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Interspecific hybridization between *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*

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Abstract *Bursaphelenchus xylophilus* is the pathogen that causes pine wilt disease, which has greatly damaged forests and ecosystems in countries of East Asia and Europe. *Bursaphelenchus mucronatus* is closely related to *B. xylophilus* in morphology and host plant specificity. A long-running debate has existed regarding whether these two species can successfully produce hybrid offspring. In the present study, we performed in the laboratory, hybridization of two *B. xylophilus* nematode isolates from China and Japan and three *B. mucronatus* isolates from China, Japan and France. Nematode isolates of *B. xylophilus* were successfully crossed with *B. mucronatus* isolates, and the rate of hybridization was relatively high; however, some hybrid offspring died. Successful hybridization occurred between *B. xylophilus* and *B. mucronatus* isolates from China, and 22 generations of hybrids were produced. All F1 hybrids could be backcrossed with their parents and produce offspring. Variation in mucro length among the hybrid offspring and their parents was observed. The hybrid offspring and their parents were inoculated into 3-months-old black pine (*Pinus tunbergii*) seedlings. Weaker pathogenicity of hybrid offspring was observed compared with that of their parents, and significantly fewer offspring nematodes than parents were reisolated from pines. Therefore, the offspring of *B. xylophilus* and *B. mucronatus* may exist in the forest and could influence disease epidemics.

Keywords *Bursaphelenchus xylophilus* · *Bursaphelenchus mucronatus* · Interspecific hybridization · Variation

Introduction

Pine wood nematode (*Bursaphelenchus xylophilus*) is the pathogen of pine wilt disease, which causes both wilting and death of pines (Yang 2003; Futai 2013). *Bursaphelenchus mucronatus* has been characterized as non-virulent or weakly pathogenic (Cheng et al. 1986; Liao et al. 2014; Ryss et al. 2018). However, because *B. mucronatus* exists widely in the pine forests of Eurasia, there has been much interest in the hybridization between *B. mucronatus* and *B. xylophilus*, and many investigations of this topic exist in the literature (Baujard 1980; Abad et al. 1991; Ailing et al. 2010). These investigations have been inconclusive, and many issues remain unresolved or unaddressed, including the pathogenicity of *B. mucronatus* and the indistinct classification characteristics of *B. mucronatus* and *B. xylophilus*. Gaining new insights in these issues will contribute to the quarantine and control of pine wilt disease.

Since *Bursaphelenchus* was first recorded by Fuchs in 1937, 62 species have been described within the genus (Fuchs 1937; Goodey 1960; Ryss et al. 2005): *B. xylophilus* was described by Steiner and Buhrer (1934) and *B. mucronatus* by Mamiya and Enda (1979) considered *B. mucronatus* to differ from *B. xylophilus* in terms of

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pathogenicity, geographic distribution and morphology. *B. xylophilus* is strongly pathogenic to pine trees, whereas *B. mucronatus* is not (Futai 2013). *Bursaphelenchus xylophilus* is distributed only in Japan (pine wilt disease was reported in 1979 only in Japan), but *B. mucronatus* is more widely distributed and is found in many countries in Asia and Europe (Braasch 2001). The *B. xylophilus* female has a broadly rounded tail tip and no caudal mucro or has a digitate terminus and a caudal mucro of approximately 1–2 μm. In contrast, the *B. mucronatus* female has a digitate terminus and a caudal mucro of more than 3 μm. In addition, the *B. xylophilus* male has an ovoid copulatory bursa, and the *B. mucronatus* male has a square shovel-shaped copulatory bursa (Kanzaki et al. 2016). Despite the established classification of the two species, issues with the pathogenicity of *B. mucronatus* and the indistinct classification characteristics of both *B. mucronatus* and *B. xylophilus* have cast doubts concerning the scientific rationale of the classification.

We performed indoor hybridization of *B. xylophilus* nematode isolates from China, Japan and *B. mucronatus* nematode isolates from China, Japan and France. Our objectives were to examine the mating ability, hybrid offspring survival and fecundity of both species.

Materials and methods

Nematodes

Two *B. xylophilus* nematode isolates and three *B. mucronatus* isolates were collected from China, Japan and France: (1) Chinese isolate of *B. xylophilus*–BxZJ, collected and separated from a susceptible *Pinus massoniana* tree in Linan County, Zhejiang Province, China by members of our laboratory; (2) Japanese isolate of *B. xylophilus*–BxJap, separated from diseased wood from Japan that was provided by Mr. Futai, Kyoto University; (3) Chinese isolate of *B. mucronatus*–BmCHN, separated from dead wood of *P. massoniana* in a non-epidemic area Yixing County, Jiangsu Province, China by members of our laboratory; (4) Japanese isolate of *B. mucronatus*–BmJap, separated from dead wood of *Pinus tunbergii* from Japan. The nematode was provided by Professor Lin Maosong, Nanjing Agricultural University; (5) French isolate of *B. mucronatus*–BmFr, separated from dead wood of *Pinus pinaster* from France and provided by Professor Lin Maosong, Nanjing Agricultural University.

Nematodes in wood were separated using the Baermann funnel method (Baujard 1980) and the isolates were identified under the microscope. Thirty female and 30 male adults were selected and placed on peptone sucrose agar (PSA) medium (200 g of potato, 18 g of sucrose, 18 g of agar, 1000 mL distilled water) containing *Botrytis cinerea* in a Petri dish and then cultured in a dark, 25 °C chamber (all cultures were under the same conditions). The nematodes were identified using specific primers 1 (5′-GATGATGCGATTGGTGACT-3′), specific primers 2 (5′-TGCGCTGCGTTGAGTCGA-3′), and specific primers 3 (5′-CAATTCACTGCGTTCTTC-3′) (Zhao et al. 2005).

Obtaining unmated female nematodes

To ensure interspecific mating instead of intraspecific mating, we obtained "unmated female nematodes" according to the following procedure: A single III- or IV-instar larva was placed in a small penicillin bottle containing 2 mL of sterile water and 1–2 mycelia of *B. cinerea* and cultured for 3 days. Using a microscope, sexed the larvae, after which they were used for mating.

Hybrid or mating methods

Hybrid method and obtaining the F1 generation

A nematode isolate of unmated females and another isolate of three active male nematodes were placed into a tube containing *B. cinerea* (with 20 replicates) and cultured for 7 days to obtain the F1 generation.

F2 generation and offspring culture

Ten larvae of the F1 generation generated by each hybrid combination were selected (the proportion of male and female nematodes was random), placed into a test tube containing *B. cinerea* mycelia (with 5 replicates) and cultured for 7 days to obtain the F2 generation.

An unmated female and another male were added to *B. cinerea* culture medium (with 5 replicates) and cultured for 5 days to obtain the F1 generation. The process was repeated using F1 male and female nematodes, and after 5 days of culture the F2 generation was obtained. The process was repeated using F2 male and female nematodes, and after 5 days of culture the F3 generation was obtained. This process was repeated until twenty-three generations were obtained.

Backcross method

A single F1 adult female nematode was selected and mated with three adult male parents on *B. cinerea* medium cultured for 14 days to obtain backcross progeny. Each treatment was repeated 10 times.

Sterile water was used to flush out nematodes of the tubes for examination and quantification. The total number of offspring, numbers of adult females and males, number

of larvae, and number of dead nematodes were obtained and used to calculate the average number of offspring and mortality as follows:

Average number of certain nematodes = total numbers found/number of test tubes with certain nematodes

Mortality rate (%) = (total number of dead nematodes/number of offspring nematodes) × 100%

Measurement of the caudal mucro of female nematodes

Nematodes were washed with sterile water, and the nematode suspension was transferred to a centrifuge tube that was placed in a water bath at 65 °C for 1 min to kill nematodes. Afterward, a 2X FA fixative (80% formalin, 20% acetic acid) was immediately added to the nematode suspension. When the nematodes were observed under the microscope, we measured the length of the female nematode caudal mucro. One hundred female nematodes were observed per treatment.

Artificial inoculation

Artificial inoculation was performed on 3-months-old black pine (*P. tunbergii*) seedlings at the end of May. A 0.8-cm-long lengthwise wound was carved into the main stem of each seedling, and a piece of cotton was placed on the wound. A nematode suspension containing 50 pine wood nematodes or sterilized water was pipetted onto the cotton and covered with parafilm. Minimum and maximum temperatures within the greenhouse ranged from 15 to 32 °C for 30 days. Seedlings continued to be watered after inoculation, but no nutrients were supplied. Pine seedlings were considered dead when seedlings wilted or turned brown. The stems of dead seedlings were cut into pieces 1 cm in length and immersed in sterilized distilled water (1 day) to confirm the presence of nematodes. The total number of extracted nematodes was counted for each seedling. Sixteen seedlings were used for inoculation, and eight seedlings were used as the control.

Results and analysis

Direct molecular detection of the nematode

Nematodes (*B. xylophilus* and *B. mucronatus*) were identified using specific primers 1, specific primers 2, and specific primers 3 (Zhao et al. 2005). The results are shown in Fig. 1. BxZJ and BxJap were identified as *B. xylophilus* (amplified fragment length of 329 bp), and BmCHN, BmJap and BmFr were identified as *B. mucronatus* (amplified fragment length of 206 bp) (Zhao et al. 2005).

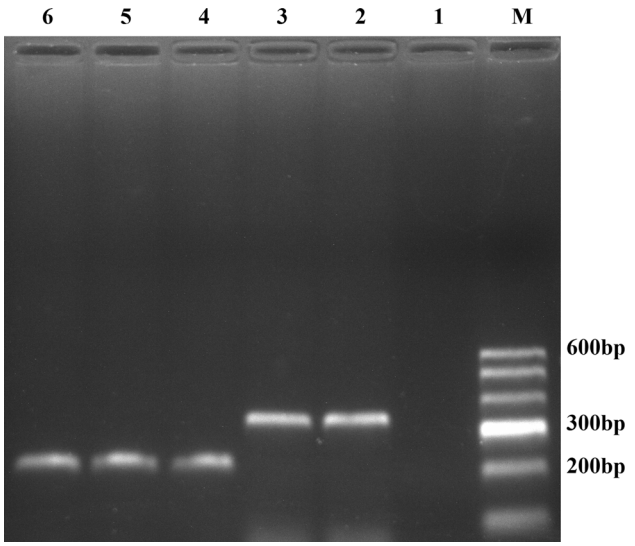

Fig. 1 Amplified profiles of nematode DNA using primer combinations of Zhao et al. (2005). Lanes 2 and 3 were identified as *B. xylophilus* (amplified fragment length of 329 bp), and lanes 4, 5 and 6 were identified as *B. mucronatus* (amplified fragment length of 206 bp). M: DNA marker, 1: Negative control, 2: BxZJ, 3: BxJap, 4: BmCHN, 5: BmJap, 6: BmFr

B. xylophilus and *B. mucronatus* interspecific and intraspecific mating conditions

Adult male and female *B. xylophilus* and *B. mucronatus* isolates were orthogonally or reverse crossed and cultivated for 7 days to observe the F1 generation; the intraspecific self-mated and single female adults were cultured as controls (Table 1). All combinations could be crossed and could generate F1 progeny, although the offspring counts varied among tubes, which ranged from 17 to 44. Compared with intraspecific self-mating, *B. xylophilus* and *B. mucronatus* produced fewer F1-generation offspring. The number of offspring produced per tube from intraspecific mating was approximately 100. However, the number of F1-generation offspring from interspecific mating was approximately 50, which is only half of that observed from intraspecific mating.

In addition, a single female nematode did not produce offspring, which suggests that this nematode might not be parthenogenetic.

Production of the F2 generation

To study the production of F2-generation nematodes, we selected 10 larvae from each F1 generation cultured in *B. cinerea* for 7 days, observed them under the microscope, and recorded the results shown in Table 2. All combinations produced an F2 generation, but the generations differed in total nematode quantity, larvae and nematode vigor (Table 2).

Table 1 Results of *B. xylophilus* and *B. mucronatus* intraspecific mating and interspecific hybridization (F1)

Hybrid combination	The number of test tubes	The number of offspring produced in vitro	The average number of offspring in vitro	Offspring mortality rate (%)
BxZJ♀ × BmCHN♂	20	11	42	1.5
BmCHN♀ × BxZJ♂	20	9	31	8.2
BxZJ♀ × BmJap♂	20	4	35	0.0
BmJap♀ × BxZJ♂	20	7	20	7.9
BxZJ♀ × BmFr♂	20	6	17	0.0
BmFr♀ × BxZJ♂	20	2	34	19.1
BxJap♀ × BmCHN♂	20	4	39	6.4
BmCHN♀ × BxJap♂	20	4	22	6.8
BxJap♀ × BmJap♂	20	8	32	12.1
BmJap♀ × BxJap♂	20	8	26	8.7
BxJap♀ × BmFr♂	20	12	43	7.4
BmFr♀ × BxJap♂	20	13	44	3.0
BxZJ♀ × BxZJ♂	3	3	100	1.0
BmCHN♀ × BmCHN♂	3	2	102	1.5
BxZJ♀ × BxJap♂	3	3	80	1.1
BxZJ♀	3	0	0	–
BmCHN♀	3	0	0	–

All combinations could be crossed and could generate F1 progeny. Compared with intraspecific self-mating, *B. xylophilus* and *B. mucronatus* produced fewer F1-generation offspring

Table 2 Results of the production of F2-generation nematodes from *B. xylophilus* and *B. mucronatus* interspecific hybridization

Hybrid combination	Total number of offspring	The number of female adults	The number of male adults	Larvae number	Larva proportion (%)	The number of dead nematodes	Mortality (%)
BxZJ♀ × BmCHN♂	213	23	18	172	80.8	22	10.3
BmCHN♀ × BxZJ♂	260	27	22	211	81.2	40	15.4
BxZJ♀ × BmJap♂	127	20	15	92	72.4	35	27.6
BmJap♀ × BxZJ♂	141	18	24	99	70.2	44	31.2
BxZJ♀ × BmFr♂	74	5	7	62	83.8	38	51.4
BmFr♀ × BxZJ♂	91	12	13	66	72.5	79	86.8
BxJap♀ × BmCHN♂	122	12	8	102	83.6	0	0.0
BmCHN♀ × BxJap♂	129	9	7	113	87.6	13	10.1
BxJap♀ × BmJap♂	167	25	25	117	70.1	33	19.8
BmJap♀ × BxJap♂	148	19	23	106	71.6	52	35.1
BxJap♀ × BmFr♂	207	27	25	155	74.9	14	6.8
BmFr♀ × BxJap♂	227	26	31	170	74.9	9	4.0

All combinations produced an F2 generation, but the generations differed in total nematode quantity, larvae and nematode vigor

The hybrid combination BmFr♀ × BxZJ♂ produced 91 offspring, but 79 died. The hybrid combination BxZJ♀ × BmFr♂ exhibited 51.4% mortality but produced fewer offspring, which shows that the breeding and survival ability of these two hybrid offspring combinations is weak. The best hybrid situation was the combination of BxJap × BmFr; this combination exhibited high fecundity regardless of the cross or backcross and produced more offspring with low mortality, which shows that the two species can hybridize and produce fertile offspring.

Breeding of hybrid offspring

To understand the reproductive capability of hybrid offspring, we obtained hybrid offspring of BxZJ × BmCHN and recorded the average number of females, males, and larvae as well as the sex ratio and mortality for each generation (Table 3). The data indicate that the hybrid combination of BxZJ × BmCH could produce 22 generations. The average number of larvae increased as the generation increased, while the average number of adults decreased. In addition, nematode mortality increased, but the sex ratio was stable between generations.

The backcross results between hybrid progenies and their parents

To understand the backcross outcomes of different crossing combinations with their parents, we mated F1-generation female adults and their parents. Each treatment was repeated in 10 tubes. The offspring reproductive data are shown in Table 4. All combinations, with the exception of (BmJap♀ × BxZJ♂)♀ × BxZJ♂, could be backcrossed with their parents and produce offspring.

Length of the caudal mucro of female nematode offspring

The length of the caudal mucro of hybrid offspring BxZJ♀ × BmCHN♂ and BmCHN♀ × BxZJ♂, backcross offspring (BxZJ♀ × BmCHN♂)♀ × BxZJ♂ and (BmCHN♀ × BxZJ♂)♂ × BmCHN♀ and their parents (BxZJ♀ and BmCHN♀) of *B. xylophilus* and *B. mucronatus* was measured. Length distribution ratios are listed in the Table 5. The length distribution of the caudal mucro of the *B. xylophilus* parent (BxZJ♀) ranged from 0 to 2 μm, and the majority were 0.5–1.5 μm. The length distribution of the caudal mucro for the *B. mucronatus* parent (BmCHN♀) ranged from 3 to 5.5 μm, and the majority were 3–4.5 μm. The length distribution for the hybrid offspring BxZJ♀ × BmCHN♂ and BmCHN♀ × BxZJ♂ ranged from 1.5 to 4 μm, and that for the backcross offspring (BxZJ♀ × BmCHN♂)♀ × BxZJ♂ ranged from 0 to 3.5 μm; the length distribution for the other backcross offspring (BmCHN♀ × BxZJ♂)♂ × BmCHN♀ ranged from 0 to 5.5 μm. The length distribution ratios of the caudal mucro of the offspring confirm the genetic law of quantitative character.

Table 3 Hybrid progeny breeding of Chinese *B. xylophilus* and *B. mucronatus* hybrid combination (BxZJ × BmCHN)

The number of offspring	BxZJ♀ × BmCHN♂					BmCHN♀ × BxZJ♂				
	The average number of females	The average number of males	The average number of larvae	Sex ratio (males: females)	Mortality rate (%)	The average number of females	The average number of males	The average number of larvae	Sex ratio (males: females)	Mortality rate (%)
F1	8.2	6.6	12.2	0.8:1	0.0	10.6	9.2	15.2	0.9:1	0.0
F2	12.4	8.8	14.6	0.7:1	2.5	11.0	11.6	16.8	1.1:1	0.0
F3	7.8	9.8	14.0	1.3:1	0.0	9.2	8.4	16.6	0.9:1	0.0
F4	7.8	6.8	11.6	0.9:1	0.0	9.4	9.4	12.2	1.0:1	0.0
F5	10.2	10.2	16.8	1.0:1	0.0	10.2	6.6	20.0	0.7:1	2.8
F6	8.2	5.4	16.2	0.7:1	7.1	11.4	9.8	15.8	0.9:1	12.1
F7	11.6	11.4	10.4	1.0:1	9.4	10.0	11.2	13.6	1.1:1	11.4
F10	8.0	5.8	14.8	0.7:1	18.5	9.0	10.2	12.4	1.1:1	10.7
F13	10.0	8.4	14.4	0.8:1	6.1	6.6	4.2	19.4	0.6:1	22.2
F17	7.0	6.8	19.4	1.0:1	21.2	4.8	6.4	14.8	1.3:1	12.0
F19	6.8	4.4	15.8	0.7:1	17.6	6.0	4.8	21.8	0.8:1	19.4
F20	5.2	2.2	17.8	0.4:1	30.4	4.4	5.2	14.0	1.2:1	34.6
F21	4.0	4.8	19.0	1.2:1	69.2	2.0	1.2	18.0	0.6:1	70.0
F22	2.4	0.4	23.4	0.2:1	77.8	1.0	0.8	19.6	0.8:1	86.4
F23	0.0	0.0	0.0	/	/	0.0	0.0	0.0	/	/

The hybrid combination of BxZJ × BmCH could produce 22 generations. The average number of larvae increased as the generation increased, while the average number of adults decreased. Nematode mortality increased, but the sex ratio was stable between generations

Table 4 Backcross results between F1 generations of different hybrid combinations and their parents

Backcross combinations (F1♀ × parents♂)	The number of test tubes	The number of offspring produced in vitro	Backcross success rate (%)	The number of offspring per tube	Offspring mortality (%)
(BxZJ♀ × BmCHN♂)♀ × BxZJ♂	10	5	50	42	17.8
(BxZJ♀ × BmCHN♂)♀ × BmCHN♂	10	5	50	31	10.6
(BmCHN♀ × BxZJ♂)♀ × BmCHN♂	10	2	20	35	7.9
(BmCHN♀ × BxZJ♂)♀ × BxZJ♂	10	3	30	20	18.3
(BxZJ♀ × BmJap♂)♀ × BxZJ♂	10	2	20	17	37.5
(BxZJ♀ × BmJap♂)♀ × BmJap♂	10	2	20	34	32.7
(BmJap♀ × BxZJ♂)♀ × BmJap♂	10	3	30	39	57.3
(BmJap♀ × BxZJ♂)♀ × BxZJ♂	10	0	0	0	–
(BxZJ♀ × BmFr♂)♀ × BxZJ♂	10	1	10	32	17.3
(BxZJ♀ × BmFr♂)♀ × BmFr♂	10	2	20	26	18.0
(BmFr♀ × BxZJ♂)♀ × BmFr♂	10	4	40	43	38.6
(BmFr♀ × BxZJ♂)♀ × BxZJ♂	10	5	50	44	35.0
(BxJap♀ × BmCHN♂)♀ × BxJap♂	10	1	10	51	14.9
(BxJap♀ × BmCHN♂)♀ × BmCHN♂	10	3	30	47	16.6
(BmCHN♀ × BxJap♂)♀ × BmCHN♂	10	2	20	44	22.3
(BmCHN♀ × BxJap♂)♀ × BxJap♂	10	3	30	53	18.0
(BxJap♀ × BmJap♂)♀ × BxJap♂	10	3	30	29	46.9
(BxJap♀ × BmJap♂)♀ × BmJap♂	10	4	40	30	52.2
(BmJap♀ × BxJap♂)♀ × BmJap♂	10	4	40	43	39.3
(BmJap♀x BxJap♂)♀ × BxZJ♂	10	4	40	33	37.7
(BxJap♀ × BmFr♂)♀ × BxJap♂	10	6	60	54	19.0
(BxJap♀ × BmFr♂)♀ × BmFr♂	10	7	70	45	19.3
(BmFr♀ × BxJap♂)♀ × BmFr♂	10	5	50	43	22.0
(BmFr♀ × BxJap♂)♀ × BxJap♂	10	5	50	51	18.5

All combinations, with the exception of (BmJap♀ × BxZJ♂)♀ × BxZJ♂, could be backcrossed with their parents and produce offspring

Table 5 Length distribution ratios of the caudal mucro for the offspring of different *B. xylophilus* and *B. mucronatus* crosses (%)

The offspring	The length range (μm)										
	< 0.5	0.5–1	1–1.5	1.5–2	2–2.5	2.5–3	3–3.5	3.5–4	4–4.5	4.5–5	> 5
BxZJ♀ × BmCHN♂	0	0	0	16	14	14	38	18	0	0	0
BmCHN♀ × BxZJ♂	0	0	0	13	25	26	17	19	0	0	0
(BxZJ♀ × BmCHN♂)♀ × BxZJ♂	22	13	16	22	18	3	6	0	0	0	0
(BmCHN♀ × BxZJ♂) ♂ × BmCHN♀	9	9	3	9	13	13	6	13	3	12	10
BxZJ♀	14	36	33	17	0	0	0	0	0	0	0
BmCHN♀	0	0	0	0	0	0	25	31	29	13	2

Pathogenicity of the nematode offspring

Three-month-old black pine seedlings were inoculated with different nematode offspring (Table 6). The offspring of *B. xylophilus* (BxZJ) was the most pathogenic, and the seedling disease rate was 100%. The offspring of *B. mucronatus* (BmCHN) was the least pathogenic, and the disease rate was 25%. The pathogenicity of the other offspring was between that of the previous two offspring and was, in the order of high to low, as follows: F5 generation of (BxZJ♀ × BmCHN♂)♀ × BxZJ♂, F5 generation of (BmCHN♀ × BxZJ♂)♂ × BmCHN♀, F10 generation of BmCHN♀ × BxZJ♂, and F15 generation of BmCHN♀ × BxZJ♂. We recovered nematodes from wilted pine seedlings; nematodes ranged from 16 to 29. The number of

Table 6 Pathogenicity of the nematode offspring inoculated to 3-months-old black pine seedlings

The nematode offspring	Number of inoculated seedlings	Number of wilted seedlings	Rate of disease (%)	The number of nematodes recovered from each wilted seedling
F10 generation of BmCHN♀ × BxZJ♂	16	12	75.0	19
F15 generation of BmCHN♀ × BxZJ♂	16	5	31.3	20
F5 generation of (BxZJ♀ × BmCHN♂)♀ × BxZJ♂	16	14	87.5	29
F5 generation of (BmCHN♀ × BxZJ♂) ♂ × BmCHN♀	16	12	75.0	16
The offspring of BxZJ	8	8	100.0	28
The offspring of BmCHN	8	2	25.0	28
Sterile water (CK)	8	0	0.0	0

The offspring of *B. xylophilus* (BxZJ) were the most pathogenic, whereas the offspring of *B. mucronatus* (BmCHN) were the least pathogenic. The pathogenicity of the other offspring was between that of the previous two offspring and was, in the order of high to low, as follows: F5 generation of (BxZJ♀ × BmCHN♂)♀ × BxZJ♂, F5 generation of (BmCHN♀ × BxZJ♂) ♂ × BmCHN♀, F10 generation of BmCHN♀ × BxZJ♂, and F15 generation of BmCHN♀ × BxZJ♂. We recovered nematodes from wilted pine seedlings; nematodes ranged from 16 to 29. The number of recovered nematodes and pathogenicity were not correlated

recovered nematodes and pathogenicity were not correlated.

Discussion

Many unanticipated factors can affect nematode hybridization success in vitro, e.g., low survival of male and female nematodes, injury to nematodes during processing and physical separation in culture. Despite some tests being unsuccessful due to other factors, single successful tests were sufficient to indicate hybridization, and unsuccessful tests due not contradict these results.

Inbreeding depression can affect the propagation of nematodes in vitro. Because nematode individuals in the same tube come from the same parents, further reproduction of these nematodes occurs through self-propagation, which could lead to inbreeding depression. Inbreeding depression may explain the lower adult density, higher larvae density and lower survivorship observed in the later generations of hybrid offspring in the present study (Queiroz 2005).

Similar to the procedures of Mamiya (1986), we used *B. xylophilus* and *B. mucronatus* nematode isolates from Japan for our study. However, unlike the reports of Mamiya, we observed that species could cross and that the hybrids could produce many generations of fertile offspring. Further examination is needed to explain the differences between our findings and those of Mamiya.

Natural nematode reproduction differs from in vitro nematode reproduction. In nature, *B. xylophilus* and *B. mucronatus* often live on the same dead tree where encounters with each other, such as hybridization and backcrossing, are possible. Hybridization or backcrossing occurs at the population level. Thus, hybridization offspring between *B. xylophilus* and *B. mucronatus* is likely to occur in nature, and their offspring viability may be strong.

The occurrence of natural phenomena suggest the existence of hybrid offspring between *B. xylophilus* and *B. mucronatus*, e.g., the "M" type of pine wood nematode isolate. Wingfield and Blanchette (1983) isolated a nematode isolate similar to the pine wood nematode from *Abies balsamea* in Minnesota of the United States. The nematode isolate has a caudal mucro and tail shape similar to those of *B. mucronatus*, but other characteristics and pathogenicity are similar to those of *B. xylophilus*. Thus, Wingfield called the nematode an "M" type pine wood nematode isolate. Later, Bergdahl (1988) obtained another nematode isolate from *Larix dahurica* that is similar to the "M" type isolate. Mamiya (1988) also recorded at least 7 "M" type isolates in Japan that are similar to the one described by Wingfield. In China, Ma et al. (1996) reported a separated nematode isolate similar to the "M" type from Sheshan of Shanghai. The female caudal mucro of this nematode isolate is 2.5–6.0 μm in length, which is very similar to that of *B. mucronatus.*

Variation also exists regarding the pine wood nematode male copulatory bursa. Mamiya and Enda (1979) and Niekie et al. (1981) initially identified the male nematode copulatory bursa to be the main character differentiating the two nematodes; the *B. xylophilus* male copulatory bursa is ovoid, and the *B. mucronatus* copulatory bursa is square.

However, despite these characteristic differences, they are not being treated as two distinguishing features. Liu and Yang (1995) observed multiple specimens of *B. xylophilus* and *B. mucronatus* and determined that the male copulatory bursa cannot distinguish the two nematodes. Zhang et al. (2008) studied 14 populations of *B. mucronatus* and 2 populations of *B. xylophilus* and reported that the *B. xylophilus* and *B. mucronatus* male copulatory bursa is complex; the author reported 12 different copulatory bursa types and no obvious differences to distinguish the two nematodes and concluded that copulatory bursa diversity and uncertainty are not appropriate as main features.

Another example is the enhancement of *B. mucronatus* pathogenicity. *Bursaphelenchus mucronatus* was initially considered not or weakly pathogenic (Mamiya and Enda 1979). However, the pathogenicity of *B. mucronatus* has recently increased. Zhang et al. (2004) tested the pathogenicity of twenty *B. mucronatus* populations from various countries on 2-years-old *Pinus thunbergii* seedlings and reported different degrees of pathogenicity among the populations. Among them, the *B. mucronatus* population from Fujian, China and three populations from France showed similar pathogenicity to *P. thunbergii* seedlings compared with those of *B. xylophilus* (Zhang et al. 2004). There were no reports of pine tree mortality over a large geographic area caused by *B. mucronatus* before pine wood nematode disease was introduced to China. However, *B. mucronatus* has recently caused large areas of pine tree mortality in various provinces of China. For example, in 2005, *B. mucronatus* caused 25,000 pine tree deaths in Huaping County, Yunnan Province, China (Li et al. 2007). *B. mucronatus* also caused a large area of pine tree deaths in Jiangxi Province in 1995 (Fang 1996). Other examples of large areas of pine tree deaths caused by nematodes exist, and these nematodes could have been the hybrids of *B. xylophilus* and *B. mucronatus.*

There have been many studies on the hybridization of *B. xylophilus* and *B. mucronatus.* The first study was conducted by Mamiya (1986), who collected and crossed 3 isolates of *B. xylophilus* and 8 isolates of *B. mucronatus* from Japan. All combinations produced F1 offspring; however, the F1s could not produce F2s, and the F1s could not backcross with the parents. The author concluded that reproductive isolation between *B. xylophilus* and *B. mucronatus* is responsible. Later, many researchers questioned the results of Mamiya. Hajaukiewicz and Myers (1988) hybridized a Japanese isolate of *B. xylophilus* and a French isolate of *B. mucronatus* and reported high potential for hybridization success exists, and the hybrid offspring could produce multiple generations and had strong reproductive ability. Bolla and Bosehert (1993) collected nematode isolates of *B. xylophilus* and *B. mucronatus* from North America, Japan and France; hybridization occurred among these isolates, and F1 offspring could generate F2 and F3 progeny. Braasch (1994) studied nematode isolates from Germany and Japan and reported that *B. xylophilus* and *B. mucronatus* can be crossed and produce pathogenic offspring. Liu and Yang (1994) collected and hybridized 5 nematode isolates of *B. xylophilus* from China, Japan and Canada and 5 *B. mucronatus* isolates from China, Japan and France; some hybridized combinations produced F2 and F3 offspring, and some backcross combinations could produce offspring. These findings were later confirmed by both Guiran and Bruguier (1989) and Riga et al. (1992). In summary, with the exception of the study of Mamiya that reported the inability of *B. xylophilus* and *B. mucronatus* to cross, other studies have various isolates of the two species can be crossed or at least partly crossed.

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