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Horizontal Transmission of *Bursaphelenchus xylophilus* between Sexes of *Monochamus alternatus*

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Abstract: Four experiments were conducted using nematode-infested and nematode-free adults of the cerambycid beetle, *Monochamus alternatus,* to determine horizontal transmission pathways of *Bursaphelenchus xylophilus.* When nematode-infested beetles of one sex and nematode-free beetles of the opposite sex were paired in containers for 48 or 72 hours, the number of nematodes carried by nematode-free beetles tended to increase with increased number of nematodes carried by nematode-infested beetles. The nematodes acquired by "nematode-free" beetles could be transmitted to pine. A female beetle that received 13 nematodes from a male transmitted one nematode to a *Pinus densiflora* bolt via an oviposition wound. When the nematode-infested and nematode-free beetles were observed continuously, it was observed that the number of nematodes carried by nematode-free beetles at the end of the first sexual mounting increased as the number of nematodes carried by nematode-infested beetles just before mounting increased. The number of nematodes transferred to nematode-free beetles was positively related to duration time of mounting. There was no difference in transmission efficacy between male-to-female transmission and female-to-male transmission. The horizontal transmission pathways are discussed relative to the persistence of *B. xylophilus* in resistant pine forests and the control of pine wilt disease.

Key words: Aphelenchoididae, *Bursaphelenchus xylophilus,* Cerambycidae, horizontal transmission, *Monochamus alternatus,* mounting, multiple infection, nematode, vector, virulence.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, is the causative agent of pine wilt disease (Kiyohara and Tokushige, 1971). This disease has devastated pine forests in eastern Asia for at least the last four decades or longer (Mamiya, 1987). This nematode is believed to be native to North America (Mamiya, 1987; Tares et al., 1992) from where it may have been first introduced into Japan in the early 1900s. Although the origin of introduction remains unclear, *B. xylophilus* was introduced into China in 1982, Taiwan in 1985, and Korea in 1988 (Enda, 1989; Mamiya, 1987). Most recently, this nematode was introduced into Portugal (Mota et al., 1999).

Pinewood nematode is transported as fourth-stage dispersal juveniles by cerambycid beetles of the genus *Monochamus* (Edwards and Linit, 1992; Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972; Wingfield and Blanchette, 1983). The nematode is harbored in the beetle tracheal system. In Japan, *M. alternatus* Hope and *M. saltuarius* (Gebler) transmit the nematode to healthy host trees of *Pinus thunbergii* Parl. and *P. densiflora* Sieb. et Zucc. (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972; Sato et al., 1987).

Three transmission pathways of *B. xylophilus* are recognized. One occurs during maturation feeding when the nematode is transferred from insect vectors to healthy pine trees via the insect feeding wounds (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972). The mature female beetle first makes slits or depressions with the mandibles in the bark and then inserts its ovipositor under the bark where the egg is laid. The second occurs during oviposition of the mature female on dead, dying, or recently cut pine trees via the oviposition wounds (Edwards and Linit, 1992; Wingfield and Blanchette, 1983). The mature male beetle searches for females on such trees for copulation. The third occurs during the mature male's search for females via wounds on the bark such as the oviposition wounds (Arakawa and Togashi, 2002). In addition, Edwards and Linit (1992) showed that *B. xylophilus* was recovered from wood surrounding oviposition wounds that nematode-free *M. carolinensis* females made with being mounted by nematode-infested males. This observation suggests that *B. xylophilus* transmission from male to female beetles occurs during mating.

The degree of virulence (nematode-induced host mortality) varies widely among populations of *B. xylophilus.* Difference in virulence is observed among populations sampled from different dead trees in the same pine stand, and among populations sampled from different beetles in the same pine stand (Kiyohara and Bolla, 1990). However, there is little variation in virulence within populations taken from a single beetle (Kiyohara and Bolla, 1990). Virulence of an established isolate does not change, at least during several years of culture in vitro when interspersed in vivo passage is included. These observations suggest that virulence is inherited and that its variation among *B. xylophilus* individuals is extremely small within insect vectors but large among different trees and vectors.

Theoretical studies have proposed that the multiple infection of an individual host by different strains of a parasite or a pathogen leads to greater virulence than would occur based on the largest reproductive rate of the parasite or pathogen. Multiple infection also leads to greater variance in virulence than single infection (Bonhoeffer and Nowak, 1994; May and Nowak, 1995; Nowak and May, 1994; van Baalen and Sabelis, 1995). Herre (1993) has shown that suppression of reproduc-

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tive ability of fig wasp species induced by nematode parasitism increases as parasite transmission increases among unrelated host individuals. That is, parasite virulence increases as horizontal transmission of parasitic nematode species between unrelated host individuals occurs more frequently. Horizontal transmission increases the frequency of multiple infection. Conversely, vertical transmission (parasite transmission from infected parent to offspring) results in a reduction of parasite virulence (Yamamura, 1993, 1996). Reproductively mature beetles concentrate on diseased trees for copulation and oviposition, and feed on twig bark of the surrounding healthy trees (Togashi, 1989, 1991). Thus, it is common for multiple infections of pine trees with *B. xylophilus* to occur. Horizontal transmission of *B. xylophilus* between heterosexual vectors enhances the level of multiple infection. This study is designed to experimentally understand the manifestation of horizontal transmission and the factors involved in the transmission.

Materials and Methods

Insects: From October to December between 1995 and 1998 more than 80 *P. densiflora* trees that had been recently killed by *B. xylophilus* were cut into 70-cm-long logs in Tokuyama City, Yamaguchi Prefecture, and hauled to a *P. densiflora* stand in Higashi-Hiroshima City, Hiroshima Prefecture. In late April to early May following the sampling year, the logs were placed in cages in the *P. densiflora* stand. *Monochamus alternatus* adults emerging from the logs were collected daily between May and August. Contamination with *B. xylophilus* was confirmed by inspection of pine twigs on which the emerging beetles fed. Beetles shown to transmit *B. xylophilus* were used as nematode-infested beetles.

Monochamus alternatus adults free from *B. xylophilus* were obtained from the 6th to 8th generations of a laboratory population originating from Shika Town, Ishikawa Prefecture, and the 2nd to 4th generations of a laboratory population originating from Tokuyama City, Yamaguchi Prefecture. Inseminated females were allowed to oviposit on about 5-cm-long, excised *P. densiflora* branches, which had been stored at 25 °C for 1 to 2 weeks. To isolate the insects from potential infestation with *B. xylophilus,* beetle eggs were collected from pine branches and placed individually in petri dishes with moistened filter paper. Newly hatched larvae were inoculated onto 70-cm-long, fresh *P. densiflora* logs. The larvae were kept at 25 °C for 4 to 5 months, followed by a 4 to 5-month storage at 10 °C to terminate the larval diapause. Then, they were returned to 25 °C to allow post-diapause larval development. Photoperiodic condition was a fixed 12-hours-light/12-hours-dark regime unless otherwise indicated.

Adult beetles used for experiments were isolated shortly after emergence and were reared individually in transparent cases (17.3 cm × 8.5 cm × 4.1 cm) on fresh *P. densiflora* and *P. thunbergii* twigs at 25 °C. All beetles, except the nematode-infested beetles used in 1999, were paired 1 to 3 weeks after emergence. Fertility was confirmed by the hatching of progeny. Nematode-infested and nematode-free beetles were 13 to 36 and 14 to 80 days old, respectively, at the start of each test. At the time of emergence the fresh weight for nematode-infested and nematode-free beetles was 168 to 484 mg and 153 to 926 mg, respectively. The Baermann funnel technique verified that samples of nematode-free beetles carried no nematodes.

Pine bolts and twigs: Healthy *P. densiflora* stems and branches were collected in Higashi-Hiroshima City, Hiroshima Prefecture. These were cut into 7-cm-long bolts without nodes to be used as beetle oviposition substrate for experiments. These bolts were soaked in hot water at about 70 °C for destruction of plant cells and then gradually cooled down to room temperature over a period of 3 to 12 hours. The bark surface was allowed to dry, and then the cut ends of pine bolts were sealed twice with paraffin (melting point, 56 to 58 °C) to prevent nematode infestation and retard desiccation. The bolts were kept in plastic bags at 25 °C for 1 week and then stored at 5 °C. They were placed at 25 °C 1 day before use when they were stored at 5 °C. Mean diameter of bolts was 3.27 cm (SD = 0.62 cm, range = 2.00 to 4.35 cm, $n = 63$), and mean bark thickness was 1.82 mm (SD = 0.57 mm, range = 1.00 to 3.35 mm, $n = 29$).

To prepare beetle food for experiments, 1-year-old, fresh *P. densiflora* twigs were defoliated and cut into 11-cm-long sections. The twig sections were washed with running tap water and placed in experimental containers as a source of food for the beetles.

Containers for experiments: Transparent plastic containers (16.0 cm × 9.0 cm at the top, 14.5 cm × 7.5 cm at the bottom, and 10.5 cm deep) were used for this experiment (Togashi and Jikumaru, 1996). The containers contained a shallow layer of water to maintain high relative humidity so the nematodes that fell to the bottom of the container could survive. Beetles, pine bolts, and twigs were held above the water by placing a stainless net above the bottom of the container. Just prior to each test, one or two excised pine twigs as food and a pine bolt for oviposition, if necessary, were placed in the appropriate container as required by the experiment. As soon as the beetles were released into the containers, any openings of the containers were covered with sheets of transparent polyethylene.

Census of pinewood nematodes: Bursaphelenchus xylophilus were separated from all samples at 25 °C for 2 to 3 days by the Baermann funnel technique.

Nematodes carried by a beetle were divided into those on the beetle body surface and those within the beetle body. To determine the number of nematodes on the beetle body surface, the beetles were placed

individually into 10 or 20-ml vials with about 5 or 10 ml of distilled water, depending on beetle size. The vials were shaken violently and quickly up and down for 10 to 20 seconds. This shaking was repeated four to five times with a short rest of 1 to 3 seconds. Subsequently, the beetles were removed and washed three times with running distilled water. Nematodes in the vial water and those in washings were separated from feces and counted. To determine the nematode number in the beetle tracheal system, the beetles were dissected with fine-tipped forceps and crushed by mortar and pestle to release the nematodes from the tracheal system. The nematodes were separated from debris and counted.

The intestinal and hypodermal cells of fourth-stage dispersal juveniles contain high concentrations of lipid droplets when they are recovered from newly emerged beetles (Kondo and Ishibashi, 1978; Stamps and Linit, 1995). When these juveniles are observed by transmitted light microscope, they show dark body color except at the head and tail (Mamiya, 1990). This dark body color is caused by the concentration of densely packed lipid droplets (Mamiya, 1990). Mean lipid content is significantly greater for the juveniles recovered from 7 to 28-day-old beetles than for those leaving the beetles (Stamps and Linit, 1998a). It decreases markedly for the juveniles recovered from 35-day-old and older beetles. Consequently, the difference in lipid content disappears between juveniles recovered from the beetles and those leaving them (Stamps and Linit, 1998a). To confirm the correlation of changes in lipid content to the length of time the nematode was associated with the beetle, body darkness was recorded for juveniles recovered from the body surfaces and tracheal systems of beetles randomly selected in three experiments 1-2, 2-1, and 2-2.

An hour after entering the trees via oviposition wounds, fourth-stage dispersal juveniles of *B. xylophilus* remain within 1.5 cm of oviposition site (Togashi and Nakayama, unpubl. data). Thus, the number of nematodes transmitted via oviposition wounds was determined as follows. Within an hour of the time that an individual beetle made an oviposition wound, the bark surface of the pine bolts containing oviposition sites was washed three times with rapidly running distilled water. Next, a sharp-edged brass tube, knife, and fine-tipped forceps were used to collect a bark disc (32-mm diam.), including the oviposition wound. A hand drill with a 24-mm-diam. bit was used to recover wood down to a depth of 1 to 2 cm under the oviposition wound. The brass tube, knife, drill bit, and forceps were flame-sterilized before each sampling. The nematodes were recovered separately from chipped bark discs and wood, and counted. Pine twigs were washed three times with rapidly running distilled water, cut into 1 to 2-mm-thick chips, and placed in the Baermann funnel to extract the nematodes. The nematodes then were counted to determine the number transmitted. Nematodes also were found in water, on the wall of the containers, and in washings from the pine bolt and twig surfaces. These were collected and counted.

Experiment 1-1 (insect male-to-female transmission of pinewood nematodes): This experiment was designed to determine if *B. xylophilus* was transmitted from male to female beetles and if the transmitted nematodes then were transferred to trees via oviposition wounds. Nematode-infested male and nematode-free female beetles were placed in pairs in containers that had two pine twigs as food. Mounting and copulation were recorded at irregular intervals. After 48 hours, female and male beetles were removed from the containers. Male beetles were processed to determine number of nematodes on the body surface and within the body. A census of nematodes also was taken for containers and for the supplied pine twigs. Female beetles soon were transferred individually to new containers with a pine bolt and a pine twig. The containers were kept in the dark for oviposition to occur. The oviposition wounds made by the female beetles were checked at 1-hour intervals. When wounds were found, the females were removed and the number of nematodes on the body surface and within the body determined. The nematode number also was determined in the bark discs that included oviposition wounds, in the wood under the wounds, and in the twigs supplied as a food source. The number of nematodes females carried at the end of pairing was calculated by summation of the nematodes on female body surface, in the body, on the inner wall of the container, on the pine bolt, in the pine twig, in washings of pine bolt and twig surfaces, and in the water in the containers where the females were released singly after being isolated from the males. The number of nematodes that left males during pairing was estimated by summing the nematodes carried by females at the end of pairing, in the pine twigs and water of the first containers, and in washings of the twig surfaces and the containers. The number of nematodes carried by males at the start of pairing was estimated by summing the nematodes carried by the males at the end of pairing and those leaving males during the pairing.

Experiment 1-2 (insect male-to-female transmission of pinewood nematodes during mating behavior): Male beetles mount females for a considerable length of time, during which they copulate with the females one or more times. To determine the relationships between duration of mounting, abundance of *B. xylophilus* carried by male beetles, and number of *B. xylophilus* transmitted from male to female beetles, nematode-infested males and nematode-free females were placed in pairs in the containers with two pine twigs and a pine bolt. Continuous observations were made to determine the duration of the first mounting by males using a stopwatch. Just after the males left the females, the number of nematodes carried was determined for the males and for the females. Three pairs showing a long mounting

of 3.35 to 8.73 hours (mean ± SD = 6.02 ± 2.69 hours) were separated artificially and the number of nematodes determined. The number of nematodes carried by males at the start of mounting was estimated by totaling the number of nematodes carried by both sexes at the end of mounting.

Experiment 2-1 (insect female-to-male transmission of pinewood nematodes): To determine if *B. xylophilus* is transmitted from female to male beetles, nematode-infested females and nematode-free males were placed in pairs in the containers with two pine twigs and a pine bolt. Mounting and copulation were recorded at irregular intervals over 72 hours. Females and males were separated from each other and the number of nematodes carried by each sex determined. The number of nematodes carried by female beetles at the start of the test was estimated by the sum of the nematodes carried by female beetles and those carried by male beetles at the end of the test.

Experiment 2-2 (insect female-to-male transmission of pinewood nematodes during mating behavior): To determine the relationships between duration of mounting, abundance of *B. xylophilus* carried by female beetles, and number of *B. xylophilus* transmitted from female to male beetles, nematode-infested females and nematode-free males were placed in pairs in the containers with two pine twigs and a pine bolt. Continuous observations were made to determine duration of the first mounting by males. The number of nematodes carried by the beetles was determined as soon as the males left the females. Three pairs of beetles that had a mounting time longer than 3 hours (mean ± SD = 3.38 ± 0.38 hours) were separated artificially and the number of nematodes determined. The number of nematodes carried by female beetles just before being mounted was estimated as the sum of nematodes carried by female beetles and those carried by male beetles at the end of mounting.

Statistical analysis: All nematode numbers were transformed into $\log_{10}(x + 1)$ to make the variance independent of the mean before analyses. Pearson's correlation coefficient was calculated and linear regression analysis was conducted to show the association between variables. In experiments 1-2 and 2-2, backward stepwise regression analysis was used to select factors explaining the variance in number of nematodes (y) transmitted to beetle horizontally at 0.05 of "*P*-to-enter" and "*P*-to-exit" values. Candidates for explaining variables were nematode-infested beetle age in days after emergence (x_1), nematode-free beetle age in days after emergence (x_2), duration of mounting in hours (x_3), number of nematodes carried by nematode-infested beetles before mounting or being mounted (x_4), number of nematodes on nematode-infested beetle body surface at the end of mounting (x_5), and number of nematodes within nematode-infested beetle body at the end of mounting (x_6). Transmission efficacy was expressed by regression lines between the nematode load of nematode-infested beetle before mounting or being mounted, number of nematodes on nematode-infested beetle body surface at the end of mounting, and number of nematodes carried by nematode-free beetle at the end of mounting. A *t*-test was conducted to compare the horizontal transmission efficacy (slope and *y*-intercept of regression line) between male-to-female vector transmission and reverse transmission (Kawabata, 1978).

Results

Experiment 1-1 (insect male-to-female transmission of pinewood nematodes): Each pair was observed 4 to 10 times (mean ± SD = 6.8 ± 1.7, $n = 16$). In 42 of 109 observations (38.5%), the male mounted the female. In the remaining 67 observations, no mounting occurred.

All 16 male beetles used carried *B. xylophilus*. The number of nematodes that left male beetles during the 48-hour test period increased as the nematode load of male beetles increased ($\log_{10}(y + 1) = -0.248 + 0.717 \log_{10}(x + 1)$, $r = 0.820$, $P < 0.001$, $n = 16$; Fig. 1A). Of 16 females paired with nematode-infested males, four carried 1 to 13 nematodes at the end of pairing while the remaining 12 carried no nematodes (Figs. 1A,B). The females that were observed to carry nematodes had been paired with males carrying more than 1,200 nematodes. The number of nematodes carried by females was inclined to increase with the number of nematodes carried by males just before pairing ($\log_{10}(y + 1) = -0.251 + 0.131 \log_{10}(x + 1)$, $r = 0.466$, $P > 0.05$, $n = 16$; Fig. 1A) and the number of nematodes on the male body surface at the end of pairing ($\log_{10}(y + 1) = -0.109 + 0.174 \log_{10}(x + 1)$, $r = 0.477$, $P > 0.05$, $n = 16$; Fig. 1B).

Females made 1 to 5 oviposition wounds (mean ± SD = 2.00 ± 1.15) 1 to 5 hours after they were released singly in containers. Of the four females receiving nematodes from males, one transmitted a nematode to a pine bolt via an oviposition wound (Fig. 2). The nematode was recovered from a bark disc containing the wound.

After oviposition, 10 nematodes were found on the body surface of one female beetle and one nematode was found on the body surface of another beetle. One nematode was in the body of a third female.

Experiment 1-2 (insect male-to-female transmission of pinewood nematodes during mating behavior): Most beetles rested after being released in the containers. Following a mean time of 57 minutes post release (range = 0 to 130 minutes, $n = 15$), males began to walk and then mounted resting females. Upon mounting, the males tried to copulate with females that were resting. Sometimes females rejected copulation by bending up the abdomen under the elytra, kicking males, or walking. Males copulated with females several times during

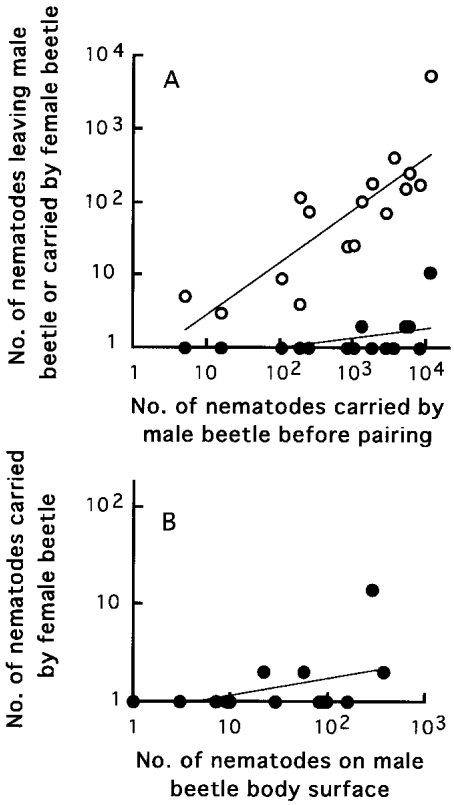

FIG. 1. Effect of the number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* males on the horizontal transmission of the nematode from males to females; relation in number between nematodes carried by the male beetle just before pairing, those leaving the male beetle (○), and those carried by the female beetle (●) at the end of pairing (A); and relation in number between nematodes on the male beetle body surface and those carried by the female beetle at the end of pairing (B). Nematode-infested males and nematode-free females were paired in containers for 48 hours, and then females were allowed to oviposit. The number of nematodes carried by males just before being paired with females, number of nematodes leaving males, and number of nematodes carried by females at the end of pairing were determined. Nematode number to which one was added is represented. Regression lines are provided.

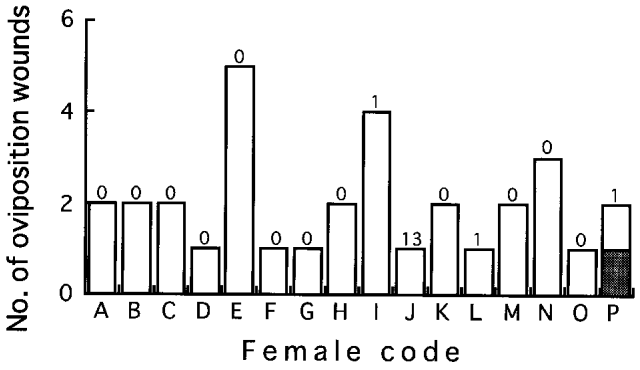

FIG. 2. Effect of the number of *Bursaphelenchus xylophilus* transmitted horizontally to *Monochamus alternatus* females by nematode-infested males on the transmission from females to *Pinus densiflora* bolts via oviposition wounds. A total of 16 nematode-free female beetles (A to P) that had been paired with nematode-infested males for 48 hours were individually introduced in containers with a pine bolt as oviposition substratum. Observation was made at 1-hour intervals. When the female made oviposition wounds, the wounds were counted and examined for nematode transmission. Shaded and open parts of columns represent the presence and absence of *B. xylophilus* at each oviposition wound. Numeral above each column represents the number of nematodes transmitted to the female beetle horizontally. Female P made two oviposition wounds and transmitted a nematode via one of two oviposition wounds.

mounting. Mounting was terminated by males spontaneously or by quick walking of females. The duration of the first mounting ranged from 10 to 524 minutes (mean ± SD = 217.9 ± 133.0 minutes, $n = 15$) including three artificial breaks of the mounting.

The number of nematodes on the male body surface at the end of mounting was related to the number of nematodes carried by males at the start of mounting ($\log_{10}(y+1) = -0.851 + 0.892 \log_{10}(x+1)$, $r = 0.933$, $P < 0.001$, $n = 15$) (Fig. 3A). The number of nematodes carried by females at the end of mounting showed a positive correlation to the number of nematodes carried by males just before mounting ($\log_{10}(y+1) = -0.790 + 0.478 \log_{10}(x+1)$, $r = 0.717$, $P < 0.01$, $n = 15$; Fig. 3A). Thus, the number of nematodes carried by females was directly proportional to the number of nematodes on the male body surface at the end of mounting ($\log_{10}(y+1) = -0.158 + 0.433 \log_{10}(x+1)$, $r = 0.621$, $P < 0.05$, $n = 15$; Fig. 3B).

Backward stepwise regression analysis showed that the number of nematodes carried by females at the end of mounting (y) was positively related to the duration of mounting (x_3) (hours) and the number of nematodes within the male body at the end of mounting (x_6) as expressed by

$$\log_{10}(y+1) = -1.0447 + 0.1105\, x_3 + 0.4321 \log_{10}(x_6+1) \quad (1)$$

After the first mounting, about 66.7% of females carried nematodes. Of 120 nematodes transmitted to female beetles, 109 were found on the female body surfaces whereas 11 were within female bodies (Fig. 4). All nematodes within female bodies had a dark body color, whereas only 1 of 24 recovered nematodes on female body surfaces was dark and the others were clear.

Experiment 2-1 (insect female-to-male transmission of pinewood nematodes): Observation was made 5 to 14 times (mean ± SD = 9.6 ± 3.4, $n = 17$) for each beetle pair during a 72-hour test period. In 19 of 163 observations (11.7%), the male mounted the female. In two observations (1.2%), the male touched a female body adjacent to him with the legs. In the remaining 142 observations (87.1%), the male and female made no contact.

Number of nematodes on female body surface was related to the number of nematodes carried by females at the start of the test ($\log_{10}(y+1) = -0.507 + 0.674 \log_{10}(x+1)$, $r = 0.650$, $P < 0.05$, $n = 17$; Fig. 5A). Ten of 17 males carried 1 to 35 nematodes (mean ± SD = 11.4 ± 14.2, $n = 10$) just after a 72-hour pairing. When nematode-free males were paired with females infested with a greater number of nematodes, males had more

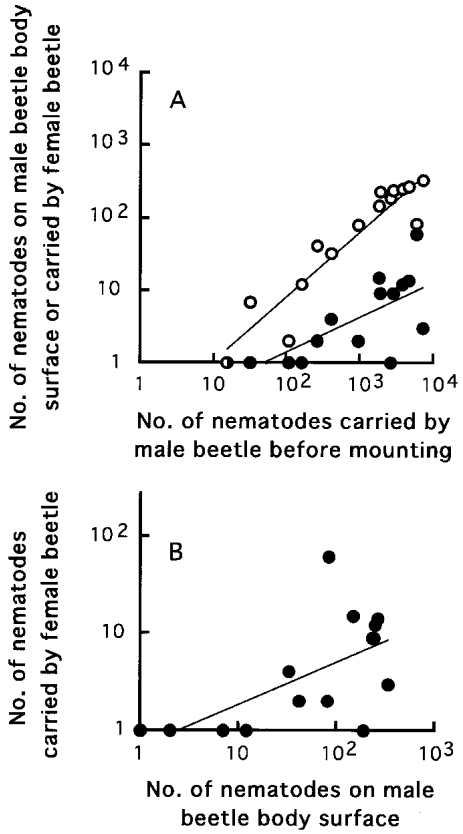

FIG. 3. Effect of the number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* males on the horizontal transmission from males to females; relations in number between nematodes carried by the male beetle just before mounting, those on the male beetle body surface (○), and those carried by the female beetle (●) at the end of mounting (A); and relation in number between nematodes on the male beetle body surface and those carried by the female beetle at the end of mounting (B). Nematode-infested males and nematode-free females were introduced in pairs into containers and observed continuously. Males mounted females for long periods of time during which time they copulated intermittently. As soon as males left females, the nematodes on beetle body surfaces and within the bodies were determined. Total number of nematodes carried by paired beetles was regarded as the number of nematodes carried by males just before mounting female. Nematode number to which one was added is represented. Regression lines are provided.

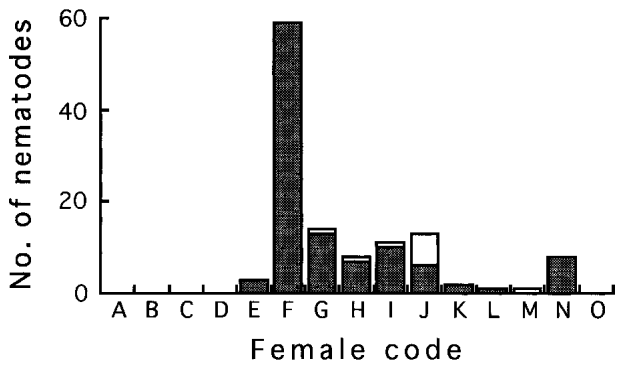

FIG. 4. Spatial distribution of horizontally transmitted *Bursaphelenchus xylophilus* on and within the body of *Monochamus alternatus* females soon after nematode-infested males stopped mounting them. The nematodes were recovered from the surface (shaded part of column) and inside (white part) of the female body separately.

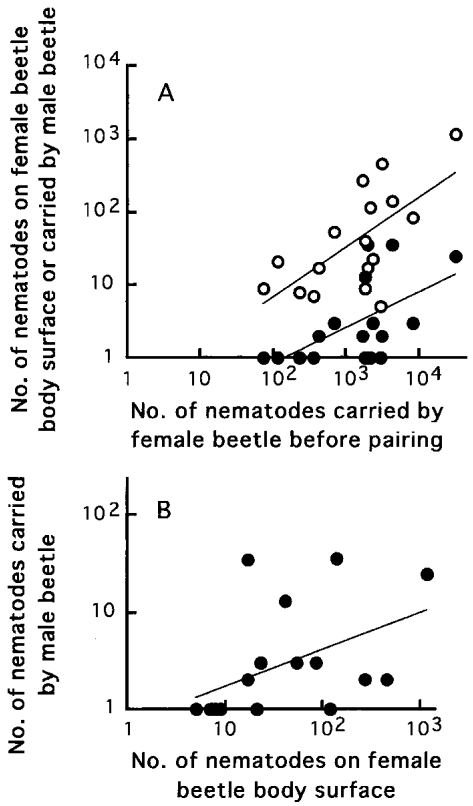

FIG. 5. Effect of the number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* females on the horizontal transmission from females to males; relations in number between nematodes carried by female beetles just before being paired, those on the female beetle body surface (○), and those carried by the male beetle (●) at the end of pairing (A); and relation in number between nematodes on the female beetle body surface and those carried by the male beetle at the end of pairing (B). Nematode-infested females and nematode-free males were paired in containers for 72 hours. After that, nematodes on the beetle body surface and within the body were determined. Total number of nematodes carried by paired beetles was regarded as the number of nematodes carried by females just before being paired with nematode-free males. Nematode number to which one was added is represented. Regression lines are provided.

nematodes at the end of pairing ($\log_{10}(y+1) = -1.060 + 0.489 \log_{10}(x+1)$, $r = 0.573$, $P < 0.05$, $n = 17$; Fig. 5A). The number of nematodes carried by males tended to increase as the number of nematodes on the female body surface increased ($\log_{10}(y+1) = -0.139 + 0.380 \log_{10}(x+1)$, $r = 0.461$, $P > 0.05$, $n = 17$; Fig. 5B).

A total of 16 nematodes were found on the body surfaces of male beetles, whereas 98 nematodes were in male bodies (Fig. 6). Of 98 nematodes in male bodies, one (1.0%) was dark in one fourth of the body and others (99.0%) showed dark body color.

Experiment 2-2 (insect female-to-male transmission of pinewood nematodes during mating behavior): The behavior of beetles was the same as that observed in experiment 1-2. Duration of first mounting ranged from 1 to 291 minutes (mean ± SD = 123.7 ± 87.4 minutes, $n = 15$) including 3 artificial breaks of mounting.

The number of nematodes on the female body surface was related to the number of nematodes carried by

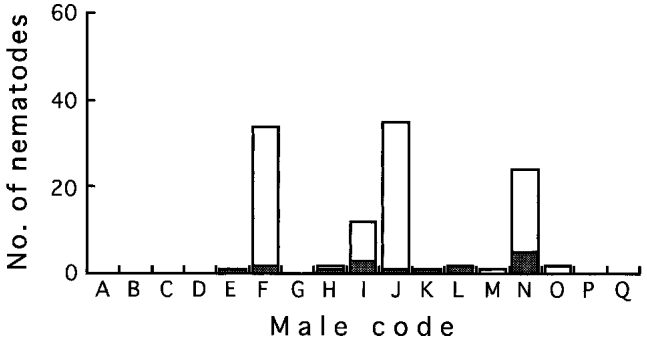

FIG. 6. Spatial distribution of horizontally transmitted *Bursaphelenchus xylophilus* on and within the body of *Monochamus alternatus* males that had been paired with nematode-infested females for 72 hours. The nematodes were recovered from the surface (shaded part of column) and inside (white part) of the male body separately.

females just before being mounted ($\log_{10}(y+1) = -0.939 + 0.822 \log_{10}(x+1)$, $r = 0.697$, $P < 0.01$, $n = 15$; Fig. 7A). The number of nematodes carried by males at the end of mounting increased significantly with increased nematodes carried by females just before being mounted ($\log_{10}(y+1) = -0.820 + 0.440 \log_{10}(x+1)$, $r = 0.549$, $P < 0.05$, $n = 15$; Fig. 7A). There was a positive relationship between the number of nematodes on the female body surface and the number of nematodes carried by the male ($\log_{10}(y+1) = -0.313 + 0.534 \log_{10}(x+1)$, $r = 0.785$, $P < 0.001$, $n = 15$; Fig. 7B).

Backward stepwise regression analysis showed that the number of nematodes carried by the male at the end of mounting (y) was positively related with duration of mounting (x_3) (hours) and the number of nematodes on the female body surface at the end of mounting (x_5) as expressed by

$$\log_{10}(y+1) = -0.6054 + 0.1714\, x_3 + 0.4917 \log_{10}(x_5+1). \quad (2)$$

After beetle mounting, 82.8% of nematodes carried by male beetles were found on male body surfaces and others were within male bodies (Fig. 8). Thirty nematodes recovered from male beetle body surfaces had a clear body. Of 16 nematodes in male bodies, two (12.5%) were almost clear and others (87.5%) showed dark body color.

Comparison of horizontal transmission efficacy between male-to-female and female-to-male beetle transmissions: The *t*-test showed no significant difference between female-to-male beetle transmission of nematodes and the reverse transmission in slope and *y*-intercept of regression line between the nematode load of nematode-infested beetle before mounting and the number of nematodes carried by nematode-free beetle after mounting (slope, $t = 0.167$, $P > 0.05$, $df = 26$; *y*-intercept, $t = 0.848$, $P > 0.05$, $df = 27$; Figs. 3A, 7A). Another *t*-test showed no significant difference between female-to-male beetle transmission and the reverse one in the regression line between the number of nematodes on nematode-infested beetle

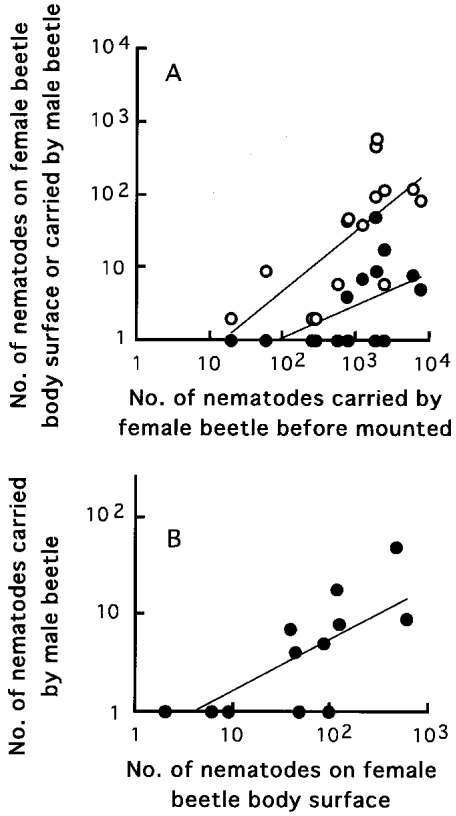

FIG. 7. Effect of the number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* females on the horizontal transmission from females to males; relations in number between nematodes carried by the female beetle just before being mounted, those on the female beetle body surface (○), and those carried by the male beetle (●) at the end of mounting (A); and relation in number between nematodes on the female beetle body surface and those carried by the male beetle at the end of mounting (B). Nematode-infested females and nematode-free males were introduced in pairs into containers and observed continuously. Males mounted females for long periods of time during which time they copulated intermittently. As soon as the males left the females, the nematodes on the beetle body surface and within the body were determined. Total number of nematodes carried by paired beetles was regarded as the number of nematodes carried by females just before being mounted by males. Nematode number to which one was added is represented. Regression lines are provided.

body surface and the number of nematodes carried by nematode-free beetles after mounting (slope, $t = -0.531$, $P > 0.05$, $df = 26$; *y*-intercept, $t = -0.034$, $P > 0.05$, $df = 27$; Figs. 3B, 7B). These indicated no statistical difference in transmission efficacy between the two heterosexual horizontal transmission pathways.

DISCUSSION

This study documented the transmission of *B. xylophilus* between sexes of *M. alternatus* adults, male-to-female and female-to-male. Edwards and Linit (1992) suggested one-way transmission of *B. xylophilus* from *M. carolinensis* males to females experimentally by recovering the nematode from the wood surrounding oviposition wounds that nematode-free females made with be-

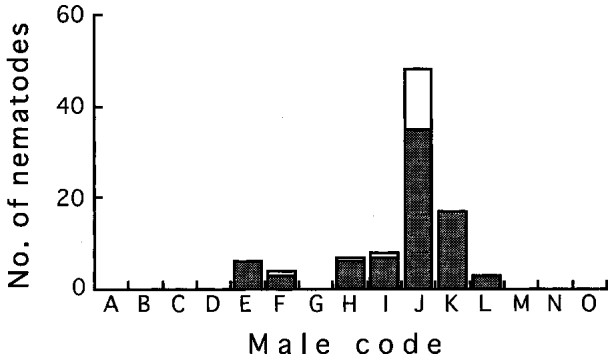

FIG. 8. Spatial distribution of horizontally transmitted *Bursaphelenchus xylophilus* on and within the body of *Monochamus alternatus* males soon after they stopped mounting nematode-infested females. The nematodes were recovered from the surface (shaded part of column) and inside (white part) of the male body separately.

ing mounted by nematode-infested males. Horizontal transmission between heterosexual vectors also was confirmed by Togashi and Jikumaru (1996), in the *Bursaphelenchus mucronatus-Monochamus saltuarius* system, who suggested that male-to-female transmission was more likely to occur than female-to-male transmission. However, no studies have been done to show quantitative relationships.

This study showed that there was a significant, positive relationship between nematode number on beetle body surface and nematode load for nematode-infested beetles (Figs. 1A, 3A, 5A, 7A). There also was a positive correlation between nematode number on nematode-infested beetle body surface and nematode number carried by nematode-free beetles following sexual mating (Figs. 3B, 7B). Surprisingly, this study also demonstrated that a nematode was transmitted from a male to female beetle and then into a pine bolt via the oviposition wound made by the female. Thus, nematode transmission from male beetles to oviposition wounds via female beetles may explain Edwards and Linit's (1992) observation that *B. xylophilus* was recovered from wood surrounding oviposition wounds made by nematode-free female beetles that had copulated with nematode-infested males. Edwards and Linit's (1992) observation also is explained by the nematode transmission to the oviposition wounds directly by male beetles (Arakawa and Togashi, 2002).

A comparison of results between experiments 1-1 and 1-2 and that between experiments 2-1 and 2-2 showed that duration of mounting was a more significant variable for horizontal transmission than was the period during which both sexes were confined in a small container. The number of nematodes transmitted to females by males and that transmitted inversely increased with increased duration of mounting (equations 1 and 2). When *Monochamus* spp. mate, males mount females for a considerable length of time during which time males repeat copulation and females sometimes oviposit (Fauziah et al., 1987; Hughes, 1979). In continuous observations of experiments 1-2 (male-to-female transmission) and 2-2 (female-to-male transmission), the mean duration of mounting was 170.8 minutes (n = 30; 217.9 minutes, n = 15 for experiment 1-2; 123.7 minutes, n = 15 for experiment 2-2). During mounting, males grasp female metathoraces with their forelegs and keep up with females while moving. They sometimes touch the female elytra with the metathoracic sternum during mounting. This action by the males may explain the horizontal transmission of *B. xylophilus* between heterosexual beetles because the nematodes exit from insect spiracles, crawl to the abdominal tip through the abdominal surface, and leave from the abdominal tip (Enda, 1977). Contact of male metathorax with female elytra might make the male-to-female transmission of *B. xylophilus* easier than the reversed transmission although the t-test showed no difference in transmission efficacy between the two transmission pathways. During copulation, the males' forelegs and middle legs grasp females while the hind legs are held onto the bark of a tree branch or trunk. Copulation also may be one of the plausible pathways of horizontal transmission of nematodes because nematodes concentrated on the beetle abdominal tips of one sex might be conveyed to the abdominal tip of the opposite sex by the contact of abdominal tips and (or) by male aedeagus.

Backward stepwise regression analysis also showed that the number of nematodes either within nematode-infested beetles or on their body surface had positive bearing on the numbers of nematodes transmitted to nematode-free beetles. Assuming that rate of nematode departure from beetle body is constant and is considerably higher than rate of nematode exit from spiracles, the number of nematodes within the body of a nematode-infested beetle is directly proportional to that on the beetle body surface. Actually, there was significant, positive correlation between the number of nematodes within and on the body of the nematode-infested beetle for the two heterosexual transmissions (Figs. 3A, 7A). That suggested that number of nematodes passing through the nematode-infested beetle body surface directly determined the number of nematodes transmitted. The result of backward stepwise regression analysis was consistent with the speculation for female-to-male transmission (equation 2) but not for male-to-female transmission, where the number of nematodes transmitted was more closely related to the number of nematodes within the beetle body (equation 1). A plausible reason for the latter result was a high correlation between the nematode numbers on the body surface and those in the body for nematode-infested beetles. A positive correlation does not always occur between the number of nematodes on the body surface and the number of nematodes within the body in beetles because Stamps and Linit (1998a) frequently observed a clump of dried nematodes in the atrium of the first

abdominal spiracles in old beetles, which would prevent nematodes from exiting the spiracles. In our study, there was no difference in age of used, nematode-infested beetles among the four experiments.

Some nematodes found within beetle bodies in the present study were transmitted during heterosexual mating between beetle vectors. Some of these nematodes showed dark body color, indicating presence of a high concentration of lipid droplets (Mamiya, 1990). Other nematodes had a clear body, indicating absence of lipid droplets. Stamps and Linit (1998 a,b) suggested that the exit behavior of the nematode from the beetle was associated with the lipid content stored by the nematode and that decrease in storage lipid may be linked to initiation of the exit process. According to Stamps and Linit (1998 a,b), nematodes without lipid droplets exit from the spiracles in response to pine volatiles. Therefore, there are two plausible mechanisms to explain nematode movement into tracheae following horizontal transmission. One is that nematodes transmitted horizontally enter the tracheae where lipid droplet formation occurred. This would imply that any nematodes on the beetle body surface have no lipid droplets. Another mechanism is that some of the nematodes on the beetle body surface have high concentrations of lipid droplets and that these are the nematodes that enter the tracheae after horizontal transmission. Actually, one nematode, recovered from the beetle body surface in experiment 1-2, had a dark body coloration. The concentration of lipid droplets needs to be examined for nematodes recovered from the beetle body surface to test the two hypotheses. In addition, dissection of beetles should be performed to find the nematodes in the beetle tracheae after horizontal transmission.

The ecological significance of horizontal transmission of nematodes varies depending on the virulence level of nematodes to host pine trees. Togashi and Jikumaru (1996) pointed out that the transmission of *B. mucronatus* by female vectors via oviposition wounds is important for nematode reproduction on dying trees because the nematode has little virulence and thus the male-to-female vector transmission enhances the probability that *B. mucronatus* on male vectors enter dying or freshly killed pine trees via beetle oviposition wounds. As *B. xylophilus* shows extremely low virulence against pine trees native to North America in natural habitats due to cool summer climate and tree resistance (Rutherford and Webster, 1987), the male-to-female vector transmission is important in that area as indicated by Edwards and Linit (1992).

Two or more *M. alternatus* beetles may transmit *B. xylophilus* to healthy pine trees prior to disease development. Reproductively mature beetles are concentrated on diseased trees to oviposit and copulate, and feed on twig bark of surrounding healthy trees (Togashi, 1989, 1991). Therefore, multiple infection occurs on individual host trees. Theoretical studies show that multiple infection by two or more strains of parasites induces an increased mean virulence level and an extended variance in virulence by rapid competitive exclusion or coexistence between strains within individual hosts (May and Nowak, 1995; Nowak and May, 1994). Two horizontal *B. xylophilus* transmission pathways (male-to-female and female-to-male beetle) and the invasion of beetles by horizontally transmitted nematodes suggest increased opportunities of multiple infection of different strains of nematodes on a given diseased tree. This should be taken into account in developing control plans for pine wilt by reforestation using resistant trees against the disease because the *B. xylophilus* transmission system may include the mechanism of the evolution toward the higher virulence. Further studies are necessary to monitor the virulence level over time and to prevent the nematode from developing higher virulence ecologically.

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