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Pinewood Nematode Species Complex: Interbreeding Potential and Chromosome Number[1]

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Abstract: Interbreeding potential, chromosome number, and host range were compared among several isolates and species of *Bursaphelenchus* from diverse geographic areas. Some isolates from North America, Japan, and France had a wide-ranging interbreeding potential, whereas others were restricted in their potential to hybridize with other isolates. Although interbreeding occurred in the laboratory between some "M" and "R" forms of *B. xylophilus*, interbreeding of *B. xylophilus* and *B. mucronatus* was rare. The hybrids had the pathogenicity of the parent with the broader host range. This fact suggests that virulence may be inherited as a dominant character or that increased virulence may have resulted from differences in hybrid vigor. The haploid chromosome number of the different isolates separated the isolates into three groups and distinguished *B. xylophilus* from *B. mucronatus*. The findings suggest that the pinewood nematode species complex consists of sibling species that have evolved by reproductive isolation, that the French isolate is a new species, and that *B. xylophilus* and *B. mucronatus* have evolved from a common ancestor.

Key words: Bursaphelenchus, chromosome, interbreeding, nematode, pinewood nematode, *Pinus*, speciation.

Many members of the genus *Bursaphelenchus* are phoretic with insects (12,14–16,21). Some species, including the pinewood nematode, *B. xylophilus* (Steiner & Buhrer), Nickle, *B. mucronatus* Mamiya & Enda, and *B. hunanensis* Yin, Fang & Tarjan either are parasitic in living conifers or mycophagous within dead conifers. These species share several morphological features, and characters differentiating them are poorly defined. Webster et al. (33) grouped these species into the pinewood nematode species complex (PWNSC), which has been given supraspecies status (10,11,31).

Bursaphelenchus xylophilus is epidemic in Japan, where it causes rapid wilting of *Pinus thunbergii* and *P. densiflora* and is the primary cause of pine forest decline in all but the northernmost prefectures and at high elevations (19). *Bursaphelenchus mucronatus*, whose range overlaps that of *B. xylophilus* in Japan, infests but does not induce wilting of pines (17,19). This nematode is differentiated from *B. xylophilus* by a mucron on the female tail and by the inability of these two species to interbreed (19,20,36). Pathogenicity of *B. hunanensis*, isolated from *P. massoniana* in Hunan Province, China (36), is unknown. *Bursaphelenchus fraudlentus* Rhüm is morphologically identical to *B. mucronatus* (29). It has been isolated from dead oak, cherry, and beech trees in Germany, where it probably feeds on fungi associated with rotting wood (29).

Bursaphelenchus xylophilus has been recovered from several pine species and from *Abies balsamae, Cedrus atlantica, C. deodara, Larix decidua, L. laricina,* and *Picea glauca* in North America (28), but it is probably pathogenic only in pine (4,19). Although not a major forest pathogen in North America, *B. xylophilus* does induce death of exotic pines planted outside of their native range where the midsummer isotherm exceeds 25 C (30). It is particularly pathogenic to *P. sylvestris* in the southern regions of the midwest (3,4). Even though *B. mucronatus* has not been found in North America, morphotypes of *B. xylophilus* with some characteristics of *B. mucronatus* have been recovered from *A. balsamae* in Minne-

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sota and in Canada (34,35). These have been called "M" forms (mucronate form) as compared to type specimen *B. xylophilus* (round tail or "R" form). Dwinell and Nickle (13) considered "M"-form isolates to be closely related to *B. mucronatus*. "M" forms also have been recovered from dead or dying conifers in France, Norway, and Siberia (19,22). The pathogenicity of these "M" forms from these areas is unclear (9, 22,25,30). For example, a French "M"-form isolate from a forest at Campet (47700 Casteljaloux) is pathogenic to 2- and 4-year-old *P. pinaster* seedlings but could not be shown to cause wilting or damage to 9-year-old *P. pinaster* in the field (9).

Both pathogenic and nonpathogenic isolates of *B. xylophilus* have been recovered from *P. densiflora* and *P. thunbergii* in Japan (17). The distribution and interbreeding behavior of the avirulent *B. xylophilus* isolates suggest they may be evolving as sibling species (17). *Bursaphelenchus xylophilus* is now spreading into the northern prefectures in Japan, where some of the most virulent isolates occur (16). Host-specific pathotypes of *B. xylophilus* have been isolated from *P. sylvestris* and *P. strobus,* in the United States (7). The biology of *B. xylophilus* varies considerably throughout North America. Not only does pathogenicity vary, but isolates that are confined at or near the infection site, as well as isolates that become systemic, have been described (3,17,23,35).

Because species and isolates often have very similar morphology, several different molecular techniques have been used to learn more about speciation within the PWNSC (1,2,7,10,11,17,26,31,33). Restriction fragment length polymorphisms of total genomic DNA have shown genetic differences between *B. xylophilus* "M" and "R" forms and between *B. xylophilus* and *B. mucronatus* (7,17,26,31,32). Ribosomal DNA nontranscribed sequence probes from *B. xylophilus* and a probe for the *unc-22* gene from *Caenorhabditis elegans* have differentiated members of the PWNSC (1,33). Differences among virulent and avirulent Japanese isolates of *B. xylophilus* have been discerned by their interbreeding potential (17,26). De Guiran and Bruguier (10) differentiated among an "M" form from *P. pinaster* in France, an "M" form from *A. balsamae* in Minnesota, a Japanese isolate of *B. xylophilus,* and a type isolate of *B. mucronatus* from Yachiyo, Chiba Prefecture in Japan by the ability of these isolates to interbreed to produce fertile hybrids. Riga et al. (26) compared several Canadian isolates of *Bursaphelenchus* with isolates from the United States and Japan by interbreeding and DNA analysis. Recently, Riga and Webster (27) compared responses of several isolates to endogenous pheromones as a taxonomic character. Although these techniques have provided considerable information about isolate and species relationships, phylogenetic relationships within the PWNSC remain unclear.

We were interested in the potential for the spread of pine wilt disease into uninfested areas or into infested areas where interbreeding of virulent isolates with avirulent isolates may lead to production of new, highly virulent hybrids. Thus, we investigated the potential of North American, Japanese, and French isolates of *B. xylophilus* and *B. mucronatus* to interbreed and produce viable offspring that persist in subsequent generations. Chromosomal analysis and virulence assays were used to evaluate relationships among these isolates.

Materials and Methods

PWNSC isolates: The isolates of *Bursaphelenchus* used are described in Table 1. *Bursaphelenchus xylophilus* and *B. mucronatus* were maintained monoxenically on *Botrytis cinerea* grown on potato dextrose agar (PDA). The insect-phoretic species *B. seani* Giblin & Kaya, *B. nitidulans* Giblin, and *B. abruptus* Giblin & Kaya were used for comparisons in some experiments (14–16). These were raised monoxenically on *Pyranochaeta mali* on PDA. All cultures were reared at 25 ± 3 C and transferred monthly. Pathogenicity of *B. xylophilus* iso-

TABLE 1. Isolates of *Bursaphelenchus*. All isolates were field isolated and maintained monoxenically on fungal mats on potato dextrose agar. "Type" refers to rounded tail ("R" form) and mucronate tail ("M" form).

Isolate	Type†	Source	Isolation site
US1 (NJPn, 23)‡	Bx "R"	*Pinus thunbergii*	Millstone, New Jersey
US2 (VPSt-1, 7)	Bx "R"	*Pinus strobus*	Burlington, Vermont
US8	Bx "R"	*Pinus sylvestris*	Lee County, Iowa
US9 (AzPh, 7)	Bx "R"	*Pinus halepensis*	Tucson, Arizona
US10	Bx "M"	*Abies balsamae*	Cloquet, Minnesota
US11	Bx "R"	*Larix larcina*	Burlington, Vermont
US12 (MPSy-1, 7)	Bx "R"	*Pinus sylvestris*	Columbia, Missouri
US12B	Bx "R"	*Pinus resinosa*	Eureka, Missouri
US13	Bx "R"	*Pinus sylvestris*	Black River Falls, Wisconsin
C1 (St Wil, 33)	Bx "R"	*Pinus sylvestris*	Ontario, Canada
C2	Bx "M"	*Abies balsamae*	Quebec, Canada
J2	Bx "M"	*Pinus thunbergii*	Nagasaki, Japan
J11	Bx "R"	*Pinus densiflora*	Ichinoseki, Iwate, Japan
J12	Bx "R"	*Monochamus alternatus*§	Tateyama, Chiba, Japan
J13 (Chiba, 33)	*B. mucronatus*	*Pinus densiflora*	Yachiyo, Chiba, Japan
J14	*B. mucronatus*	*Pinus densiflora*	Takahagi, Ibaraki Japan
S10	Bx "R"	*Pinus densiflora*	Hirose, Shimane, Japan
C14-5	Avirulent Bx "R"	*Pinus densiflora*	Ichinomiya, Chiba, Japan
OK2	Avirulent Bx "R"	*Pinus lushness*	Onna, Okinawa, Japan
F1	Bx "M"	*Pinus pinaster*	Saint Symphorien, France
B. kevini	Insect phoretic	Nitidulid beetles	Sonoma County, California
B. seani	Insect phoretic	*Anthophora bomboides*	Sonoma County, California
B. abruptus	Insect phoretic	*Anthophora abrupta*	Sonoma County, California

† Bx "R" is a round tail morphotype of *B. xylophilus;* Bx "M" is a mucronate tail form; "avirulent" designates isolates of *B. xylophilus* reportedly uninfective towards *Pinus thunbergii, P. densiflora,* and *P sylvestris* in greenhouse tests (17); "insect-phoretic" refers to mycophagous species of *Bursaphelenchus* that live in association with and are carried by *Anthophora* bees and that are not associated with conifers.

‡ Designation used in other publications.

§ Insect vector for *B. xylophilus* and *B. mucronatus* in Japan.

lates was maintained as described by Kiyohara and Bolla (17).

Pathogenicity assays: Pathogenicity of each isolate of *B. xylophilus* and *B. mucronatus* was determined in 4-year-old *P. sylvestris, P. strobus, P. nigra, P. taeda,* and *P. jeffreyi* seedlings, raised in the greenhouse at 22 ± 3 C with a 12 hour light–dark cycle. They were watered as needed and fertilized monthly with a liquid fertilizer. Seedlings were infected through a 0.5-cm^2 abrasion of the bark at the midpoint of the trunk (7). Pathogenicity was defined by the number of seedlings that wilted within the time required for isolate US12 to induce wilting of 100% of the inoculated *P. sylvestris* seedlings. Wounded seedlings and seedlings inoculated with a supernatant fraction from the fungal cultures were controls. Seedlings that wilted were harvested and chipped. Nematodes were extracted in a modified Baermann funnel and the number of nematodes per gram dry weight of wood was determined.

In other pathogenicity assays, forty 4-year-old seedlings were inoculated with each isolate. Ten seedlings from each group were harvested 5, 10, 15, and 30 days after inoculation. The seedlings were chipped and the nematodes were extracted in a modified Baermann funnel. The number of nematodes per gram of wood dry weight was estimated to determine isolates changes as a function of time after inoculation, nematode isolate, and pine species.

Interbreeding potential: The interbreeding potential of *Bursaphelenchus* isolates was compared in single-pair reciprocal interbreedings. Third-stage juvenile females were identified by development of the genital anlagen (12). Individual females were interbred to adult males on 1-day-old cultures of *B. cinerea* grown on PDA. Adult

males and females were removed 5 days after the cultures were started. Five days later the nematodes were collected from the cultures in a modified Baermann funnel apparatus and counted. Each attempted interbreeding was repeated 60 times. F_1 generations were interbred by recovering a third-stage juvenile female and a third-stage juvenile male from these cultures. These were interbred, the nematodes were collected, and the F_2 isolate size was determined. We considered a interbreeding successful only when either the parental interbreeding produced more than an average of 30 F_1 offspring per 60 matings or the interbreeding of the F_1 generation produced a viable F_2 generation that sustained itself through successive inbreeding. An inadvertent inclusion of an adult fertilized female in the experimental series was noted when fewer than 30 offspring were produced in a breeding experiment.

Chromosome number: Adult male and female nematodes of each isolate were incubated overnight in 0.8 µg/ml colchicine (5). The nematodes were then squashed on glass slides and stained with propionic acid–orcein as described by Triantaphyllou (32). Stained preparations were mounted in Euparal (5), and the chromosomes in reproductive cells were counted. Thirty counts were made on 8 to 10 slides for each isolate.

Results

Pathogenicity: Different species of four-year-old pine seedlings responded differently to the isolates of *B. xylophilus* and *B. mucronatus* (Table 2). *Pinus thunbergii* was the most susceptible pine species, followed in order by *P. sylvestris, P. strobus,* and *P. nigra. Pinus taeda* was susceptible to only isolates J2, J11, and J12, and *P. jeffreyi* was resistant or tolerant to all isolates. *Pinus strobus, P. nigra,* and *P. thunbergii* were moderately susceptible to isolate F1. This "M"-form isolate did not induce wilting in any other species, although the number of nematodes in the seedlings increased through 15 days after inoculation. *Bur-*

Table 2. Host range† (and population size increases‡) as determined by the response of *Pinus* spp. to inoculation with different isolates of *Bursaphelenchus.* Forty 4-year-old seedlings of each species were inoculated in each greenhouse assay.

	P. sylvestris	*P. strobus*	*P. nigra*	*P. taeda*	*P. jeffreyi*	*P. thunbergii*
US1	S (+)	R (−)	S (+)	T (±)	T (±)	S (+)
US2	T (±)	S (+)	T (±)	R (−)	R (−)	S (+)
US8	S (+)	T (±)	T (±)	R (−)	R (−)	S (+)
US9	MS (±)	MS (±)	MS (±)	T (±)	R (−)	S (+)
US10	T (±)	T (±)	T (±)	T (±)	R (−)	MS (±)
US11	S (+)	S (+)	S (+)	T (±)	R (−)	S (+)
C1	S (+)	S (+)	S (+)	T (±)	R (−)	S (+)
C2	T (±)	T (±)	T (±)	T (±)	R (−)	MS (±)
J2	S (+)	S (+)	S (+)	S (+)	T (±)	S (+)
J11	S (+)	S (+)	S (+)	S (+)	T (±)	S (+)
J12	S (+)	S (+)	S (+)	S (+)	T (±)	S (+)
J13	R (−)	R (−)	R (−)	R (−)	R (−)	R (−)
J14	R (−)	R (−)	R (−)	R (−)	R (−)	R (−)
S10	S (+)	S (+)	S (+)	R (−)	R (−)	S (+)
C14-5	T (±)	T (±)	T (±)	T (±)	R (−)	R (±)
OK2	T (±)	T (±)	R (−)	R (−)	R (−)	R (±)
F1	T (±)	MS (±)	MS (±)	T (±)	T (±)	MS (±)

† S = susceptible (i.e., 80–100% wilted within 30 days after inoculation); MS = moderately susceptible (i.e., 50–75% wilted in 30 days); T = tolerant (i.e., although nematode population size increased through 15 days after infection, <10% of the seedlings wilted); R = resistant (i.e., no population size increase and no wilted seedlings).

‡ + = Population size increased through the time the seedlings wilted; ± = population size increased through 15 days after inoculation then declined; − = no change in population size or no nematodes isolated from the seedlings 30 days after inoculation.

saphelenchus mucronatus isolates J13 and J14 did not induce wilting of any of the tested pines. Isolates OK2 and C14-5 of *B. xylophilus* were avirulent, and isolates S-10 and C1 induced wilting of *P. sylvestris, P. nigra, P. strobus,* and *P. thunbergii* (Table 2). The *B. xylophilus* "M" forms US10 and C2 increased initially in all pine seedlings except *P. jeffreyi,* but only *P. thunbergii* had moderate susceptibility to these isolates. Isolates J2, J11, and J12 were among the most pathogenic ones tested; they rapidly induced wilting of all pines except *P. jeffreyi.*

Interbreeding potential: The geographically isolated Japanese *B. mucronatus* isolates (J13 and J14) did not interbreed with the French "M" form (F1) nor with each other (Table 3). However, males of each isolate interbred with females of a Japanese "R" form J11 and a Canadian "R" form C1. The *B. mucronatus* isolate from Chiba (J13) also interbred with the *B. xylophilus* "R" forms US1 and US2. Isolate US1 males did not interbreed with US12 females; however, the reciprocal interbreeding did occur. The F_1 generation from the hybridization of US1 females × US12 males produced a viable F_2 generation that persisted in culture (Tables 3, 4).

The avirulent Japanese "R" forms OK2 and C14-5 interbred with both *B. mucronatus* isolates from Japan and with the "R" form US12 to produce a viable and fertile first generation (Table 5). They did not interbreed with "M"-form isolates US10 and F1. Males of the Canadian "M" form, C2, did not interbreed with the "R" form C1 females but did mate with "R" forms US1 from New Jersey and US 13 from Wisconsin. Interbreeding potential of C2 females was less restrictive than that of the males. They interbred with US12, US13, and C1 (Table 3).

Isolate US10 males (*B. xylophilus* "M" form, Minnesota) interbred with US8, US9, US11, and US12 (*B. xylophilus* "R" form, Iowa, Arizona, Vermont, and Missouri) but did not interbreed with US13 (*B. xylophilus* "R" form, Wisconsin). US10 females did not interbreed with the US12 isolate. The French "M" form did not interbreed with any other "M" forms, nor with *B. mucronatus* from Japan. Males of F1 interbred with females of the "R"-form isolates US2 and US8 and also with the Japanese "R"-form isolate J12, but the offspring were not reproductively viable and F_2 generations were not produced. F1 females interbred with males of US2 but not with males of US8.

The Vermont "R" forms (US2 and US11) interbred to produce reproductively viable hybrid offspring (Tables 3, 4) but differed in their ability to interbreed with US9, J13, and F1. Interbreeding of US11 with US12 produced a reproductively viable F_1 generation that persisted in culture. Isolate US2 also interbred with US12, but the F_1 were not fertile and an F_2 generation was not produced.

Several other attempted reciprocal matings resulted in F_1 generations that were infertile and did not produce a F_2 generation. These were: US11 males × J2 females, US13 males × US2 females, US13 males × J2 females, US13 males × J13 females, C1 males × J11 females, J13 males × C1 females, and F1 males × J12 females (Tables 3, 4).

Data presented in Table 4 demonstrate that reciprocal interbreedings were not always consistent, i.e., males from isolate A and females of isolate B may have produced a viable and sustainable F_1 generation, but not A females and B males. For example, males of isolate US8 interbred with females of C1, but females of US8 did not interbreed with C1 males; US10 males interbred with US12 females, but US12 males did not interbreed with US10 females; and F1 males interbred with US8 females, but US8 males did not interbreed with F1 females.

The insect-phoretic species *B. nitidulans, B. seani,* and *B. abruptus* did not interbreed with each other or with *B. xylophilus* or *B. mucronatus.*

Hybrid pathogenicity: Pathogenicity of hybrids from reciprocal interbreedings varied and was approximately that of the most virulent parental isolate (Table 6). The F_1 generations from interbreeding of the

TABLE 3. Interbreeding potential of different populations of *Bursaphelenchus*, as determined by the average number of offspring produced from 60 reciprocal single pair matings. Interbreedings that produced on average 30 F_1/60 interbreedings were considered negative.

Female parent	Male parent															
	US1	US2	US8	US9	US10	US11	US12	US13	C1	C2	J2	J11	J12	J13	J14	F-1
US1	654	65	117	0	0	130	65	78	0	91	0	78	0	117	0	0
US2	52	456	91	156	0	195	91	56	91	0	0	260	52	208	0	325
US8	130	104	532	234	260	299	130	78	26	0	0	91	130	0	0	234
US9	0	78	268	567	221	0	169	156	317	0	546	117	0	0	0	0
US10	0	0	234	247	653	260	0	0	0	0	0	0	0	0	0	0
US11	104	143	299	0	91	789	156	364	0	0	104	143	0	0	0	0
US12	0	39	48	221	104	104	428	91	234	0	156	104	0	728	0	0
US13	52	65	754	65	0	52	169	589	130	104	195	923	39	26	0	26
C1	0	104	65	91	0	0	221	0	423	0	182	39	0	52	65	0
C2	0	0	0	0	0	0	143	416	169	325	0	0	0	0	0	0
J2	0	117	338	130	0	52	130	39	156	0	598	0	0	0	0	0
J11	0	494	78	156	0	234	65	364	52	0	0	879	0	65	78	0
J12	0	39	78	0	0	0	91	39	26	0	0	26	824	0	0	52
J13	0	117	0	0	0	0	390	52	0	0	0	0	0	821	0	0
J14	0	0	0	0	0	0	0	130	169	0	0	117	0	0	763	0
F1	0	182	0	0	0	0	0	0	0	0	0	0	0	0	0	897

TABLE 4. Establishment of viable F_2 generations by inbreeding single males and females from an F_1 generation of *Bursaphelenchus* hybrids. The male and female parents indicate the original isolates that generated the F_1. Thus, a mating indicated by US-1 male × US2 female describes inbreeding of the F_1 population established from this interbreeding. Values are the mean of 20 reciprocal single pair matings.

Female parent of F_1	Male parent of F_1															
	US1	US2	US8	US9	US10	US11	US12	US13	C1	C2	J2	J11	J12	J13	J14	F-1
US1	386	33	104	0	0	172	78	65	0	77	0	55	0	65	0	0
US2	82	339	87	144	0	221	65	26	114	0	0	198	40	65	0	114
US8	104	91	299	148	117	166	104	65	22	0	0	191	182	0	0	99
US9	0	78	208	387	13	0	182	104	96	0	100	96	0	0	0	0
US10	0	0	234	55	453	169	0	0	0	0	0	0	0	0	0	0
US11	39	156	96	0	77	549	78	221	0	0	55	117	0	0	0	0
US12	0	77	91	143	78	98	552	78	90	0	134	64	0	338	0	0
US13	66	55	165	62	0	156	91	332	75	89	169	364	26	26	43	65
C1	0	117	65	88	0	0	91	0	219	0	87	52	0	36	67	0
C2	0	0	0	0	0	0	117	208	117	337	0	0	0	0	0	0
J2	0	76	130	100	0	39	66	26	128	0	420	0	0	0	0	0
J11	0	234	78	124	0	310	87	247	20	0	0	228	0	55	57	0
J12	0	26	139	0	0	0	39	13	20	0	0	22	433	0	0	20
J13	0	91	0	0	0	0	195	44	0	0	0	0	0	327	0	0
J14	0	0	0	0	0	0	0	87	78	0	0	69	0	0	149	0
F1	0	104	0	0	0	0	0	0	0	0	0	0	0	0	0	196

TABLE 5. Haploid chromosome number and interbreeding potential of *Bursaphelenchus* isolates with males of *B. xylophilus* "R" form US12, "M" form US10, and two *B. mucronatus* isolates, J13 and J14.

Female parent	Chromosome number	Interbreeding potential†				
		US12	J13	J14	F1	US10
US1	3	+	+	−	−	−
JS9	3	+	−	−	−	+
US10	3	−	−	−	−	+
US11	3	+	−	−	+	+
US13	3	+	−	−	−	−
F1	3	−	−	−	+	−
J2	3	+	−	−	−	−
J11	3	+	+	+	−	−
J12	3	+	−	−	−	+
C2	5	+	−	−	−	−
J13	5	−	+	−	−	−
J14	5	−	−	+	−	−
OK2	6	+	+	+	−	−
C14-5	6	+	+	+	−	−
C1	6	+	+	+	−	−
US2	6	+	+	−	+	−
US8	6	+	−	−	+	+
US12	6	+	+	−	−	+
US12B	6	+	+	−	−	+
S10	6	+	−	−	−	+
B. nitidulus	4	−	−	−	−	−
B. seani	8	−	−	−	−	−
B. abruptus	14	−	−	−	−	−

† Males of isolates US12, US10, J13, J14, and F1 were interbred with females of the isolates indicated in the table. Interbreedings were scored as positive (+) only if the hybrids from an initial mating could be inbred to produce a second generation that persisted in culture. − = lack of viable and fertile F_1. Any interbreeding that produced on the average <30 F_1/60 interbreedings was considered negative.

avirulent Japanese isolates OK2 or C14-5 with virulent isolates US2, US12, and S10 were more virulent than the parents. When OK2 or C14-5 were interbred with US12, the host specificity of the US12 parent was partially lost. The host specificity of US12 and US2 was lost in offspring from their interbreedings with the virulent Japanese isolate S10. The offspring from this interbreeding were more virulent than either of the parental isolates. The F_1 from hybridization of US12 with either C1 or C2 were more virulent in *P. strobus* than were the parental isolates. Offspring from interbreedings of F1 with US12 or US2 had the virulence characteristics of US12 and US2. Host specificity was retained in the offspring. The F_1 from interbreeding *B. mucronatus* J13 × US12 were virulent.

Chromosome number: The PWNSC clearly segregated into at least three groups based on haploid chromosome number (Table 5). Group 1 had a haploid chromosome number of 3 and included "M" forms US10 and F1. The second group had a haploid chromosome number of 5 and included the Canadian "M" form isolate C2 and *B. mucronatus* isolates J13 and J14. The third group had a haploid chromosome number of 6 and included no "M" form or *B. mucronatus* isolates. The avirulent Japanese isolates OK2 and C14-5 fell into this group. Both of these isolates interbred with the *B. mucronatus* isolates J13 and J14.

DISCUSSION

The population of *Bursaphelenchus* individuals within a single tree may be a product of the hybridization of several different isolates deposited in the tree during the maturation feeding or oviposition of several individual beetles. However, the nematodes within a single tree can be con-

TABLE 6. Pathogenicity of selected first-generation isolates from matings between *Bursaphelenchus xylophilus* and *B. mucronatus* in 4-year-old *Pinus sylvestris* and *P. strobus* seedlings. Results are expressed as the percentage of 40 infected pine seedlings wilted within 30 days of inoculation with 100 F_1 juveniles

Parental isolates		Mean percentage of seedlings wilted	
Male	Female	*P. sylvestris*	*P. strobus*
US2	US2	0	100
US2	C1	100	100
US2	C2	100	100
US2	S10	100	100
US2	F1	0	76
US12	US2	100	100
US12	US12	100	0
US12	C1	100	100
US12	C2	100	100
US12	J13	100	80
US12	S10	100	100
US12	C14-5	70	50
US12	OK2	70	50
US12	F1	100	0
C1	C1	100	60
C2	C2	0	0
J13	J13	0	0
S10	S10	30	30
OK2	OK2	0	0
C14-5	C14-5	0	0
F1	F1	0	20

sidered as an individual population; it is confined to that tree and dissemination for interbreeding with other populations requires transport by a vector insect (19). Kiyohara and Bolla (17) demonstrated that populations within a tree were homogenous. If there is not a continuous range of susceptible hosts, gene flow between isolates could become very restricted and sibling species could develop. This may be seen in the significant difference in pathogenicity among isolates throughout the range of *B. xylophilus* in Japan and the lack of variation within isolates from individual pines or from within an isolated pine stand (17). Such reproductive isolation may be the underlying force for development of the PWNSC. The range of pathogenicity among isolates (17) and among hybrids derived from interbreeding virulent isolates, avirulent with virulent isolates, and "M"- and "R"-form isolates suggests complicated genetics and the involvement of more than a single gene in the determination of pathogenicity.

It cannot be generalized that all *Bursaphelenchus* isolates from conifers in the United States, Canada, Japan, and Europe are reproductively isolated, because under laboratory conditions interbreedings can be forced between many isolates and between *B. xylophilus* and *B. mucronatus*. The hybrids of some of these interbreedings were viable and persisted in culture for at least several generations. Whether any of these interbreedings actually could occur in nature is open to conjecture; there is obvious potential for many *B. xylophilus* isolates to interbreed with other *B. xylophilus* or *B. mucronatus* isolates from the same area. It is clear that some isolates are reproductively isolated, however, and are only distantly related to other isolates within the species complex (2,10,17,33). Thus *B. mucronatus* J13 and J14 are geographically separated in Japan and do not appear to interbreed with each other; hence either may be subspecies within a *B. mucronatus* group or they may be separate sibling species. Another example of reproductive isolation occurs upon comparing the reproductive potential of "M"-form isolates of *B. xylophilus* with each other or with *B. mucronatus* isolates. We were unable to obtain interbreeding of the "M" form from *A. balsamae* in Minnesota, US10, with any other "M"-form isolate or with *B. mucronatus* from Japan. The US10 isolate did interbreed with North American *B. xylophilus* "R" form but not with the Japanese *B. xylophilus* we used. These results place US10 closer to *B. xylophilus* than to *B. mucronatus*, in agreement with other reports (2,10,33). Unlike Riga and Webster (26), we were unable to obtain interbreedings between the French isolate and *B. mucronatus* from Japan. This difference could be attributable in part to technical differences in the way interbreedings were done.

Several theories might explain why some populations did not interbreed reciprocally. For example, it might be suggested that some isolates reproduce by pseudog-

amy or that hybrid dysgenesis occurs (26). Pseudogamy is found in several nematode species and occurs within genera of amphimitic nematodes in which some species reproduce by gamete fusion (23). Pseudogamy may be an intermediate step in the evolution towards parthenogenesis (32); however, no parthenogenic populations of *Bursaphelenchus* spp. have been found. (10). Riga et al. (26) proposed that reciprocal matings failed because of hybrid dysgenesis and that this might be a step towards reproductive isolation. This hypothesis is supported by the report of de Guiran and Bruguier (10) that abnormal juveniles were produced from matings of *B. xylophilus* with *B. mucronatus* (10). To prove hybrid dysgenesis there must be evidence of maternally inherited mobile genetic elements.

Investigators have used several techniques to develop relationships within the PWNSC. Whether based on interbreeding potential, differences in DNA sequence, virulence, pheromone attraction, or morphology, separation into distinct *B. xylophilus* and *B. mucronatus* groups occurs. Some isolates lie outside these groups and are clearly distinct from but related to members of these groups (10,31). Our studies on interbreeding potential, pathogenicity, host preference, and chromosome number support the idea of two major groups: a *B. xylophilus* group and a *B. mucronatus* group. The *B. xylophilus* group can be further divided into diploid (2n = 6) and tetraploid (2n = 12) subgroups. Interbreeding potential suggests that these groups are closely related and are derived from a common ancestor. Unlike Mamiya (19), we obtained viable hybrids from interbreedings between some *B. xylophilus* isolates and *B. mucronatus*.

On the basis of interbreeding potential and virulence, the French isolate (F1) is clearly separated from both the *B. xylophilus* and *B. mucronatus* groups. It is more closely related to the *B. xylophilus* group, as it interbred with some isolates within this group and has a chromosome number associating it with this group. Unlike other investigators (11,31) we were unable to obtain interbreedings of F1 with *B. mucronatus* or *B. xylophilus* from Japan. This discrepancy could have resulted from our use of single-pair mating experiments as opposed to the larger number of pairs (10–50) used by others (10,26). The F1 isolate also did not interbreed with the "M" form US10. Separation of F1 as an independent isolate based on interbreeding potential supports the assignment of separate species status to F1 based on differences in DNA sequences (2). Although "M" form C2 from Canada did not interbreed with *B. mucronatus*, J13 and J14, the chromosome number and pathogenicity of C2 place it within the *B. mucronatus* group. DNA sequence comparisons also place this isolate with *B. mucronatus* (12,33).

The two avirulent isolates from Japan, C14-5 and OK2, have chromosome numbers placing them in the *B. xylophilus* tetraploid subgroup. They have the potential to interbreed with isolates from both the *B. xylophilus* and *B. mucronatus* groups, but they do not interbreed with each other (6). This suggests that these are sibling species derived from a common ancestor.

It is difficult to compare our results with those of others because the source of the different isolates may not be identical. By comparing our results with others, we propose that *Bursaphelenchus* from conifers is a complex of sibling species that have evolved through reproductive isolation, that the French isolate is probably a new species, and that, as suggested (2,26), the *B. xylophilus* and *B. mucronatus* groups are derived from a common ancestor. Results from reciprocal interbreedings also support the contention that the Japanese isolates of *Bursaphelenchus* originated from North American isolates (2,19).

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