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Is there a relationship between the intrinsic rate of propagation and *in-vitro* migration and virulence of the pinewood nematode, *Bursaphelenchus xylophilus*?

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Abstract We investigated if rates of propagation and migration were related with the level of virulence in the pinewood nematode *Bursaphelenchus xylophilus* using 17 offspring lines from the F_2 crosses between virulent and avirulent isolates. Virulence was tested by inoculating seedlings of *Pinus thunbergii* with the nematodes. The proportion of dead seedlings ranged from 13.3% to 77.8%, 20 weeks after inoculation. Migration rate of the nematodes was estimated by measuring their migration distance per unit time in an artificial substrate that imitated pathways in pine trees. Migration rate varied from 0.85 to 3.53 mm min^{-1}. Propagation rate was determined based on population growth on the fungus *Botrytis cinerea*, and it ranged between $10^{3.88}$ and $10^{4.99}$ per 12 days. Statistical analyses revealed that virulence was not correlated with migration rate, but was negatively correlated with propagation rate on *Botrytis cinerea*, suggesting that the nematodes paid some cost for virulence. Also, there was no relationship between rates of migration and propagation. Cluster analysis showed that the biological parameters varied between crossbred lines, with no kinship bias, suggesting the absence of sex-linked inheritance in virulence and rates of propagation and migration.

Keywords Behavioural analysis · Cross breeding · Micro-moulded structure · Pine wilt disease · *Pinus thunbergii*

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Introduction

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, is the causal agent of pine wilt disease, inciting worldwide concern about forest ecosystems (Mota and Vieira 2008). This nematode has caused great economic losses and prompted stringent governmental interventions and regulations for the control of pine wilt disease. The local spread of the nematode is steady and devastating in several Asian countries including China, Japan and South Korea (Suzuki 2002), and the disease is also present in Europe (Mota et al. 1999; Suzuki 2002).

Virulence is one of the key factors affecting the epidemiology of plant diseases where plants infested and killed by pathogens play the major role as sources of disease transmission by vectors, such as pine wilt. The virulence of pathogens is governed by their propagation and migration within host plants (e.g., Bogs et al. 2004). In fact, it has been suggested that the development of pine wilt disease consists of early and advanced stages characterised by limited migration and accelerated propagation of *B. xylophilus*, respectively (Fukuda 1997). Parasites with higher propagation rates may kill their hosts more rapidly and thus shorten their longevity; there is a highly significant correlation between virulence and population density in *B. xylophilus* extracted from killed seedlings (Dwinell 1985). The nematode migrates through cortical tissue and resin canals (Ichihara et al. 2000), and rapid exploitation requires rapid migration to undamaged tissues to acquire new food resources. *B. mucronatus,* a less virulent but phylogenetically very close species, is restricted in its dispersal and propagation as compared to *B. xylophilus* (Odani et al. 1985).

Pine species and their breeding lines with varying levels of susceptibility to the nematode have been used to study the significance of propagation and migration. Nematode migration and propagation were slow in the tissues of resistant lines and species of pines (Kuroda et al. 1991; Kuroda 2004). Resistant pine species have been suggested to have higher activity of chemicals which immobilise nematodes (Yamada and Ito 1993).

The relationship of virulence with both propagation and migration has rarely been examined simultaneously. Few nematode isolates have been compared in relation to propagation and migration (Odani et al. 1985; Togashi and Matsunaga 2003), and the number of isolates used for comparison was insufficient to determine the relationship between virulence and other parameters. Moreover, some researchers pointed out genetic divergence between nematode isolates that represented virulent and avirulent ones (Iwahori et al. 1998; Takemoto and Futai 2007). These findings implied that 'phylogenetic constraints' (Harvey and Pagel 1991) should cast doubt on the scheme of the previous comparative studies; association between virulence and other characteristics could simply have reflected phylogenetic association. An effective strategy to overcome this drawback entails crossing isolates with different levels of virulence to establish breeding lines for experiments. The aim of this study was to determine if high rates of propagation and migration were essential for virulence by using 17 crossbred lines of *B. xylophilus* with a certain genetic variation among them.

Materials and methods

Crossbred lines of *B. xylophilus*

Virulent (Ka-4) and avirulent (OKD-1) isolates of *B. xylophilus* provided by Dr. T. Aikawa, Forestry and Forest Products Research Institute (FFPRI) were used as parental isolates to establish crossbred lines. Breeding was performed as follows: 10–50 individuals of the parental isolates were pre-cultured for about 1 week on 7-day-old *Botrytis cinerea* Pers. colonies grown on 0.05% malt extract agar plats in 3.5-mm diameter Petri dishes at 23°C in the dark. Female juveniles at the third or fourth stage which were easily discriminated from the males were transferred onto new 7-day-old *B. cinerea* colonies on small agar blocks (ca. 1.5×1.5 mm) and incubated for 2 days. Virgin females were transferred individually into a 0.5-μl droplet of sterile, distilled water attached inside the lid of new Petri dishes in which 1-day-old fungal colonies were growing. Single male adults were subsequently transferred to the water droplets containing single females to facilitate mating (Ka-4 female × OKD-1 male or OKD-1 female × Ka-4 male). After 1-day incubation, a piece of agar (ca. 5×5 mm) cut from a fungal colony was carefully stamped on the droplet to collect the male and female on the agar surface. Each agar piece with a pair of nematodes was returned onto the plate so that the F_1 generation would grow on the colony. Sib mating (i.e., mating between offspring derived from the same parents) was repeated twice more in the same manner to obtain F_2 pairs, and their offspring were used as founder populations of 17 breeding lines, i.e., KO1-1, KO1-2, KO1-3, KO1-4, KO2-2, KO2-3, KO3-1, KO4-1, KO4-2, KO6-1, KO6-2, OK6-1, OK7-1, OK7-2, OK8-2, OK9-1, and OK9-2. 'KO' indicates that the line was derived from the pairing of a Ka-4 female and an OKD-1 male. Similarly, 'OK' indicates offspring lines from the pairing of an OKD-1 female

and a Ka-4 male. The 2-digit combination following 'KO' or 'OK' indicates a certain parental combination at the F_1 and F_2 generations, respectively. For instance, KO1-1 and KO1-2 shared the same ancestral pair at the F_1 generation, but differed in their ancestral pairs at the F_2 generation. Stochastic predictions indicate that one-fourth part of the genome of an individual nematode in the crossbred lines comprises allele pairs both from Ka-4 and another one-fourth from OKD-1, and that the other half has allele pairs, with one allele originating from Ka-4 and the other allele from OKD-1.

Experiment 1. Virulence test

Seedlings of Japanese black pine, *Pinus thunbergii* Parl., were prepared as follows. Seeds, provided by Dr. T. Aikawa, FFPRI, were placed on agar plates for germination at 23°C. Germinated seedlings were transplanted into test tubes (10 ml) filled with Kanuma soil, a soft yellowish pumice, (about 2–5 mm in diameter; Iwamoto, Tochigi, Japan) with small holes in the bottom. Young plants were incubated at 22–28°C under natural light until their stem diameter reached ca. 1.7 mm. To inoculate *B. xylophilus*, stems of 14-week-old seedlings were cut vertically for ca 1 cm with a razor blade ca 3 cm above the soil surface and inserted with a 2 cm-long, thin triangular piece of filter paper (Advantec, 5C, Tokyo, Japan) (Asai and Futai 2002). Water suspension (20 μl) containing 200 individuals of each crossbred line was absorbed in the paper. Forty-five seedlings were inoculated for each line, and distilled water was used as a control. Test tubes with seedlings were dipped in a 3-cm-deep plate filled with a nutrient solution (1:2000 diluted Hyponex; Hyponex Inc., Tokyo, Japan) and incubated at 23°C under a 12/12-h light-dark cycle. Seedlings with discoloured top or wilt symptoms were regarded as dead.

Experiment 2. Propagation rate

Nematodes were propagated in *B. cinerea* grown in 6-cm diameter Petri dishes containing 5 ml of potato-dextrose agar. Each of 5-cm diameter colonies of *B. cinerea* received 300 individuals and was incubated at 23°C. Nematodes were collected from the plates 6 and 12 days after incubation with a Baermann funnel for 48 h and counted.

Experiment 3. Migration rate

The micro-moulded substrate method described by Otobe et al. (2004) was used to determine migration rate using flexible polydimethylsiloxane (PDMS) (approximately 5 mm×5 mm). Migration distance during a period of 30–60 s was measured in the straight artificial pathways (60 μm×50 μm×2 mm). The tested nematodes varied in larval stage and were 500–700-μm long. All experiments were conducted at 23°C, and movement of 54 individuals was recorded by a microscope (MIC-D, Olympus, Japan). The distance and time were calculated by image-analysis software (Video Capture 6.5, Ulead Systems Inc., Japan).

Statistical analysis

The number of individuals (X) was transformed into $\log_{10}(X+1)$ in experiment 2. The correlation coefficient was calculated to investigate the relationship between virulence, propagation, and migration. Cluster analysis on the crossbred lines with respect to virulence (at 20 weeks), propagation (at 12 weeks), and migration speed was done to investigate if these properties varied among the lines depending on kinship and sex-related inheritance. The data of propagation and migration speed were analysed with ANOVA and Tukey's test. These calculations were performed with SAS v9.1 (SAS Institute Inc., Cary, NC, USA).

Results

The proportion of dead seedlings inoculated with the crossbred lines ranged from 2.2–71.1% after 4 weeks and 13.3–77.8% after 20 weeks (Table 1). The level of virulence of each crossbred line was consistent during the period of observation, with highly positive correlations between them ($r>0.781$, $P<0.001$). Propagation of the 17 lines was not significantly different both at day 6 ($F=0.81$, $P=0.728$) and day 12 ($F=0.48$, $P=0.981$).

Migration rate varied significantly among lines ($P<0.01$), with the highest rate 3.53 mm min^{-1} for OK8-2 and the lowest 0.82 for KO1-3 (Table 1), indicating that nematodes can potentially migrate 118.0–508.3 cm per day. Migration rate varied between 0.82 and 2.83 mm min^{-1} for the KO lines and between 1.12 and 3.53 mm min^{-1} for the OK lines.

Table 1 Virulence, propagation rate, and migration rate of the 17 crossbred lines of *Bursaphelenchus xylophilus*

Line	Seedlings killed (%) [a]			Propagation rate $\log_{10}(X+1)$ [b,c]		Migration rate [c]
	4 wk	12 wk	20 wk	6 d	12 d	(mm min^{-1})
KO1-1	26.7	31.1	31.1	3.68±0.21	4.78±0.05	2.02±0.13
KO1-2	2.2	4.4	13.3	3.86±0.23	4.89±0.16	1.45±0.10
KO1-3	4.4	6.7	13.3	4.20±0.05	4.98±0.12	0.82±0.07
KO1-4	71.1	73.3	77.8	3.09±0.38	3.88±0.30	2.03±0.13
KO2-2	57.8	62.2	62.2	3.18±0.18	4.71±0.16	1.91±0.12
KO2-3	26.7	33.3	33.3	3.88±0.22	5.05±0.03	1.38±0.07
KO3-1	6.7	15.6	20.0	3.60±0.12	4.74±0.12	1.40±0.09
KO4-1	2.2	28.9	35.6	3.79±0.18	4.92±0.09	2.83±0.21
KO4-2	35.6	42.2	46.7	4.04±0.08	4.25±0.17	1.61±0.10
KO6-1	22.2	22.2	28.9	4.26±0.07	4.29±0.13	1.65±0.13
KO6-2	11.1	20.0	28.9	3.35±0.11	4.52±0.13	1.75±0.15
OK6-1	22.2	40.0	40.0	3.58±0.40	4.69±0.21	2.18±0.16
OK7-1	24.4	71.1	75.6	4.12±0.11	4.57±0.16	2.37±0.11
OK7-2	6.7	33.3	37.8	4.00±0.07	4.99±0.13	1.12±0.07
OK8-2	22.2	24.4	33.3	3.71±0.20	4.99±0.09	3.53±0.22
OK9-1	4.4	11.1	28.9	3.53±0.09	4.90±0.08	1.42±0.10
OK9-2	24.4	37.8	44.4	3.64±0.15	4.94±0.08	1.85±0.19
Control	0.0	2.2	2.2			

[a] Forty-five inoculated seedlings were used for each line, and figures represent % killed seedlings

[b] The number of nematodes (X) collected from 6- and 12-day-old cultures on *Botrytis cinerea* colonies in 6-cm-diam Petri dishes transformed to $\log_{10}(X+1)$. Data are indicated as mean ± standard error

[c] Migration rate of nematodes passing through water-filled, long and straight pathways (60 μm×50 μm×2 mm) moulded on a micro substrate. Data are indicated as mean ± standard error

Cluster analysis revealed no kinship bias in the parameters of the 17 lines (Fig. 1). For example, there was a large difference among KO1-1, KO1-2, KO1-3, and KO1-4 even though the 4 lines originated from the same F_1 pairing.

Discussion

As expected, virulence and other biological characteristics varied among the nematode lines crossbred from virulent and avirulent isolates, implying that genetic

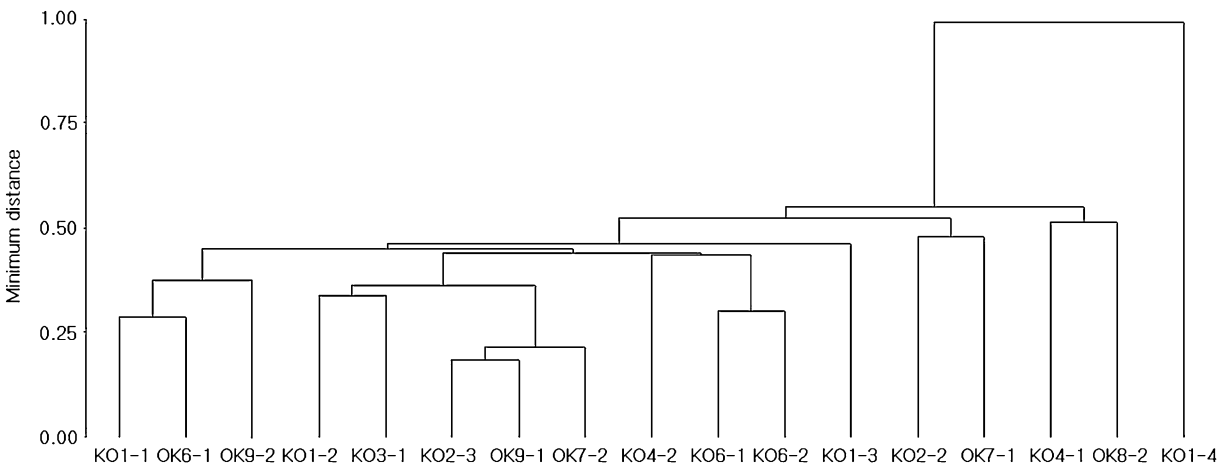

Fig. 1 Dendrogram generated by cluster analysis on the 17 crossbred lines of *Bursaphelenchus xylophilus* with minimum distance based on the data of virulence (20 wk), propagation rate (averages at 12 d), and migration rate, showing no kinship bias in these parameters. Similar results were obtained when the other combinations of virulence (4 wk and 12 wk) and propagation (6 d) data were tested (data not shown)

Table 2 Cross-correlation coefficient between virulence, propagation rate, and migration rate for the 17 crossbred lines of *Bursaphelenchus xylophilus*

Significance levels of correlation are indicated with * ($P<0.05$) and ** ($P<0.01$)

		Virulence			Propagation rate	
		4 wk	12 wk	20 wk	6 d	12 d
Propagation rate	6 d	−0.504*	−0.322	−0.346		
	12 d	−0.641**	−0.520*	−0.546*	0.233	
Migration rate		0.208	0.324	0.350	−0.223	−0.038

variation was sufficiently maintained during the generation progress until the end of the experiments. We found little difference in virulence between lines derived from the reciprocal cross between Ka-4 and OKD-1: the rate of dead seedlings was 13.3–77.8% for KO lines and 26.7–75.6% for OK lines. This implies that there was no maternal or paternal inheritance of virulence, as was reported by Kiyohara and Bolla (1990). The use of a set of crossbred lines differing in virulence should provide insights into the pathogenesis of *B. xylophilus*. The prevalence of whole-genome analyses should increasingly requires well-managed experimental lines with genetic and pedigree information. Each of the crossbred lines generated by us was heterozygous, and yet had the limitation with respect to its genetic structure being affected by drift and selection pressure. For a detailed elucidation of the mechanism of pathogenesis in future studies, recombinant inbred lines should be established from parental inbred lines with different levels of virulence (Shinya et al. 2010), as were those in mice established for genetic studies (Bailey 1971).

In cultures of the fungus *B. cinerea*, population increase was greater in isolates with higher virulence than in those with lower virulence (Kiyohara and Bolla 1990; Wang et al. 2005). The negative correlation between virulence and propagation found here (Table 2) contradicts previous findings, possibly due to the differences in the nematode populations used for experiments. The present study used a set of offspring lines originated from well-managed crosses between virulent and avirulent isolates, and this allowed us to assess essential associations between virulence and other characteristics, excluding confounding factors such as phylogenetic constraints (Harvey and Pagel 1991). The findings of this study imply that more virulent nematodes pay a higher cost for virulence which otherwise could be spent on a higher fecundity. If this trade-off applies to the process of population growth of the nematode within single trees, 'uncooperative' phenotype which would not pay a cost for pathogenesis to kill the host plants may occasionally be adaptive on disease development. This can prevent maximization of nematode virulence in single trees. The origin of the cost for pathogenesis was not disclosed in the present study, but previous reports suggested that the nematode produced additional materials such as phytotoxins, cellulase, and terpenoids which could play a part in pathogenesis (Wang et al. 2010). The activity of cellulase was found to be correlated with virulence (Kojima et al. 1994). Virulent isolates have slightly different metabolic profiles (Bolla et al. 1987) and DNA sequences (Iwahori et al. 1998).

It is important to elucidate how migration influences the virulence of *B. xylophilus*. The nematode is suggested to migrate to all parts of plants and propagate in search of nutrient sources and favourable environments (Melakeberhan and Webster 1990). Several studies have found that virulent isolates of *B. xylophilus* migrate faster than avirulent ones and an allied avirulent species *B. mucronatus*, although exceptions exist (Iwahori and Futai 1996; Togashi and Matsunaga 2003). In this study, migration rate did not appear to be an important factor governing virulence (Table 2). The results from this study may represent the behavioural response of *B. xylophilus* against the physical structure of resin canals. The structure of the experimental device was similar to the resin duct, with a long and straight pathway, and migration in the devise may represent intrinsic migration ability (Eo et al. 2010). Movement of the nematode in living tissues might not be reproduced in modified structures because of the presence of inhibitive chemical substances (Hanawa et al. 2001) such as viscous oleoresin in resin canals. Iwahori and Futai (1996) found that nematodes migrated through stems where resin was vigorously exuded and that nematode population increased as resin exudation reduced. Further research is necessary to elucidate the relationship between virulence and responses against

viscous oleoresin and other chemical substances of living pine trees.

A limitation of the present study is that the virulence of nematodes was evaluated on small pine seedlings without considering the size effect of host plants: the rate of migration may be more important for virulence in larger host plants. Anyway, the present system for measuring migration rate has the potential for prompt analysis of migration ability. Our study represents an initial step in understanding how virulence and other characteristics of *B. xylophilus* essentially interact with each other.

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