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Taxonomic relationships of *Bursaphelenchus xylophilus* and *B. mucronatus* based on interspecific and intraspecific cross-hybridization and DNA analysis

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Summary — Cross-hybridization between five geographic isolates from North America of *Bursaphelenchus xylophilus* (St. John, St. William, MSP4, Q1426) and Japan (Ibaraki) and two isolates (Japanese and French) of *B. mucronatus*, members of the pinewood nematode species complex (PWNSC), was done under controlled laboratory conditions. Intraspecific hybridization among isolates of *B. xylophilus* from Japan and North America produced fertile offspring. Cross-hybridization among isolates of *B. mucronatus* from France (female) and Japan (male) produced fertile F_n generations, however the reciprocal cross died out. Interspecific hybridization among isolates of *B. mucronatus* and *B. xylophilus* produced an F_1 that died out in most of the crosses in which the female was from *B. mucronatus* and the male was from *B. xylophilus*. Genomic DNA from *B. xylophilus*, *B. mucronatus* and their hybrid-crosses were Southern blotted and probed with a ribosomal gene clone which produced band pattern differences between the isolates of *B. xylophilus* and of *B. mucronatus* as well as between the French and Japanese isolates of *B. mucronatus*. The results support the distinct species status of *B. xylophilus* and *B. mucronatus* and suggest also that *B. xylophilus*, and *B. mucronatus* from France and Japan come from a common ancestor. In addition, they provide evidence for separate species status for the French and Japanese isolates of *B. mucronatus*.

Résumé — ***Relations taxonomiques entre* Bursaphelenchus xylophilus *et* B. mucronatus *fondées sur des hybridations interspécifiques et intraspécifiques croisées et l'analyse de l'ADN*** — Des hybridations croisées entre cinq isolats géographiques de *Bursaphelenchus xylophilus* provenant d'Amérique du Nord (St. John, St. William, Q1426 et MSP4) et du Japon (Ibaraki), et deux isolats (japonais et français) de *B. mucronatus*, membres du complexe d'espèces du nématode du bois de pin (PWNSC), ont été réalisées en conditions contrôlées au laboratoire. L'hybridation intraspécifique entre isolats de *B. xylophilus* venant du Japon et d'Amérique du Nord produit des descendants fertiles. L'hybridation entre isolats de *B. mucronatus* venant de France (femelle) et du Japon (mâle) produit des générations fertiles F_n, mais la descendance de l'hybridation réciproque s'est éteinte. L'hybridation interspécifique entre isolats de *B. mucronatus* et de *B. xylophilus* produit une F_1 qui meurt dans la plupart des cas où la femelle appartient à *B. mucronatus* et le mâle à *B. xylophilus*. L'hybridation d'une sonde d'ADN ribosomique avec les ADN génomiques de *B. xylophilus*, *B. mucronatus* et de leurs hybrides montre des différences dans les profils d'hybridation entre les isolats de *B. xylophilus* et ceux de *B. mucronatus*, ainsi qu'entre les isolats français et japonais de *B. mucronatus*. Ces résultats confirment le statut d'espèces distinctes de *B. xylophilus* et *B. mucronatus* et suggèrent également que *B. xylophilus* et *B. mucronatus* de France et du Japon proviennent d'un ancêtre commun. De plus, ils fournissent des arguments en faveur d'un statut d'espèces distinctes pour les isolats français et japonais de *B. mucronatus*.

Key-words : *Bursaphelenchus*, hybridation, DNA, *Pinus*.

Bursaphelenchus xylophilus (Steiner & Buhrer) Nickle, the pinewood nematode, and its close relative *B. mucronatus* Mamiya & Enda, both members of the pinewood nematode species complex (PWNSC) (Webster *et al.*, 1990), are important parasites of pines and some related species. Both species have attracted interest because of their economic importance to lumber trading nations, their relative pathogenicity and uncertainty regarding their respective taxonomic status. Differentiating between some PWNSC isolates, based solely on morphological characters, can sometimes be inconclusive because the characters of some isolates are similar and at the same time variable. Cross-hybridization experiments have been done to provide additional data. Mamiya (1986) concluded that *B. xylophilus* and *B. mucronatus* in Japan are distinct species because the F_1 generation of their interspecific hybrid is not viable thus fulfilling an essential criterion of a biological species. However, de Guiran and Boulbria (1986) found that fertile offspring were produced from crosses of a French isolate, identified as *B. mucronatus*, with a Japanese isolate of *B. mucronatus* and with a Japanese isolate of *B. xylophilus*. The French isolate also cross-hybridized with a North American isolate of *B. xylophilus* from Minnesota (USA) but only when the parental generation was left with the F_1 offspring. They concluded that *B. xylophilus* and

B. mucronatus are members of a " super species ". Subsequently, Rutherford *et al.* (1990) referred to *B. xylophilus* and *B. mucronatus* as being members of the pine wood nematode species complex and cited biological evidence to support this claim. The results of cross-hybridization experiments differ from one laboratory to another, probably in part due to differences in the prevailing environmental conditions (Dobzhansky *et al.*, 1977) or to the isolates used.

Webster *et al.* (1990), using recombinant DNA analysis provided evidence that *B. mucronatus* and *B. xylophilus* are distinct from each other and that a French isolate tested was genetically more similar to *B. mucronatus* than to *B. xylophilus*. The objective of our study was to collate information derived from intra- and interspecific cross-hybridizations and from DNA analysis of the parent and offspring in order to clarify the biological and taxonomic relationships between *B. xylophilus* and *B. mucronatus* and between the French and Japanese isolates of *B. mucronatus*.

Materials and methods

Five geographic isolates of *B. xylophilus*, three Canadian, namely St. John (SJ), St. William (SW), Q1426 (Q14), one Japanese, Ibaraki (Ib), and one from the USA, MSP4 (MSP4) and two of *B. mucronatus*, a Japanese, Chiba (Ch) and a French (Fr) isolate (see Webster *et al.*, 1990 for details of source) were reared over several generations, without changing pathogenicity status to *Pinus sylvestris* seedlings (Riga *et al.*, 1991), on *Botrytis cinerea* on 1 % potato dextrose agar (PDA) plates. Nematodes were washed off the lids of the plates with sterile distilled water, and ten single, fourth stage juveniles (J4) of each sex from each of the seven isolates were individually crossed. Intra- and interspecific hybridizations and reciprocal crosses were performed with all seven isolates on *B. cinerea* plates (15 g agar and 0.64 g potato dextrose broth) and maintained at 29 °C. Successful cross-hybridizations were repeated ten times while unsuccessful crosses (i.e. where the F_1 or a subsequent generation died out) were replicated twenty times. Intra-isolate crosses, also replicated ten times, served as a control and standard for judging fecundity of the parental stocks. The parents were removed from the plate 4 days after the first day of egg-laying in order to avoid back-crossing with the F_1 progeny. Subsequent inbred generations were reared under the same conditions and monitored. In this paper the male parent is named first and the female second in all crosses. For logistical reasons and to ensure that there was consistency for all matings the number of progeny per mating was measured as the number of eggs layed in the 24 hours following the first egg recorded. Data on the number of progeny of the F_1 generation were analysed using the non-parametric Mann-Whitney-Test (Sokal & Rohlf, 1969), and significance is stated at the 95 % confidence level.

Genomic DNA was extracted from several nematode plates of each of the parental isolates and of each of the following successful hybrids, all of which had been tested for their ability to infect *P. sylvestris* seedlings (Riga *et al.*, 1991), SJ × SW, MSP4 × SW, Fr × SW, Ch × SW, Ch × Fr, Q14 × SW, Ib × SW, and Ch × SJ was extracted and purified (as described in Webster *et al.*, 1990). Approximately 2 µg of DNA was restricted with HindII and SalI, size fractionated on 0.7 %

Table 1. Mean number (± SE) of juveniles produced per cross during the first day of egg laying following the intra- or interspecific cross-hybridization of single pairs of *Bursaphelenchus xylophilus* and *B. mucronatus* isolates.

Males	Females						
	B. xylophilus					*B. mucronatus*	
	St. John	St. William	Ibaraki	Q1426	MSP4	French	Chiba
St. John	13.0 ± 3.1	6.8 ± 1.8	17.8 ± 3.3	7.2 ± 2.1	6.9 ± 2.1	1.8 ± 1.2*	1.3 ± 0.2*
St. William	6.0 ± 1.6	10.0 ± 3.5	14.0 ± 3.4	6.0 ± 1.9	10.9 ± 2.6	0.9 ± 0.5*	5.0 ± 1.6**
Ibaraki	3.6 ± 1.0	11.5 ± 3.3	7.9 ± 1.6	11.9 ± 2.6	13.2 ± 2.9	10.4 ± 2.4	1.1 ± 0.6*
Q1426	8.0 ± 1.5	7.4 ± 2.1	11.4 ± 2.0	11.3 ± 1.7	8.2 ± 2.0	5.2 ± 1.5*	3.0 ± 1.3*
MSP4	3.5 ± 1.5	5.5 ± 1.4	9.0 ± 1.9	2.9 ± 1.3	14.8 ± 2.2	3.5 ± 1.1	1.3 ± 0.6*
French	2.9 ± 1.2	9.7 ± 1.6	3.8 ± 1.3	8.9 ± 1.7	10.5 ± 1.8	12.5 ± 2.1	10.6 ± 1.8*
Chiba	2.1 ± 0.8	2.9 ± 1.3	4.5 ± 1.7	5.3 ± 1.8	9.1 ± 1.9	14.0 ± 2.5	8.0 ± 1.7*

* These cross hybridizations produced an F_1 that died out; ** This cross hybridization produced a fertile F_1 whose progeny died out after ten generations; all cross-hybridizations were repeated ten times while all unsuccessful cross-hybridizations (*) and (**) were repeated twenty times.

agarose electrophoretic gels (Davis *et al.*, 1980), transferred to nylon membranes using the bidirectional transfer method of Smith and Summers (1980) and hybridized to the labelled ribosomal gene clone pB × 2 (Webster *et al.*, 1990) from the Japanese isolate, *B. xylophilus* Ibaraki. The hybridization conditions were 62 °C in 5 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM $(Na_{1.5})PO_4$, 1 mM Na_2EDTA pH(7.0), 0.3 % SDS, and 5 × Denhardts (1 × Denhardts = 0.02 % w/v of bovine serum albumin, Ficoll 400, and polyvinyl pyrrolidone 40) (Davis *et al.*, 1980). The filters were washed at 45 °C in 2 × SSPE + 0.2 % SDS, air dried, and exposed to X-ray film for 24 hours. This DNA analysis was repeated three times.

Results

Cross-hybridization experiments

Bidirectional crosses of sexes of all the *B. xylophilus* isolates produced fertile offspring (F_1). The average number of F_1 offspring, newly hatched juveniles, produced on the first day of egg laying ranged from 2.9 to 17.8 (Table 1). The F_1 gave rise to subsequent F_n generations which established a population of several million individuals. There was no significant difference between the mean number of nematodes produced on the first day of egg laying resulting from SJ, SW, Ib and Q14 parents and the intraspecific crosses of the parent isolates. However, there was a significant difference between the mean number of nematodes produced on the first day of egg laying by the MSP4 parents as compared with those from the MSP4 × SJ and MSP4 × SW intraspecific hybrid crosses (P = 0.05), but not between the MSP4 parents and the other intraspecific crosses. The cross-hybridization of the male Ch to the female Fr, both putative *B. mucronatus* isolates produced subsequent F_n generations. There was no significant difference between the mean number of nematodes produced on the first day of egg laying resulting from the intraspecific cross of the parental Ch isolate and the Ch × Fr hybrid. However, their reciprocal cross, Fr × Ch, produced an F_1 generation that died out (Table 1).

Males of *B. mucronatus*, Ch isolate mated successfully with females of each *B. xylophilus* isolate producing several generation. There was no significant difference between the mean number of nematodes produced on the first day of egg laying resulting from Ch parents in comparison with those from the Ch × Ib, Ch × Q14 and Ch × MSP4 intraspecific crosses, but there was a significant difference between Ch parents compared with the Ch × SJ and Ch × SW intraspecific crosses (P = 0.05). However, the reciprocal crosses between a female *B. mucronatus*, Ch isolate and a male of each of the *B. xylophilus* isolates failed to become established and the F_1 died out except for the SW × Ch cross in which the F_1 gave rise to individuals that persisted for at least ten generations. There was a significant difference between the mean number of nematodes produced on the first day of egg laying by the SJ, Ib, Q14 and MSP4 parents as compared with their interspecific crosses with the Ch parent (P = 0.05), with the exception of the number of progeny derived from the SW parent in comparison with those from the SW × Ch interspecific cross. Single females of *B. mucronatus*, Fr isolate when mated with single males of each of the three Canadian *B. xylophilus* isolates failed to produce viable F_1 progeny, but interbred successfully with *B. xylophilus* males from the United States (MSP4) and Japan (Ib) isolates (Table 1). Significantly less nematodes were produced on the first day of egg laying resulting from the crosses between SJ, SW, Q14 and MSP4 parents and each of their interspecific crosses with the Fr parent (P = 0.05). However, the MSP4 × Fr hybrid produced viable F_1 progeny while the others did not. There was no significant difference between the mean number of nematodes produced on the first day of egg laying resulting from the Ib parents in comparison with that from the Ib × Fr interspecific cross.

DNA analysis

The ribosomal gene probe detected restriction fragment length polymorphism (RFLP) between the *B. xylophilus*, Q14, MSP4, SJ, SW, and Ib populations, and between *B. mucronatus*, Ch and Fr populations. These RFLP's were used to determine whether the components of the genome from both parental populations were present in the inbred hybrid progeny (F_n) (Fig. 1).

DNA from the *B. xylophilus* intraspecific hybrids, Q14 × SW, Ib × SW, SJ × SW, and MSP4 × SW showed RFLP patterns similar to that of both of the respective parental populations. The DNA from *B. mucronatus* intraspecific cross, Ch × Fr, showed mostly the Fr RFLP pattern, but one band is shared between the two isolates (Fig. 1). The DNA from the interspecific hybrid crosses Ch × SJ and Ch × SW *(B. mucronatus* × *B. xylophilus)* showed only the RFLP pattern from the *B. xylophilus* parent and the interspecific hybrid Fr × SW showed the RFLP pattern from both *B. xylophilus* (very faintly) and *B. mucronatus* parents (Fig. 1).

Discussion

The parental isolates *B. xylophilus* and *B. mucronatus* are readily distinguished as two groups by the RFLP's labelled by the ribosomal gene probe. These results concur with those reported by Webster *et al.* (1990) and Abad *et al.* (1991). The DNA analysis of the *B. xylophilus* hybrids showed that in the area of the genome where the probe hybridizes, the hybrid crosses inherit their DNA from both of the parents (Fig. 1). For example, the *B. xylophilus* hybrid, Ib × SW, shows banding from both parental isolates. The DNA analysis of the *B. mu-*

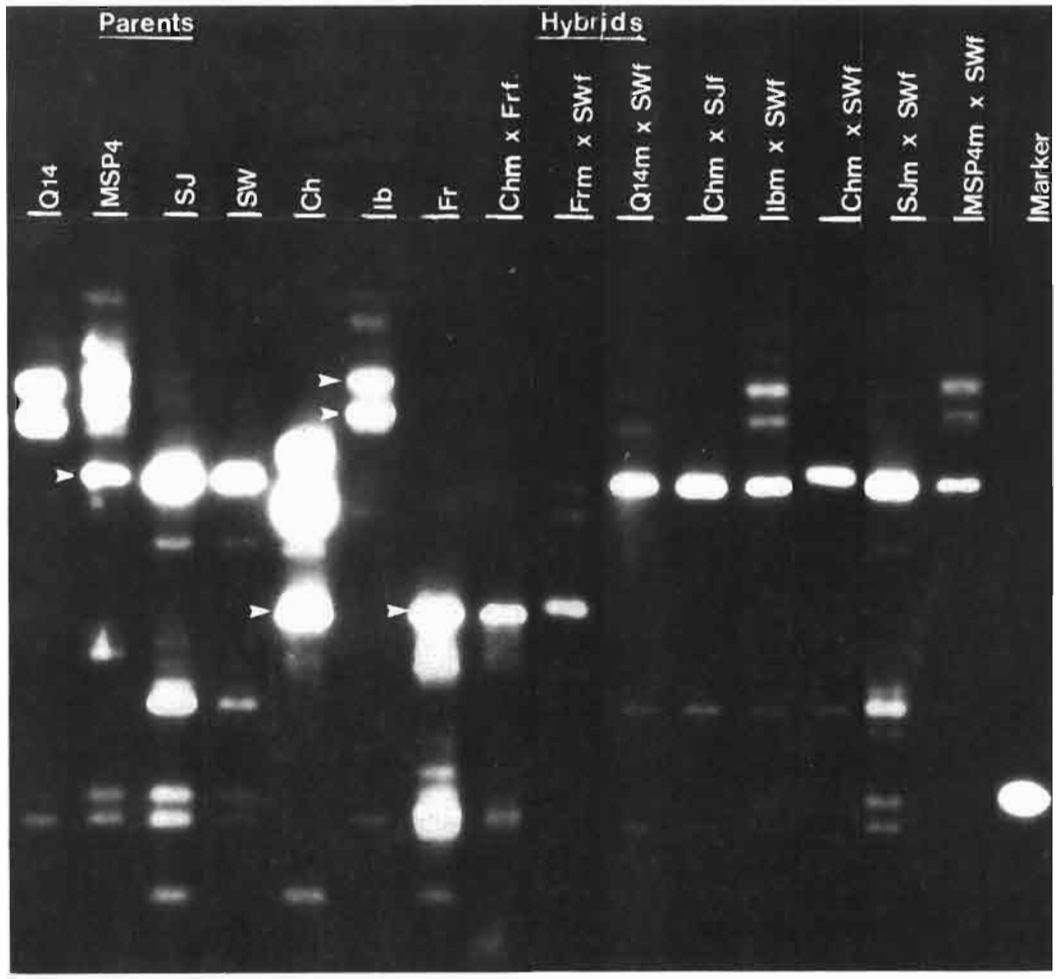

Fig. 1. Composite of total DNA from *Bursaphelenchus xylophilus* (Q14, MSP4, SJ, SW and Ib), and *B. mucronatus* (Ch and Fr) isolates and their hybrids digested with HindIII and SalI, then probed with the total ribosomal gene clone pB × 2. The first seven lanes from the left are the parental isolates and the remaining lanes are the intra- and interspecific hybrids. The arrows indicate bands that can be used to identify parental genome types.

cronatus Ch × Fr hybrid shows that hybrid cross inherited the DNA mainly from the Fr parent but may have obtained some from the Ch parent in view of the shared band (Fig. 1). The DNA analysis of the interspecific hybrids shows the banding pattern from the *B. xylophilus* parent, for example in the Ch × SW hybrid (Fig. 1). These DNA banding patterns suggest that under our laboratory conditions it appears that the *B. xylophilus* genome is favoured over the *B. mucronatus,* Ch genome. Similarly, the *B. mucronatus,* Fr genome appears to be favoured over the Ch genome. In addition, the *B. xylophilus* and the Fr genome appear to be segregating randomly.

The reciprocal hybridization crosses of *B. xylophilus* isolates Q14, SJ, SW, MSP4 and Ib from Canada, United States and Japan produced fertile progeny that reproduced vigorously over several generations. This concurs with the results of similar experiments, with different geographic isolates, reported by Mamiya (1986) and de Guiran and Bruguier (1989).

Although interspecific hybridization between males of the *B. mucronatus* isolates and females of all the *B. xylophilus* isolates was successful, some of the reciprocal crosses were not. Similarly, Mamiya (1986) reported that F_1 hybrids of *B. xylophilus* males × *B. mucronatus* females failed to produce offspring. However, de Guiran and Bruguier (1989) reported that the French *B. mucronatus* isolate gave fertile hybrids in reciprocal crosses with *B. xylophilus.* In our experiments crosses of the Japanese female *B. mucronatus* isolate with male *B. xylophilus* failed to produce fertile hybrids. The ability of *B. xylophilus* and *B. mucronatus* to interbreed and

produce viable offspring in some but not all sex combinations of isolates, suggests that these two species come from a common ancestor. As a consequence, matings in nature between these species may result in progeny having hybrid or unstable morphological or physiological traits which may influence morphological and pathogenic expression.

The fact that some of the interspecific crosses are non-reciprocal can be used to postulate two theories. Firstly, that we are dealing with three very closely related but distinct species, in which gene flow in the wild does not exist and which have not yet developed complete reproductive isolation. This could allow successful crosses to be generated under laboratory conditions. Alternatively, these results could be generated by a hybrid-dysgenesis-like phenomenon (Bregliano & Kidwell, 1983). In this case the maternal cytoplasm of the female *B. mucronatus* would contain or fail to contain an unknown component which causes abnormal development in heterogenous crosses. For the *B. mucronatus* Fr isolate this effect is not as stable or as predictable as that in *B. mucronatus* Ch isolate. It is also possible that the unknown component is different between *B. mucronatus* Fr and Ch because when the Ch female was crossed with either the Fr male or *B. xylophilus* males no progeny was produced. These hypotheses are not mutually exclusive and it is possible that the hybrid-dysgenesis-like phenomenon is a step towards reproductive isolation between new species.

This paper provides molecular and biological evidence to support separate species status for *B. xylophilus* and *B. mucronatus*. In addition, our evidence supports the de Guiran and Bruguier (1989) hypothesis that these species come from a common ancestor. We also provide evidence that suggests separate species status for the French and Japanese isolates of *B. mucronatus* and this is supported by results from the use of polymerase chain reaction technology and direct sequencing (Beckenbach, *et al.*, in press).

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