Polymorphism of Japanese isolates of the pinewood nematode, *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae), at heat-shock protein 70A locus and the field detection of polymorphic populations

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Abstract

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) pattern of heat-shock protein 70A gene (*hsp70A*) was examined for 29 isolates of the pinewood nematode. Among them, two known avirulent and five virulent isolates generated banding patterns identical within each of the two isolate groups but distinctive between the two. On the other hand, field populations were collected in two geographical areas, around Kasama City, Ibaraki Prefecture and the southern part of Kujukurihama coast, Chiba Prefecture, Japan. PCR-RFLP image analysis revealed that some of the populations possessed both of the two alleles. The geographical distribution of the alleles was random in the two sampling areas. On the basis of the results, we hypothesize that the avirulent strain might have a certain contribution to the gene pool of the pinewood nematode, although there has been no report on the collection of an avirulent population from Japan recently.

Key words: Avirulent isolate; forest disease; genetic polymorphism

INTRODUCTION

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, the causal agent of pine wilt disease (Kiyohara and Tokushige, 1971), is supposed to have originated in North America (de Guiran and Bruguir, 1989). Since 1905, when pine wilt disease was first reported, the area damaged by this disease has continued to spread throughout Japan except for the northernmost prefectures, Aomori and Hokkaido. Pine wilt disease has also destroyed huge areas of pine forests in other East Asian countries such as China and Korea, and has recently been found in Portugal (Mota et al., 1999), becoming a possible threat to European countries as an invasive forest pest.

Among the many PWN populations collected in Japan, several populations from different geographical origins are known to have only slight or no pathogenicity to host pine trees (e.g., Kiyohara and Bolla, 1990), and are generally called ‘aviru-

lent’ isolates. The avirulent isolates have a lower potential to invade the bark tissues of pine shoots (Asai, 2002), and lower ability to disperse (Ichihara et al., 2000) and propagate (Kiyohara and Bolla, 1990) within healthy pine trees. The number of nematodes of avirulent isolates carried by the vector beetle, *Monochamus alternatus* Hope, is far fewer than that of the virulent isolates (Aikawa et al., 2003b).

Although no reproductive isolation has developed between the two groups of isolates (e.g., Kiyohara and Bolla, 1990; Aikawa et al., 2003a), some studies have suggested that the two groups may have a different origin. For example, a molecular phylogenetic study with the internal transcribed spacer (ITS) region of ribosomal DNA showed that virulent and avirulent isolates belong to different clades in a phylogenetic tree of *B. xylophilus* and related species (Iwahori et al., 1998). In case of heat-shock protein 70A gene (*hsp70A*) as well, Takemoto et al. (2005) showed that an avirulent isolate generates a banding pattern different from

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that of a virulent isolate in their polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. However, they tested only one avirulent and one virulent isolate, and no information on allele frequency in natural pine stands has been given so far. In this study, we therefore investigated the frequency of *hsp70A* alleles in 29 populations subcultured in the laboratory and others collected from natural pine stands. A new primer specific to *hsp70A* alleles of *B. xylophilus* was designed to avoid nonspecific amplification from other wood-inhabiting nematodes.

MATERIALS AND METHODS

Primer set specific to *B. xylophilus*. The partial sequence of *hsp70A* of a Japanese isolate of *B. mucronatus*, which is the most closely related of the known species (Kanzaki and Futai, 2002) and possibly inhabits dead pine trees together with *B. xylophilus* (Mamiya, 1979), was determined. Firstly, a DNA fragment of *hsp70A* was amplified by PCR with a set of primers, *hsp70f* (5'-GACACCGA-GCGTCTAATCGGAG-3') and *hsp70r* (5'-GTAC-CACCAAGATCGAAG-3') originally described in Beckenbach et al. (1992). PCR amplifications were performed in 25 μ l reaction mixtures containing: 10 ng/ μ l of DNA template, 1.5 μ l; 10 μ M of each primer, 1.3 μ l; 1.25 mM dNTP, 2.5 μ l; Gene Taq NT (Nippon Gene, Japan), 0.1 μ l (0.5 U); 10 $\times$ reaction buffer, 2.5 μ l; autoclaved distilled water, 15.8 μ l. The PCR program consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 15 s and elongation at 72°C for 60 s, and concluded with a final elongation at 72°C for 10 min. All PCR amplifications were run on a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems). PCR products were purified by recovering the DNA fragment after electrophoresis in 0.7% agarose gel. Purified PCR products were used as templates for PCR direct sequencing using an ABI 3130 Genetic Analyzer with a reaction kit (BigDye® Terminator Cycle Sequencing Kit v1.0, Applied Biosystems) following the manufacturer's recommendations. To design a primer set specific to *B. xylophilus*, the resulting *hsp70A* sequence of *B. mucronatus* was aligned together with those of two *B. xylophilus* isolates, C14-5 and S10, deposited in the DNA Databank of Japan under ac-

cession numbers AB211292 and AB211293, respectively.

Nematodes. Allele frequencies of *hsp70A* were analyzed for 29 PWN isolates, collected from all over Japan. These isolates were maintained as laboratory cultures (Table 1). Among the 29 isolates examined, some have been well investigated for their pathogenicity: C14-5 and OKD1 have considerably low pathogenicity while Ka4, NS (a subculture of an isolate 'Shimabara' in Ikeda et al. (1994)), S10, S6-1 and T4 have rather high pathogenicity against host pine species (e.g., Kiyohara and Bolla, 1990; Ikeda et al., 1994; Aikawa et al., 2003a). As one isolate from Kasama City was found to have the rarer of the two alleles found in the 29 isolates, field populations were again collected from this locality (Table 2). In addition, other field populations were collected in the southern part of Kujukurihama Coast, where an avirulent isolate, C14-5, had been collected. In these two areas, in early April 2004, wood samples were taken from a dead part of a host tree (*Pinus thunbergii* or *P. densiflora*) or a recently killed host tree. In this report, PWN individuals collected from one tree are regarded as a subpopulation. All samples were placed in a Baermann funnel to extract nematodes for 1 d. Pinewood nematodes and other nematodes were extracted and counted, and then stored in 100% ethanol at -20°C until DNA extraction.

Preparation of DNA templates and PCR amplification. DNA templates were prepared using the method of Iwahori et al. (1998) for each of the isolates and from field populations sampled at the two above-mentioned locations. The numbers of nematodes used for DNA extraction were more than 100 for the field populations (Table 2) and ca. 10,000 for the isolates. PCR amplifications were performed in 12.5 μ l reaction mixtures with the same composition as mentioned above. To amplify the *hsp70A* alleles, we used a set of two primers, *hsp70f-bx.sp.* (5'-CGTATTTGGTAAGCTTTTG-3') designed in this study and *hsp70r* originally described in Beckenbach et al. (1992). All PCR amplifications were run under the same condition as mentioned above.

RFLP analysis. Ten microliters of PCR products were immediately digested by adding 0.4 μ l (4 U) of *AluI* (New England Biolabs) and 1.2 μ l of NEBuffer 2 (10 $\times$) (New England Biolabs), and incubated at 37°C overnight. Those digested PCR

Table 1. PWN isolates used in this study

Code	Isolate name	Location	Sampling year	Resource
1	C14-5	Ichinomiya, Chiba Pref.	1975	<i>M. alternatus</i> adult
2	S6-1	Mito, Ibaraki Pref.	1975	<i>M. alternatus</i> adult
3	NS	Shimabara, Nagasaki Pref.	1975?	Dead <i>P. densiflora</i>
4	S10	Yasugi (old Hirose), Shimane Pref.	1982	Dead <i>P. densiflora</i>
5	OKD1	Okayama, Okayama Pref.	1984	
6	T4	Ichinoseki, Iwate Pref.	1992	Dead <i>P. densiflora</i>
7	Ka1	Kasama, Ibaraki Pref.	1990's?	
8	Ka4	Kasama, Ibaraki Pref.	1994	Dead <i>P. densiflora</i>
9	Hachiro1	Lake Hachirogata, Akita Pref.	1995	
10	Hachiro2	Lake Hachirogata, Akita Pref.	1995	
11	Oomori3	Yokote (old Oomori), Akita Pref.	1995	
12	TN1	Akita, Akita Pref.	1995	
13	TN3	Murakami, Niigata Pref.	1995	
14	Ei	Imabari, Ehime Pref.	1996	Dead <i>P. thunbergii</i>
15	Kg	Kyoto, Kyoto Pref.	1996	Dead <i>P. thunbergii</i>
16	KK	Kyoto, Kyoto Pref.	1996	Dead <i>P. densiflora</i>
17	Yt1	Shunan (old Tokuyama), Yamaguchi Pref.	1996	Dead <i>P. thunbergii</i>
18	Yt238	Shunan (old Tokuyama), Yamaguchi Pref.	1996	Dead <i>P. thunbergii</i>
19	Karatsu3	Karatsu, Saga Pref.	1997	Dead <i>P. thunbergii</i>
20	Kotobikihama	Kyotango (old Amino), Kyoto Pref.	1997	Dead <i>P. thunbergii</i>
21	Kumihama	Kyotango (old Kumihama), Kyoto Pref.	1997	Dead <i>P. thunbergii</i>
22	OKN3	Kunigami, Okinawa Pref.	1997	Dead <i>P. luchuensis</i>
23	Sa-black1	Sado Island, Niigata Pref.	1997	Dead <i>P. thunbergii</i>
24	Sa-black2	Sado Island, Niigata Pref.	1997	Dead <i>P. thunbergii</i>
25	Sa-red	Sado Island, Niigata Pref.	1997	Dead <i>P. densiflora</i>
26	Tt1	Tottori, Tottori Pref.	1997	Dead <i>P. thunbergii</i>
27	Tt2	Tottori, Tottori Pref.	1997	Dead <i>P. thunbergii</i>
28	Tt5	Tottori, Tottori Pref.	1997	Dead <i>P. thunbergii</i>
29	St1	Otsu, Shiga Pref.	1998	Dead <i>P. thunbergii</i>

Table 2. Field populations of the PWN analyzed in this study

Code	Sampling date	Location	Resource	Symptom development on the tree ^a	Epidemic in the stand ^b	Disease control ^c	Nematode number/g dw. ^d	Nematode number used for DNA extraction
30	2004.4.6	Kasama, Ibaraki Pref.	<i>P. densiflora</i>	little	slight	no	53.7	741
31	2004.4.6	Kasama, Ibaraki Pref.	<i>P. densiflora</i>	complete	severe	no	559.2	4,194
32	2004.4.6	Nanakai, Ibaraki Pref.	<i>P. densiflora</i>	complete	slight	no	62.6	651
33	2004.4.6	Nanakai, Ibaraki Pref.	<i>P. densiflora</i>	complete	slight	no	517.4	5,226
34	2004.4.6	Nanakai, Ibaraki Pref.	<i>P. densiflora</i>	complete	slight	no	451.9	6,552
35	2004.4.6	Nanakai, Ibaraki Pref.	<i>P. densiflora</i>	complete	slight	no	403.4	3,348
36	2004.4.6	Nanakai, Ibaraki Pref.	<i>P. densiflora</i>	complete	slight	no	494.3	6,970
37	2004.4.6	Nanakai, Ibaraki Pref.	<i>P. densiflora</i>	complete	slight	no	473.8	4,312
38	2004.4.7	Ichinomiya, Chiba Pref.	<i>P. thunbergii</i>	complete	medium	no	188.8	3,612
39	2004.4.7	Ichinomiya, Chiba Pref.	<i>P. thunbergii</i>	half	slight	yes	20.9	473
40	2004.4.7	Ichinomiya, Chiba Pref.	<i>P. thunbergii</i>	little	slight	yes	725.7	16,677
41	2004.4.7	Ichinomiya, Chiba Pref.	<i>P. thunbergii</i>	complete	slight	yes	23.3	568

^a Disease symptoms on a host tree with foliage wilt of <0–10, 10–30, 30–70, 70–90 and 90–100% are ranked as ‘little’, ‘partial’, ‘half’, ‘most’ and ‘complete’, respectively.

^b Severity of disease epidemic in a stand with killed host trees in the proportion of <0–30, 30–70 and 70–100% are ranked as ‘slight’, ‘medium’ and ‘severe’, respectively.

^c ‘yes’ or ‘no’ indicates whether a stand was under disease control.

^d ‘dw.’ is the abbreviation of ‘dry weight of a sample’.

products were resolved by electrophoresis in 5% polyacrylamide gel in Tris-Borate-EDTA (TBE) buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 50 min at 100 V. The gels were stained with 2.5 mg/l ethidium bromide over 30 min and photographed under UV light.

Image analysis and estimation of allele frequencies. An image analysis method was used to estimate allele frequencies in the subpopulations as performed by Takemoto et al. (2005). The brightness of PCR-RFLP bands specific to *S* (ca. 270 bp) and *C* alleles (ca. 150 and 130 bp) were scanned under UV light after staining with ethidium bromide. *S* and *C* alleles were previously reported by Takemoto et al. (2005) as alleles specific to S10 (virulent isolate) and C14-5 (avirulent isolate), respectively. All of the populations analyzed were characterized by either the *S* or *C* allele or a mixture of the two. 'Peak height ratio' was determined by dividing the peak height of the band specific to the *S* allele by the total peak height of the two bands specific to the *C* allele. 'Peak area ratio' was determined by dividing the peak area of the band specific to the *S* allele by the total peak area of the two bands specific to the *C* allele. Allele frequencies were then estimated with standard lines determined using a series of DNA templates with a known ratio of S10 to C14-5. In this study, *S*-allele frequency in a subpopulation was given as the average of two estimates from 'Peak height ratio' and 'Peak area ratio'.

RESULTS

Primer set specific to *B. xylophilus*

Figure 1 shows the alignment of the *hsp70A* sequences of two *B. xylophilus* isolates (C14-5 and S10) and one *B. mucronatus* isolate (M). Thirty-eight (11%) substitutions were found in the comparable 338 bp of the *hsp70A* sequences, especially in the intron. The sequence of *B. mucronatus* was deposited in the DNA Databank of Japan under accession number AB284381. A new forward primer, *hsp70f*-bx.sp., for the specific amplification of *hsp70A* of *B. xylophilus*, was designed based on the difference in the 5'-end region of the intron. The combination of this primer and the reverse primer, *hsp70r*, specifically amplified the *hsp70A* fragment of *B. xylophilus*, while that of *B. mucronatus*, a closely related species, was not amplified (Fig. 2).

<i>B. xylophilus</i> (C14-5)	1	-----CCCTCAAAAC	10
<i>B. xylophilus</i> (S10)	1	-----CCCTCAAAAC	10
<i>B. mucronatus</i> (M)	1	GACACCGAGCGTCTAATCGGAGATGCCGTAAGAACAGGTCCCATGAA.....	61
<i>B. xylophilus</i> (C14-5)	11	ACCGTATTGGTAAGCTTTGACTGAGTATTATTTTATGCG-ATTAAAGTACTTTA	69
<i>B. xylophilus</i> (S10)	11	-----	69
<i>B. mucronatus</i> (M)	62	.T.....G..A..-C.....ATATAGAA...TG.....C	117
<i>B. xylophilus</i> (C14-5)	70	TATAAGATGTCTTTTACAGATGCCAAGCGTTTATTGCGCCAAATTCGATGAACCCACA	129
<i>B. xylophilus</i> (S10)	70	-----	129
<i>B. mucronatus</i> (M)	118	A...CATGA.T...G.....A.....T.....G.....	177
<i>B. xylophilus</i> (C14-5)	130	GTTCAAGCCGACATGAAGCACTGCCATTCAAGTCGTCCAGCCGAAGGTGACGTCTCT	189
<i>B. xylophilus</i> (S10)	130	-----	189
<i>B. mucronatus</i> (M)	178	C.....A.....	237
<i>B. xylophilus</i> (C14-5)	190	AAGGTTCAAGTCGAATCAAGAGAGACCAAGAGCTTCTTCCCTGAAGATCTCATCT	249
<i>B. xylophilus</i> (S10)	190	-----	249
<i>B. mucronatus</i> (M)	238	G.....G.....	297
<i>B. xylophilus</i> (C14-5)	250	ATGGTCTTATCAAGATGAAGAAACCGCAAGCTTCTCCGATCTGAAGTCAAGGAT	309
<i>B. xylophilus</i> (S10)	250	-----	309
<i>B. mucronatus</i> (M)	298	T.....A.....A..C	357
<i>B. xylophilus</i> (C14-5)	310	GCCGTGTACCGTCCAGCTCACTCAAGCTCTCAGCTCAGGCTACGAAGACGCC	369
<i>B. xylophilus</i> (S10)	310	-----	369
<i>B. mucronatus</i> (M)	358	G.....C.....	417
<i>B. xylophilus</i> (C14-5)	370	GGAGCTATTGCTGTTTGAATGTTCTTCAATCATCAATGAGCCGACCGCGCTCCATC	429
<i>B. xylophilus</i> (S10)	370	G.....	429
<i>B. mucronatus</i> (M)	418	-----	427
<i>B. xylophilus</i> (C14-5)	430	G	430
<i>B. xylophilus</i> (S10)	430	.	430
<i>B. mucronatus</i> (M)	427	-	427

Fig. 1. Alignment of the *hsp70A* sequences of two *B. xylophilus* isolates (C14-5 and S10) and one *B. mucronatus* isolate (M). In the underlined sequence, a primer specific to *B. xylophilus* was designed. Italicized sequences are intron bases. Dashes indicate gaps introduced to maximize the alignment. Dots mean bases identical to those of the query sequence.

Fig. 2. Specificity of the newly designed primer *hsp70f*-bx.sp. Electrophoresis was conducted in 1.5% agarose gel at 50 V for 80 min. As a size marker, 100 bp DNA Ladder (New England Biolabs) was used. †: primer sets used for PCR, ††: Bx, Bm and NC stand for *B. xylophilus*, *B. mucronatus* and negative control (distilled water), respectively.

Genotyping of Japanese isolates of PWN

Only three types of PCR-RFLP banding patterns were detected from the 29 PWN isolates tested (Fig. 3A): *S*, *C* and mixed type of the two, but no other types were found. Among the 29 isolates, both of the two avirulent isolates, C14-5 and

OKD1, generated a *C*-type banding pattern, and all of the isolates known as virulent isolates, NS, S10, S6-1, T4 and Ka4, generated an *S*-type banding pattern. Almost all of the other isolates generated an *S*-type banding pattern, but only one isolate, Ka1, generated a mixed-type pattern (Fig. 3B) with the *C* allele as 9%.

Frequency of *hsp70A* alleles in field populations

Twenty-three and 13 wood samples were col-

Fig. 3. PCR-RFLP patterns of *hsp70A* gene fragment digested with *AluI* for the PWN isolates analyzed in preliminary research. Digestions were resolved using 5% acylamide gel electrophoresis at 100 V for 50 min. As a size marker, 100 bp DNA Ladder (New England Biolabs) was used. A: PCR-RFLP patterns of 29 PWN isolates. The same code number as that in Table 1 is used to denote each isolate. B: PCR-RFLP pattern of an isolate, Ka1, in comparison with that of a virulent (S10) and an avirulent isolate (C14-5).

lected from 18 and 13 geographical points around Kasama City, Ibaraki Prefecture and in the southern part of Kujukurihama Coast, Chiba Prefecture, respectively. Since little or no PWN was extracted from several samples, only eight and four subpopulations (Table 2) from the two respective areas were used for subsequent analysis. The PCR-RFLP patterns were *S*, *C* and mixed type of the two, and no other types were found (Fig. 4). Figure 5 shows the geographical distribution of *hsp70A* alleles. Allele frequencies, which were estimated using image analysis of their PCR-RFLP banding pattern, varied among the subpopulations.

DISCUSSION

Takemoto et al. (2005) mixed a virulent and avirulent isolate and cultured the mixture, finding that the frequency of the *S* allele converged at ca. 0.7 over time, showing apparent heterosis at the *hsp70A* locus or another locus closely linked with it; however, the subpopulations collected from the region around Kasama City had the *S* allele at various frequencies, which had not converged. The observed allele frequencies may reflect the effect of random factors such as genetic drift rather than natural selection because the *S* and *C* alleles themselves are identical at the amino acid level (Takemoto et al., 2005).

Among the 29 isolates examined in our present study, some had been well investigated for their pathogenicity: C14-5 and OKD1 have considerably

Kasama and Nanakai								Kujukurihama Coast			
30	31	32	33	34	35	36	37	38	39	40	41

Fig. 4. PCR-RFLP patterns of *hsp70A* gene fragment digested with *AluI* for PWN subpopulations analyzed in the main research. Digestions were resolved using 5% acylamide gel electrophoresis at 100 V for 50 min. As a size marker, 100 bp DNA Ladder (New England Biolabs) was used. The same code number as that in Table 2 is used to denote each of the PWN subpopulations analyzed.

Fig. 5. Geographical distribution of *hsp70A* alleles in sampling areas. The frequency of the *S* allele was estimated with PCR-RFLP image analysis. Black and white portions in each pie graph correspond to *S* and *C* alleles, respectively. The same code numbers as those in Table 2 are used for PWN subpopulations. Bars are 1 km. A: Region around Kasama City, Ibaraki Prefecture. B: Southern part of Kujukurihama Coast, Chiba Prefecture.

low pathogenicity while Ka4, NS (a subculture of an isolate 'Shimabara' in Ikeda et al. (1994)), S10, S6-1 and T4 have rather high pathogenicity against host pine species (e.g., Kiyohara and Bolla, 1990; Ikeda et al., 1994; Aikawa et al., 2003a). According to the PCR-RFLP pattern of *hsp70A*, two isolates, C14-5 and OKD1, were typed into one group, and five isolates, Ka4, NS, S10, S6-1, and T4 into another (Fig. 3A). Aikawa et al. (2003a) also reported that PCR-RFLP of the ITS region enabled the discrimination of five virulent isolates (Ka4, S10, T4, S6-1 and No. 375) from two avirulent isolates (C14-5 and OKD1). If these isolates can be regarded as random samples, the null hypothesis that there is no relationship between their pathogenicity and PCR-RFLP patterns is rejected with a significance level of $p=0.0476$ (Fischer's exact probability test) for each of the two loci, *hsp70A* and ITS. It is therefore considered to be improbable that their PCR-RFLP pattern matched their traits by chance. We therefore hypothesize that avirulent and virulent isolates were derived from different ancestral populations characterized with *C* and *S* alleles, respectively. This hypothesis

is in agreement with Iwahori et al. (1998), who discussed that Japanese avirulent isolates (C14-5 and OK-2) may have a different origin from Japanese virulent isolates (S10, S6-1 and T4) based on ITS sequences.

There has been no report on the collection of an avirulent PWN isolate since 1984, when OKD1 was isolated (Kawazu et al., 1996); however, some subpopulations had both *S* and *C* alleles in the two areas, the region around Kasama City and the southern part of Kujukurihama Coast (Fig. 5). If the ancestral population of avirulent isolates is supposed to have been characterized with the *C* allele as discussed above, the persistence of the allele in the subpopulations may indicate the genetic contribution of the avirulent ancestor to the gene pool of Japanese PWN by introgressive hybridization with the virulent ancestor. If no relationship between their genotype and pathogenicity is supposed, the genetic polymorphism found in the present study may simply reflect the original polymorphism in the ancestral populations, and we must consider a superficial relationship between the genotype and the pathogenicity of the seven Japanese isolates (C14-5, Ka4, NS, OKD-1, S10, S6-1 and T4) as caused by some sampling bias (e.g., biased distribution of the alleles after the artificial introduction of local populations to other places). Since the number of samples was too few and the research sites were too limited to prove this hypothesis, populations should be collected from other regions. In particular, if avirulent populations are dominant in some regions, their DNA polymorphism should be examined. Investigations on other genetic loci with segregation analysis among them are also needed.

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REFERENCES

- Aikawa, T., T. Kikuchi and H. Kosaka (2003a) Demonstration of interbreeding between virulent and avirulent populations of *Bursaphelenchus xylophilus* (Nematoda:

- Aphelenchoididae) by PCR-RFLP method. *Appl. Entomol. Zool.* 38: 565–569.
- Aikawa, T., K. Togashi and H. Kosaka (2003b) Different developmental responses of virulent and avirulent isolates of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), to the insect vector, *Monochamus alternatus* (Coleoptera: Cerambycidae). *Environ. Entomol.* 32: 96–102.
- Asai, E. (2002) The effect of simulated acid rain on the development of pine wilt disease. PhD thesis, Kyoto University, Kyoto, Japan. 122 pp.
- Beckenbach, K., M. J. Smith and J. M. Webster (1992) Taxonomic affinities and intra- and interspecific variation in *Bursaphelenchus* spp. as determined by polymerase chain reaction. *J. Nematol.* 24: 140–147.
- de Guiran, G. and N. Bruguir (1989) Hybridization and phylogeny of the pine wood nematode (*Bursaphelenchus* spp.). *Nematologica* 35: 321–330.
- Ichihara, Y., K. Fukuda and K. Suzuki (2000) Early symptom development and histological changes associated with migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. *Plant Dis.* 84: 675–680.
- Ikeda, T., T. Toda and M. Tajima (1994) Histological responses of different resistant families of *Pinus thunbergii* and *P. densiflora* to an invasion of pine wood nematode. *Ann. Phytopath. Soc. Japan* 60: 540–542.
- Iwahori, H., K. Tsuda, N. Kanzaki, K. Izui and K. Futai (1998) PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundam. Appl. Nematol.* 21: 655–666.
- Kanzaki, N. and K. Futai (2002) A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology* 4: 35–41.
- Kawazu, K., H. Zhang, H. Yamashita, H. Kanzaki (1996) Relationship between the pathogenicity of the pine wood nematode, *Bursaphelenchus xylophilus*, and phenylacetic acid production. *Biosci. Biotechnol. Biochem.* 60: 1413–1415.
- Kiyohara, T. and R. I. Bolla (1990) Pathogenic variability among populations of the pinewood nematode, *Bursaphelenchus xylophilus*. *Forest Science* 36: 1061–1076.
- Kiyohara, T. and Y. Tokushige (1971) Inoculation experiment of a nematode, *Bursaphelenchus* sp., onto pine trees. *J. Jpn. For. Soc.* 53: 210–218 (in Japanese with English summary).
- Mamiya, Y. (1979) *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and its biology and pathogenicity to pine trees. *Nematologica* 25: 353–361.
- Mota, M. M., H. Braasch, M. A. Bravo, A. C. Penas, W. Burgermeister, K. Metge and E. Sousa (1999) First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1: 727–734.
- Takemoto, S., N. Kanzaki and K. Futai (2005) PCR-RFLP image analysis—a practical method for estimating isolate-specific allele frequency in a population consisting of two different strains of the pinewood nematode, *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae). *Appl. Entomol. Zool.* 40: 529–535.