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Ribosomal DNA haplotype distribution of *Bursaphelenchus xylophilus* in Kyushu and Okinawa islands, Japan

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Abstract: Ribosomal DNA region sequences (partial 18S, 28S and complete ITS1, 5.8S, and ITS2) of the pinewood nematode (*Bursaphelenchus xylophilus*) were obtained from DNA extracted directly from wood pieces collected from wilted pine trees throughout the Kyushu and Okinawa islands, Japan. Either a 2569bp or 2573bp sequence was obtained from 88 of 143 samples. Together with the 45 rDNA sequences of pinewood nematode isolates previously reported, there were eight single nucleotide polymorphisms and two indels of two bases. Based on these mutations, nine haplotypes were estimated. The haplotype frequencies differed among regions in Kyushu island (northwest, northeast and center, southeast, and southwest), and the distribution was consistent with the invasion and spreading routes of the pinewood nematode previously estimated from past records of pine wilt and wood importation. There was no significant difference in haplotype frequencies among the collection sites on Okinawa island.

Key words: Bursaphelenchus xylophilus, haplotype, invasive species, pine wilt disease, pinewood nematode, rDNA, spread route.

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970) vectored by the cerambycid beetle *Monochamus* spp. has caused serious damage to pine forests in East Asia, including Japan, China, Korea, and Taiwan (Kiyohara and Tokushige, 1971; Sun, 1982; Enda, 1988, 1989; Enda and Taketani, 1992). In 1999, PWN was also detected in dead pine stands in Portugal (Mota et al., 1999). It subsequently became a widespread threat to pine forests, and many countries have established programs to cope with this disease (Toda, 1997, 2004, 2006; Mota and Vieira, 2008).

In Japan, the first incidence of pine wilt disease was reported in Nagasaki city, Nagasaki Prefecture in 1905 (Yano, 1913). The PWN first spread to the area where an industry reliant on wilted pine trees was located (lumber mill, pulp mill, shipyard, and coal mine), and it has been expanding throughout the surrounding areas (Kishi, 1988; Togashi and Jikumaru, 2007). According to the Japanese Forestry Agency, the damage has expanded during the last five decades, with the volume of damaged trees surpassing 2 million m^3 in 1978, and peaking in 1979 due to high temperatures and low precipitation in the summer. The damage occurred throughout Japan, except in the two Northern-most prefectures of Aomori and Hokkaido, which have cool summer climates (Togashi and Jikumaru, 2007). The Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) and Japanese black pine (*P. thunbergii* Parl.) which are mainly distributed in the Honshu, Shikoku, and Kyushu islands, and the Luchu pine (*P. luchuensis* Mayr.) in the Okinawa islands have been damaged by PWN.

The origin and spreading routes of PWN have been estimated by several molecular biology techniques. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the ribosomal DNA region (rDNA) has been used for the discrimination of isolates (Iwahori et al., 1998). Analysis of the data indicated that virulent Japanese, US, and Chinese isolates formed one cluster, while Canadian isolates formed another. These results supported the hypothesis that PWN was probably introduced to Japan from the US (Tarès et al., 1993; Harmey and Harmey, 1993). Amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and sequences of the 28S and Internal Transcribed Spacer (ITS) of rDNA have shown that Chinese PWN populations are closer to Japanese populations than to American populations (Cheng et al., 2008; Zhang et al., 2008), and two major invasion pathways from Guangdong within China have been estimated (Cheng et al., 2008). The results of inter-simple sequence repeats (ISSR) and RAPD indicated that there was low variability among Portuguese isolates, and founder(s) of the Portuguese populations most likely were transported one or two times to Portugal from sites in East Asia, but not from North America (Metge and Burgermeister, 2006; Vieira et al., 2007).

Several invasion and spreading routes in Japan have been estimated from past records of pine wilt and wood transportation (Kishi, 1988). The PWN genotype of each region was presumed to reflect the invasion and spread routes. Previous studies have indicated that there was sequence variability in the rDNA region among PWN isolates (Iwahori et al., 1998; Beckenbach et al., 1999; Kanzaki and Futai, 2002; Mota et al., 2006; Zhang et al., 2008), and six rDNA haplotypes were estimated from the

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TABLE 1. Sampling sites of *Bursaphelenchus xylophilus* on Kyushu and Okinawa islands; code of nematode samples, SNPs and indels, and estimated rDNA haplotypes. Numbers indicate the position of the SNPs and indels in total sequence data in this study. ':' indicates deletion. 'ND' indicates no sequence data. '*' indicates the collection site in which samples were analyzed in Nose et al. (submitted).

sampling site			sample code	18S		ITS1				ITS2						haplotype
island	prefecture	collection site (code)		80	651	1755	1813	1824	1967	2251	2320	2328	2329	2357	2358	
Kyushu	Fukuoka	Kanesaki	Fk1	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Fk)	Fk3	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Fk4	T	A	A	G	T	A	G	C	G	G	G	C	D
			Fk5	T	A	A	G	T	A	G	C	G	G	G	C	D
		Yukuhashi	Fy1	T	A	A	T	T	A	A	C	G	G	G	C	B
		(Fy)	Fy2	T	A	A	G	T	A	G	C	G	G	G	C	D
			Fy3	T	A	A	G	T	A	G	C	G	G	G	C	D
			Fy4	T	A	A	G	T	A	G	C	G	G	G	C	D
			Fy5	T	A	A	G	T	A	G	C	G	G	G	C	D
			Fy6	T	A	A	T	C	A	A	T	:	:	:	:	F
	Saga	Chinzei*	Sc1	T	A	A	T	T	A	A	T	:	:	:	:	A
		(Sc)	Sc3	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Sc6	T	A	A	T	T	A	A	T	:	:	:	:	A
			Sc9	T	A	A	T	T	A	A	T	:	:	:	:	A
			Sc11	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Sc12	T	A	A	T	T	A	A	T	:	:	:	:	A
		Karatsu Aiga*	Sks1	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Sks)	Sks2	T	A	A	G	T	A	G	C	G	G	G	C	D
		Karatsu Minato*	Skm1	T	A	A	T	T	A	A	T	:	:	:	:	A
		(Skm)	Skm2	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Skm3	T	A	A	T	T	A	A	T	:	:	:	:	A
			Skm5	T	A	A	T	T	A	R	Y	ND	ND	ND	ND	AC
			Skm6	T	A	A	T	T	A	A	C	G	G	G	C	B
			Skm8	T	A	A	T	T	A	A	C	G	G	G	C	B
			Skm9	T	A	A	T	T	A	A	T	:	:	:	:	A
	Nagasaki	Sasebo	Nss1	T	A	A	T	T	A	A	T	:	:	:	:	A
		(Nss)	Nss2	T	A	A	T	T	A	A	C	G	G	G	C	B
			Nss3	T	A	A	T	T	A	A	C	G	G	G	C	B
			Nss4	T	A	A	T	T	A	A	T	:	:	:	:	A
			Nss5	T	A	A	T	T	A	R	C	G	G	G	C	BC
			Nss6	T	A	A	T	T	A	A	T	:	:	:	:	A
		Unzen	Nu1	T	A	A	T	T	A	A	C	G	G	G	C	B
		(Nu)	Nu2	T	A	A	T	T	A	A	C	G	G	G	C	B
			Nu3	T	A	A	T	T	A	A	C	G	G	G	C	B
			Nu4	T	A	A	G	T	A	G	C	G	G	G	C	D
			Nu6	T	A	A	K	Y	A	R	Y	ND	ND	ND	ND	DF
		Shimabara	Ns2	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Ns)	Ns3	T	A	A	G	T	A	G	C	G	G	G	C	D
			Ns4	T	A	A	G	T	A	G	C	G	G	G	C	D
	Kumamoto	Nishigoushi* (Kn)	Kn1	T	A	A	G	T	A	G	C	G	G	G	C	D
		Kousa*	Kk1	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Kk)	Kk2	T	A	A	G	T	A	G	C	G	G	G	C	D
			Kk3	T	A	A	G	T	A	G	C	G	G	G	C	D
			Kk5	T	A	A	G	T	A	G	C	G	G	G	C	D
			Kk6	T	A	A	K	Y	A	R	Y	ND	ND	ND	ND	DF
			Kk8	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Kk9	T	A	A	G	T	A	G	C	G	G	G	C	D
			Kk12	T	A	A	G	T	A	G	C	G	G	G	C	D
		Kamishima,	Kak2	C	A	A	T	T	A	A	C	G	G	G	C	G
		Amakusa	Kak3	T	A	A	T	C	A	A	T	:	:	:	:	F
		(Kak)	Kak4	T	A	A	T	C	A	A	T	:	:	:	:	F
			Kak6	T	A	A	T	Y	A	A	T	:	:	:	:	AF
			Kak9	T	A	A	G	T	A	G	C	G	G	G	C	D
			Kak10	T	A	A	G	T	A	G	C	G	G	G	C	D
		Shimoshima,	Kas2	T	A	A	T	C	A	A	T	:	:	:	:	F
		Amakusa	Kas5	T	A	A	K	T	A	R	Y	ND	ND	ND	ND	AD
		(Kas)	Kas6	T	A	A	T	Y	A	A	T	:	:	:	:	AF
			Kas10	T	A	W	T	T	A	A	Y	ND	ND	ND	ND	AH_1 / BH_2
	Oita	Ajimu	Oaj1	T	A	A	T	T	A	G	C	G	G	G	C	C
		(Oaj)	Oaj3	T	A	A	G	T	A	G	C	G	G	G	C	D
			Oaj4	T	A	A	G	T	A	G	C	G	G	G	C	D
		Saeki	Os1	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Os)	Os2	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os3	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os4	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os5	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os6	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os7	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os8	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os9	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os10	T	A	A	G	T	A	G	C	G	G	G	C	D
			Os12	T	A	A	G	T	A	G	C	G	G	G	C	D

(*Continued*)

TABLE 1. Continued

sampling site			sample code	18S		ITS1				ITS2						haplotype
island	prefecture	collection site (code)		80	651	1755	1813	1824	1967	2251	2320	2328	2329	2357	2358	
Kyushu	Miyazaki	Shiiba*	Ms2	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Ms)	Ms5	T	A	A	G	T	A	G	C	G	G	G	C	D
			Ms-mix	T	A	A	G	T	A	G	C	G	G	G	C	D
		Morotsuka*	Mm1	T	A	A	K	T	A	R	C	G	G	G	C	BD
		(Mm)	Mm2	T	A	A	K	T	A	R	C	G	G	G	C	BD
			Mm3	T	A	A	G	T	A	G	C	G	G	G	C	D
			Mm4	T	A	A	G	T	A	G	C	G	G	G	C	D
		Hyuga*	Mh2	T	M	A	T	T	A	A	T	:	:	:	:	AE
		(Mh)	Mh3	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Mh4	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Mh5	T	A	A	T	T	A	A	C	G	G	G	C	B
			Mh9	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Mh10	T	A	A	T	T	A	G	C	G	G	G	C	C
		Hitotsuba	Mht1	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
		(Mht)	Mht4	T	A	A	T	T	A	A	C	G	G	G	C	B
			Mht6	T	A	A	T	T	A	A	T	:	:	:	:	A
		Nichinan	Mnc1	T	A	A	T	T	M	R	C	G	G	G	C	BI_1 / CI_2
		(Mnc)	Mnc2	T	A	A	G	T	A	G	C	G	G	G	C	D
			Mnc7	T	A	A	T	T	A	A	C	G	G	G	C	B
			Mnc10	T	A	A	T	T	M	R	C	G	G	G	C	BI_1 / CI_2
		Kushima	Mkm3	T	A	A	T	T	A	A	C	G	G	G	C	B
		(Mkm)	Mkm4	T	A	A	G	T	A	G	C	G	G	G	C	D
			Mkm5	T	A	A	T	T	A	A	C	G	G	G	C	B
			Mkm6	T	A	A	G	T	A	G	C	G	G	G	C	D
	Kagoshima	Sakurajima*	Ks1	T	A	A	T	T	A	A	C	G	G	G	C	B
		(Ks)	Ks2	T	A	A	T	T	A	A	C	G	G	G	C	B
			Ks3	T	A	A	K	T	A	R	Y	ND	ND	ND	ND	AD
			Ks4	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Ks5	T	A	A	T	T	A	R	C	G	G	G	C	BC
			Ks8	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Ks9	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Ks10	T	A	A	T	T	A	A	Y	ND	ND	ND	ND	AB
			Ks11	T	A	A	T	T	A	A	C	G	G	G	C	B
		Sendai	Ksn1	T	A	A	T	T	A	A	C	G	G	G	C	B
		(Ksn)	Ksn2	T	A	A	T	T	A	A	C	G	G	G	C	B
			Ksn3	T	A	A	T	C	A	A	T	:	:	:	:	F
			Ksn4	T	A	A	T	C	A	A	T	:	:	:	:	F
		Ibusuki	Ki1	T	A	A	T	C	A	A	T	:	:	:	:	F
		(Ki)	Ki3	T	A	A	T	C	A	A	T	:	:	:	:	F
			Ki4	T	A	A	T	C	A	A	T	:	:	:	:	F
			Ki5	T	A	A	T	C	A	A	T	:	:	:	:	F
Okinawa	Okinawa	Kayou	Oky5	T	A	A	G	T	A	G	C	G	G	G	C	D
		(Oky)	Oky6	T	A	A	G	T	A	G	C	G	G	G	C	D
		Kanna	Okn1	T	A	A	T	C	A	A	T	:	:	:	:	F
		(Okn)	Okn2	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Okn3	T	A	A	T	T	A	R	Y	ND	ND	ND	ND	AC
			Okn4	T	A	A	K	Y	A	R	Y	ND	ND	ND	ND	DF
			Okn5	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Okn6	T	A	A	T	C	A	A	T	:	:	:	:	F
		Yaka	Oy1	T	A	A	T	C	A	A	T	:	:	:	:	F
		(Oy)	Oy2	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Oy5	T	A	A	T	C	A	A	T	:	:	:	:	F
		Ishikawa	Oi1	T	A	A	T	C	A	A	T	:	:	:	:	F
		(Oi)	Oi2	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Oi3	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Oi4	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Oi5	T	A	A	K	T	A	G	C	G	G	G	C	CD
		Zakimi	Oz1	T	A	A	K	Y	A	R	Y	ND	ND	ND	ND	DF
		(Oz)	Oz2	T	A	A	K	Y	A	R	Y	ND	ND	ND	ND	DF
			Oz3	T	A	A	K	T	A	G	C	G	G	G	C	CD
			Oz4	T	A	A	K	Y	A	R	Y	ND	ND	ND	ND	DF
			Oz5	T	A	A	K	T	A	G	C	G	G	G	C	CD

sequences of PWN isolates collected on Kyushu island (Nose et al., unpublished). In this study, we collected a large number of PWN samples throughout the Kyushu and Okinawa islands, and DNA was extracted directly from wilted pine wood pieces using a new simple method. The geographical distribution of haplotypes was investigated by sequencing the rDNA region (partial 18S, 28S, and complete ITS1, 5.8S, and ITS2).

Materials and Methods

Samples: Between July 2006 and January 2007, small wood pieces were collected with a drill from 110 wilted Japanese black pines and Japanese red pines on Kyushu island, and from 33 Luchu pines on the main island of Okinawa. The samples were stored at -20°C until use.

DNA extraction: The wood pieces were freeze-dried, and 100mg was transferred to a 2ml tube with two stainless steel beads (ϕ 6mm), and crushed into powder using an MM300 (Retsch) crusher. One mL of extraction buffer (100 mM Tris pH8.5, 5 mM EDTA, 200 mM NaCl, 0.2 % SDS, 100 ng/μL Proteinase K (USB)) was added to 100mg of the wood powder, and the mixture was incubated at 55°C overnight. The mixture was cooled to 4°C, and 300μl of 5M potassium acetate was added. After centrifugation, the supernatant was purified using MagneSil RED (Promega) and used as a template DNA for PCR.

Sequencing: PCR was performed using four primer pairs amplifying the rDNA region (Primer 1, F: TTAAGC CATGCATGTCTAAGTGGAG, R: AAATGCCTTCGCTGT AGGACG; Primer 2, F: GGCTAAAACAATGGTTAACAG GAACAG, R: ATGATCCAGCCGAAGGTTCAC; Primer 3, F: ACTAGTTTAATCGCAGTGGCTTGAAC, R: AAAGCC CAAGAGCGCAATATG; Primer 4, F: GTCGATGAAGAA CGCAGTGAATTG R: CGTTTCACTCGCAGTTACTCAG G), amplifying four products of 921 bp, 987 bp, 549 bp, and 577-581 bp, respectively.

The reaction conditions were an initial denaturation at 94°C for 30 sec, 30 cycles of 94°C for 30 sec, 63°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 120sec. The samples that did not amplify under these PCR conditions were amplified using touch-down PCR. The reaction consisted of an initial 60 sec denaturation step for 94°C, 35 cycles of 94°C for 30 sec, 63-57°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 120sec (for the first 10 cycles, the annealing temperature was lowered 0.6°C each cycle from 63°C; for the next 25 cycles, annealing was carried out at 57°C). Five μL of the PCR product was electrophoresed using 1.2% agarose gel. The remaining PCR product was treated with 1.67 units Exonuclease I (USB) and 0.67 units Shrimp Alkaline Phosphatase (USB), mixed well and incubated at 37°C for 1 hour, and subsequently incubated at 75°C for 15 minutes to inactive the enzymes. The sequence reaction was carried out with -21M13 / M13 Rev primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The product was purified with CleanSEQ (Agencourt) and sequenced with an ABI PRIZM 3130 Genetic Analyzer (Applied Biosystems).

Data Analysis: The sequences were assembled and aligned with Sequencher 4.2.2 (GeneCodes). Haplotypes and haplotype frequencies were estimated based on the sequence data under a hypothesis that a sample consists of one or two haplotypes. A Neighbor-joining (NJ) dendrogram of the haplotype was constructed with the sister species *B. mucronatus* as an outgroup using the genetic distance model (Tamura and Nei, 1993) in Geneious Pro 4.5.4 (Biomatters Ltd). The sequence of *B. mucronatus* was obtained from an isolate which had been subcultured at the Kyushu Regional Breeding Office, Forest Tree Breeding Center, Forestry and Forest Products Research Institute, using the same sequencing method described above. Pairwise F_{st} (Reynolds et al., 1983; Slatkin, 1995) was calculated from the haplotype

Table 2. Putative rDNA haplotypes and haplotype frequencies of *Bursaphelenchus xylophilus* detected from Kyushu and Okinawa islands. Number indicates the position of the SNPs and indels in the total sequence nucleotide in this study. ':' indicates deletion.

putative haplotype	18S		ITS1				ITS2						haplotype ferquency (%)
	80	651	1755	1813	1824	1967	2251	2320	2328	2329	2357	2358	
A	T	A	A	T	T	A	A	T	:	:	:	:	15.3
B	T	A	A	T	T	A	A	C	G	G	G	C	19.5
C	T	A	A	T	T	A	G	C	G	G	G	C	7.3
D	T	A	A	G	T	A	G	C	G	G	G	C	42.4
E	T	C	A	T	T	A	A	T	:	:	:	:	0.4
F	T	A	A	T	C	A	A	T	:	:	:	:	14.5
G	C	A	A	T	T	A	A	C	G	G	G	C	0.8
H_1	T	A	T	T	T	A	A	C	G	G	G	C	-
H_2	T	A	T	T	T	A	A	T	:	:	:	:	-
I_1	T	A	A	T	T	C	G	C	G	G	G	C	-
I_2	T	A	A	T	T	C	A	C	G	G	G	C	-

frequencies in ARLEQUIN v. 3.1 (Laurent Excoffier) to analyze the relationship among collection sites. P-values smaller than a 0.05 significance level were detected with 10,000 permutations.

Results

DNA was extracted from the 143 samples, and the full sequence (2569-2573bp) was successfully obtained from 88 of them (Table 1). We could not obtain the full sequence data from the other 55 DNA samples. The rDNA sequences of 88 samples in this study and 45 PWN isolates collected on Kyushu island in a previous study were compared, and eight single nucleotide polymorphisms (SNP) and two indels (IND) of two bases were detected (Table 2). Referring to the PWN sequences in previous reports and DDBJ, the SNPs at nucleotide positions 80, 1755 and 1967 (SNP_{80}, SNP_{1755} and SNP_{1967}) were detected for the first time. One SNP and 2 INDs in the ITS2 region (SNP_{2320} - $IND_{2328\text{-}2329}$ - $IND_{2357\text{-}2358}$) showed 2 types of combinations, that is '*C*- *GG*- *GC*' and '*T*- : : - : : (: indicates deletion)'. Among the 88 samples in this study, 58 samples were homogeneous for the former haplotype, and 21 samples were homogeneous for the latter. In the remaining 8 samples, the sequences from the 2,328th to 2358th nucleotides were not determined, because the downstream sequences of the 2,328th nucleotide in forward sequencing and upstream of the 2,358th nucleotide in reverse sequencing were defective (Table 1, indicated by 'ND'). Since these samples possessed both *T* and *C* peaks at the 2320th nucleotide position (SNP_{2320}), they were estimated to have heterogeneity with the two haplotypes.

Based on the sequence mutations, nine rDNA haplotypes were estimated (Table 2). Eighty-four samples in this study were divided into the six haplotypes A-F previously reported (Nose et al., submitted). New haplotypes (G, H and I) were detected from the other four samples

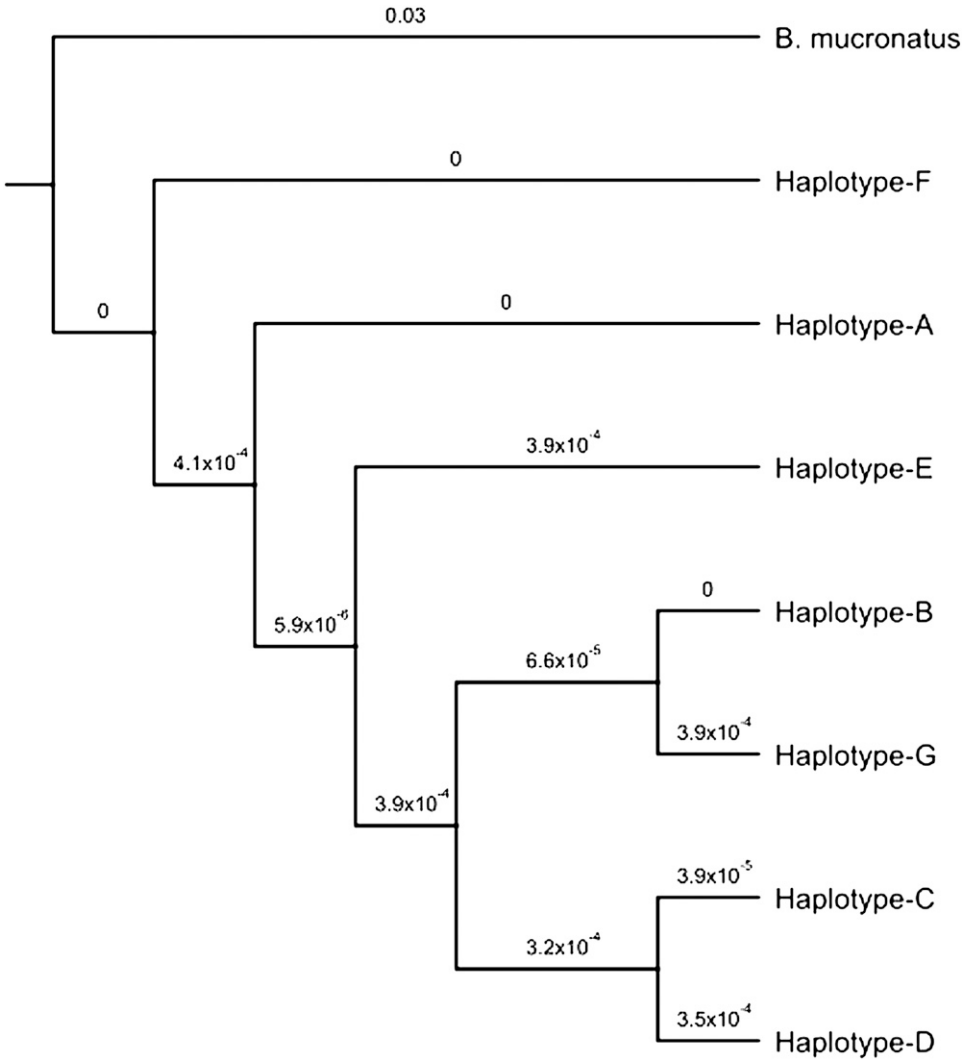

Fig. 1. Relationship between the seven rDNA haplotypes of *Bursaphelenchus xylophilus* and an outgroup (*B. mucronatus*). The dendrogram was generated based on the Neighbor-joining cluster analysis from genetic distance, as calculated in Tamura and Nei (1993). Numerical values indicates substitutions per site of the rDNA sequence (partial 18S, 28S, and complete 5.8S, ITS1, ITS2).

(Table 1: Kak2, Kas10, Mnc1, Mnc10). Kas10 was assumed to have a combination of two haplotypes, either AH_1 or BH_2. Mnc1 and Mnc10 were either the BI_1 or CI_2 haplotypes. The haplotype frequencies were calculated from the 130 samples (Table 2) consisting of the 45 PWN isolates collected from Kyushu island in the previous study (Nose et al., submitted) and the 85 samples collected in this study, excluding three samples (Kas10, Mnc1, Mnc10) for which it was impossible to estimate the haplotype. The major haplotype D accounted for 41.9% of the samples, while minor haplotypes E and G accounted for 0.4% and 0.8%, respectively.

The geographic distribution of the rDNA haplotypes differed among regions (Fig. 2 and Table 3). On Kyushu island, haplotype D was mainly detected in the northeast and center regions (Fig. 2 and Table 1; collection site Co.; Nu, Sks, Fk, Fy, Oaj, Os, Ns, Kn, Kk, Ms, Mm). Haplotypes A and B were detected mainly in the northwest (Sc, Skm, Nss), and haplotype B in the southeast (Mm, Mh, Mht, Mnc, Mkm, Ks). In the southwest (Ksn, Ki, Kak, Kas), haplotype F, which was rare in the other regions, was frequently detected. On Okinawa island (Oky, Okn, Oy, Oi, Oz), haplotypes C, D and F were mainly detected, but haplotypes A and B were rare. The results of the population comparison in ARLEQUIN v. 3.1 indicated the relationship between genetic and geographic distance (Table 3). Within the region, the pairwise F_{st} calculated from the haplotype frequencies was low, and no significance difference was detected among the collection sites. On the other hand, there was a tendency for significant difference to be seen among the regions. However, there was no significant difference between the northwest and southeast regions of Kyushu island.

The NJ dendrogram of PWN haplotypes based on the sequences of the rDNA region is shown in Fig. 1. The dendrogram indicated a critical separation between PWN and *B. mucronatus*. Among PWN haplotypes, the dendrogram separated F haplotypes, which were detected mainly in the southeastern part of Kyushu island. Haplotype B was the most distant genetically from haplotype F. The haplotypes A and B, which were detected frequently in the same area, were genetically distinct within the PWN haplotypes.

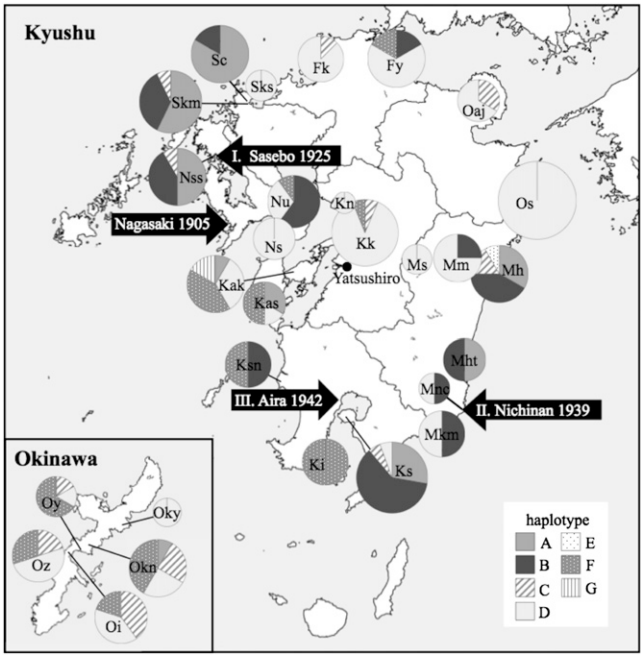

FIG. 2. Distribution of rDNA haplotypes of *Bursaphelenchus xylophilus* on Kyushu and Okinawa islands. Pie chart diameter indicates the proportion of rDNA haplotypes within the collection site. Diameter indicates the sample size of the collection site. Arrow indicates the site and year of the pinewood nematode invasion reported by Kishi (1988).

DISCUSSION

In this study, DNA was directly extracted from the wood powder using a simple method which only requires immersing the sample in extraction buffer and incubating it overnight. Since there is no need to extract nematodes in Baermann funnels from wood tissues as in the conventional method, the sample can be frozen or dried, and thus preserved until use. The sequences of the rDNA region were successfully obtained from 88 out of the 144 DNA samples (61.1%) extracted using this method.

The haplotype frequencies differed among the studied regions, and the geographical distribution of the haplotype frequencies matched the putative invasion and spreading routes of PWN on Kyushu island estimated by Kishi (1988) from past records of pine wilt and wood importation (Fig. 2 and Table 3). Following suppression of the first incidence of disease in Nagasaki in 1905 (Yano, 1913), three main spread routes within Kyushu island (I, II, and III) were reported by Kishi (1988). I. The first route started at Sasebo (Nss) in 1925, and spread north according to Kishi (1988). The disease also extended to the central region, since the wilted pine trees were imported from Nagasaki Prefecture to a pulp mill in Yatsushiro (Kishi, 1988). These areas are consistent with the regions where haplotype D (northeast and center; Nu, Sks, Fk, Fy, Oaj, Os, Ns, Kn, Kk, Ms, Mm) and A/B (northwest; Sc, Skm, Nss) were mainly detected (Fig. 2). It was assumed that PWN with haplotype A/B may have invaded Sasebo (Nss) after haplotype D, or PWN with haplotype D may have invaded from Nagasaki and haplotype A/B from Sasebo (Nss). II. A second spreading route started at Nichinan (Mnc) in 1939 due to the wood importation of a pulp mill, and expanded to the north and south along the coast (Kishi, 1988), where haplotype B was predominant (southeast; Mm, Mh, Mht, Mnc, Mkm, Ks). III. The third route started at Aira in 1942 and expanded to Sendai (Ksn) and Ibusuki (Ki, Kishi 1988), where haplotype F was frequently detected (southwest). This haplotype was rare in the other regions of Kyushu

TABLE 3. Genetic distance of *Bursaphelenchus xylophilus* collection site based on rDNA haplotype frequencies. The diagonal above shows the results of the permutation test (+; P<0.05, -; P>0.05), and the diagonal below shows pairwise F_{st}.

			Kyushu																						Okinawa				
			northwest				northeast & center									southeast						southwest								
			Sc	Skm	Nss	Nu	Sks	Fk	Fy	Oaj	Os	Ns	Kn	Kk	Ms	Mm	Mh	Mht	Mnc	Mkm	Ks	Ksn	Ki	Kak	Kas	Oky	Okn	Oy	Oi	Oz
	northwest	Sc	0.000	−	−	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	+	+	+	−	+	+	+	+	+
		Skm	−0.114	0.000	−	−	−	+	+	+	+	+	−	+	+	+	−	−	−	−	−	−	+	+	−	−	+	+	+	+
		Nss	0.067	−0.133	0.000	−	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+
		Nu	0.440	0.235	0.074	0.000	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	northeast & center	Sks	0.735	0.586	0.538	0.367	0.000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Fk	0.636	0.528	0.493	0.345	−0.290	0.000	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	−	−
		Fy	0.520	0.400	0.345	0.117	−0.200	−0.078	0.000	−	−	−	−	−	−	−	+	+	−	−	+	−	+	−	−	−	−	−	−	−
		Oaj	0.554	0.419	0.376	0.208	−0.200	−0.299	−0.130	0.000	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
		Os	0.884	0.809	0.784	0.711	0.000	0.170	0.224	0.441	0.000	−	−	−	−	−	+	+	−	−	+	+	+	+	+	−	+	+	+	−
		Ns	0.771	0.642	0.600	0.459	0.000	−0.132	−0.059	0.000	0.000	0.000	−	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	−	−
		Kn	0.667	0.467	0.400	0.125	0.000	−1.000	−0.800	−1.000	0.000	0.000	0.000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Kk	0.714	0.626	0.596	0.471	−0.318	−0.168	−0.001	−0.114	0.042	−0.175	−1.000	0.000	−	−	+	+	−	−	+	+	+	+	+	−	+	−	−	−
Kyusyu		Ms	0.771	0.642	0.600	0.459	0.000	−0.132	−0.059	0.000	0.000	0.000	0.000	−0.175	0.000	−	+	+	−	−	+	+	+	−	−	−	−	−	−	−
		Mm	0.581	0.433	0.363	0.133	−0.263	−0.119	−0.207	−0.153	0.271	−0.091	−1.000	−0.045	−0.091	0.000	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	southeast	Mh	0.165	−0.036	−0.143	−0.025	0.445	0.402	0.273	0.250	0.731	0.518	0.267	0.525	0.518	0.277	0.000	−	−	−	−	−	+	+	−	−	+	−	−	+
		Mht	0.033	−0.191	−0.263	0.022	0.529	0.480	0.314	0.333	0.825	0.613	0.333	0.603	0.613	0.333	−0.204	0.000	−	−	−	−	+	+	−	−	+	−	+	+
		Mnc	0.461	0.205	0.040	−0.348	0.000	0.015	−0.263	−0.200	0.711	0.250	−1.000	0.218	0.250	−0.379	−0.091	−0.043	0.000	−	−	−	−	−	−	−	−	−	−	−
		Mkm	0.485	0.291	0.161	−0.140	0.111	0.126	−0.081	0.000	0.593	0.250	−0.333	0.262	0.250	−0.167	0.062	0.111	−0.600	0.000	−	−	+	−	−	−	−	−	−	−
		Ks	0.185	0.007	−0.097	−0.038	0.377	0.347	0.221	0.226	0.619	0.438	0.210	0.448	0.438	0.212	−0.140	−0.152	−0.142	0.011	0.000	−	+	+	−	−	+	+	−	+
		Ksn	0.485	0.291	0.161	−0.140	0.529	0.480	0.245	0.333	0.825	0.613	0.333	0.603	0.613	0.333	0.062	0.111	−0.043	0.111	0.076	0.000	−	−	−	−	−	−	−	−
	southwest	Ki	0.797	0.680	0.642	0.518	1.000	0.775	0.574	0.724	1.000	1.000	1.000	0.831	1.000	0.750	0.567	0.667	0.724	0.667	0.524	0.333	0.000	−	−	−	−	−	+	−
		Kak	0.467	0.367	0.333	0.139	0.205	0.215	0.040	0.091	0.574	0.305	−0.100	0.335	0.305	0.159	0.267	0.295	0.023	0.155	0.254	0.062	0.178	0.000	−	−	−	−	−	−
		Kas	0.308	0.203	0.198	0.103	0.245	0.250	0.061	0.089	0.678	0.368	−0.111	0.397	0.368	0.179	0.152	0.143	−0.018	0.143	0.157	0.000	0.167	−0.170	0.000	−	−	−	−	−
		Oky	0.735	0.586	0.538	0.367	0.000	−0.290	−0.200	−0.200	0.000	0.000	0.000	−0.318	0.000	−0.263	0.445	0.529	0.000	0.111	0.377	0.529	1.000	0.205	0.245	0.000	−	−	−	−
		Okn	0.441	0.347	0.315	0.144	0.214	0.165	0.074	−0.014	0.554	0.306	−0.067	0.305	0.306	0.171	0.214	0.277	0.030	0.158	0.221	0.083	0.222	−0.083	−0.112	0.214	0.000	−	−	−
	Okinawa	Oy	0.458	0.339	0.300	0.103	0.245	0.199	0.061	−0.011	0.678	0.368	−0.111	0.369	0.368	0.179	0.189	0.250	−0.018	0.143	0.202	0.000	0.167	−0.170	−0.212	0.245	−0.237	0.000	−	−
		Oi	0.450	0.342	0.306	0.148	0.080	0.000	0.005	−0.235	0.519	0.205	−0.333	0.178	0.205	0.058	0.180	0.261	−0.082	0.078	0.186	0.180	0.452	0.002	−0.020	0.080	−0.138	−0.166	0.000	−
		Oz	0.497	0.389	0.353	0.167	−0.157	−0.146	−0.145	−0.285	0.313	−0.010	−0.750	−0.016	−0.010	−0.100	0.258	0.315	−0.145	0.022	0.232	0.239	0.518	−0.025	−0.017	−0.157	−0.068	−0.091	−0.172	0.000

island. In Amakusa (Kak, Kas), haplotypes D and F were predominant. The PWN with haplotype D might have invaded from the north, and F from the south. Kishi (1988) hypothesized that PWN had invaded both northwest and southeast regions from Hyogo prefecture, where the pine forest had been damaged earlier. The results of the population comparison supported this hypothesis. The pairwise F_{st} showed that there was no significant difference between the northwest and southeast regions, where haplotype B was frequently detected (Table 3).

The low temperatures in the mountains of Kyushu island may be one of the factors that caused the difference of haplotype frequencies among the regions. Previous reports suggested that there was a relationship between disease occurrence and temperature. Rutherford and Webster (1987) reported that in the presence of both PWN and its vector, the disease occurrence was confined to warm climates where the mean air temperature exceeds 20°C for protracted periods in Japan. Also Taketani et al. (1975) indicated that pine wilt was more severe at higher temperatures. The PWN tended to be more active and fecund the higher the temperature (Rutherford et al., 1992). For this reason, occurrence of the disease might be rare in high mountains, because of its low temperature. Indeed, Hashimoto et al. (1974) reported that there was little damage caused by PWN at altitudes over 800m, and that PWN might have habitat limits of around 700-750m on Kyushu island. The mountains on Kyushu island might thus have prevented the occurrence and expansion of disease, and obstructed the dispersal of PWN. Accordingly, the haplotype frequencies of the founder population might have been maintained in each area. Moreover, the dispersal of Japanese pine sawyer (*M. alternatus* Hope) may also play an important role in the PWN haplotype distribution, since PWN is mainly transmitted by the beetle in Japan. The mean of total distance traveled was estimated to be 10.6-12.3m during their lifetime (Shibata 1986), and a laboratory experiment with a flight mill recorded a maximum distance of 3.3km (Enda 1985). The dispersal of beetles is estimated to dependent on stand density, number of beetles emerging from individual dead trees, maximum air temperature, and precipitation (Togashi 1990; Takasu 2000).

The PWN was introduced to Okinawa island, since wilted pine trees were transported from throughout Kyushu island for engineering projects (Kuniyoshi, 1974; Ganeko, 1974). The first damage was found at the northern part in 1973 (Kuniyoshi, 1974; Ganeko, 1974), and the next year, PWN was detected from wilted pine stands at the northern and central areas (Ganeko, 1976). In this study, there was no distinct difference in haplotypes within Okinawa island (Fig. 2 and Table 3; Oky, Okn, Oy, Oi, Oz). Haplotypes C, D and F were frequently detected, while haplotype B, which was predominant on Kyushu island, was not found on Okinawa, and few haplotype A were detected. Haplotypes C, D and F were assumed to have spread without anything disturbing their migration due to the high temperatures on Okinawa island. According to the Japan Meteorological Agency, the mean monthly temperature from 1971 to 2000 exceeded 15°C throughout most of the year on Okinawa island, unlike on Kyushu.

As shown by the NJ dendrogram (Fig. 1), genetically distinct haplotypes were detected within a collection site, such as A/B at the northwest of Kyushu island and C/D/F on Okinawa. Since there is little possibility that these haplotypes were established after the PWN entered Japan, they might have been introduced from several original places. Investigating the haplotypes in North America, particularly in the US, may lead to information about the place of origin. However, there is still insufficient data on the distribution and genetic characterization of PWN in North America. Since the PWN from different origins may show different pathogenicity behaviors, it is important to obtain a clear picture of the PWN strain in each region indicated by haplotype distributions to carry out effective management measures. Global warming is predicted to change the distribution of animals and plants in the future, and pine wilt disease may expand to larger areas as a result (Kamata, 2003). Continuous research on the invasion and spreading routes of PWN is needed for disease control and pest management.

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