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Molecular Phylogeny of Geographical Isolates of *Bursaphelenchus xylophilus*: Implications on the Origin and Spread of this Species in China and Worldwide

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Abstract: The genetic diversity and phylogeny of 26 isolates of *Bursaphelenchus xylophilus* from China, Japan, Portugal and North America were investigated based on the D2/3 domain of 28S rDNA, nuclear ribosomal Internal Transcribed Spacer (ITS) sequences, and random amplified polymorphic DNA (RAPD) analysis. The genetic diversity analysis showed that the D2/3 domain of 28S rDNA of isolates of *B. xylophilus* from China, Portugal, Japan and the US were identical and differed at one to three nucleotides compared to those from Canada. ITS sequences of isolates from China and Portugal were the same; they differed at one or two nucleotides compared to those of Japanese isolates and at four and 23 nucleotides compared to those from the US and Canada, respectively. The phylogenetic analysis indicated that Chinese isolates share a common ancestor with one of the two Japanese clades and that the Canadian isolates form a sister group of the clade comprised of isolates from China, Portugal, Japan, and the US. The relationship between Japanese isolates and those from China was closer than with the American isolates. The Canadian isolates were the basal group of *B. xylophilus*. This suggests that *B. xylophilus* originated in North America and that the *B. xylophilus* that occurs in China could have been first introduced from Japan. Further analysis based on RAPD analysis revealed that the relationship among isolates from Guangdong, Zhejiang, Shandong, Anhui provinces and Nanjing was the closest, which suggests that pine wilt disease in these Chinese locales was probably dispersed from Nanjing, where this disease first occurred in China.

Key words: Bursaphelenchus xylophilus, ITS, phylogeny, pinewood nematode, RAPD, systematics, D2/3 regions of 28S rDNA.

Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle, 1970, the causal agent of pine wilt disease (PWD) (Mamiya, 1984), is one of the most important invasive organisms worldwide and has devastated pine forests in Eastern Asia (Kiyohara and Tokushige, 1971; Yang and Wang, 1988; Chio et al., 2006). It is believed that *B. xylophilus* is the greatest threat to pine forests of some countries in Europe and the southern hemisphere (Dwinell, 1997; Webster, 1998; Mota et al., 1999; Matsunaga and Togashi, 2004). *Bursaphelenchus xylophilus* was first recorded in Japan in 1905 and was thought to be native to North America (Yano, 1913; Dropkin and Foudin, 1979; Knowles et al., 1983; Dwinell, 1993; Kanzaki and Futai, 2002). The dispersal of this nematode from one continent to another is thought to occur by transportation of infested logs, packing boards and wood chips (Evans et al., 1996; Dwinell, 1997). To date, *B. xylophilus* has been reported in Japan, the US, Canada, China, Mexico, South Korea and Portugal (Yano, 1913; Dropkin and Foudin, 1979; Cheng et al., 1983; Knowles et al., 1983; Yi et al., 1989; Dwinell, 1993; Mota et al., 1999). The first occurrence of *B. xylophilus* in China was reported in 1982 in the Sun-Yat-Sen Mausoleum Landscapes of Nanjing (Cheng et al., 1983). Later, extensive surveys revealed the widespread occurrence of the pine wood nematode throughout the county of Nanjing. Currently, this nematode has been found in 94 areas of nine provinces in China (China National Forestry Department Announcement, 2006). Since *B. xylophilus* was placed on the list of quarantined organisms of China, this nematode has been often detected by Chinese inspectors in imported wooden packing boxes from Japan, Canada, the US and other epidemic-stricken areas (Xu et al., 1995; Du et al., 1999; Li, 2005). *Bursaphelenchus xylophilus* has been also detected in native Chinese wooden packing boxes (Yang et al., 2004). Phylogenetic and biogeographic investigation of these isolates of *B. xylophilus* is therefore important to understand the origin and spread of this species in China and worldwide.

It has been demonstrated that the 28S rRNA gene and nuclear ribosomal DNA containing the first internal-transcribed spacer (ITS1), 5.8S rRNA and second internal transcribed spacer (ITS2) regions are informative for molecular diagnostics of nematodes and in the phylogenetic relationship analyses between nematodes from population to class level (Adams et al., 1998; Kampfer et al., 1998; Iwahori et al., 1998; Beckenbach et al., 1999; De Giorgi et al., 2002; Wang et al., 2005; Nadler et al., 2006). As a fast and sensitive molecular marker, random amplified polymorphic DNA (RAPD) has been widely used to explore nematode relationships at inter and intra-specific levels (Williams et al., 1990; Guirao et al., 1995; Da Conceição et al., 2003; Wu et al., 2005; Vieira et al., 2007). These three molecular

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markers have been used to study the genetic variability within and among species of the genus *Bursaphelenchus* or as a molecular identification tool for *B. xylophilus* (Mota et al., 1999; Beckenbach et al., 1999; Kanzaki and Futai, 2002; Zheng et al., 2003; Metge and Burgermeister, 2005; Wu et al., 2005; Wang et al., 2005; Zhang et al., 2006; Vieira et al., 2007). However, there is no report that combines the use of the three markers for the phylogenetic analysis of geographical isolates of *B. xylophilus* from different countries. In this study, the D2/3 region of 28S rRNA gene, ITS region sequences and RAPD data were used to investigate the genetic diversity and phylogenetic affinities of different geographical isolates of *B. xylophilus* from China, Japan, the US, Canada and Portugal in order to provide molecular evidence to understand the origin and spread of this nematode in China and worldwide.

Materials and Methods

Sampling: A total of 26 isolates of *B. xylophilus* were studied, including nine from five provinces of China, eight from Japan, four from Portugal, two from the US and three from Canada. Three isolates of *B. mucronatus* were used as out-group. Twenty-five new sequences were generated in this study. The material from which these new sequences were obtained is stored as voucher specimens in Prof. Maosong Lin's lab in Nanjing Agricultural University. All other sequences used in this study were obtained from GenBank. Nematode isolate codes, sources and GenBank accession numbers appear in Table 1.

DNA extraction: DNA from mixed life stages was extracted according to the method described by Harmey and Harmey (1993) and used as template for RAPD

Table 1. Codes, sources, and accession numbers for the taxa used in this study.

Isolate code	Source	GenBank Accession No. ITS	GenBank Accession No. 28S rDNA
B. xylophilus			
BxJ1[a]	Japan (Provided by Mr. P. Y. Shen, Nanjing Entry-exit Inspection and Quarantine Bureau, China)	EF446943[b]	EF446934[b]
BxJT4	Japan (Zheng et al., 2003)	—	DQ356002
BxJ18	Japan (Aichi, Yokoyama et al.)	AB294736	—
BxJ186	Japan (Mito, Ye et al.)	—	AY508106
BxJOK	Japan (Okinawa, Mota et al.)	AB277205	—
BxJCH	Japan (Chiba, Mota et al.)	AB277203	—
BxJIW	Japan (Iwate, Mota et al.)	AB277207	—
BxJSH	Japan (Shimane, Mota et al.)	AB277206	—
BxPT1W	Portugal (Marateca, Metge et al.)	—	AM396580
BxPSP	Portugal (Setubal peninsula, Mota et al.)	AB277208	—
BxPHF	Portugal (Herdade de Ferraria, Mota et al.)	AB277204	—
BxPP	Portugal (Metge et al.)	AM157747	—
BxUSA1	US (Provided by Prof. J. M. Webster, Simon Fraser University, Canada)	EF446951[b]	EF446940[b]
BxUSA2	US (Provided by Prof. J. M. Webster, Simon Fraser University, Canada)	EF446951[b]	EF446940[b]
BxCanada	Canada (Provided by Prof. J. M. Webster, Simon Fraser University, Canada)	EF446946[b]	EF446935[b]
BxCA187	Canada (New Brunswick, Ye et al.)	—	AY508107
BxCA188	Canada (Quebec, Ye et al.)	—	AY508108
BxCNJ1[a]	China (Nanjing Sun-Yat-Sen Mausoleum, Jiangsu Province, collected by our lab)	—	EF446930[b]
BxCNJ2[a]	China (Nanjing Sun-Yat-Sen Mausoleum, Jiangsu Province, collected by our lab)	EF446948[b]	EF446941[b]
BxCNJ3[a]	China (Nanjing Jiangning Forest, Jiangsu Province, collected by our lab)	EF446944[b]	EF446929[b]
BxCNJ4[a]	China (Nanjing Jiangning Forest, Jiangsu Province, collected by our lab)	—	EF446931[b]
BxCAS[a]	China (South Anhui province, provided by Prof. R. Z. Yang, Anhui Agricultural University, China)	—	—
BxCAJ[a]	China (Mingguang, Anhui Province, provided by Prof. R. Z. Yang, Anhui Agricultural University, China)	EF446945[b]	EF446942[b]
BxCZD[a]	China (Daishan, Zhegiang Provinces, collected by our lab)	EF446952[b]	EF446937[b]
BxCSC[a]	China (Changdao, Shandong Province, provided by Prof. B. J. Yang, Chinese Academy of Forestry)	EF446947[b]	EF446932[b]
BxCGD[a]	China (Dongguan, Guangdong Province, provided by Prof. X. B. Gao, South China Agricultural University)	EF446950[b]	EF446933[b]
B. mucronatus			
BmJ2[a]	Japan (Provided by Mr. P. Y. Shen, Nanjing Entry-exit Inspection and Quarantine Bureau, China)	—	—
BmRussian	Russia (Provided by Mr. Z. Y. Zhang, Qingdao Entry-exit Inspection and Quarantine Bureau, China)	—	EF446939[b]
BmCSC	China (Zhoushan, Zhejiang Province, collected by our lab)	EF446953[b]	EF446938[b]

[a] Isolate with RAPD polymorphism data.
[b] New sequences determined in this study

amplification. Nematode pellets were ground in liquid nitrogen to a fine powder. Six volumes of extraction buffer (200 mM NaCl, 5% SDS, 2 mg/ml Proteinase K, 50 mM EDTA, 100 mM Tris-Cl, pH 8.0) were added, and the mixture was incubated at 55°C for 60 min with occasional mixing. The DNA was extracted once with an equal volume of phenol and twice with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated by adding 2.5 volumes of absolute ethanol followed by centrifugation, then resuspended in TE (10 mM Tris, 1mM EDTA, pH8.0) and stored at −70°C.

DNA extracted from one individual nematode was used for 28S rDNA and ITS amplification (Joyce et al., 1994). A single nematode was placed on a 70% ethanol swabbed microscope slide in 10 µl of worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45 Tween 20, 0.01% gelatin and 60 µg/ml proteinase K). NP40 and Tween 20 (Amresco) detergents were used to aid in the degradation of the nematode proteins. The nematode was cut in half, and the pieces in the lysis buffer were transferred to a sterile 0.5 ml microcentrifuge tube on ice. The tubes containing worm lysates were frozen at −80°C for 10 min and then incubated at 65°C for 1 hr, followed by 10 min at 95°C to inactivate the proteinase K. The lysates were cooled on ice and centrifuged at 12,000*g* for 2 min, and 2.5 µl of the supernatant was used in the PCR reaction. It was important not to add too much of the debris at the bottom of the tube after centrifugation as this could inhibit the *Taq* polymerase (Hominick et al., 1997).

PCR amplification and sequencing: Sequences of the partial 28S rDNA, the ITS region of the ribosomal DNA and RAPD data of pinewood nematode were used for the genetic diversity and phylogenetic analyses in this study. The primers used for amplification of the 28S rRNA gene fragment were designed by Qiu et al. (2005), corresponding to nucleotide positions 2,987–3,528 of the rDNA tandem repeat of *C. elegans* (Ellis et al., 1986). The 28S rDNA primers were 5′-CGA TAG CGA ACA AGT ACC GAG AG-3′ (forward primer) and 5′-CCT GCT CAG GCA TAG TTC ACC ATC-3′ (reverse primer). The forward primer is located upstream of the D2 region, and the reverse primer is located downstream of the D3 region, based on the secondary structure model of *C. elegans* 28S rRNA (Ellis et al., 1986). PCR amplification was conducted in a 50 µl PCR mixture containing 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTPs, 1 µl of 5 mM forward and reverse primers, respectively, 0.5 µl of *Taq* polymerase, 33 µl of double-distilled water and 2.5 µl DNA. All PCR reactions were conducted in a Px2 Thermal Cycler (Thermo Electron Co.), and the following cycling profile was used: 1 cycle at 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min; the last step was 72°C for 10 min. Two 5′ primers for the amplification of ITS were designed by our lab based on reported ITS sequences of *B. xylophilus* and related nematodes from GenBank. The sequences are 5′-TTG ATT ACG TCCC TGC CCT TT-3′ and 5′-TTT CAC TCG CCG TTA CTA AGG -3′. The reverse primer was reported by Vrain et al. (1992). The PCR reaction parameters are the same as described above for 28S rDNA amplification. The PCR products were sequenced by Invitrogen Corporation (Shanghai, China).

Forty oligonucleotide decamer primers were used for RAPD amplification, 11 of which amplified distinct and reproducible DNA bands (Table 2). PCR amplification was performed using 25 ng of genomic DNA in a standard 10 µl PCR mixture (500 mM KCl, 100 mM Tris-HCl, 3.0 mM $MgCl_2$, 0.1% gelatin (w/v), 1% Triton x-100, 200–400 µM dNTP and 10 nM primer) under the following thermal cycling program: 95°C for 4 min, 35 cycles of 94°C for 30 sec, 37°C for 50 sec and 72°C for 2 min, with a final extension step of 72°C for 10 min.

Phylogenetic analysis: Sequences were assembled with Sequencing Analysis 3.0 and aligned with CLUSTAL X (Thompson et al., 1997), initially under the default alignment parameters. Molecular phylogenetic relationships between isolates were reconstructed by Bayesian inference (BI), maximum parsimony (MP) and neighbor-joining methods (NJ). Bayesian phylogenetic reconstruction was performed by using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). The model that best fitted the data was identified by GTR + G model test using the program MrModeltest 2.0 (Nylander, 2004). Four cold Metropolis-coupled Markov chains Monte Carlo (MCMCMC) for 10,000,000 generations were run, and one tree was retained every 100 generations and a burn-in of 2,000 generations (Huelsenbeck and Ronquist, 2001). Parsimony analysis was performed via the close-neighbor-interchange (CNI) searching algorithm of MEGA 4 (Tamura et al., 2007) with uniform (standard parsimony) weighing methods, using all sites, and random addition trees. Character states (nucleotides) were specified as unordered. MEGA 4 was also used for NJ analysis. Relative support of clades in the analyses was assessed in a bootstrap analysis (Felsen-

TABLE 2. RAPD-PCR primer sequences and amplified bands among *Bursaphelenchus xylophilus* isolates.

Primer	Primer sequences (5′— 3′)	Total of bands (m)	Polymorphic bands (n)	Polymorphism % (n/m × 100)
S344	CCGAACACGG	12	7	58.3
S24	AATCGGGCTG	8	4	50.0
S356	CTGCTTAGGG	13	10	76.9
S351	ACTCCTGCGA	9	3	33.3
S348	CATACCGTGG	12	6	50.0
S347	CCTCTCGACA	17	13	76.4
S350	AAGCCCGAGG	11	7	63.6
S23	AGTCAGCCAC	10	5	50.0
S346	TCGTTCCGCA	8	6	75.0
S360	AAGCGGCCTT	10	7	70.0
S357	ACGCCAGTTC	12	5	41.6

stein, 1985) with 1,000 replicates. *Bursaphelenchus mucronatus* was used as out-group.

The distinct RAPD products of each primer were amplified and run electrophoretically twice to make sure that no bands were artifacts. For RAPD data analysis, the presence or absence of DNA fragments was visually scored for all isolates. A data matrix of "1" and "0" was constructed based on the presence or absence of each RAPD marker. The resulting matrix was used as input data to MEGA 4 and NTSYSpc 2.1 (Rohlf, 2000) to conduct phylogenetic trees by UPGMA with P-distance model and with similarity model in the two kinds of software packages, respectively.

Genetic diversity and isolate differentiation analysis were calculated using Software MEGA 4. Genetic differentiation between different geographical isolates was assessed by comparing the number of pairwise differences among those isolates.

Results

Sequence statistics: 28S rDNA sequences of 12 isolates of *B. xylophilus* and closely related species *B. mucronatus* were new to this study. ITS sequences of 10 isolates of *B. xylophilus* from China, Japan, the US and Canada were newly determined. The other sequences were obtained from GenBank. The GenBank accession numbers of those sequences are listed in Table 1. All the 28S rDNA sequences and ITS regions of the ribosomal DNA obtained were 519 to 638 bp and 784 to 785 bp long, respectively. Alignment of partial 28S rDNA sequences yielded a consensus length of 519 bp, of which 20 were variable and eight were parsimony-informative. The ITS region of the ribosomal DNA of *B. xylophilus* was 784 bp in length, of which 308 bp was ITS1 and 316bp was ITS2. The alignments of ITS regions were 813 bp, of which 106 sites were variable and six sites were parsimony-informative.

RAPD: Of 40 random primers, 11 primers producing clear and reproducible banding patterns with all isolates were selected as RAPD molecular markers. These markers generated a unique set of products ranging from 200 bp to 1,600 bp. Statistical data showed a total of 122 fragments were produced by the 11 primers, and 73 segments showed polymorphism (Table 2).

Genetic diversity: The D2/3 domain and ITS region nucleotide sequences of the isolates from China were identical (Table 3). The nucleotide sequences of the D2/3 domain of 28S rDNA of isolates from Japan and the US were the same as those of isolates from China, and isolates from these countries are termed GroupCPJU. The D2/3 domain of GroupCPJU was one base different from those of isolates from Canada (Table 3). ITS region sequence analysis showed that genetic diversity was significant among geographical isolates from different countries, but not significant among Chinese isolates (Table 4). ITS sequences of isolates from China and Portugal were identical and composed GroupCP.

Table 3. Genetic distances among *Bursaphelenchus xylophilus* and *B. mucronatus* groups based on 28S rDNA sequence data.

		BxUSA1-2	BxCNJ1-4	BxCSC	BxCZD	BxCGD	BxCAJ	BxPT1W	BxJ1	BxJ186	BxJT4	BxCanada	BxCA187	BxCA188	BmRussian	BmCHN
GroupCPJU	BxUSA1-2		0	0	0	0	0	0	0	0	0	1	2	3	10	19
	BxCNJ1-4	0.000		0	0	0	0	0	0	0	0	1	2	3	10	19
	BxCSC	0.000	0.000		0	0	0	0	0	0	0	1	2	3	10	19
	BxCZD	0.000	0.000	0.000		0	0	0	0	0	0	1	2	3	10	19
	BxCGD	0.000	0.000	0.000	0.000		0	0	0	0	0	1	2	3	10	19
	BxCAJ	0.000	0.000	0.000	0.000	0.000		0	0	0	0	1	2	3	10	19
	BxPT1W	0.000	0.000	0.000	0.000	0.000	0.000		0	0	0	1	2	3	10	19
	BxJ1	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0	0	1	2	3	10	19
	BxJ186	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0	1	2	3	10	19
	BxJT4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		1	2	3	10	19
	BxCanada	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002		1	2	9	18
	BxCA187	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.002		1	10	17
	BxCA188	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.002		11	16
	BmRussian	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.017	0.019	0.021		11
	BmCHN	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.035	0.033	0.031	0.021	

Note: Above diagonal shows the number of different sites, and below diagonal shows genetic distances.

TABLE 4. Genetic distances among *Bursaphelenchus xylophilus* and *B. mucronatus* groups based on ITS sequence data.

		GroupCP								JapancladI			JapancladII					
		BxCNJ2-3	BxCAJ	BxCSC	BxCZD	BxCGD	BxPSP	BxPHF	BxPP	BxJ1	BxJIW	BxJ18	BxJSH	BxJCH	BxJOK	BxUSA1-2	BxCanada	BxCHN
GroupCP	BxCNJ2-3		0	0	0	0	0	0	0	1	1	1	2	2	2	4	23	88
	BxCAJ	0.000		0	0	0	0	0	0	1	1	1	2	2	2	4	23	88
	BxCSC	0.000	0.000		0	0	0	0	0	1	1	1	2	2	2	4	23	88
	BxCZD	0.000	0.000	0.000		0	0	0	0	1	1	1	2	2	2	4	23	88
	BxCGD	0.000	0.000	0.000	0.000		0	0	0	1	1	1	2	2	2	4	23	88
	BxPSP	0.000	0.000	0.000	0.000	0.000		0	0	1	1	1	2	2	2	4	23	88
	BxPHF	0.000	0.000	0.000	0.000	0.000	0.000		0	1	1	1	2	2	2	4	23	88
	BxPP	0.000	0.000	0.000	0.000	0.000	0.000	0.000		1	1	1	2	2	2	4	23	88
JapancladI	BxJ1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		0	0	2	2	2	3	24	89
	BxJIW	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000		0	2	2	2	3	24	89
	BxJ18	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000		2	2	2	3	24	
JapancladII	BxJSH	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002		0	0	1	22	
	BxJCH	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000		0	1	22	87
	BxJOK	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000		1	22	87
BxUSA1-2		0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.004	0.004	0.004	0.001	0.001	0.001		21	86
BxCanada		0.029	0.029	0.029	0.029	0.029	0.029	0.029	0.029	0.031	0.031	0.031	0.028	0.028	0.028	0.027		97
BmCHN		0.120	0.120	0.120	0.120	0.120	0.120	0.120	0.120	0.112	0.112	0.112	0.119	0.119	0.119	0.118	0.134	

Note: Above diagonal shows the number of different sites, and below diagonal shows genetic distances.

TABLE 5. Genetic distances among *Bursaphelenchus xylophilus* and *B. mucronatus* groups based on RAPD-PCR analysis.

	BxCSC	BxCAJ	BxCAS	BxCNJ2	BxCGD	BxCZD	BxCNJ1	BxCNJ4	BxCNJ3	BmJ2	BxJ1
BxCSC		27	16	10	19	12	12	18	23	67	38
BxCAJ	0.221		23	19	32	29	23	31	32	58	49
BxCAS	0.131	0.189		8	19	14	12	18	19	61	36
BxCNJ2	0.082	0.156	0.066		15	12	6	18	17	65	36
BxCGD	0.156	0.262	0.156	0.123		13	13	21	22	62	33
BxCZD	0.098	0.238	0.115	0.098	0.107		8	18	19	65	30
BxCNJ1	0.098	0.189	0.098	0.049	0.107	0.066		16	17	65	34
BxCNJ4	0.148	0.254	0.148	0.148	0.172	0.148	0.131		27	71	42
BxCNJ3	0.189	0.262	0.156	0.139	0.180	0.156	0.139	0.221		62	37
BmJ2	0.549	0.475	0.500	0.533	0.508	0.533	0.533	0.582	0.508		65
BxJ1	0.311	0.402	0.295	0.295	0.270	0.246	0.279	0.344	0.303	0.533	

Note: Above diagonal shows the number of different sites, and below diagonal shows genetic distances.

Japanese isolates of *B. xylophilus* were divided into two groups, JapancladI and JapancladII, based on ITS sequences. JapancladI comprised BxJIW (Japan isolate from Iwate) and BxJ1, a Japanese isolate detected by Customs of Nanjing Entry-Exit Inspection and Quarantine Bureau in packing boards from Japan. The other Japanese isolates formed JapancladII. GroupCP was one and two bases different from JapancladI and JapancladII, respectively, in ITS sequences, while they were four and 23 bases different from US and Canadian isolates, respectively, in ITS sequences (Table 4). RAPD analysis revealed that genetic distances among Chinese isolates ranged from 0.066 to 0.262, and no correlation with geographical origin was found (Table 5).

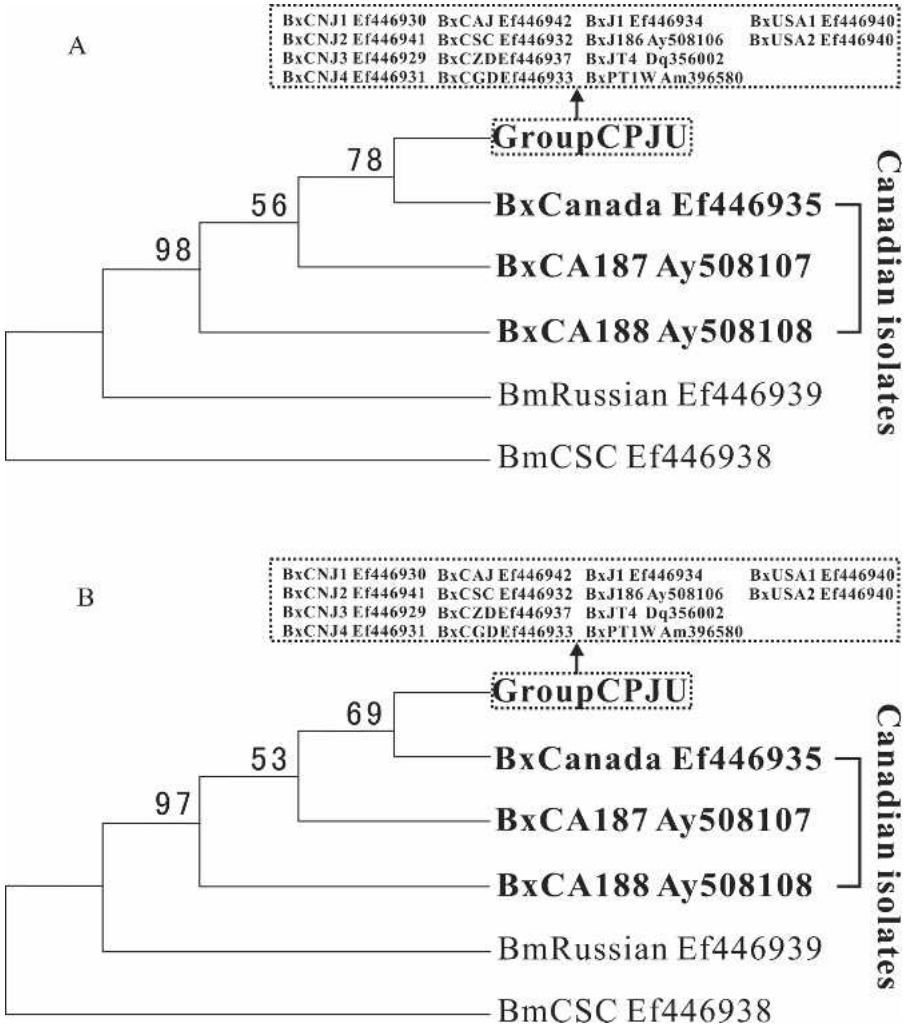

FIG. 1. Phylogenetic trees based on D2/3 of 28S rDNA sequences of *Bursaphelenchus xylophilus*. The numbers above nodes denote bootstrap values. A: Neighbor-joining tree, B: Maximum Parsimony tree.

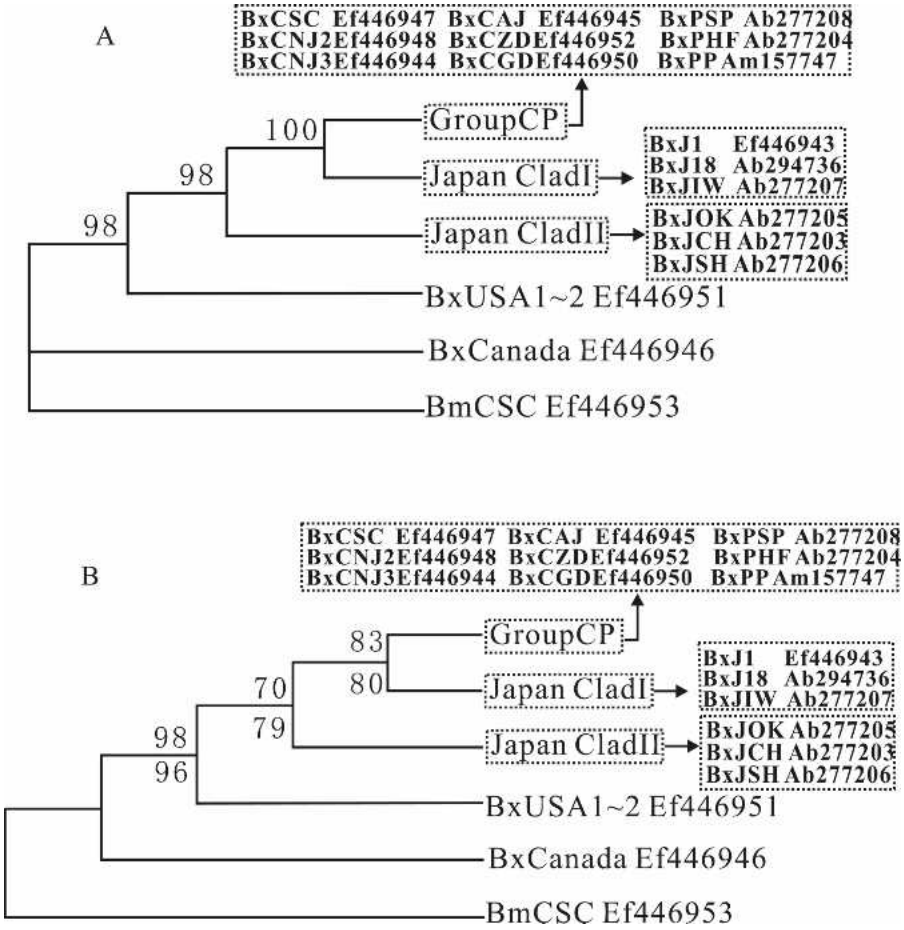

FIG. 2. Phylogenetic trees based on ITS sequences of *Bursaphelenchus xylophilus*. The numbers in the figure denote bootstrap values. A: Bayes interference tree, B: Maximum Parsimony/Neighbor-joining tree

Phylogenetic relationships among isolates of B. xylophilus: 28S rDNA phylogenetic trees were constructed using MP and NJ methods. The two phylogenetic trees based on the D2/3 domain of 28S rDNA showed that all the isolates of *B. xylophilus* formed a well supported monophyletic clade (≥97% bootstrap values), in which the *B. xylophilus* isolate BxCA188 collected from Quebec, Canada, was the basal clade of *B. xylophilus* (Fig. 1A,B). The two US isolates clustered together with the Asian and Portuguese isolates sharing the same D2/3 domain sequences and comprised GroupCPJU, which was the sister group of one of the other two Canadian isolates (Fig. 1). Three BI, MP and NJ phylogenetic trees based on ITS sequences support that Chinese and Portuguese (GroupCP) and Japanese and US isolates composed a monophyletic clade with high bayesian posterior probability and bootstrap values (0.98, 98% and 96%, respectively) and that the isolate BxCanada from Canada was the basal isolate of *B. xylophilus* (Fig. 2). Japanese isolates were split into two non-sister groups, JapancladI and JapancladII. The closer relationship between GroupCP (Chinese and Portuguese isolates) and JapancladI was suggested with high bayesian posterior probability and bootstrap values (1.00, 83% and 80%, respectively) (Fig. 2). The two US isolates formed the sister group ([GroupCP + JapancladI] + JapancladII), which was strongly supported with high bootstrap values and bayesian posterior probability (0.98, 98% and 96%, respectively) (Fig. 2). The results were in accordance with those based on 28S rDNA, both supporting a closer relationship between isolates of *B. xylophilus* from China, Japan and the US, which provides significant hints to the origin and spread of the pine wilt disease caused by *B. xylophilus* worldwide (Fig. 3).

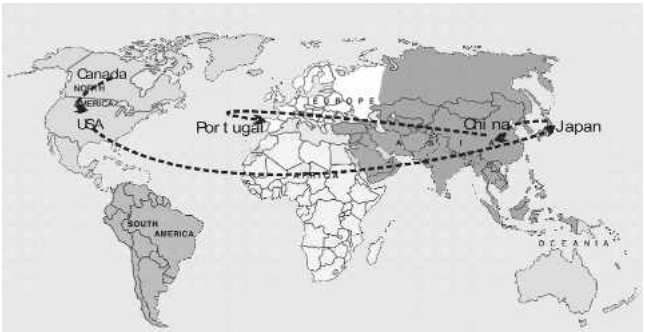

FIG. 3. Possible routes for spread of *Bursaphelenchus xylophilus* based on D2/3 of 28S rDNA and ITS sequence data analysis. The arrows denote possible dispersal directions.

Data analysis based on RAPD: To overcome the disadvantage of reproducibility of RAPD, only the 11 primers that produced consistent amplification products were selected from the 40 random primers by the optimal RAPD-PCR system developed in our previous study (Zhang et al., 2006). The 11 primers produced a total of 122 fragments, 73 of which were polymorphic. RAPD analysis revealed that genetic diversity was distinct among different isolates of *B. xylophilus* (Tables 5,6). The average genetic variation between Chinese and Japanese isolates was significantly higher than the average genetic distance among Chinese isolates (Table 6). The genetic distance among isolates of *B. xylophilus* from China was variable (from 0.066 to 0.262, Table 5). The smallest genetic distances were between isolate BxCNJ1 from Sun Yat-sen's tomb of Nanjing and BxCZD from Daishan, Zhejiang province, and between BxCNJ2 from Sun Yat-sen's tomb of Nanjing and BxCAS from south of Anhui province. The greatest genetic distance was between isolate BxCAJ from Mingguang, Anhui province, and BxCGD from Dongguan, Guangdong province, and BxCNJ3 from Nanjing Jiangning Forest. The genetic diversity among isolates from Nanjing was richer than that among other Chinese isolates (except for isolate BxCAJ) (Table 6). Two UPGMA trees showed the same topology and indicated that *B. xylophilus* was a monophyletic group. The isolate BxJ1 from Japan was the basal clade of *B. xylophilus* (Fig. 4). Bootstrap analysis (bootstrap values >50%) significantly suggested that Chinese isolates were monophyletic and divided into two clades: Mingguang (BxCAJ) and Nanjing groups, in which one Nanjing isolate (BxCNJ3) was the basal subclade, and all the other Chinese isolates, including three Nanjing isolates form the other subclade (Fig. 4A). RAPD analysis confirmed the closer relationship between Nanjing isolates and some other isolates of *B. xylophilus* from China, which provides significant evidence to the origin of the pine wilt disease caused by *B. xylophilus* in some locales of China (Fig. 5).

Discussion

We selected representative geographical isolates from five provinces of China and from other countries where pine wilt disease occurs in order to investigate the possible origins of *B. xylophilus* in China and worldwide. Kanzaki and Futai (2002) studied the relationship among *Bursaphelenchus* species based on ITS regions and COI gene sequences and found that *B. xylophilus* probably originated in North America from an isolate of *B. mucronatus* from Canada. Thereafter, *B. mucronatus* was used as out-group to reveal the systematic relationships among different geographical isolates of *B. xylophilus* from China and other countries.

Several molecular markers, such as homologous DNA probes, ITS-RFLP and RAPD, have been used to demonstrate the variability of different geographical isolates of *B. xylophilus*. By using homologous DNA probes, Tares et al. (1992) divided *B. xylophilus* into US, Canadian and Japanese subtypes. The US and Japanese subtypes shared a common ancestor. Iwahori et al. (1998) found that the ITS-RFLP map of Japanese, Chinese and US isolates were highly homologous. Lu et al. (2001) reported that the restriction enzyme map of ITS regions of *B. xylophilus* isolates from China and Japan were the same, but different from the Canadian. We used two molecular markers with different evolutionary rates to discuss the variability of geographical isolates of *B. xylophilus* and obtained results similar to those of previous studies. Genetic diversity analysis revealed that *B. xylophilus* isolates from China, Japan and the US share identical sequences in the D2/3 region of 28S rDNA and similar nucleotide sequences in the ITS region, which suggests that isolates from these countries probably share a common ancestor. The low genetic diversity among Chinese, Japanese and US isolates of *B. xylophilus* suggests that *B. xylophilus* in those countries originated from a single introduction of the pest.

Further phylogenetic analysis also showed that isolates from China, Japan and the US formed a monophyletic group which was the sister group of the clade composed by Canadian isolates. Our results confirm the hypothesis that *B. xylophilus* originated in North America (Knowles et al., 1983; Dwinell, 1993; Mota et al., 1999; Kanzaki and Futai, 2002). The sequences of the ITS region of six Chinese isolates of *B. xylophilus* are identical to those of three Portuguese isolates. This is consistent with the lower genetic variability observed by Vieira et al. (2007) in isolates from the two countries relative to an isolate from the US, as revealed by RAPD, and suggests an East Asian origin of the Portuguese *B. xylophilus*. Japanese isolates of *B. xylophilus* were paraphyletic groups with one clade more closely related to the Chinese and Portuguses groups. The two clades together form a sister group with another Japanese clade. This suggests that the *B. xylophilus* present in China may have spread from Japan.

The D2/3 region of 28S rDNA and ITS regions provided some useful evidence for understanding the origin and spread of *B. xylophilus* among different countries, but no useful data for the spread of *B. xylophilus*

Table 6. Average genetic distances among *Bursaphelenchus xylophilus* isolates based on RAPD-PCR data.

Isolates	Average genetic distances
Between Chinese and Japanese isolates	0.305
Between Chinese isolates	0.150
Between Chinese isolates (not including Nanjing isolates and BxCAJ)	0.127
Between Nanjing isolates	0.138
Between BxCAJ and Nanjing isolates	0.215
Between BxCAJ and other Chinese isolates (not including Nanjing isolates)	0.228

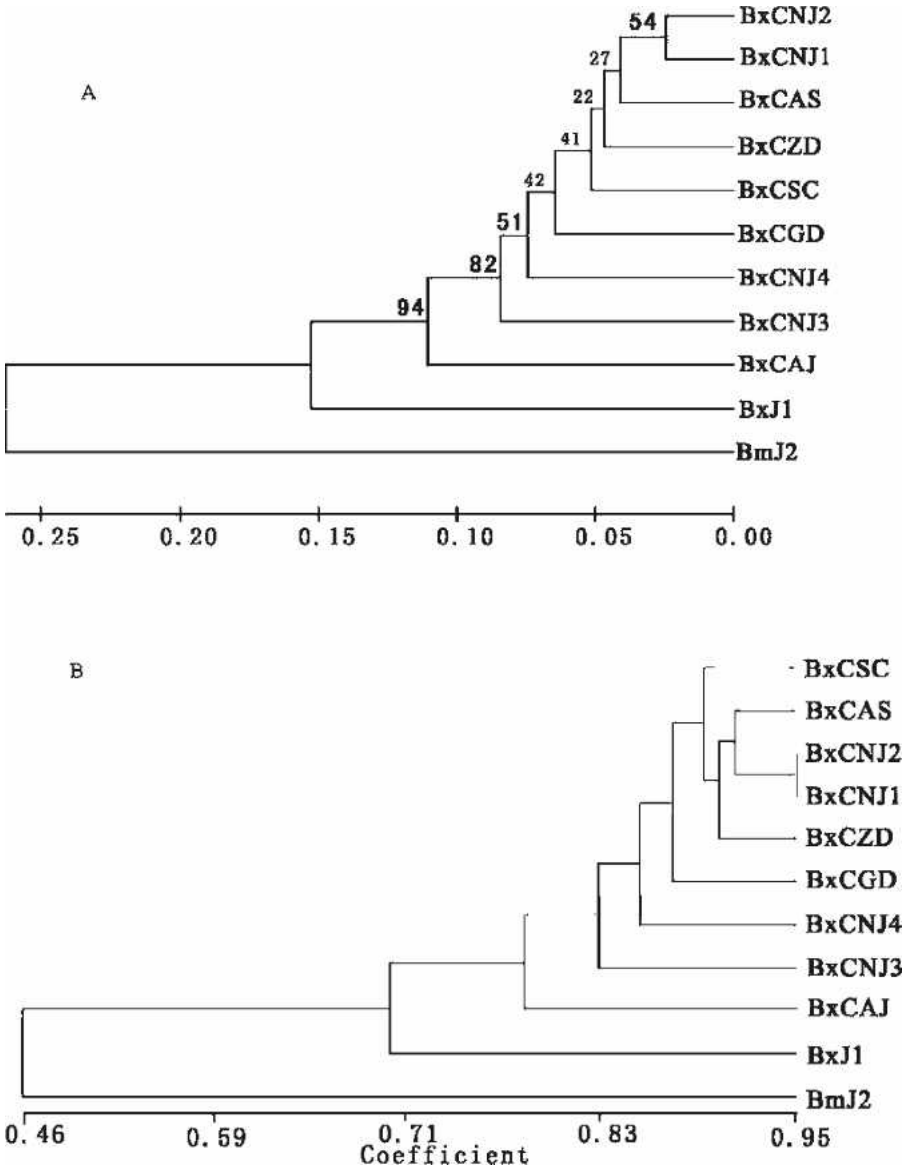

FIG. 4. UPGMA trees of Chinese isolates of *Bursaphelenchus xylophilus* based on RAPD-PCR data. A: UPGMA tree using MEGA 4 with P-distance model and bootstrap analysis. The numbers above each node are bootstrap values. The scale denotes branch length value. B: UPGMA tree constructed using NTSYSpc 2.1 with similarity model.

within China. For this reason, RAPD was utilized to investigate the genetic diversity of *B. xylophilus* isolates from China. The average genetic variation of *B. xylophilus* in China was 0.150, which is higher than that of *B. xylophilus* in Portugal (<0.1) as revealed by RAPD. This was consistent with the occurrence history of *B. xylophilus* in the two countries. The genetic variation between Chinese isolate BxCAJ and other Chinese isolates was higher than the average genetic variation among all Chinese isolates. RAPD data also showed that BxCAJ, an isolate from Mingguang, Anhui, forms a group distinct from the main Chinese isolates. Surprisingly, although Mingguang is adjacent to Nanjing, the relationships between isolates of *B. xylophilus* collected from these two places was farther apart than those between Nanjing isolates and all the other Chinese isolates. This may imply that the isolate of *B. xylophilus* in Mingguang was introduced from abroad independently. The average genetic diversity among Nanjing isolates (0.138) was higher than that (0.129) among other Chinese isolates (not including BxCAJ), which could indicate that Nanjing was the first occurrence place of *B. xylophilus* in China. Phylogenetic analysis based on RAPD showed that *B. xylophilus* isolates from South of Anhui, Shandong, Zhejiang and Guangdong provinces were closely related to Nanjing isolates. This suggests that pine wilt disease caused by *B. xylophilus* in some locales of China was probably dispersed from Nanjing.

Three molecular markers revealed that the relationship between Chinese isolates of *B. xylophilus* and those from Japan are closest, suggesting that *B. xylophilus* could have been first introduced to China from Japan

FIG. 5. Possible routes for spread of *Bursaphelenchus xylophilus* in China based on RAPD-PCR and ITS data analysis. The arrows in the figure denote possible directions. The Δ symbol indicates collection sites for the *Bursaphelenchus xylophilus* isolates.

(Fig. 3). Canadian isolates are in the basal branch of *B. xylophilus*, which confirms that *B. xylophilus* probably originated in North America (Fig. 3). Further RAPD phylogenetic analysis revealing the closer relationship between Nanjing isolates of *B. xylophilus* and other Chinese isolates suggests that pine wilt disease in those parts of China was probably dispersed from Nanjing, where pine wilt disease first occurred in China (Fig. 5).

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