4 cm tall, Slide Magazine 36 capacity, Cabin Industrial Co., Tokyo, Japan) were placed side by side in each container. A 3 to 8-mm-deep layer of distilled water was added to the containers to maintain high relative humidity (R. H. ca 100%) and then the containers were sealed with a polyethylene sheet.

Inoculated bolts were stored in large transparent plastic containers (39.1 cm × 23.8 cm at the top, 36.1 cm × 20.8 cm at the bottom, × 27.4 cm deep). Containers also held distilled water 3 to 5 mm deep. Two plastic plates (16.5 cm × 19.0 cm × 1.0 cm tall, Biofilter, Nisso, Tokyo, Japan) were set on six bottles (10.8 cm tall) that had been placed in each container. These plates contained a large number of slits and were positioned to hold the inoculated bolts about 11 cm above the water surface. The containers were sealed with a polyethylene sheet.

Census of nematodes: The number of nematodes on a beetle body surface after the end of a test was determined by placing male beetles individually in 10 or 20-ml vials containing 5 to 10 ml, respectively, distilled water. The vials were sealed with screw caps and then shaken vigorously and quickly for 10 to 20 seconds. Shaking was repeated 4 to 5 times with a short rest of 1 to 3 seconds interspersed during this time interval. This rendered the beetles motionless. The beetles were removed from the vials and washed three times with rapidly running distilled water. Nematodes from the insect body surface were recovered from the vial water and the washings, which included beetle feces, by using a Baermann funnel for 2 to 3 days at 25 °C. The washed beetles were dissected with forceps and homogenized with pestle and mortar. Nematodes were extracted in a Baermann funnel (25 °C for 2 to 3 days) to determine the number of nematodes within the insect tracheal system. The number of nematodes carried by male beetles (nematode load) prior to introduction into experimental containers was estimated by summing nematode number on and in the beetle body after the test.

Wood was removed from each pine bolt 1 cm or more from the cut end to determine the nematode population density per gram of dried wood. A hand drill with a 24-mm or 14-mm bit was used for experiments 1 and 2, respectively. Nematodes were extracted from the wood sample in a Baermann funnel for 2 to 3 days. Wood samples then were dried at 80 °C for 5 or more days and weighed. The dry weight of the wood samples taken in experiments 1 and 2 was 3.4 to 6.2 g (n = 24) and 7.7 to 17.0 g (n = 40), respectively.

Experiment 1 (transmission of pinewood nematodes from male insects to pine trees via oviposition wounds): Nematode-free female beetles were introduced individually into small containers with a single pine bolt and allowed to oviposit in the dark at 25 °C. The bolt was checked hourly for the presence of oviposition wounds. Bolts were transferred to new containers when the wounds were found. Seventeen to 40-day-old male beetles, infested with *B. xylophilus,* were anesthetized on ice and their mouthparts fixed with synthetic glue (Aron-alpha, Konishi, Osaka, Japan). These male beetles were then individually released into the containers with a pine bolt on which the females had oviposited. The position of the male within the container was recorded at intervals of about 6 hours during a 48-hour period at 25 °C under a fixed 12 hour light-12 hour dark photoperiod. The males then were removed from the containers and the nematode load determined. The bolts were incubated at 25 °C for an additional month, and the nematode population density in the bolts was determined.

As a control, eight pine bolts without oviposition wounds were presented to nematode-infested males with fixed mouthparts for 48 hours. The bolts were incubated for about 1 month at 25 °C, and the nematode population density in the bolts was determined.

Experiment 2 (movement of pinewood nematodes on pine bark surface): Individual holes were made through the pine bark on 17-cm-long pine bolts using a punch across 2 mm. A hole was made 1, 6, 11, or 16 cm from one of the cut ends of each bolt. The bolts were laid singly on each slide rack in the experimental containers. The holes were kept on the top side of the bolts, and the containers were maintained at 25 °C for 2 to 5 hours before nematode inoculation.

Fourth-stage dispersal juveniles of *B. xylophilus* were isolated following criteria described by Stamps and Linit (1998 a,b; 2001). Nematode-infested beetles, 29 to 66 days old, were individually washed with distilled water as in experiment 1. Washings of 3 to 7 beetles were combined, and nematodes were extracted in the Baermann funnel for only 1 hour at 25 °C to prevent damage to the nematodes. Twenty milliliters of water was collected from the bottom of the funnel into two glass tubes. Nematodes were collected by centrifugation at 1,500 rpm for 90 seconds. The supernatant was decanted and the nematodes were resuspended in the remaining 1.0 ml water. An 0.8-ml-aliquot of the suspension was drawn into a 7-cm-long, 1.0-mm and 7.4-mm top and bottom i.d., respectively, tip (Quality Scientific Plastics, Petaluma, CA) on a 100 to 1,000-μl micropipet (Benchmate, Nichiryo, Tokyo, Japan). The micropipet containing the nematode suspension was

held vertically to concentrate the nematodes at the small opening of the pipet tip. When water almost dripped from the pipet tip, the volume adjuster of pipet was changed to increase the inner volume of the pipet slightly. After 1 hour, approximately 20 µl of suspension containing nematodes was delivered onto a 4 to 5-mm disc of absorbent cotton that had been placed on the bolts 1 cm from the cut end. The suspension did not flow from the absorbent-cotton disc. The remainder of the water was expelled from the pipet tip and the tip washed three times. The washings were mixed with the material expelled from the pipet tip and the number of nematodes determined. The number of nematodes also was determined in the suspension (0.2 ml) remaining in the centrifuge tube. The number of nematodes placed on each cotton disc was determined by subtracting the number of nematodes remaining in the pipet from four times the number of nematodes remaining in the centrifuge tube. Using *B. xylophilus* grown on a fungus *Botrytis cinerea* Persoon at 25 °C in the laboratory, the high accuracy of this method was indicated preliminarily by a regression line— (estimated nematode number) = 0.9094 × (actual nematode number) ($n = 9$, $R^2 = 0.951$).

After 3 days at 25 °C, the pine bolts inoculated with nematodes were placed in large containers for incubation. At transfer of the pine bolts, cotton discs had dried completely. The nematode population density was determined about 1 month later.

Statistical analyses: In experiment 1, all nematode counts (x) were transformed into $\log_{10}(x + 1)$ to make the variance independent of the mean before analysis. Addition of unity to nematode count was conducted to make transformed values non-negative. Pearson's correlation coefficient was calculated to reveal any association between variables. The *t*-test was used to compare the nematode load on beetles. This test also was used to determine the difference in the number of oviposition wounds per pine bolt between pine bolts showing successful nematode reproduction after 1 month and those bolts that did not provide evidence of successful nematode infestation. Backward stepwise regression analysis was used in experiment 2 to select factors explaining the variance of successful nematode reproduction in pine bolts at 0.05 of "*P*-to-enter" and "*P*-to-exit" values. Nematode reproduction (y) was successful (1) or failed (0). Factors explaining the variance included: the log-transformed values of number of inoculated nematodes ($x_1 \geqq 2$ because of amphimictic reproduction), distance between nematode inoculation point and artificial hole (x_2), number of days after the pine bolts were prepared (x_3), and relative evaluation of the type or degree of bark surface texture (x_4). Fisher's exact probability in a contingency table was used to compare the proportion of pine bolts with successful nematode reproduction dependent on three levels of bark texture.

Results

Experiment 1 (transmission of pinewood nematodes from male insects to pine trees via oviposition wounds): Estimated *B. xylophilus* load of the males just before this test ranged from 0 to 9,010 (mean ± SD = 1,603 ± 2,680, $n = 16$). *Bursaphelenchus xylophilus*-infested male beetles were found 79.7% of the time (94 of 118 observations) on pine bolts having oviposition wounds. After exposure of the males to these pine bolts for 48 hours, the number of nematodes on male body surfaces (y) increased as the estimated nematode load of the beetle (x) just before a test increased ($\log_{10}(y + 1) = -0.009 + 0.498 \log_{10}(x + 1)$, $r = 0.857$, $P < 0.001$, $n = 16$, Fig. 2).

Bursaphelenchus xylophilus populations were established in some pine bolts that were exposed to male beetles with a large number of nematodes on the body surface (Fig. 3A; Table 1) and with a substantial nematode load prior to the test (Fig. 4A; Table 1). The nematode density (y) in pine bolt 1 month after the test was significantly correlated to the number of nematodes (x) on the body surface of a male beetle at the end of the test ($\log_{10}(y + 1) = -0.162 + 1.070 \log_{10}(x + 1)$, $r = 0.721$, $P < 0.001$, $n = 16$, Fig. 3B) and to the total number of nematodes (x) carried by a male beetle at the end of test ($\log_{10}(y + 1) = -0.254 + 0.573 \log_{10}(x + 1)$, $r = 0.664$, $P < 0.01$, $n = 16$, Fig. 4B). The number of oviposition wounds on a pine bolt had no influence on the establishment of *B. xylophilus* (Figs. 3A and 4A; Table 1). No establishment of nematode population occurred in pine bolts without oviposition wounds, even if such bolts were exposed to eight male beetles with a mean load of 368 nematodes (SD = 366, range 12-903).
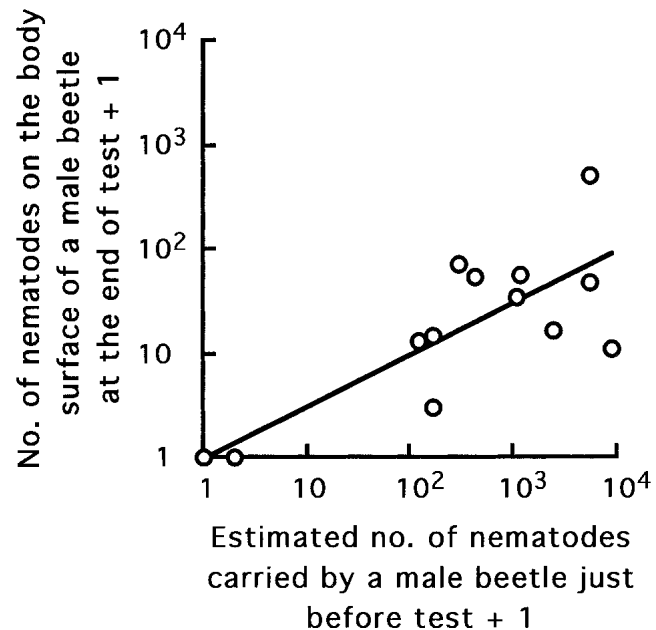

FIG. 2. Relationship between the number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* males just before the test and the number of nematodes on the body surface of the males at the end of the test.
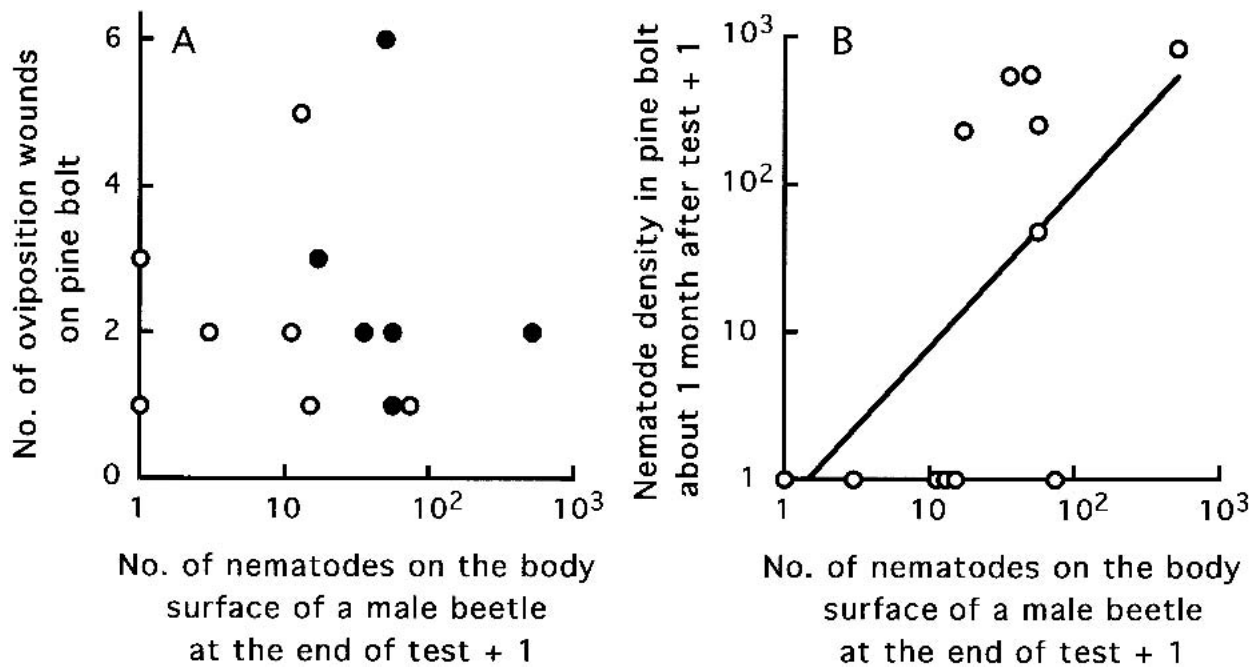
FIG. 3. Effect of nematode number on body surface of *Monochamus alternatus* males and the number of oviposition wounds on *Pinus densiflora* bolts on A) the establishment of the nematode population within the bolts about 1 month after exposure of the pine bolts to the infested beetles. The nematodes established a population in some bolts (●) but not in others (○). B) Relationship between the number of nematodes on the body surface of male beetles and the nematode density per gram of dried wood in pine bolts after about 1 month.

Experiment 2 (movement of pinewood nematodes on pine bark surface): The number of nematodes inoculated onto individual pine bolts from infested beetles ranged from 1 to 184 (Table 2). Nematode populations were found in some pine bolts about 1 month after the nematodes were inoculated onto the bark surface of pine bolts, at distances of 0, 5, and 10 cm away from a hole made by the punch. The proportion of bolts with nematode population decreased with increased distance between the inoculation point and artificial hole (Table 2). The densities of nematodes, including juveniles and adults, were low 1 month after inoculation (Table 2). Even excluding inoculation of single nematodes because of amphimictic reproduction, the proportion of pine bolts with a nematode population decreased with increased distance between inoculation point and artificial hole (Fig. 5).

Backward stepwise regression analysis showed that the distance between nematode inoculation point and artificial hole (x_2) explained the successful nematode reproduction (y) ($y = 0.413 - 0.029\ x_2$). However, the number of nematodes inoculated (x_1), conditions of pine bolts expressed by the number of days after cutting (x_3), or the texture of pine bark surface (x_4) did not affect nematode reproduction.

No relationship was observed between bark surface texture and proportion of pine bolts with successful nematode reproduction (Fig. 6) (Fisher's exact probability $P > 0.05$ for any combination of different bark textures).

TABLE 1. Relationship between *Bursaphelenchus xylophilus* load on *Monochamus alternatus* males, beetle oviposition wounds on *Pinus densiflora* bolts, and success or failure in nematode reproduction in pine bolts exposed to male beetles individually for 48 hours.

	Pine bolts with		
	Successful nematode reproduction	Unsuccessful nematode reproduction	*P* value
No. of pine bolts used	6	10	—
No. of nematodes on body surface of beetle (range)[a,b]	119.8 ± 192.7 (16–512)	11.0 ± 22.2 (0–72)	<0.01
Total no. of nematodes carried by beetle (range)[a,b]	2,649.7 ± 2,254.5 (414–5,499)	975.2 ± 2,825.0 (0–9,010)	<0.01
No. of oviposition wounds per pine bolt (range)[a]	2.7 ± 1.8 (1–6)	2.0 ± 1.3 (1–5)	>0.05

[a] mean ± SD.
[b] *t*-test was conducted after transformation with $\log_{10}(x + 1)$.

FIG. 4.   Effect of the number of *Bursaphelenchus xylophilus* nematodes carried by *Monochamus alternatus* males and the number of oviposition wounds on *Pinus densiflora* bolts A) on the establishment of the nematode population within the bolts about 1 month after the exposure of the pine bolts to the infested beetles. The nematodes established a population in some bolts (●) but not in others (○). B) Relationship between the number of nematodes carried by the male beetles and the nematode density per gram of dried wood in pine bolts about 1 month after test.

DISCUSSION

A new transmission pathway from male beetles to pine trees via conspecific oviposition wounds was found in the *B. xylophilus-M. alternatus* system. The establishment of *B. xylophilus* in pine trees by this transmission pathway tended to increase as the number of nematodes carried by male beetles increased (Figs. 4A,B). The nematodes emerge from spiracles of the beetles, crawl along the beetle's body surface to the abdominal tip, and then drop (Enda, 1977). Thus, the number of nematodes on the body surface of a beetle might be expected to correlate more closely with successful nematode reproduction in pine bolts than the total number of nematodes carried by beetles (nematode load, total number of nematodes on body surface and in tracheal system). We determined the number of nematodes on the body surface only at the end of test (experiment 1, Figs. 2 and 3), and the number of nematodes leaving the beetle's body during the 48-hour test period was unknown. Consequently, the correlation between nematodes on the body surface and nematode reproduction in the bolts is probably on the conservative side.

There are two plausible mechanisms explaining the newly discovered transmission pathway of *B. xylophilus* from male insects to pine bolts via pre-existing oviposition wounds. One is that the nematodes drop from the male beetles when the abdominal tip is positioned on or over the oviposition wounds. Males have been observed to rest or sometimes walk around on pine bolts searching for mates (Fauziah et al., 1987). The other is that the nematodes move along the bark surface to oviposition wounds from the location at which they left

TABLE 2.   Effect of distance on *Bursaphelenchus xylophilus* infection on *Pinus densiflora* bolts.

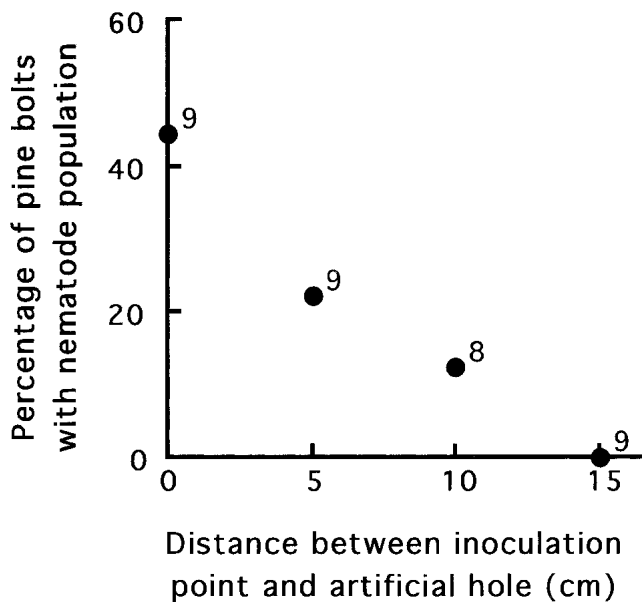| Distance between inoculation point and artificial hole on the bark surface (cm)	0	5	10	15
No. of pine bolts used	10	10	10	10
No. of nematodes inoculated per pine bolt (range)[a]	19.5 ± 18.6 (1–52)	35.9 ± 45.9 (1–144)	43.0 ± 54.7 (1–184)	31.7 ± 48.2 (1–164)
No. of pine bolts with nematode population increase	4	2	1	0
Nematode density per g dry wood in pine bolts with successful establishment of nematode population (range)	3.2 ± 2.8 (0.6–6.5)	3.8 ± 2.9 (1.7–5.8)	8.6 (8.6)	—

[a] mean ± SD.

FIG. 5. Relationship of the distance between the *Bursaphelenchus xylophilus* inoculation point and artificial hole on the *Pinus densiflora* bark surface to the successful nematode reproduction within the bolts inoculated with two or more nematodes. Number near each dot represents the number of pine bolts inoculated with two or more nematodes.

the beetle. These mechanisms are not exclusive and may both occur on a single pine bolt. We observed that *B. xylophilus* crawled on the tree bark surface and entered into the pine wood via artificial wounds (the second mechanism, Table 2; Fig. 5). This observation suggests that *B. xylophilus* may enter dying trees via any wounds that reach into the inner bark. We also observed that movement of nematodes occurred when bark surface was not wet as long as the relative humidity was very high. In Japan, *M. alternatus* is nocturnal (Nishimura, 1973) and transmits the nematode primarily during the rainy season. Consequently, this newly observed type of transmission probably occurs in the field.

Unsuccessful transmission occurred in some bolts when the nematodes were applied to artificial holes via a cotton disc (Table 2; Fig. 5). When this was done, the water was absorbed quickly into the wood exposed by the damage to the bark so that the nematodes could have been trapped by cotton fibers. If this occurred, the movement of the nematodes out of the cotton was difficult. In contrast, when the cotton disc—filled with nematode suspension—was placed on undamaged bark, the disc remained moist. Thus, the water in the cotton disc would allow the nematodes to easily crawl out of the cotton onto the bark surface. Successful nematode transmission decreased with an increased distance between inoculation point and artificial hole. No transmission occurred when inoculation was 15 cm away from the artificial hole (Fig. 5). Interestingly, transmission had no relationship to bark surface texture or inoculum quantity (Fig. 6).

The mechanism of transmission we have reported in this paper is potentially important for *B. xylophilus* in North America. There is less virulence of this nematode to pines native to North America because of tree resistance in the South and a cool summer climate in the North or at high elevations (Rutherford and Webster, 1987). Thus, *B. xylophilus* must be transmitted to dying and recently killed trees in order for it to persist in the pine forest ecosystem. Wingfield and Blanchette (1983) suggested that the *B. xylophilus* transmission via oviposition wounds was much more significant than transmission via feeding wounds to healthy trees. This would mean that *B. xylophilus* carried by male beetles does not contribute significantly to the persistence of the population (Edwards and Linit, 1992). In other words, *B. xylophilus* individuals have no fitness when carried by male beetles. However, nematode transmission from male to female beetles during mating and then to pine trees via oviposition wounds made by the infested females has been observed (Edwards and Linit, 1992; Togashi and Arakawa, unpubl. data). Nematode transmission directly from male beetles to recently killed pine trees via oviposition wounds documented herein means that *B. xylophilus* carried by male beetles may contribute to the persistence of *B. xylophilus* in the pine forest ecosystem in North America. As there is no difference in the initial nematode load between male and female beetles (Linit et al., 1983; Wingfield and Blanchette, 1983), the male-to-tree pathway might explain why there has been no selection pressure on the nematode to favor entering female beetles over male beetles. This inference would be reinforced by demonstrating the presence of male-to-tree transmission of *B. xylophilus* via pre-existing oviposition wounds in the beetle vectors native to North America.

This new transmission pathway we have reported might affect the establishment of the nematode in Eu-
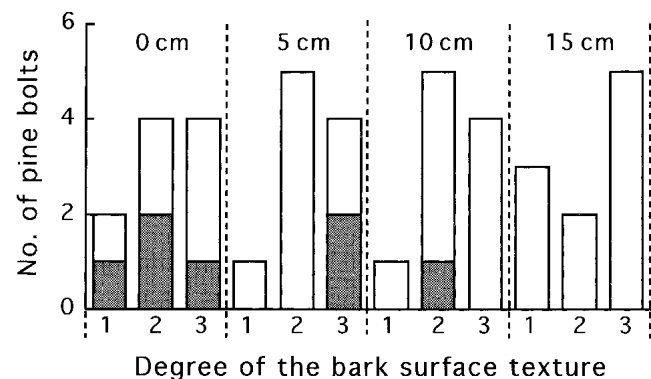

FIG. 6. Effect of distance between the *Bursaphelenchus xylophilus* inoculation point and artificial hole on the bark surface of *Pinus densiflora* bolts and the bark surface texture on successful nematode reproduction about 1 month after inoculation. Numbers in the figure represent the distance between the nematode inoculation point and the artificial hole. Shaded and open parts of columns indicate successful and unsuccessful nematode reproduction in pine bolts, respectively.

rope. Previously, the major threat of establishment was for a female beetle carrying the nematodes to get loose and oviposit in a pine forest in Europe (Evans et al., 1996). Now, as demonstrated in this paper, the ability of an introduced male beetle to transfer the nematodes to dead or dying pine trees where potential native beetle vectors may be developing increases the risk of nematode establishment. In contrast, the role of reproductively mature beetle vectors might be trivial in the spread of pine wilt disease in the epidemic areas of East Asia, where pine trees die from the nematode transmission by beetle vectors via feeding wounds and then attract the reproductively mature beetles. A mathematical model showed that the proportion of *M. alternatus* beetles travelling a long distance determines largely the spread rate of the disease per year without artificial transportation of logs and wood products infested with the beetles and the nematode (Takasu et al., 2000). Flight activity of *M. alternatus* was higher before reproductive maturation than after (Ito, 1982; Togashi, 1990), and there are a large number of dying trees within the disease infestation range. Thus, both sexes of reproductive mature beetles infested with the nematodes might rarely reproduce beyond the front of the disease infestation range.

Theoretical studies predict that multiple infection with different strains of a parasite selects more virulent strains and a great variation in virulence for a parasite population (Baalen and Sabelis, 1995; Bonhoeffer and Nowak, 1994; Leung and Forbes, 1998; May and Nowak, 1995; Nowak and May, 1994). To date, four transmission pathways of *B. xylophilus* have been described. The nematode is transmitted from insect vectors to pine twigs via insect feeding wounds (Linit, 1990; Mamiya and Enda, 1972; Wingfield and Blanchette, 1983). Secondly, *B. xylophilus* is transmitted from female insects to pine trees via oviposition wounds (Edwards and Linit, 1992; Wingfield, 1983). Third is the insect male-to-female and female-to-male transmission of *B. xylophilus* during mating, resulting in nematode transmission to pine trees via feeding and(or) oviposition wounds (Edwards and Linit, 1992; Togashi and Arakawa, unpubl. data). Fourth, as is shown in this study, *B. xylophilus* can be transmitted directly from infested male beetles to dead or dying pine through wounds traversing the bark or through oviposition sites. Reproductively mature insect vectors are concentrated on dying or recently killed pine trees to copulate and oviposit (Togashi, 1989). They feed on twig bark of the surrounding healthy trees (Togashi, 1991). Thus, the four transmission pathways provide multiple infection opportunities for *B. xylophilus* into a single pine. This inference is based on a small genetic variance among nematodes within individual insect vectors but a great variance among nematodes carried by different vectors. This was estimated by a lack of variation in virulence among isolates separated from individual insect vectors but a

great variation among isolates from different vectors (Kiyohara and Bolla, 1990). The results of our study may suggest that *B. xylophilus* populations are inclined to select higher virulence and maintain greater variance in virulence to pine trees through multiple pathways of transmission.

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