Genetic Structure of Populations of the Pinewood Nematode *Bursaphelenchus xylophilus*, the Pathogen of Pine Wilt Disease, Between and Within Pine Forests

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Accepted for publication 1 September 2006.

ABSTRACT

Zhou, Z.-H., Sakaue, D., Wu, B.-Y., and Hogetsu, T. 2007. Genetic structure of populations of the pinewood nematode *Bursaphelenchus xylophilus*, the pathogen of pine wilt disease, between and within pine forests. *Phytopathology* 97:304-310.

We analyzed the genetic structure of *Bursaphelenchus xylophilus* populations within individual trees (subpopulations) in three distant pine forests (Tanashi, Tsukuba, and Chiba in Japan) based on the polymorphism of four microsatellite (SSR) markers. Most of the nematodes from subpopulations in Tanashi showed the same genotype over 2 years, indicating that nematodes of that genotype dominated there for years. In contrast, 16 and 15 genotypes were identified in nematode populations

from Tsukuba and Chiba, respectively. Despite the high genetic diversity within the Tsukuba and Chiba populations, extremely low genetic diversity was observed within the subpopulations. The genetic difference between the Tsukuba and Chiba populations was significantly smaller than that between Tanashi and either Tsukuba or Chiba. Observed heterozygosity was significantly less than expected based on Hardy-Weinberg equilibrium. These findings are best explained by a founder effect, geographic isolation between populations, explosive nematode multiplication from a small number within individual trees, and the Wahlund effect.

Additional keywords: Japanese pine sawyer, *Monochamus alternatus*, *Pinus densiflora*, *P. thunbergii*.

Pine wilt disease, caused by the pinewood nematode *Bursaphelenchus xylophilus* (Steiner et Buhner) Nickle, kills large numbers of several pine species in Asia and elsewhere. Outbreaks of pine wilt disease have been a major threat to pine forests in Japan and, more recently, in China, Korea, and Portugal (27). As late as 2004 in Japan, the nematode caused an annual loss of 0.7 million m³ of pine wood (8). Although enormous efforts have been made to control pine wilt disease in the past decades, the pinewood nematode remains a serious pathogen.

The spread of pine wilt disease among trees and the development of disease within trees have been described (2,6,15,16,27). The pathogenic nematodes are carried in the tracheal system of the Japanese pine sawyer beetle, *Monochamus alternatus* Hope, and are introduced into healthy pine trees in early summer via wounds caused by sawyer beetle maturation feeding. The introduced nematodes kill the epithelial cells in the resin canals and then invade the surrounding tissues, killing the parenchymal cells. As a result, the hydraulic conductivity of the tree is reduced. Once this happens, the nematodes reproduce and rapidly increase to large numbers within the xylem; by autumn, the tree wilts and dies. Sawyer beetles oviposit on the affected and dead trees, and their larvae develop under the bark. The next year, in early summer, nematodes move into the tracheal system of the adult beetle just before its flight from the dead tree and then are transmitted to healthy pine trees through maturation feeding by the

sawyer beetle. The reiteration of this cycle rapidly spreads the nematode and pine wilt disease throughout the forest.

Although the general features of *B. xylophilus* reproduction are known, many details remain unclear. Thus, we lack a detailed understanding of how the nematode infects and multiplies within trees in nature and how the sawyer beetle migrates, feeds, oviposits, and transmits nematodes in nature. Because these detailed aspects of nematode and beetle ecology are likely to determine the genetic structure of a *B. xylophilus* population within an individual host tree or a forest, we can infer the details of nematode reproduction in nature by investigating the genetic structure of natural nematode populations. Little currently is known about the genetic structure between and within nematode populations, but such information should increase our understanding of the disease.

To analyze the population genetic structure in various organisms, researchers recently have used highly polymorphic microsatellite (SSR) markers (11,14,21,23,28,29,31-33). Such markers also should be useful in genetic analyses of pinewood nematode populations. Thus, in the present study, we isolated a set of polymorphic SSR markers for *B. xylophilus* and then used the markers to analyze the genetic structure of *B. xylophilus* populations in three pine forests.

MATERIALS AND METHODS

Collection and preparation of nematodes. *B. xylophilus* was collected from three pine forests suffering from pine wilt disease in the Kanto district, Japan: the Experimental Station at Tanashi, the University of Tokyo (Tanashi forest); Mount Tsukuba

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(Tsukuba forest); and the University Forest in Chiba, the University of Tokyo (Chiba forest) (Fig. 1A). The Tanashi forest is located in a western suburb of Tokyo metropolis and is surrounded by residential areas. Mountainous Tsukuba and Chiba forests are located in the northeastern part of the Kanto Plain, Ibaraki Prefecture, and in the southern part of the Boso Peninsula, Chiba Prefecture, respectively. In each forest, wood chips were collected from individual *Pinus densiflora* Sieb. et Zucc. (Japanese red pine) and *P. thunbergii* Parl. (Japanese black pine) trees killed by natural infection of the pinewood nematode. Chips were collected by drilling portions (18 mm in diameter) from the trunk (three portions per trunk) at ≈ 1.3 m height; the three portions were combined to form one sample per tree with ≈ 60 cm³ of wood chips per sample. In Tanashi, we sampled six *P. densiflora* and three *P. thunbergii* trees in 2001, and four *P. densiflora* and one *P. thunbergii* tree in 2002. Twelve trees sampled from Tanashi were within an area of ≈ 200 by 300 m in the Experimental Station, and the other two *P. densiflora* trees were in a temple garden ≈ 0.7 km from the Experimental Station. In 2001, we sampled 10 *P. densiflora* trees in Tsukuba (Fig. 1B) and seven *P. densiflora* and two *P. thunbergii* trees in Chiba (Fig. 1C).

Nematodes were extracted from ≈ 30 cm³ of wood chips of each pine tree using a Baermann funnel (20). Nematodes extracted from a single tree were regarded as a subpopulation, and subpopulations from Tanashi, Tsukuba, and Chiba were labeled with a prefix (TA, TS, and CH, respectively) followed by a number. All subpopulations in a forest were collectively considered to represent the forest population. Extracted nematodes were used immediately for DNA extraction without any multiplication step.

Fig. 1. A, Location of the studied forests, and sampling sites of *Bursaphelenchus xylophilus* subpopulations in B, Tsukuba and C, Chiba. The nematodes extracted from an individual tree were regarded as a subpopulation, and subpopulations from Tsukuba and Chiba were labeled with a prefix (TS and CH, respectively) followed by a number.

To test the SSR markers (see below), we used nine *B. xylophilus* isolates obtained from many different areas in Japan (Table 1). Seven of the nine isolates (isolates 67, C14-5, Ka-4, OKD-1, S6-1, S-10, and T4) were provided by the Forestry and Forest Products Research Institute, Japan. Two of the nine *B. xylophilus* isolates (TES-2 and TES-4) were obtained from dead pine trees in Tanashi in 2000. Five other nematode species (including *B. mucronatus*, *Aphelenchoides* spp., *Aphelenchus* spp., and a species of Rhabditida) also were used to test the SSR markers. An isolate of *B. mucronatus* was provided by the Forestry and Forest Products Research Institute, Japan. Isolates of an *Aphelenchoides* sp. (*Aphelenchoides* sp. 1) and an *Aphelenchus* sp. were obtained from dead pine trees in 1997. In 2001, nematodes of another *Aphelenchoides* sp. (*Aphelenchoides* sp. 2) and a Rhabditida species were extracted from a dead pine tree in Tanashi and in Chiba, respectively, and directly used for DNA extraction. Other than these latter two nematode species, the nematodes (nine isolates of *B. xylophilus* and one isolate each of *B. mucronatus*, *Aphelenchoides* sp. 1, and *Aphelenchus* sp.) were maintained and multiplied on the fungus *Botrytis cinerea* growing on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI). Nematodes were collected from the PDA using a Baermann funnel (20) before DNA extraction.

DNA extraction. Isolate T4 of *Bursaphelenchus xylophilus* was used for mass DNA extraction to develop SSR markers. Nematodes multiplied on *Botrytis cinerea* cultures were suspended in 200 μ l of water, frozen with liquid nitrogen, and ground into powder using a mortar and pestle. The powder then was suspended in 500 μ l of lysis buffer (10 mM Tris-HCl, 100 mM EDTA, 20 ng of RNase A/ml, and 0.5% sodium dodecyl sulfate) and incubated at 37°C for 1 h. After addition of proteinase K to a final concentration of 100 μ g/ml, the mixture was incubated at 50°C for over 3 h. After twice partitioning with a chloroform/isoamyl alcohol mixture (24:1, vol/vol), DNA in the upper phase was precipitated by adding three volumes of 99.5% ethanol and 1/10 volume of 3 M NaOAc (pH 5.2). Precipitated DNA was dissolved in Tris-EDTA (TE) buffer and stored at -30°C until use.

For SSR polymorphism analyses, DNA was extracted from individual nematodes that were directly extracted from wood chips. A nematode was picked up using a fine needle (a modified dental root canal needle; Pierce, Tokyo) under a dissection microscope and transferred to a drop of TE buffer (6 μ l) on a glass slide. The nematode was smashed by covering it with a coverslip (8 by 8 mm) and tapping lightly on the slip with a metal probe under the dissection microscope. Tissue of the smashed nematode was scraped into the TE buffer and collected in a microtube. This tissue suspension was used directly as the DNA template for polymerase chain reaction (PCR).

PCR amplification. SSR markers were amplified by PCR in 10 μ l of a reaction solution containing 1 μ l of template DNA solution, 0.4 mM each dNTP, 1 $\times$ buffer (Takara Shuzo Co., Shiga,

TABLE 1. Microsatellite (SSR) genotypes of nine *Bursaphelenchus xylophilus* isolates

Isolate	Collection site ^b	Sizes of PCR products at each locus ^a							
		Bx01	Bx02	Bx03	Bx04	Bx05	Bx06	Bx07	Bx08
67	Ashikita Town, Kumamoto Pref.	140/142	72	196/320	95	149/153	139	150/154	109/110
C14-5	Ichinomiya Town, Chiba Pref.	142	—	228	99	153	135	154	109
Ka-4	Kasama City, Ibaraki Pref.	140	84	214	135	149	141	156	114
OKD-1	Okayama City, Okayama Pref.	142	108	200	103	153	137	152	109
S6-1	Mito City, Ibaraki Pref.	146	76	214	135	149	141	155	109
S-10	Hirose Town, Shimane Pref.	146	76	214	135	149	141	155	109
T4	Ichinoseki City, Iwate Pref.	140	84	214	131	149	139	157	114
TES-2	Nishitokyo City, Tokyo Metropolis	146	84	320	135	149	139	155	114
TES-4	Nishitokyo City, Tokyo Metropolis	146	84	320	135	149	139	155	114

^a Values represent the base pair number of the polymerase chain reaction (PCR) products. In most cases, PCR produced a single band, suggesting that the locus was homozygous, and only one value is listed. In a few cases, PCR produced two bands, indicating that the locus was heterozygous, and two values separated by a slash are listed; — indicates that no band was produced.

^b Collection sites were widely distributed over those areas in Japan infested with *B. xylophilus*; Pref. = Prefecture.

Japan), which included 2.5 mM Mg²⁺ and 0.5 U of LA *Taq* DNA polymerase (Takara Shuzo Co.), and 0.4 µM of a primer pair in which the reverse primer was labeled with Texas Red. PCR was performed in a thermal cycler (TP3000; Takara Shuzo Co.) using 29 cycles of 1 min at 94°C, 30 s at annealing temperature (Ta), and 1 min at 72°C, followed by one cycle of 1 min at 94°C, 30 s at Ta, and 5 min at 72°C.

Development of SSR markers. SSR markers were isolated using a dual suppression PCR method (14). Five restriction-fragment libraries were constructed by digesting DNA from isolate T4 using the restriction enzymes *AfaI*, *AluI*, *EcoRV*, *HaeIII*, and *SspI* (Takara Shuzo Co.). The digested DNA fragments then were ligated with an uneven-length adaptor (26). In the first step, the library of DNA fragments digested by *EcoRV* was used as a template for PCR using an SSR motif primer, (CA)₁₀ or (GA)₁₀, and an adaptor primer. Amplified fragments were subcloned, and several clones were sequenced. Two specific primers for nested PCR in the second step were designed from the region flanking the SSR motif in each sequence. In the second step, nested PCR was performed on every restriction-fragment library with the two primers designed in the first step and two adaptor primers designed for nested PCR. Nested PCR products of a restriction-fragment library that showed a single clear band on agarose gel were subcloned and sequenced. An SSR marker, (i.e., a primer pair for amplifying the region containing an SSR) was designed from the sequence (Table 2).

Polymorphism analysis. Each SSR marker was amplified by PCR of nematode DNA using the SSR primer pair. A portion of the PCR product was denatured at 94°C for 5 min and separated on a sequencing gel made of 6% Long Ranger acrylamide (FMC BioProducts, Rockland, ME), 6.1 M urea, and 1.2× TBE (0.1 M Tris [hydroxymethyl] aminomethane, 3.0 mM EDTA, and 0.1 M boric acid) using a sequencer (SQ-5500; Hitachi, Tokyo). Banding pattern polymorphisms were analyzed using FRAGLYS 3.0 software (Hitachi Electronics Engineering, Tokyo). When two bands were detected, each band was considered to represent an allele at the locus; when one band was detected, it was considered to represent a homozygous allele pair. Samples with identical banding patterns at every locus were assumed to belong to the same genotype.

Statistics. The observed heterozygosity (*H*_o) at each SSR locus was obtained by adding *H*_o values of all subpopulations and dividing by 33, the number of subpopulations. The expected heterozygosity (*H*_e) was calculated from allele frequencies at each SSR locus by Nei's equation (19). For calculating *H*_e, each allele frequency was calculated by adding frequencies of the allele in all subpopulations and dividing by the number of subpopulations. Deviation of *H*_o from *H*_e (i.e., from Hardy-Weinberg equilibrium) was tested statistically by a χ^2 test at *P* < 0.05 (19).

Nei's average number of differences and mean number of pairwise differences were calculated from genotypes of individual nematodes by ARLEQUIN version 2.0 (25) as genetic differences between and within subpopulations or populations.

A dendrogram was produced with a neighbor-joining method based on Nei's average number of differences between subpopulations using Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 software (13).

Population structure based on genotypic data also was inferred with STRUCTURE version 2 (22). In this program, which uses a model-based clustering method, the number of original populations (*K*) is arbitrarily presumed. For each value of *K*, STRUCTURE then calculates the probability of the actual genotypic frequencies. The *K* value with the highest probability and clearest genetic structure is considered to best represent reality. The program can assign individuals to the original populations and can identify migrants or admixed individuals. In this study, the program was run with the admixture model and was used without prior population information. Each value of *K* (2 to 5) was run for 50,000 iterations (with a burn-in period of 50,000 iterations).

RESULTS

Isolation and characterization of SSR markers for the pinewood nematode. In the first step, 12 fragments with GT or CT repeats on one side were sequenced; in the second step, specific primers for nested PCR were designed for 9 of the 12 fragments. Eight SSR markers were successfully developed and designated as Bx01, Bx02, Bx03, Bx04, Bx05, Bx06, Bx07, and Bx08 (Table 2).

PCR amplifications of all eight SSR markers yielded positive electrophoresis bands from the DNA of all nine *B. xylophilus* isolates. DNA templates from all isolates except isolate 67 produced single electrophoresis bands for every marker, indicating that every marker was homozygous in all isolates (Table 1). For all nine isolates of *B. xylophilus*, three alleles were identified for Bx01, four for Bx02, five for Bx03, five for Bx04, two for Bx05, four for Bx06, six for Bx07, and three for Bx08 (Table 1). The nine isolates were separated into seven different genotypes by the eight markers; S6-1 and S-10, and TES-2 and TES-4 showed the same genotypes. Because the quantity of DNA from a single nematode often was insufficient for analysis by more than four markers, and because the seven genotypes could be distinguished by four markers (Bx02, Bx03, Bx04, and Bx07), these four markers were used for subsequent polymorphism analyses.

To determine the specificity of the SSR markers to *B. xylophilus*, PCR amplification of six loci (Bx02, Bx03, Bx04, Bx06, Bx07, and Bx08) was performed for templates from five nematode species other than *B. xylophilus*: *B. mucronatus*, two

TABLE 2. Microsatellite (SSR) markers for *Bursaphelenchus xylophilus*^a

Locus name	Sequences of primer pair (5'–3')	Repeat	Annealing temperature (°C)
Bx01	F-CTGACAATCTTCTGTCGTATG R-CCGTCTACTAATATACTCGT	(GT) ₁₄ ...(AG) ₇	53
Bx02	F-TCTGATTCTCTCACACCCTC R-AAAGGGAAGTGACGAAAGCG	(CT) ₁₃	56
Bx03	F-GCCTCAAGACATTCCGAATACC R-TCTGGTACCACGCACGTTTTC	(TC) ₄₀	58
Bx04	F-TTTCTTCTGGCATGGCAAAG R-AAGATATTGAACGATCTTTGTGGC	(TC) ₂₄	55
Bx05	F-CATTGAATAATTACACGCGGC R-TCTTCTACCGCCACTTTCTTTAAC	(TG) ₈	55
Bx06	F-CGAAGAAGATCTTTAATCATCCG R-TGAGAAATTGCAAAGATCCTCCG	(GT) ₈	55
Bx07	F-AACGGAAGAGAGCTTCACG R-TAGGCCCTCCTTGACAAAAGC	(TC) ₁₀	56
Bx08	F-CTGCTATTTTCGACTTCTC R-CAAGGATCGTGTCCTCTTTTGT	(CT) ₁₀	55

^a These SSR markers were developed from isolate T4 of *B. xylophilus*; ... indicates that there was an intervening sequence.

Aphelenchoides spp., an *Aphelenchus* sp., and a species of Rhabditida. Most of these species occur with *B. xylophilus* in nature (12). PCR amplification of templates from these nematodes did not yield any products. Based on these limited data, the six markers seemed to be specific to *B. xylophilus*.

In total, six alleles for Bx02, nine for Bx03, four for Bx04, and three for Bx07 were detected from the three forests. No more than

three alleles were identified at each locus in any subpopulation (Table 3). *Ho* significantly deviated from *He* at every locus (Table 4).

Genetic structure of nematode subpopulations in Tanashi.

In all, 420 individual nematodes (30 from every subpopulation) from Tanashi were analyzed using the four SSR markers; 418 individuals showed the same genotype, with complete homozy-

TABLE 3. Microsatellite (SSR) genotypes of *Bursaphelenchus xylophilus* in subpopulations^a

Site, tree number ^c	Sampling year	Tree species	Number of nematodes	Sizes of PCR products in each locus ^b			
				Bx02	Bx03	Bx04	Bx07
Tanashi							
TA-1	2001	<i>Pinus densiflora</i>	30	84	320	135	155
TA-2	2001	<i>P. densiflora</i>	30	84	320	135	155
TA-3	2001	<i>P. densiflora</i>	30	84	320	135	155
TA-4	2001	<i>P. densiflora</i>	30	84	320	135	155
TA-5	2001	<i>P. densiflora</i>	30	84	320	135	155
TA-6	2001	<i>P. densiflora</i>	30	84	320	135	155
TA-7	2001	<i>P. thunbergii</i>	30	84	320	135	155
TA-8	2001	<i>P. thunbergii</i>	30	84	320	135	155
TA-9	2001	<i>P. thunbergii</i>	30	84	320	135	155
TA-10	2002	<i>P. densiflora</i>	30	84	320	135	155
TA-11	2002	<i>P. thunbergii</i>	30	84	320	135	155
TA-12	2002	<i>P. densiflora</i>	1	76/84	320	135	155
			1	84	322	135	155
			28	84	320	135	155
TA-13	2002	<i>P. densiflora</i>	30	84	320	135	155
TA-14	2002	<i>P. densiflora</i>	30	84	320	135	155
Tsukuba							
TS-1	2001	<i>P. densiflora</i>	26	84	214	117	155
TS-2	2001	<i>P. densiflora</i>	2	72/76	214	135	147
			8	84	214	135	147
			1	84	214	135	147/155
TS-3	2001	<i>P. densiflora</i>	48	84	214	135	155
TS-4	2001	<i>P. densiflora</i>	26	86	214	135	155
TS-5	2001	<i>P. densiflora</i>	4	84	214	135	155
			24	84	214/320	135	155
			2	84	320	135	155
TS-6	2001	<i>P. densiflora</i>	8	84	214	135	155
			15	76	214	135	155
			13	76/84	214	135	155
TS-7	2001	<i>P. densiflora</i>	20	86	214	135	155
			8	86	214	135	147/155
			8	86	338	135	155
			3	78/86	214/338	135	155
TS-8	2001	<i>P. densiflora</i>	26	76	214	135	155
TS-9	2001	<i>P. densiflora</i>	8	72	214/320	135	155
			14	72	320	135	155
			8	72	214	135	155
TS-10	2001	<i>P. densiflora</i>	21	84	214	135	155
			5	76/84	214	135	155
Chiba							
CH-1	2001	<i>P. densiflora</i>	48	84	214	135	155
CH-2	2001	<i>P. densiflora</i>	30	108	214	131	155
CH-3	2001	<i>P. densiflora</i>	2	84/108	196/214	135	155
			3	108	214	135	155
			12	84	196	135	155
			10	84	214	135	155
CH-4	2001	<i>P. densiflora</i>	28	—	212	135	155
CH-5	2001	<i>P. densiflora</i>	35	86	228	135	157
CH-6	2001	<i>P. densiflora</i>	28	76	214	135	155
CH-7	2001	<i>P. densiflora</i>	13	78	230/336	137	155
			9	78	230	137	155
			4	78	336	137	155
CH-8	2001	<i>P. thunbergii</i>	40	78	—	135	155
CH-9	2001	<i>P. thunbergii</i>	15	78	214	135	147/155
			8	78	214	135	155
			3	78	214	135	147

^a Four markers (Bx02, Bx03, Bx04, and Bx07) were used for genotypic polymorphism analyses of individual *B. xylophilus*.

^b Values in the columns represent the base pair number of the polymerase chain reaction (PCR) products. In most cases, PCR produced a single band, indicating that the locus was homozygous, and only one value is listed. In a few cases, PCR produced two bands, indicating that the locus was heterozygous, and two values separated by a slash are listed. A dash indicates that no band was produced.

^c Each sampled tree (subpopulation) was labeled with a prefix (TA, TS, or CH representing Tanashi, Tsukuba, and Chiba forest, respectively) followed by a number.

gosity of all markers (Table 3). The homozygous band sizes of Bx02, Bx03, Bx04, and Bx07 were 84, 320, 135, and 155 bp, respectively. Although the two exceptional individuals in subpopulation TA-12 had the same monomorphic allele composition at three loci as the other individuals, one showed a different homozygous allele pair of 322 bp at locus Bx03, and the other showed a heterozygous allele pair of 84 and 76 bp at Bx02. Two nematode subpopulations (TA-13 and TA-14) from the temple garden ≈ 0.7 km from the Experimental Station also showed the same genotype as subpopulations from the Experimental Station.

Genetic structure of nematode subpopulations in Tsukuba and Chiba. Ten and nine nematode subpopulations were extracted from dead pine trees in Tsukuba and Chiba, respectively. Genotypes of 11 to 48 individuals in each subpopulation were analyzed using the four markers. Five alleles for Bx02, three for Bx03, two for Bx04, and two for Bx07 were detected in *B. xylophilus* from Tsukuba (Table 3). Five alleles for Bx02, six for Bx03, three for Bx04, and three for Bx07 were detected in *B. xylophilus* from Chiba (Table 3).

Individuals from 10 subpopulations (TS-1, TS-3, TS-4, TS-8, CH-1, CH-2, CH-4, CH-5, CH-6, and CH-8) in Tsukuba and Chiba were monomorphic and homozygous at the four loci. Some

individuals in the other nine subpopulations were heterozygous. Six (TS-5, TS-6, TS-9, TS-10, CH-7, and CH-9) were heterozygous for only one locus (Bx02, Bx03, or Bx07), two (TS-2 and CH-3) were heterozygous for two loci (Bx02 and Bx07, or Bx02 and Bx03), and one (TS-7) was heterozygous for three loci (Bx02, Bx03, and Bx07) (Table 3).

In these two forests, 29 genotypes (16 from Tsukuba and 15 from Chiba) were distinguished, and the major genotype in each subpopulation differed from that in Tanashi (Table 3). Two individuals from a subpopulation in Tsukuba (TS-5) showed the same genotype as that in Tanashi. Several genotypes were shared by subpopulations in Tsukuba and Chiba. One genotype was found in four Tsukuba and two Chiba subpopulations (TS-3, TS-5, TS-6, TS-10, CH-1, and CH-3). Another genotype occurred in two Tsukuba and one Chiba subpopulation (TS-6, TS-8, and CH-6).

The logs of the probabilities calculated by the STRUCTURE program were $-3,941$ ($K = 2$), $-2,992$ ($K = 3$), $-2,956$ ($K = 4$), and $-2,482$ ($K = 5$). Because it was the smallest value that captured the major structure in the data, a K value of 3 was chosen (i.e., the nematode individuals were assigned to three origins [clusters], designated A, B, and C). Most of the nematodes from Tanashi were assigned to cluster A (Fig. 2). Although some individuals from two subpopulations in Tsukuba and one subpopulation in Chiba also were assigned to cluster A, all nematodes from two subpopulations in Tsukuba and five in Chiba were assigned to cluster B with high probabilities. All nematodes from six subpopulations in Tsukuba and three in Chiba were assigned to cluster C with high probabilities. Interestingly, some subpopulations (TS-5, TS-9, and CH-3) contained nematode individuals in cluster A, C, and a mixed A/C cluster. This may indicate that nematodes of cluster A and C were introduced to the same host tree, mated, and produced hybrid individuals.

Nematode genetic differences between subpopulations and between populations. A neighbor-joining dendrogram based on the genetic differences between subpopulations was created (Fig. 3). Nematode subpopulations in Tanashi belonged to a group that clearly was separated from those in Tsukuba and Chiba, but subpopulations in Tsukuba and Chiba were not separated from each other. In Chiba and Tsukuba, the genetic differences between subpopulations in the same forest were significantly larger than those between the two populations (Table 5). Genetic differences

TABLE 4. Allele numbers and heterozygosities of the four microsatellite (SSR) markers in *Bursaphelenchus xylophilus* populations^a

Locus	Allele number	Heterozygosity ^b	
		<i>Ho</i>	<i>He</i>
Bx02	6	0.028	0.578
Bx03	9	0.052	0.638
Bx04	4	0.000	0.173
Bx07	3	0.026	0.143

^a Values were obtained from SSR genotypes of *B. xylophilus* from 33 subpopulations in three forests (Tanashi, Tsukuba, and Chiba). The genotypes were analyzed using four SSR markers (Bx02, Bx03, Bx04, and Bx07).

^b The observed heterozygosity (*Ho*) at each SSR locus was obtained by adding *Ho* values of all subpopulations and dividing by 33, the number of subpopulations. The expected heterozygosity (*He*) was calculated from allele frequencies at each SSR locus by Nei's equation. For calculating *He*, each allele frequency was calculated by adding frequencies of the allele in all subpopulations and dividing by the number of subpopulations. At each locus, *Ho* significantly deviated from *He* (i.e., deviated from Hardy-Weinberg equilibrium) as determined by a χ^2 test at $P < 0.05$.

Fig. 2. Cluster analysis of *Bursaphelenchus xylophilus* populations in Tanashi, Tsukuba, and Chiba using STRUCTURE version 2. An individual nematode is represented by a vertical bar. Such bars of the same subpopulation are assembled in parallel, forming a rectangular assemblage discriminated by vertical black lines. Subpopulations are arranged sequentially as TA-1 to TA-14 in Tanashi, TS-1 to TS-10 in Tsukuba, and CH-1 to CH-9 in Chiba. Based on genotype, the program probabilistically assigned each individual nematode to admixed clusters of cluster A, B, and C. The proportion of each cluster in each bar, representing the probability of assignment for the nematode to the cluster, is indicated by white, light-gray, and dark-gray shading.

between the Tanashi population and either Chiba or Tsukuba population were much larger than the genetic difference between the Chiba and Tsukuba populations. Genetic difference was not correlated with geographic distance among subpopulations in Tsukuba and Chiba.

DISCUSSION

Polymorphic and species-specific SSR markers of the pinewood nematode. Although the SSR markers that we developed for the pinewood nematode showed intraspecific polymorphism, the degree of SSR polymorphism seems rather small compared with that exhibited by SSR markers of other organisms (11,14, 21,23,28,29,31–33). This low degree of polymorphism may partly result from *B. xylophilus* being an introduced species that has spread very rapidly through Japanese forests (27).

In this study, SSR markers were useful as intraspecific markers for identification of *B. xylophilus* genotypes. Because the nine nematode isolates used to test the makers were collected from a wide range throughout Japan, the genetic diversity among these isolates should well represent that of all *B. xylophilus* nematodes in Japan. In addition, PCR amplification of SSR markers for the pinewood nematode also was species specific to some extent. Several researchers previously have used PCR techniques other than SSR polymorphism analysis for specific and intraspecific identification of *B. xylophilus* (1,3,4,7,9,10,18). The present SSR markers also could be used for *B. xylophilus* identification.

Genetic differences within and between nematode populations of the three forests. All nematode subpopulations extracted from dead pine trees in Tanashi had the same genotype, except for two individuals; this genotype was maintained for at least 2 years, suggesting that the same genotype has dominated the Tanashi region for years. Because Tanashi forest is fragmented and isolated from nearby forests by a large urban residential zone, this

genotypic simplicity may be the result of a founder effect and genetic drift. Pine wilt disease was first recognized in Tanashi in 1988; thus, the founder effect and genetic drift over the 13 intervening years may have resulted in genotypic simplification.

Compared with the Tanashi population, the Tsukuba and Chiba populations had substantially higher genotypic diversity. The Chiba and Tsukuba forests are larger and are less fragmented by residential regions than the Tanashi forest. Thus, the higher allele diversity may be attributable partly to larger nematode and sawyer beetle population sizes that reduced the founder effect and genetic drift.

The dendrogram based on genetic differences clearly distinguished Tanashi subpopulations from those in Tsukuba and Chiba, and the major genotype in Tanashi rarely was found in the other two forests. The genetic isolation of the Tanashi population from the other two populations may result from its geographic isolation (i.e., from its being surrounded by a large residential area). Because the spread of *B. xylophilus* relies on the migration of its vector, the pine sawyer beetle, the genetic isolation of the Tanashi nematode population may result from limited migration of the sawyer over the metropolis.

In contrast, the Tsukuba and Chiba subpopulations were intermingled in the dendrogram, and several genotypes commonly were observed in both forests. Because both Chiba and Tsukuba forests are large and seldom fragmented by residential regions, relatively large sawyer populations may inhabit these areas and migrate within and between the populations. This may result in significant nematode gene flow between the populations.

The cluster analysis suggests that nematodes in the three forests were derived from three different origins. The Tsukuba and Chiba nematode populations that were admixed with two clusters seem to have come from two origins (i.e., populations that differed from the Tanashi origin). This inference is consistent with the dendrogram (Fig. 3) showing that the Tanashi population is isolated from the Tsukuba and Chiba populations, and that the Tsukuba and Chiba populations are genetically indistinguishable.

Genetic difference between nematode subpopulations from individual trees. The genetic difference was much less within subpopulations than between subpopulations. Genotypic frequency distribution in each *B. xylophilus* subpopulation in a tree is highly affected by the genotypes of the founder nematodes introduced by the sawyer beetles and the mode of nematode proliferation within a tree. Nematodes are introduced into the tree via the wounds formed by sawyer maturation feeding (15). There is no report showing that the sawyer beetle keeps a territory for maturation feeding by driving other individuals from the tree. If a sawyer does not maintain such a territory after maturation feeding and if other sawyers can freely access the pine tree, the genotypic frequency distribution of nematodes would tend to be similar among trees. Thus, the mode of nematode introduction to the tree could not be the main cause of the relative genetic uniformity of individuals within the same tree.

Fig. 3. Neighbor-joining dendrogram (using the Molecular Evolutionary Genetics Analysis software) based on the genetic differences between *Bursaphelenchus xylophilus* subpopulations in the three forests. Genetic differences were assessed using Nei's average number of differences, which were calculated using ARLEQUIN version 2. Every subpopulation was labeled with a prefix (TA, TS, or CH representing Tanashi, Tsukuba, and Chiba forests, respectively) followed by a number.

TABLE 5. Genetic difference indices between and within *Bursaphelenchus xylophilus* populations^a

	Tanashi	Tsukuba	Chiba
Tanashi	0.006 ^b	...	...
Tsukuba	0.955 ^c	1.259 ^b	...
Chiba	1.100 ^c	0.257 ^c	2.141 ^b

^a Genetic differences between and within three populations (Tanashi, Tsukuba, and Chiba) were assessed from microsatellite genotypes of individual nematodes using the ARLEQUIN program.

^b Each diagonal element is the mean number of pairwise differences within the population, an index of genetic difference within the population.

^c Each element below the diagonal is Nei's average number of differences between populations, an index of genetic difference between populations. For the calculation, the allele frequency in each forest was calculated as the average of frequencies of a given allele in all subpopulations in the forest.

Experiments have shown that most *B. xylophilus* inoculated onto a pine tree are unable to invade the host pine tissues and those that do invade persist at an extremely low number until the beginning of symptom development (16,17). Proliferation from such a small number of nematodes may produce a founder effect and early genetic drift, and result in a much simpler genotypic frequency distribution than that of the originally introduced nematodes.

Another remarkable characteristic of the *B. xylophilus* genotype was the excess of homozygous allele pairs. The Wahlund effect and inbreeding can explain an excess of homozygous allele pairs (5,24,30). The Wahlund effect predicts that H_o is smaller than H_e in a population under Hardy-Weinberg equilibrium in the presence of subpopulations with different allele frequencies. The present analyses of genetic differences and clusters between subpopulations indicated the independency between subpopulations. Therefore, the deviation between H_o and H_e could be mostly explained by the Wahlund effect. Because only a small number of nematodes may be present before the explosive multiplication stage, inbreeding also might contribute to the deviation.

ACKNOWLEDGMENTS

We thank T. Kiyohara, formerly of the Forestry and Forest Products Research Institute, Japan, for providing pinewood nematode isolates; and S. Ugawa, Graduate School of Frontier Sciences, the University of Tokyo, and T. Yonemichi, University Forest in Chiba, the University of Tokyo, for their support in collecting wood samples. This work was partly supported by a Grant-in-Aid for Scientific Research (16101008) from the Ministry of Education, Science, Sports, and Culture of Japan, and a grant (03DZ19317) from Science and Technology Commission of Shanghai Municipality, China.

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