

## 博士論文（要約）

### 論文題目

Effects of reproductive interference between *Bursaphelenchus xylophilus* and *B. mucronatus* on the development of pine wilt disease

(マツノザイセンチュウとニセマツノザイセンチュウの繁殖干渉がマツ材線虫病に及ぼす影響)

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## Summary

Pine wilt disease is an infectious disease of pine trees. It is caused by the nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and is transmitted by cerambycid beetles in the genus *Monochamus*. The pathogenic nematode native to North America has been introduced to East Asia and westernmost Europe, where the non-pathogenic, closely-related nematode *B. mucronatus* is distributed widely. Once *B. xylophilus* invades susceptible pine forests, *B. mucronatus* is displaced rapidly by *B. xylophilus*. The species displacement has been implicitly considered to result from the interspecific competition for limited resources.

Reproductive interference is caused by incomplete recognition of the other species and reduces the fitness through mating attempts and copulation with the other species. Interspecific competition through reproductive interference is characterized by asymmetric, frequency-dependent outcomes and brings several ecological and evolutionary consequences on species. *Bursaphelenchus xylophilus* and *B. mucronatus* are known to produce F<sub>1</sub> hybrids that cannot establish populations in most cases, suggesting the presence of reproductive interference between the two species. The aim of this study was to determine the interspecific competition through reproductive interference in the two nematode species and effects on the development of pine wilt disease and dispersal of the pathogenic nematodes.

### **Suppressive effects of *B. mucronatus* on pine wilt disease development and *B. xylophilus* populations in pine seedlings**

Theories predict that one of the two competing species populations quickly displaces the other through reproductive interference in a frequency-dependent

manner. Thus it is anticipated that *B. mucronatus* suppresses the virulence of *B. xylophilus* against pine trees when *B. mucronatus* was inoculated at greater proportions than *B. xylophilus*.

To determine the suppressive effects of *B. mucronatus* on pine wilt disease development, 15 000 nematodes including an isolate of *B. xylophilus*, T4, and an isolate of *B. mucronatus*, Srf, whose hybrids break down, were inoculated on each of 30 3-year-old *Pinus thunbergii* seedlings at three combinations of different numbers in early August. As control, *B. xylophilus* alone was inoculated on 30 other seedlings. Inoculation of *B. mucronatus* significantly retarded the speed of foliage discoloration and significantly prolonged the survival time of seedlings.

*Bursaphelenchus mucronatus* significantly reduced the nematode density in xylem. Analysis of rDNA genotypes showed 1846 *B. xylophilus*, no *B. mucronatus* and one hybrid.

To determine when the interspecific competition occurred in pine trees, I inoculated 4 000 T4 nematodes, 11 000 Srf nematodes, or 15 000 nematodes of mingled isolates on 3-year-old *P. thunbergii* seedlings in late July and sampled them 4, 8, 12, and 16 weeks after the inoculation. Genotyping of rDNA showed extremely small proportions of *B. mucronatus* and hybrids in Week 4 and their disappearance in Week 8, indicating that the interspecific competition almost finished within four weeks of the inoculation. However, the simultaneous inoculation of *B. mucronatus* made the population size of *B. xylophilus* small even after its disappearance, i.e. the mean nematode densities in seedlings were smaller when the two species were inoculated simultaneously than when *B. xylophilus* alone.

### **Interspecific competition between *B. xylophilus* and *B. mucronatus***

Reproductive interference is caused by incomplete recognition of heterospecific opposite sex and the production of inviable and sterile hybrids. It is called “pseudocompetition” because the basic difference between resource competition and reproductive interference is the presence or absence of a shared limited resource. Theoretically, one species population takes longer to displace the other species population by the exploitative competition than the competition through reproductive interference, in which species displacement occurs in frequency-dependent manner. Using isolates of the two *Bursaphelenchus* species whose hybrid populations do not persist, I investigated the relationship between the outcomes of competition and their population traits. Values of intrinsic rate of natural increase,  $r$ , and carrying capacity,  $K$ , were estimated to be 0.854/ day and 72 361, 0.509/ day and 28 272, and 1.10/ day and 120 062 for isolates T4, Srf, and TBm119, respectively on *Botrytis cinerea* fungal mat in 50-mm-diameter Petri dishes at 25 °C. When 50 nematodes were placed on the *B. cinerea* fungal mat at seven different ratios of isolates T4 and Srf of 10:0, 9:1, 7:3, 5:5, 3:7, 1:9 and 0:10, molecular techniques showed the occurrence of the hybrids in a week. Those also revealed that T4 displaced Srf in 1-2 and 2-4 weeks when being inoculated at proportions of 0.9 and 0.7, respectively, whereas Srf displaced T4 in two of the three dishes in 4 and 4-5 weeks when being inoculated at proportions of 0.9 and 0.7, respectively. On the other hand, T4 displaced another *B. mucronatus* isolate TBm119 in 1-2 weeks when being inoculated at a proportion of 0.9, while TBm119 displaced T4 in 2-4, 1-4, and 1-2 weeks when being inoculated at proportions of 0.5, 0.7, and 0.9.

Quick, frequency-dependent species displacement between *B. xylophilus* and *B. mucronatus* were characteristics of interspecific competition through reproductive interference. Based on mathematical models of reproductive interference and resource

competition by Kuno (1992), zero-growth isoclines were depicted using estimates of parameters  $b$ ,  $d$ ,  $h$ ,  $c$ , by the above-mentioned experiments. Comparing the observations and the predictions indicated the interspecific competition through reproductive interference rather than competition for resources. Difference in species displacement patterns between two *B. mucronatus* isolates was explained in part by smaller  $r$  and  $K$  values in Srf than in T4 and by larger  $r$  and  $K$  values in TBm119 than in T4.

### **Interspecific copulation between *B. xylophilus* and *B. mucronatus***

To determine the recognition ability between conspecific and heterospecific opposite sexes in copulation, a virgin adult of one sex and two virgin adults of the opposite sex of two nematode species were placed on PAD medium containing *Nectria viridescens*. Genotyping of rDNA of F<sub>1</sub> offspring showed the difference in sex-specific recognition ability between nematode isolates. Females of the two species had higher abilities of species recognition than the males; males copulated with heterospecific opposite sex more frequently than the females in the presence of those of the same species. In the absence of conspecific males, *B. mucronatus* females accepted *B. xylophilus* males more frequently than *B. xylophilus* females accepted the heterospecific males. Especially, Srf females (15/ 16) more often accepted T4 males in copulation compared with TBm119 females (18/ 22). On the other hand, there was no difference in copulation rate with the heterospecific females between the males of the two species in the absence of the conspecific females.

### **Frequency-dependent inhibitory effects of *B. mucronatus* on the number of *B. xylophilus* carried by *Monochamus alternatus***

The number of *B. xylophilus* carried by newly-emerged *Monochamus alternatus* adults, the initial nematode load, determines the transmission ability of nematodes; even one beetle with heavy nematode loads can transmit an enough number of nematodes to induce pine wilt disease. To determine the effects of *B. mucronatus* on the boarding of *B. xylophilus* onto *M. alternatus* beetles, beetle larvae were singly into artificial holes of pine bolts together with 2 000 nematodes at seven different ratios of the two nematode species. Mean initial nematode loads were smaller when bolts were inoculated with the two nematode species than when with *B. xylophilus* alone, indicating the inhibition of *B. xylophilus* boarding by *B. mucronatus*. Proportions of *B. xylophilus* in the nematode loads were more than 0.8 when the percentage of *B. xylophilus* in the inoculum was 50 % or more, whereas those were less than 0.1 when it was 30 % or less. That was caused by the frequency-dependent inhibition of *B. xylophilus* population growth by *B. mucronatus* in pine bolts and higher boarding abilities of *B. xylophilus*. However, higher boarding rates of *B. xylophilus* in small populations in xylem than in huge populations may explain the difficulty of exclusion of *B. xylophilus* under field conditions.

### **General discussion**

This thesis showed that interspecific competition between *B. xylophilus* and *B. mucronatus* occurs through reproductive interference when their hybrids cannot establish a population. Laboratory experiments using the two nematode species revealed quick displacement of a species population and its frequency dependency, which are characteristics of competition through reproductive interference. Frequency-dependent competition patterns differed depending on nematode isolates used. That was explained in part by differences in the intrinsic rate of natural

increase, carrying capacity, and incomplete recognition of heterospecific opposite sex. Interspecific crossing studies so far conducted show the hybrid breakdown in most cases. My experiments showed that this type of competition affected the development of pine wilt disease and the initial nematode load of insect vectors in frequency-dependent manner. Thus, competition through reproductive interference is considered to be responsible in part for the displacement of *B. mucronatus* by *B. xylophilus* observed in the field.

# Chapter 1

## Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner *et al.* Buhner) Nickle, is the causative agent of pine wilt disease (PWD) (Tokushige & Kiyohara, 1969). The nematode kills the trees of susceptible pine species such as *Pinus densiflora*, *P. thunbergii* and *P. pinaster* (Mamiya, 1987; Zhao *et al.*, 2003; Robertson *et al.*, 2011). Pine wilt disease is one of the four serious diseases in the world. Since being recorded in Nagasaki, Japan in 1905 (Yano, 1913; Mamiya, 1983; Kishi, 1995), PWD has spread throughout Japan except Hokkaido (Mamiya, 2001; Kimura *et al.*, 2011) and during 1980s it has expanded to East Asia and invaded the westernmost continent in Europe in 1999 (Mamiya, 1987; de Guiran & Bruguier, 1989; Rutherford & Webster, 1987; Mota *et al.*, 1999; Robertson *et al.*, 2011).

Tree mortality from PWD and the rate of the disease development increase with the increasing number of nematodes inoculated on healthy trees (Kiyohara & Tokushige, 1971; Hashimoto & Sanui, 1974). *Bursaphelenchus xylophilus* is transported by adult cerambycid beetles in the genus *Monochamus*; *M. alternatus* in East Asia and *M. galloprovincialis* in Europe (Mamiya & Enda, 1972; Sousa *et al.*, 2001). When vector adults feed on the twig bark of healthy pine trees, the nematodes enter the trees through the feeding wounds (Mamiya & Enda, 1972). The number of nematodes transmitted by an insect vector varies depending on the initial number of carried nematodes (initial nematode load) and the vector's age (Togashi, 1985). The nematodes proliferate on blue stain fungi in newly dead trees (Kiyohara & Tokushige, 1971; Kobayashi *et al.*, 1974). Females of the vector beetles deposit the eggs into dying and newly dead pine trees from the infection of *B. xylophilus*.

Vector females and males also transmit the nematodes into such trees *via* the oviposition wounds and others (Wingfield, 1983; Edwards & Linit, 1992; Arakawa & Togashi, 2002). Beetle larvae feed on the inner bark. Nematodes, *B. xylophilus* live in the resin cannal. The second stage propagative juveniles (J2s) will develop into the third stage propagative juveniles (J3s) or the third stage dispersal juveniles (JIIIIs). The proportion of the JIIIIs of *B. xylophilus* increases as the season advances (Aikawa, 2006; Mamiya, 1983). However, the JIIIIs may develop back into the fourth stage propagative juveniles (J4s) if no insect vectors exist. Otherwise, the JIIIIs aggregated around the pupal chambers of insect vectors in xylem and molt to the fourth stage dispersal juveniles (JIVs), the special stage for transfer, immediately after beetle pupae eclose to adults. The JIVs enter the tracheal system of newly-eclosed beetle adults and gain entrance to healthy pine trees through the feeding wounds made by the adult beetles (Kiyohara & Mamiya, 1972; Mamiya & Enda, 1979; Linit, 1988; Jikumaru & Togashi, 2001). The JIVs molt to adults in the trees and the adult females deposit the eggs after copulating with the males, repeating the infectious cycle.

The pathogenic nematode, *B. xylophilus*, is native to North America. *Bursaphelenchus mucronatus*, the close relative to *B. xylophilus*, is widely distributed across Eurasia (Braasch *et al.*, 2011) and has similar biology to *B. xylophilus* except for its avirulence against pine trees (Mamiya & Enda, 1979). When invading *P. densiflora* and *P. thunbergii* stands that *B. mucronatus* inhabits, *B. xylophilus* displaces *B. mucronatus* in the course of pine wilt epidemics in Japan (Kishi, 1995; Mamiya, 2006). This is the case in China (Cheng *et al.*, 2009). Actually both species were rarely recovered from a dead pine tree (Vincent *et al.*, 2008; Kishi, 1995). However, it is reported that a *P. densiflora* stand, where *B. mucronatus* and

its vector *M. saltuarius* live, inhibits the colonization of *B. xylophilus* in a cool summer area in Japan (Jikumaru & Togashi, 2008). The interaction between *B. xylophilus* and *B. mucronatus* may bring a development of biological control techniques against expansion of PWD.

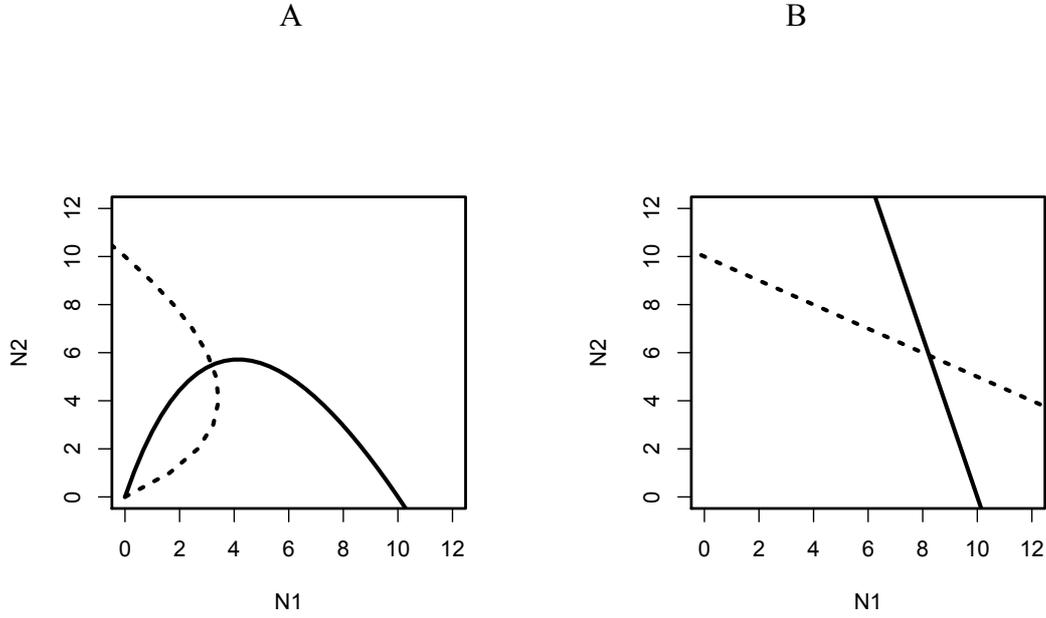
Sexual interaction, which is also called 'reproductive interference', between animal species brings several ecological and evolutionary consequences on species involved (Gröning & Hochkirch, 2008). In its original sense, interference is defined as a type of competition (Birch, 1957). However the basic difference between competition and interference is the presence or absence of a shared limited resource (Begon *et al.*, 1996). Compared with competition for conspecific mates, the shared resource, heterospecific sexual interaction is more of a wasting time, energy, nutrients, or gametes, leading a fitness loss to the species. Reproductive interference is caused by incompletely distinguishing the opposite sex of the same species from that of different species and results in enhanced reproductive cost due to unsuccessful embryonic development, infertile hybrids and so on (Gröning & Hochkirch, 2008), which deeply affects the population dynamics of competing species. On the other hand, resource competition results in the displacement of the subordinate species by dominant species through more efficient use of resources (Tilman, 1982).

Theoretical studies show that reproductive interference makes one of two competing species populations displace the other in frequency-dependent manner whereas resource competition is likely to do that depending on the relative strength between intraspecific and interspecific competitions (Fig. 1-1). They also indicate that reproductive interference requires much shorter time to displace one species population than resource competition (Ribeiro & Spielman, 1986; Kuno, 1992).

The features are confirmed in several animals (Nishida *et al.*, 2012; Kishi *et al.*, 2009;

Thum, 2007; Takafuji *et al.*, 1997).

Many interspecific crossing experiments between *B. mucronatus* and *B. xylophilus* show that it is easy to produce F<sub>1</sub> hybrids but difficult to establish populations originating from hybrids (Mamiya, 1986; de Guiran & Bruguier, 1989; Riga *et al.*, 1992; Bolla & Boschert, 1993; Taga *et al.*, 2011). The results suggest the possibility of reproductive interference between the two species under mingled conditions. However, the details of reproductive interference between *B. xylophilus* and *B. mucronatus* are unknown. So, I aimed at determining the effects of reproductive interference between *B. xylophilus* and *B. mucronatus* on the several stages of infectious cycles of *B. xylophilus*. In Chapter 3, I described the suppressive effects of *B. mucronatus* on the development of PWD. In Chapter 4, I determined frequency-dependent outcome of interspecific competition between *B. xylophilus* and *B. mucronatus*. In Chapter 5, I showed the asymmetric recognition ability between *B. xylophilus* and *B. mucronatus*. In Chapter 6, I determined the inhibitory effects of *B. mucronatus* on boarding of *B. xylophilus* on adult vector. Finally, I discussed the effects of sexual interaction between the two species on the expansion of *B. xylophilus* range.



$$\begin{aligned} \frac{dN_1}{dt} &= \left\{ b_1 \left( \frac{N_1}{N_1 + c_{12}N_2} \right) - d_1 \right\} N_1 - h_1 N_1^2 & \frac{dN_1}{dt} &= (b_1 - d_1)N_1 - h_1(N_1 + c_{12}N_2)N_1 \\ \frac{dN_2}{dt} &= \left\{ b_2 \left( \frac{N_2}{N_2 + c_{21}N_1} \right) - d_2 \right\} N_2 - h_2 N_2^2 & \frac{dN_2}{dt} &= (b_2 - d_2)N_2 - h_2(N_2 + c_{21}N_1)N_2 \end{aligned}$$

Fig. 1-1. Comparison of the zero-growth isoclines for assessing the competition outcome between the models for the reproductive interference (A) and for the resource competition (B). Mathematical models by Kuno (1992) are shown under each figure. The meanings of parameters are given as follows.  $N$ : the number of species;  $t$ : time;  $b$ : instantaneous birth rate;  $d$ : instantaneous death rate;  $h$ : crowding effect to be imposed per individual on others.  $c$ : competition coefficient. Isoclines are calculated from equations under the figure respectively using the same values of parameters ( $c_{12} = 0.3$ ,  $c_{21} = 0.5$ ,  $b_1 = b_2 = 2$ ,  $d_1 = d_2 = 1$ ,  $h_1 = h_2 = 0.1$ ). The solid and the broken lines represent the isoclines for  $N_1$  and  $N_2$ , respectively.

## Chapter 2

### Materials and Methods

Four experiments were executed in this study, where common materials and methods were used. So I describe the common materials and methods herein.

Those peculiar to experiments are described in respective chapters.

**Culture media of fungi:** Potato Dextrose Agar (Difco, Becton, Dickenson and Company, Sparks, MD, US) (PDA) and barley grains were used for fungal culture. Suspension of 39g PDA and 1L of deionised water was autoclaved at 121 °C for 20 min, and then poured into 35mm, 50mm or 90 mm diam. Petri dishes. As another medium, 10 ml of barley grains and 10 ml of deionised water were placed in 50-ml Erlenmeyer flasks and autoclaved at 121 °C for 20 min.

**Fungi:** Three fungal species were used in this study; *Botrytis cinerea*, *Ophiostoma minus* and *Nectria viridescens*. Each fungus was subcultured in 90 mm diam. Petri dishes with PDA medium at 25 °C and then held at 5 °C till use.

**Nematodes:** One isolate of *Bursaphelenchus xylophilus*, T-4, and two isolates of *B. mucronatus*, Srf and TBm119, were used in this study (Table 2-1). *B. mucronatus* Srf and TBm119 are closest to *B. m. kolymensis* from Portugal and Poland, respectively, in the molecular sequences of ITS-region (Kanzaki, personal communication).

Each isolate of nematodes was subcultured monoxenically on *B. cinerea* mycelial mat in 50 ml Erlenmeyer flasks at 25 °C in the dark, and then was held at

5 °C in the dark when the mycelial mat was consumed completely. To maintain the nematode isolates, 3-10 barley grains with nematodes were transferred onto new *B. cinerea* mycelial mat after 3-6 months of storage at 5 °C in the dark.

**Extracting and counting nematodes:** The Baermann funnel technique was used to extract nematodes from media at 25 °C for one to three days according to experiments. The technique used 90 mm diam. funnel and JK wiper (150-S) (Nippon paper crecia Co., LTD., hitotsubashi, Chiyoudaku, Tokyo, Japan).

Nematode suspensions extracted were centrifuged at 1 500 rpm (251.78 ×g) for 90 sec. The nematodes precipitated were counted.

**Genotyping of rDNA:** Genotyping of nuclear rDNA was performed for individual nematodes following a modified method of Matsunaga & Togashi (2004). To obtain DNA template, nematodes were placed in a small glass Petri dish (3.6 cm in diameter) with M9 buffer (Sulston & Hodgkin, 1988). After being rinsed in M9 buffer at *ca* 25°C for *ca* 2 h, nematodes were individually placed in 10 µl of lysis buffer (mixture of 9.17 µl of TE buffer at pH 8.0, 0.46 µl of ‘enzyme solution’ and 0.37 µl of ‘lysis solution’ in a commercial kit named ISOHAIR manufactured by Nippon Gene, Tokyo, Japan) in 0.2 ml reaction tubes with flat cap (Molecular BioProducts, San Diego, CA, USA) (Kikuchi *et al.*, 2009; Tanaka *et al.*, 2012) and frozen at –70°C or below for *ca* 30 min (Barstead *et al.*, 1991). After thawing at room temperature, the DNA solution was heated at 55°C for 30 min. Solution including the DNA templates was held at –20°C until use.

PCR amplification was performed in a total volume of 10 µl or 5µl using a thermal cycler (GeneAmp PCR system 9 600, Applied Biosystems, Foster City, CA,

USA). In one method of PCR amplification, 10  $\mu$ l contained 5% of a total individual nematode lysate, 2.5 pmol of each of two primer pairs for two species, 5  $\mu$ l of 'MasterMix solution' (AmpliTaq Gold PCR MasterMix, Applied Biosystems, Foster City, CA, USA) and 4.1  $\mu$ l of ultra-pure water. In another method, 5  $\mu$ l contained 10% of a total individual nematode lysate, 1.0 pmol of each of two primer pairs for two the species, 1.55  $\mu$ l of 'BIOTAQ™ DNA Polymerase' (0.5  $\mu$ l of 10 $\times$ NH<sub>4</sub> Reaction buffer, 0.8  $\mu$ l of 10mM dNTP Mix, 0.25  $\mu$ l of 50mM MgCl<sub>2</sub> solution) (BIOTAQ™ DNA Polymerase, Nippon Genetics Co. Ltd, Tokyo, Japan) and 2.7  $\mu$ l of ultra-pure water. DNA sequences of primer pairs were TCCGGCCATATCTCTACGAC (MF) and GTTTC AACCAATTCCGAACC (MR) for *B. mucronatus*, and ACGATGATGCGATTGGTGAC (XF) and TATTGGTCGCGGAACAAACC (XR) for *B. xylophilus* (Matsunaga & Togashi, 1997). PCR amplification using 10  $\mu$ l of solution was performed under the following conditions; initial denaturation at 95°C for 7 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 55.9°C for 30 s and extension at 72°C for 1 min, followed by a final incubation at 72°C for 6 min. In the case of 5  $\mu$ l solution, PCR amplification was performed as follows; initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 56.0°C for 30 s and extension at 72°C for 30 s, followed by a final incubation at 72°C for 10 min. PCR products were resolved electrophoretically using 1.5% agarose gel (Agarose, Code: 5003, Takara Bio Inc., 3-4-1 Seta, Otsu, Shiga, Japan) with 1  $\times$  TAE buffer at pH 8.0. The gel included a commercial stain (GelRed Nucleic Acid Stain, 10 000  $\times$  in water, Biotium, San Francisco, CA, US) at a rate of 0.5  $\times$  10<sup>-4</sup> (v/ v). Band pattern was photographed under UV light of 312 nm wavelength (Print Graph, AE – 6932GXES –U, Atto, Tokyo, Japan). Molecular size was evaluated using a commercial marker (100 bp DNA ladder, Takara Bio Inc., Otsu,

Japan). PCR products were expected to be 210 bp and 557 bp in length for *B. mucronatus* and *B. xylophilus*, respectively. Thus, the two-band image determined the genotype of nematode to be *Bm/Bx* when *Bm* and *Bx* represent genes of *B. mucronatus* and *B. xylophilus*, respectively.

Table 2-1. *Bursaphelenchus xylophilus* and *B. mucronatus* isolates used in this study

Species	Isolate	Source	Location	Year	Pathogenicity	Remarks
<i>B. xylophilus</i>	T-4	<i>Monochamus alternatus</i> adult	Ichinoseki, Iwate Pref.	1992	Virulent	
<i>B. mucronatus</i>	Srf TBm119	<i>Monochamus urussovi</i> adult <i>Monochamus saltuarius</i> adult	Furano, Hokkaido <sup>a</sup> Takano, Hiroshima Pref.	2005 1998	Avirulent Avirulent	<i>B. m. kohymensis</i> ( <i>European type-Portugal</i> ) <sup>b</sup> <i>B. m. kohymensis</i> ( <i>European type-Poland</i> ) <sup>b</sup>

<sup>a</sup> Togashi (2008).

<sup>b</sup> Information by Dr. Kanzaki.

### Chapter 3

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## Chapter 4

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## Chapter 5

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## Chapter 6

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## Chapter 7

### General discussion

This study first revealed the suppressive effects of *B. mucronatus* on the development of pine wilt disease (PWD) by the simultaneous inoculation of *P. thunbergii* with *B. xylophilus* and *B. mucronatus*. The suppressive effects increased as the frequency of *B. mucronatus* in a definite number of inoculated nematodes increased (Chapter 3). When simultaneous inoculation of the two *Bursaphelenchus* species caused pine seedlings to die, *B. mucronatus* populations and interspecific hybrids were recovered at extremely low densities 4 weeks later and disappeared 8 weeks later. By contrast, nematodes showing genotype *Bx/Bx* of rDNA persisted. The population size was greater when *B. xylophilus* was inoculated alone than when inoculated together with *B. mucronatus*. Two nematode isolates used are known to form inviable or sterile hybrid offspring (Taga *et al.*, 2011). Thus, the suppressive effects of *B. mucronatus* on PWD development were considered to be caused by reproductive interference (sexual interaction) between the nematode species. On the other hand, it is difficult to consider indirect effects of *B. mucronatus* on the *B. xylophilus* populations *via* the response of pine seedlings, because *B. mucronatus* does not cause the induced resistance of pine seedlings (Kiyohara, 1982; Kiyohara *et al.*, 1989, 1990).

Reproductive interference causes frequency-independent outcomes in the interspecific competition; one species populations displace the other when the former is included at a high frequency at first, whereas it is displaced by the other when at a small frequency. Laboratory experiments showed frequency-dependent species displacement in the competition between the two *Bursaphelenchus* species (Chapter

4). The species displacement occurred rapidly. Analysis using mathematical models indicated that the two *Bursaphelenchus* species competed through reproductive interference. A great difference in interspecific competition coefficients lead to extremely short time of coexistence (Chapter 4).

Comparing the results between field and laboratory experiments indicates that living pine trees modify the interspecific competition through reproductive interference because of unsuccessful colonization of *B. mucronatus* in living pine seedlings.

Reproductive interference is caused by the incomplete species recognition (Gröning & Hochkirch, 2008). Heterospecific copulation and hybridization are the most costly types of reproductive interference (Liou & Price, 1994; Fitzpatrick & Shaffer, 2007; Pfennig, 2007; Gröning & Hochkirch, 2008). When a pair of virgin conspecific adults and a virgin heterospecific adult were placed in a medium, a small proportion of females copulated with heterospecific males in the presence of conspecific males. By contrast, substantial proportions of females copulated with the heterospecific males in the absence of a conspecific male. In the latter case, *B. mucronatus* females tended to more often accept the heterospecific males than *B. xylophilus* females. Especially, almost all the Srf females accepted T4 males in copulation unlike TBm119 females, which may explain the higher ability of species displacement of TBm119 than Srf isolate in addition to a higher intrinsic rate of natural increase and a greater carrying capacity.

*Bursaphelenchus xylophilus* and *B. mucronatus* have a specific transmission stage in the infection cycles. When the interspecific competition occurs in pine trees through reproductive interference, different ability of nematodes to get on and off the beetles may affect the spread of pine wilt disease in pine forests. Experiments of

nematode boarding indicated that the number of *Bx/Bx* nematodes carried by *M. alternatus* adults decreased in a stepwise manner, as the proportion of *B. mucronatus* nematodes in inoculum increased. Though interspecific competition in Petri dishes indicated the displacement of isolate T4 by isolate TBm119 when the initial proportions of TBm119 nematodes were 0.5 to 0.9 (Chapter 4), boarding experiment showed that T4 persisted in pine bolts irrespective of the initial proportions of TBm119. This suggests the heterogenic habitats within pine stems may allow T-4 populations to persist.

*Bursaphelenchus xylophilus* displaces *B. mucronatus* in epidemic areas of pine wilt disease, although *B. mucronatus* is reported to inhibit the invasion of *B. xylophilus* in a cool-summer area. In this study, I indicated that *B. mucronatus* inhibits the PWD development when it is inoculated together with *B. xylophilus* on pine trees. Displacement of *B. xylophilus* by *B. mucronatus* was shown in frequency-dependent manner in laboratory. However, *B. mucronatus* almost disappeared in pine trees one month after the inoculation, although it suppressed the population growth of *B. xylophilus* after its disappearance. In addition, *B. xylophilus* persisted within dead pine stems even when a large number of *B. mucronatus* were inoculated. Such ecological traits of *B. xylophilus* and *B. mucronatus* are considered to greatly contribute to the rapid spread of *B. xylophilus* range although *B. mucronatus* has an ability to displace *B. xylophilus*.

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