

High Mitochondrial Genome Diversity and Intricate Population Structure of *Bursaphelenchus xylophilus* in Kyushu, Japan

Hanyong Zhang,¹ Erika Okii,² Eiji Gotoh,^{2*} and Susumu Shiraishi²

¹Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakoza-ki, Higashi-ku, Fukuoka 812-8581, Japan.

²Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.

*E-mail: eiji.gotoh@agr.kyushu-u.ac.jp.

This paper was edited by Axel Elling.

Received for publication January 9, 2018

Abstract

Mitogenomic diversity and genetic population structure of the pinewood nematode (PWN) *Bursaphelenchus xylophilus* inhabiting Kyushu, Japan were analyzed. A method for performing long PCR using single nematodes and sequencing nematode mitochondrial genomes individually is presented here. About 8kb (~55%) of the complete mitochondrial genome was successfully obtained from 285 individuals collected from 12 populations. The 158 single nucleotide polymorphisms detected corresponded to 30 haplotypes, clearly classified into two clades. Haplotype diversity was 0.83, evidencing a remarkable high diversity within Kyushu. The high genetic differentiation among the 12 populations (0.331) might be due to past invasion and expansion routes of PWN in northeastern and southeastern Kyushu. The distinct genetic composition of populations within the northwestern, central western, and southwestern Kyushu seems to be mostly related to the extinction of pine forests and long-range migration of PWN due to human activity. Overall, direct long PCR and sequencing of single nematode individuals are effective methods for investigating mitochondrial polymorphisms, and these are effective tools for PWN population genetics and other intraspecific studies.

Key words

Bursaphelenchus xylophilus, Haplotype diversity, Genomics, Mitochondrial polymorphism, Pinewood nematode, Population structure, Sequence polymorphism.

Pine wilt disease (PWD), one of the most serious forest problems worldwide, originated in North American countries (Dwinell, 1997) spreading to many others during the 20th century. In the U.S.A and Canada, PWD is not considered as a primary pathogen of native pines (Leal et al., 2013), whereas in invasion areas, such as Japan, Korea, China, and Portugal, it exerts serious damage to forest ecosystems (Yun et al., 2012).

In Japan, PWD was first recorded at Nagasaki prefecture, northwestern part of Kyushu, in 1905. During the last century, this disease has been responsible for the yearly loss of 700,000m³ of pinewood (Mamiya and Shoji, 2009). In China, PWD was first observed in Nanjing in 1982, but it rapidly expanded (Yang, 1995;

Zhang and Luo, 2003; Wu, 2004), and over one million hectares of pine forests have died due to PWD (Zhao, 2008). In South Korea, PWD was first identified in the Gumjung Mountain Region of Busan in 1988 (Yi et al., 1989), but it widely spread to 57 cities and prefectures until 2010 (Jung et al., 2010a). In Europe, PWD was first reported in Portugal in 1999 (Mota et al., 1999). During the next decade, nearly 510,000ha of Portuguese pine forests were destroyed due to PWD (Valadas et al., 2012). By 2008, the disease spread across the country (Rodrigues, 2008) and, more recently, the PWD was detected in Madeira Island, 1000km southwest of mainland Portugal (Fonseca et al., 2012). PWD has also been detected in Spain (Abelleira et al., 2011; Robertson et al., 2011), and, throughout the world,

44 *Pinus* species have been infected by PWD under natural conditions (Shi et al., 2013).

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, Nickle, 1970 (Steiner and Buhner, 1934) (Nematoda: *Aphelenchoididae*), which is a kind of plant parasite, is the causal agent of PWD (Kiyohara and Tokushige, 1971). This nematode can survive in healthy and in dead trees. In healthy host trees, the PWN causes cell destruction leading to host death in a few months (Cardoso et al., 2012). Although it has been listed as a quarantine pest in more than 40 countries (Mota et al., 1999; Schrader and Unger, 2003), the lack of an effective method to inhibit its expansion is still a serious problem worldwide. Because the PWN is present in the logs, lumber, and wooden packaging material used in commercial transportation (Hu et al., 2013), it is difficult to inhibit PWN expansion, except for the transportation performed by its vectors, the beetles within the genus *Monochamus*. Understanding its transmission routes and dispersal mechanisms could be effective for controlling the propagation of the PWN, especially to uninfected regions (Jung et al., 2010b).

Molecular markers showing sufficient genetic polymorphism are thought to help in understanding the epidemiology of PWD (Jung et al., 2010b), and many studies have focused on PWN population analysis. Cheng et al. (2008) studied the genetic variation during the invasive process of PWN in China and successfully inferred its possible spread routes using amplified fragment length polymorphism (AFLP). Iwahori et al. (1998) used an internal transcribed spacer (ITS)-Restriction Fragment Length Polymorphism (RFLP) map to identify the relationship among Japanese, Chinese, Canadian, and US PWN isolates. Random amplified polymorphic DNA (RAPD)-PCR and ITS-RFLP have been used to examine PWN populations in mainland Portugal (Vieira et al., 2007) and in Madeira Island (Fonseca et al., 2012), respectively. Zhou et al. (2007) reported that microsatellite markers might be useful for studying PWD, and they used such markers to evaluate the genetic structure of PWN populations among and within pine forests. The polymorphism of Japanese PWN isolates from 29 populations sub-cultured in the laboratory and collected from natural pine stands was analyzed based on variations at the heat-shock protein 70 locus (Takemoto and Futai, 2007). All these DNA-based techniques provide an attractive solution for examining PWN populations, despite their limitations: the resolution level of RAPD and RFLP markers is low; few of these markers can be applied to a single PWN individual due to its extremely small body size; and determination of intraspecific variability is difficult because sample sizes are too small (Valadas et al., 2013).

Mitochondrial DNA (mtDNA) is an excellent marker for the study of phylogenetic relationships due to its high copy number in a cell (Valadas et al., 2013). In recent years, mitochondrial genes have been used as markers for intraspecific variation studies in nematodes (Madani et al., 2010). They were employed to investigate genetic relationships among Peruvian and Canadian populations of *Globodera pallida* (Picard et al., 2007; Plantard et al., 2008; Madani et al., 2010), and the mitochondrial gene cytochrome b (*cytb*) successfully confirmed the origin of new populations of this species (Plantard et al., 2008). These results suggested that mitochondrial genes might be useful for studying intraspecific variability in the genus *Bursaphelenchus*. Furthermore, Sultana et al. (2013) reported the whole genome sequence of PWN, thereby providing valid information for studying PWN populations' variability based on mitochondrial genome sequencing and analysis of its polymorphisms. In addition to reporting the complete mitochondrial genome of PWN, Sultana et al. (2013) also compared the entire mitochondrial genomes of *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* (Mamiya and Enda, 1979), and developed a molecular tool to identify these two species. Similarly, Pereira et al. (2013) clarified the intraspecific phylogeny of *Bursaphelenchus xylophilus* isolates from different world regions and of *Bursaphelenchus mucronatus* isolates using three mitochondrial genes: cytochrome c oxidase subunit 1 (*cox1*), NADH dehydrogenase subunit 5 (*nad5*), and small subunit ribosomal RNA (*rrnS*). A previous study also reported the phylogenetic relationships among *Bursaphelenchus* species inferred from mtDNA and nuclear ribosomal sequence data (Ye et al., 2007).

All the above-mentioned studies suggest that mitochondrial genome information might be a new and efficient tool for examining population genetic variability in PWN. However, PWN mitochondrial genome diversity is not well understood, and using this information to evaluate PWN population diversity has not advanced to date. Thus, in the present study, the partial mitochondrial genome was sequenced for individual PWNs, and the sequence polymorphism and genetic population structure in PWN were investigated in detail.

Materials and methods

Nematode collection and preparation

The nematode samples used in this study were collected from 12 different forests in Kyushu, Japan where PWD was reported from 2012 to 2014 (Fig. 1). Pine chips were obtained from five points in the trunk, using a drill with a diameter of 16mm. Nematodes

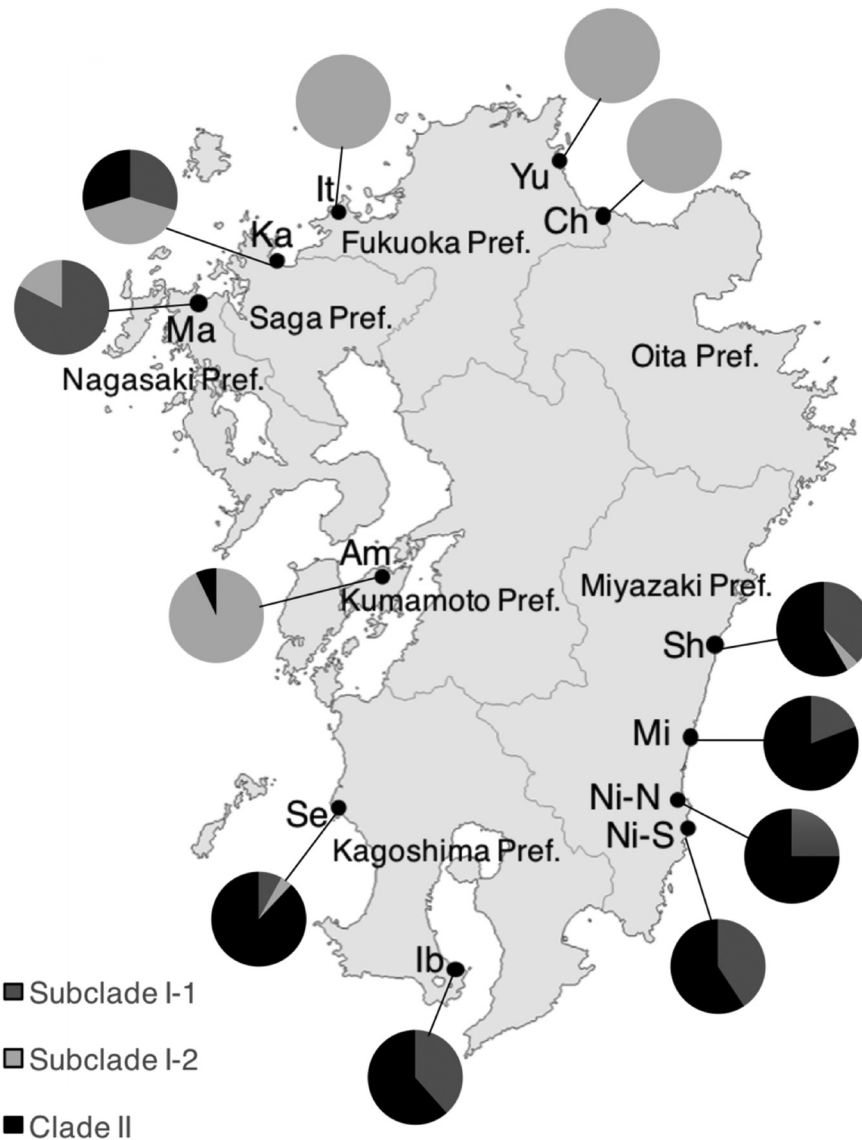


Figure 1: Location of the studied populations of *Bursaphelenchus xylophilus* (black dots) in Kyushu and the group of haplotype distribution of each population. The names of populations are written in code as indicated in Table 1. The classification of haplotypes is presented as in Figure 2.

were extracted from the chips using the Baermann funnel method (Iwahori and Futai, 1993), cultured on *Botrytis cinerea* Pers. (1794) grown on barley culture medium, and incubated at 25°C for 10 d to 20 d. After successful rearing, nematodes were removed from the culture medium, and stored in 5-ml tubes containing distilled water, at 4°C until use. Mitochondrial DNA (mtDNA) sequencing was performed for each individual nematode. The number of nematode individuals in each population for which mtDNA sequences were successfully obtained and used in further analysis are listed in Table 1. The number of trees in each

population that became the source of the sequenced nematodes are also listed.

PCR amplification and DNA sequencing

A primer pair for long PCR was designed in Oligo7 (Molecular Biology Insights), based on the complete mitochondrial genome sequence of *Bursaphelenchus xylophilus* (14,778 bp; NCBI Accession No GQ332424). The forward (5'-TCCTCCATTAAGAACTTTAGGGC AT-3') and reverse (5'-TACAGTCAAAGCAATAGGAC-GAGA-3') primers designed produced an amplicon

Table 1. Summary information for populations in Kyushu.

Population	Code	N ^a	No. of trees ^b	Location	Prefecture
Matsuura	Ma	23	16	33.3°N, 129.7°E	Nagasaki
Karatsu	Ka	27	10	33.5°N, 129.9°E	Saga
Itoshima	It	25	25	33.6°N, 130.2°E	Fukuoka
Yukuhashi	Yu	14	14	33.7°N, 131.0°E	Fukuoka
Chikujo	Ch	12	12	33.7°N, 131.1°E	Fukuoka
Amakusa	Am	28	10	32.6°N, 130.4°E	Kumamoto
Shintomi	Sh	24	20	32.1°N, 131.5°E	Miyazaki
Miyazaki	Mi	26	20	31.9°N, 131.4°E	Miyazaki
North Nichinan	Ni-N	28	4	31.6°N, 131.4°E	Miyazaki
South Nichinan	Ni-S	27	20	31.6°N, 131.4°E	Miyazaki
Sendai	Se	25	20	31.8°N, 130.2°E	Kagoshima
Ibusuki	Ib	26	20	31.2°N, 130.6°E	Kagoshima

^aNumber of individuals analyzed in each population. Total number of individuals analyzed = 285; ^bThe number of trees in each population that became the source of the sequenced nematodes.

about 8kb long that covered more than 50% of the mitochondrial genome.

Each nematode was used for direct amplification of mtDNA. Under a binocular stereomicroscope, each individual was separated from the nematode suspension using a needle and divided into two halves using a scalpel. Both halves were then transferred to a 25- μ l PCR mixture [1 \times Gflex PCR Buffer (Takara), 0.43 μ M each primer, 0.625 units Tks Gflex DNA Polymerase (Takara)]. The PCR reaction was carried out in the SureCycler 8800 (Agilent Technologies) device and comprised an initial denaturation at 94°C for 1 min, followed by 30 cycles at 98°C for 10s and 68°C for 8min. The resulting amplicon was electrophoresed on 1% agarose gel with ethidium bromide, and the ~8-kb-long fragment obtained was excised from the gel with the MagExtractor[®]-PCR & Gel clean up (Toyobo), following the manufacturer's instructions. Using the excised fragment as DNA template, a sequencing library was prepared using the TruSeq Nano DNA Library Prep Kit (Illumina) and mtDNA sequencing was carried out in the Illumina MiSeq (Illumina) platform, following the manufacturer's protocols.

Sequence data analyses

The quality of the obtained sequences was evaluated. Only the sequences with quality over Q20 were ex-

tracted and were used for assembly into the mtDNA sequence (ca. 8kb) on GS Reference Mapper Software (Roche). The corresponding region of the complete mitochondrial genome sequence of *Bursaphelenchus xylophilus* (GQ332424) was used as reference. Contigs with an average depth of 451 for the 285 individuals were used for sequence alignment and for the identification of single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) in Sequencher (Gene Codes). The exact coding region of each gene was determined based on the annotation listed for GQ332424 and on basic local alignment search tool (BLAST) analysis conducted on the National Center for Biotechnology Information (NCBI) web platform (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Mitochondrial DNA polymorphism analysis

The evaluation of genetic polymorphisms, including the number of polymorphic sites, nucleotide substitutions, and transitions and transversions without indels, was carried out in DnaSP (Librado and Rozas, 2009). This software was also used to determine the number of haplotypes (Nh) and nucleotide and haplotype diversities. Haplotype diversity (Hd: Nei and Tajima, 1981) and Nh were computed to evaluate mitog-

enomic diversity in each population. MEGA (Kumar et al., 2016) was used to translate protein-coding genes based on the invertebrate mitochondrial codon usage table, and synonymous and non-synonymous substitutions in protein-coding regions were identified. The number of variations, including nucleotide substitutions in genes and non-coding regions, and variations in amino acid sequences, were also determined. The significant differences in SNP densities between genes or regions were examined by Student's *t*-test in Excel (Gosset, 1908).

Phylogenetic analysis

Haplotype distribution and diversity (Hd) in each population were investigated independently. Haplotype sequence data were imported into MEGA to build a phylogenetic tree of haplotypes using the maximum likelihood (ML) method based on the Kimura 2-parameter model (Kimura, 1980), with 1,000 bootstrap replications. Phylogenetic relationships among the 12 populations in Kyushu were analyzed using haplotype frequencies in populations. Haplotype composition and frequency of haplotypes in each population were used to calculate genetic differentiation (*Gst*), gene flow (*Nm*), and genetic distances (*Ds*) among populations. A phenogram was also drawn for the 12 populations based on the neighbor-joining (NJ) method (Saitou and Nei, 1987).

Results

Sequence statistics

A fragment of about 8,060bp, corresponding to 54.5% of the PWN mitochondrial genome (ca. 14.8kb), was sequenced. In the PWN mitochondrial genome, there is an extremely AT-rich and extensive non-coding region of about 1.7kb (13,129-14,778 of GQ332424), while all mitochondrial genes are placed in the remaining 13.1kb (Sultana et al., 2013). The sequence decoded in the present study corresponds to 61.4% of the latter fragment.

Analysis of SNPs

A total of 158 SNPs were detected in mtDNA sequences, corresponding to one SNP per 51bp, on average (Table 2). In total, 22 genes (8 protein-coding genes, 12 transfer RNA (tRNA) genes, and 2 rRNA genes) and 6 non-coding regions were confirmed in the 8,060bp sequence. The number of SNPs in each gene/region is shown in Table 2. Nine tRNA genes (*trnD*, *trnG*, *trnH*, *trnA*, *trnP*, *trnV*, *trnW*, *trnE*, and *trnY*)

showed no SNPs. Thus, the 158 SNPs corresponded to 139 SNPs from protein-coding genes, 3 SNPs from the remaining 3 tRNA genes, 13 SNPs from 2 rRNA genes, and 3 SNPs from non-coding regions.

A total of 88% (139/158) of the SNPs were found in the protein-coding genes, leading to an average occurrence of one SNP/41bp. Most of these SNPs (40 SNPs, 1 SNP/39bp) were found in *nad5*, but *cox1* showed the highest average occurrence of SNPs (39 SNPs, 1 SNP/30bp). Genes coding for NADH dehydrogenase subunits 1 and 6 (*nad1* and *nad6*) presented high number and average occurrence of SNPs (22 SNPs, 1 SNP/40bp and 13 SNPs, 1 SNP/33bp, respectively). Genes coding for NADH dehydrogenase subunit 3 (*nad3*) and ATP synthase subunit 6 (*atp6*) showed a relatively low average occurrence of SNPs, as only six (1 SNP/57bp) and seven (1 SNP/60bp) SNPs were found in each sequence, respectively. Cytochrome *c* oxidase subunit 2 (*cox2*) was the most conservative among the eight protein-coding genes (seven SNPs, 1 SNP/99bp). Because only three SNPs were detected in 3 of the 12 tRNAs for which nucleotide sequences were decoded (i.e., *trnC*, *trnM*, and *trnS*), the average occurrence of SNPs in tRNA genes was extremely low (1 SNP/223bp). According to the predicted secondary structure of mitochondrial tRNA genes, 20 of the 22 tRNA genes identified in PWN have a unique structure; a loop of variable size (TV-replacement loop; 6-12bp) is thus displayed instead of the TΨC arm loop (Sultana et al., 2013). In the present study, the SNPs of *trnC* and *trnS* occurred in the TV-replacement loop and TΨC arm loop, respectively; the SNP of *trnM* occurred close to its 5' extremity. The two rRNA genes (*rns* and large subunit rRNA gene (*rnl*)) found here contained six and seven SNPs, respectively, thereby showing a lower average occurrence of SNPs than the protein-coding genes (1 SNP/116bp for *rns* and 1 SNP/134bp for *rnl*). The total length of the six non-coding regions identified here was 50bp (1-20bp in each region). Generally, non-coding regions are the most variable in the genome (Van der Veer and De Vries, 2004), which was also revealed for the partial mitochondrial genome of PWN. For example, the region comprising *cox1-trnC* was only 15bp but contained two SNPs, thereby presenting an extremely high occurrence rate of SNPs (1 SNP/7.5bp). In the *trnD-trnG* region, one SNP was detected in three base pairs. The differences in SNP densities between protein-coding genes and tRNA genes or rRNA genes were significant ($P \leq 0.01$) according to the *t*-test.

The 158 SNPs corresponded to 122 transitions (Ts) and 36 transversions (Tvs), leading to a Ts/Tv ratio of 3.4 (Table 2). In total, 114 of the 122 Ts were found in the protein-coding genes. These genes presented

Table 2. Sequence polymorphism and substitutions identified in the mitochondrial genome of *Bursaphelenchus xylophilus*.

Gene/region	Length ^a (bp)	Sequence polymorphism		Substitution					Amino acid residue		Syn ^d / Non-syn ^d ratio	
		No. Indels	Indels/ bp ^b	No. SNPs	SNPs/ bp ^b	No. Ts ^c	No. Tv ^c	Ts ^c / Tv ^c ratio	No. Syn ^d	No. Non-syn ^d		
Protein-coding gene	<i>cox1</i>	1,156		39	1/30	35	4	8.8	34	5	6.8	
	<i>cox2</i>	690		7	1/99	7	0	–	6	1	6.0	
	<i>nad3</i>	342		6	1/57	5	1	5.0	4	2	2.0	
	<i>nad5</i>	1,569		40	1/39	30	10	3.0	30	9	3.3	
	<i>nad6</i>	435		13	1/33	11	2	5.5	11	2	5.5	
	<i>nad4L</i>	234		5	1/47	4	1	4.0	5	0	–	
	<i>nad1</i>	873		22	1/40	17	5	3.4	21	1	21.0	
	<i>atp6</i>	417		7	1/60	5	2	2.5	7	0	–	
	subtotal	5,716		139	1/41	114	25	4.6	118	20	5.9	
tRNA	<i>trnC</i>	54		1	1/54	0	1	–				
	<i>trnM</i>	54		1	1/54	0	1	–				
	<i>trnD</i>	55										
	<i>trnG</i>	54										
	<i>trnH</i>	55										
	<i>trnA</i>	55										
	<i>trnP</i>	57										
	<i>trnV</i>	57										
	<i>trnW</i>	55										
	<i>trnE</i>	55										
	<i>trnS</i>	55		1	1/55	0	1	–				
	<i>trnY</i>	63	1	1/63								
	subtotal	669	1	1/669	3	1/223	0	3	–			
	rRNA	<i>rrnL</i>	937		7	1/134	4	3	1.3			
<i>rrnS</i>		697		6	1/116	3	3	1.0				
subtotal		1,634		13	1/126	7	6	1.2				
Non-coding region	<i>cox1-trnC</i>	15	1	1/15	2	1/7.5	0	2	–			

<i>trnC-trnM</i>	7										
<i>trnM-trnD</i>	20										
<i>trnD-trnG</i>	3	–	–	1	1/3	1	0	–			
<i>nad3-nad5</i>	1										
<i>rrnS-trnS</i>	4										
subtotal	50	1	1/50	3	1/17	1	2	0.5			
Total	8,060 ^e	2	1/4030	158	1/51	122	36	3.4	118	20	5.9

^aThe nucleotide sequence length for that gene or region; ^bThe average occurrence of single nucleotide polymorphisms (SNPs) or insertions/deletions (Indels) in that specific part of the sequenced mitochondrial genome; ^cTs, transitions; Tv, transversions; ^dSyn, synonymous substitution; Non-syn, non-synonymous substitution; ^eThe total length contains 9bp overlapping genes or regions; Blank cells indicate no value; – means the value cannot be calculated.

Ts/Tv ratios of 2.5-8.8 (average 4.6), which indicated that Ts were more frequent than Tv. The *cox1* gene contained most Ts and showed the highest Ts/Tv ratio (8.8). *Nad5*, which harbored the largest number of SNPs, held more Tv than *cox1* and therefore showed a lower Ts/Tv ratio (3.0). Only one Tv was identified among the five SNPs observed in NADH dehydrogenase subunit 4L gene (*nad4L*). On the other hand, no Tv appeared in the *cox2*. The Ts/Tv ratio of the two rRNA genes was 1.2, which means that the number of Ts and Tv was almost identical. Only Tv were detected in all of the tRNA genes, while in the non-coding region there were two Tv in *cox1-trnC* and one Ts in *trnD-trnG* (Table 2).

Amino acid variation was examined for all SNPs detected in the protein-coding genes. The numbers of synonymous and non-synonymous substitutions (Syn and Non-syn, respectively) in amino acids were 118 and 20, respectively (Table 2). Within the 118 Syn, 104 were Ts and 14 were Tv. A total of 97% (115) of the Syn were due to mutations at the third position of the codon, while the remaining three Syn were caused by mutations at the first codon position (C/T substitution in *nad5*, C/T substitution in *nad6*, and C/T substitution in *nad1*). In all, 10 (50%) of the 20 Non-syn were caused by mutations at the second codon position, 7 were due to mutations at the first codon position, and 2 were due to mutations at the third codon position. One Non-syn recognized in *nad5* corresponded to simultaneous changes in the second and the third positions of the codon. The overall Syn/Non-syn ratio was 5:9. Thus, Syn occurred about six times more frequently than Non-syn, which means that most variations in the partial mitochondrial genome of PWN

could be considered neutral (Kimura, 1983). However, the Syn/Non-syn ratio in *nad3* (2.0) and *nad5* (3.3) were lower than in other genes, suggesting that relatively high selective effects might have occurred in these two genes.

Analysis of indels

Because the mitochondrial genome of PWN is extremely AT-rich (Sultana et al., 2013), it is difficult to accurately estimate the length variation of long homopolymer (poly-A and poly-T) regions (Linnertz et al., 2012). Therefore, a PCR was employed in the present study to allow the occurrence of artificial length variation due to possible PCR slippage in simple sequence repeats (Hauge and Litt, 1993; Murray et al., 1993). All indels (length variation) recognized in homopolymer regions were excluded due to the low quality of the identification of nucleobases (i.e., Q score) of their last parts in many individuals. Only two indels were detected outside homopolymer regions and no indels were found in the eight protein-coding genes (Table 2). Only 1 of the 12 tRNA genes (*trnY*) displayed one-base deletion in its anticodon stem, which was found in 1 of the 285 nematodes investigated. In the *cox1-trnC* inter-genic spacer region, one four-base deletion was observed (Table 2).

Haplotype distribution and diversity

Haplotype analysis was performed for the 285 PWNs sampled, and 30 haplotypes (hts-01–30 in Table 3) were detected based on 160 polymorphic sites that

consisted of 158 SNPs and two indels. Polymorphic sites of haplotypes and the position of each SNP in relation to the reference sequence (GQ332424) are displayed in Table S1.

The frequency of each haplotype detected in each of the 12 investigated populations is shown in Table 3. The most frequent haplotype in Kyushu was ht-21 (34.4%, 98 nematodes), although ht-01 (15.1%, 43 nematodes) and ht-13 (14.7%, 42 nematodes) also showed relatively high frequency. On the other hand, 14 haplotypes (ht-05, hts-08–09, hts-11–12, hts-16–19, ht-23, hts-25–26, and hts-29–30) were identified as extremely rare, as they were detected in only one nematode. In total, 20 of 30 haplotypes were population-specific, while the remaining 10 haplotypes were distributed in multiple populations. The most frequent haplotype (ht-21) was observed in 7 of the 12 populations, including all 6 populations in the southern region of Kyushu (Shintomi, Miyazaki, North Nichinan, South Nichinan, Sendai, and Ibusuki) and in the central western region (Amakusa). Haplotypes 10, 13, and 14 were found in the northern region (Karatsu, Itoshima, Yukuhashi, and Chikujō) and central western region (Amakusa), and four haplotypes (hts-01–04) were detected in the northern and southern Kyushu regions. Therefore, haplotype composition notably differed among regions.

Haplotype diversity in the 12 populations varied from 0.30 to 0.83, and its average was 0.55 (Table 3); in eight populations it was above 0.5. Particularly, populations from Amakusa, Karatsu, and Ibusuki showed H_d above 0.8, which is considered high haplotype polymorphism, while populations from Sendai, Miyazaki, and Itoshima showed H_d below 0.4 and N_h below 4. The haplotype diversity for the entire Kyushu region was 0.83.

The haplotypes found in three populations from northeastern Kyushu (Itoshima, Yukuhashi, and Chikujō) were similar to each other, and common haplotypes were also detected in four populations from southeastern Kyushu (Shintomi, Miyazaki, North Nichinan, and South Nichinan).

Phylogenetic analysis of haplotype data

A phylogenetic tree for the 30 haplotypes was constructed based on the ML method, using the 158 SNPs identified in the present study (Fig. 2). The closely related *Bursaphelenchus mucronatus* (Accession GU177865) was employed as outgroup to determine the root of the phylogenetic tree.

The dendrogram clustered the 30 haplotypes into two Clades (Clade I and Clade II). A major phylogenetic difference was observed between the two clades;

there was a minimum difference of 58 SNP sites (between ht-26 of Clade I and ht-25 of Clade II) and a maximum difference of 105 sites (between ht-27 of Clade I and hts-02/03/21 of Clade II). In contrast, no significant difference emerged among haplotypes within each clade. Among the 24 haplotypes within Clade I, 91 sites differed between hts-10 and 30, while only four sites differed between hts-25 and 28, among the six haplotypes within Clade II. Clade I was further divided into two subclades (Subclades I-1 and I-2) and three single haplotypes (hts-17, 26, and 10). The sequences of these three haplotypes differed from that of the other 21 haplotypes contained in Clade I. Subclades I-1 and I-2 consisted of 11 and 10 haplotypes, respectively. However, the maximum disagreement within Clade I consisted of only 16 SNP sites between ht-30 (Subclade I-1) and ht-12 (Subclade I-2), and no significant difference was identified between the two subclades. Most of the haplotypes within Subclade I-1 were distributed in the northern Kyushu region, haplotypes within Clade II were mainly distributed in southern Kyushu region, and haplotypes in Subclade I-2 were detected in both northern and southern Kyushu regions.

Phylogenetic relationships among the mitogenomic sequences of six isolates (Ibaraki, Japan: AP017463; Korea: GQ332424 and NC023208; and Portugal: JQ429761, JQ514067, and JQ514068) were also analyzed. No sequence polymorphisms were identified for isolate within the same area in Korea and Portugal and, therefore, a phylogenetic tree was built after aligning three mitogenomic sequences (Ibaraki: AP017463, Korea: GQ332424, and Portugal: JQ429761) and the 30 haplotype sequences from Kyushu (hts-01–30). As a result, the Ibaraki (East Japan) sequence was assigned to Subclade I-1, whereas sequences from Korea and Portugal were allocated to Clade II. Sequences from Ibaraki and Portugal were highly similar to ht-13 and hts-21/23, respectively. The Korean sequence was not closely related to seven of the haplotypes within Clade II (hts-02, 03, 21, 23, 25, 28, and JQ429761). Comparing the Korean sequence with the other 32 sequences (haplotype and mitochondrial sequences) revealed 35 SNPs and 8 indels in 2 rRNA genes, and no significant differences for the 8 protein-coding genes and 12 tRNA genes.

Genetic relationships among populations

Genetic distances among populations were 0.14 to 0.92, based on haplotype frequency in each population (Table 4). The maximum distance was obtained between Miyazaki (southeastern region) and Karatsu

Table 3. Haplotype distribution within in each population.

Population	Haplotype																														Nh ^a	Hd ^b
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
Matsuura	16	3	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	0.50
Karatsu	-	-	8	3	1	8	2	1	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	0.82
Itoshima	-	-	-	-	-	-	-	-	-	1	1	-	20	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	0.36
Yukuhashi	-	-	-	-	-	-	-	-	-	6	-	7	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	0.60
Chikujo	-	-	-	-	-	-	-	-	-	3	-	1	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	0.53
Amakusa	-	-	-	-	-	-	-	-	-	-	-	7	4	2	1	1	1	1	1	9	2	-	-	-	-	-	-	-	-	-	9	0.83
Shintomi	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	14	1	-	-	-	-	-	-	-	-	4	0.57	
Miyazaki	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21	-	-	-	-	-	-	-	-	-	2	0.32	
Nichinan-N	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21	3	-	-	-	-	-	-	-	-	3	0.42	
Nichinan-S	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	1	1	-	-	-	-	-	-	-	4	0.57	
Sendai	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21	-	-	2	1	1	-	-	-	-	4	0.30	
Ibusuki	-	9	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	7	2	1	1	8	0.80
Total	43	12	10	7	1	8	2	1	1	13	1	1	42	8	2	1	1	1	10	98	5	1	2	1	1	1	7	2	1	1	-	0.83
Frequency (%)	15.1	4.2	3.5	2.5	0.4	2.8	0.7	0.4	0.4	4.6	0.4	0.4	14.7	2.8	0.7	0.4	0.4	0.4	3.5	34.4	1.8	0.4	0.7	0.4	0.4	0.4	2.5	0.7	0.4	0.4	-	-

Numbers indicate the number of individuals present in each haplotype. - indicate that no individual presented the haplotype. ^aNumber of haplotypes detected in the population; ^bHaplotype diversity, $Hd = (1 - \sum x_i^2) n / (n-1)$, where x_i is the frequency of the i th haplotype and n is the sample size (Nei and Tajima, 1981).

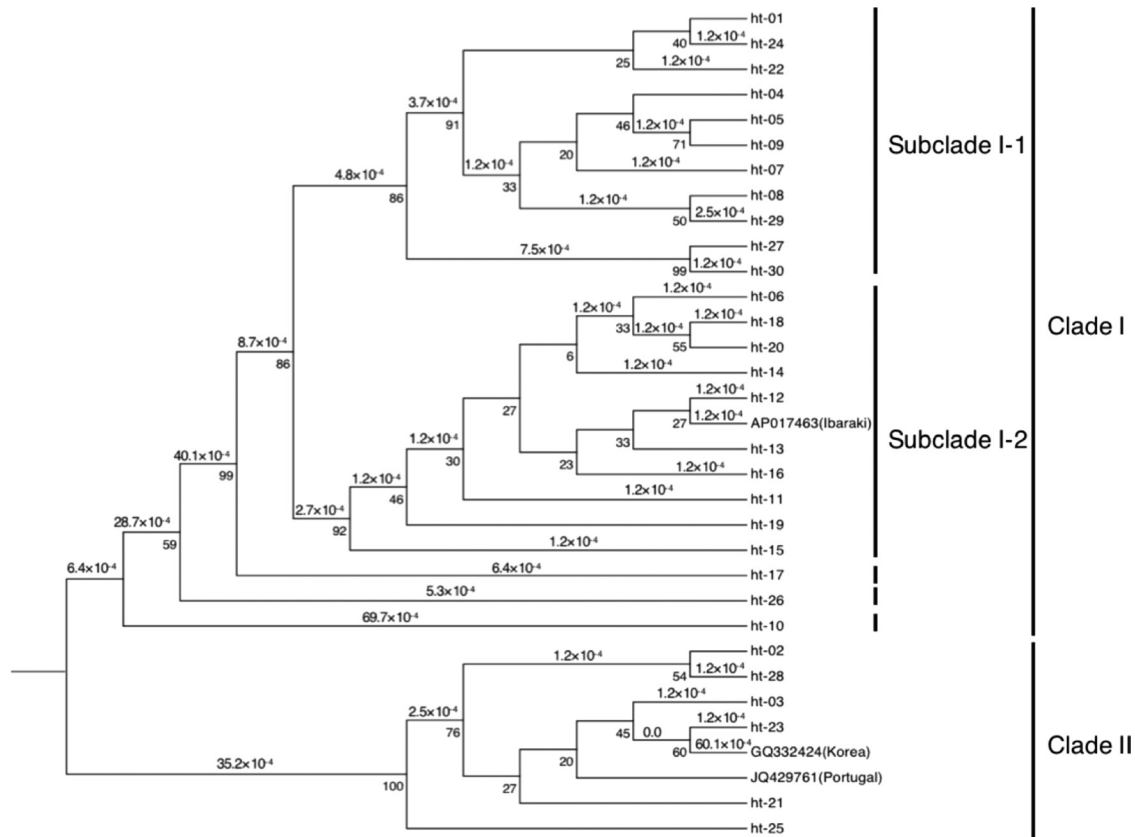


Figure 2: Phylogenetic tree for 33 mitochondrial haplotypes of *Bursaphelenchus xylophilus* containing 30 from Kyushu and 3 from previous studies was inferred by using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model. Numbers under the branches represent support values from bootstrap replications of 1,000. Evolutionary distances greater than 0.0001 are shown above branches. Wide bars indicate the major clades clustering.

(northwestern region), and no common haplotypes were detected. On the other hand, the Ds among three populations in northeastern Kyushu (Itoshima, Yukuhashi, and Chikujo) and the Ds among four populations in southeastern Kyushu (Shintomi, Miyazaki, North Nichinan, and South Nichinan) were 0.14 to 0.37 and 0.14 to 0.35, respectively. Geographical distances among populations within each group were very small.

The NJ tree constructed for the 12 populations based on Ds (Fig. 3) revealed that the three populations in northeastern Kyushu and the four populations in southeastern Kyushu formed cohesive clades. Three populations, located in the northwestern region (Matsura and Karatsu) and southernmost region (Ibusuki), formed a loose clade. The other two populations (Amakusa in the central western region and Sendai in the southwestern region) were clade-independent.

The *Gst* and *Nm* among populations were 0.33 and 1.01, respectively.

Discussion

In the present study, a method for performing long PCR using single nematodes was directly developed for individually sequencing each mitogenome. Sequences of about 8kb, corresponding to 55% of the whole mitochondrial genome of PWN were obtained for 285 nematode individuals using this method. A total of 160 polymorphic sites were detected in these sequences, leading to a frequency of polymorphism as high as one polymorphic site per 51 bp. However, a low SNP frequency was detected in tRNA genes, although this is consistent with tRNAs gene sequences being more conservative than that of other genes (Van der Veer and De Vries, 2004). The mitogenomic polymorphisms of the PWN have been mainly used in interspecies comparative studies (Pereira et al., 2013; Sultana et al., 2013). Their application to intraspecific studies is limited, and a lack of polymorphisms' evaluation is evident.

Table 4. Pairwise genetic distances between the 12 populations examined based on haplotype frequency.

Population	Ma	Ka	It	Yu	Ch	Am	Sh	Mi	Ni-N	Ni-S	Se
Matsuura (Ma)	–	–	–	–	–	–	–	–	–	–	–
Karatsu (Ka)	0.570	–	–	–	–	–	–	–	–	–	–
Itoshima (It)	0.693	0.665	–	–	–	–	–	–	–	–	–
Yukuhashi (Yu)	0.703	0.738	0.144	–	–	–	–	–	–	–	–
Chikujo (Ch)	0.703	0.714	0.367	0.288	–	–	–	–	–	–	–
Amakusa (Am)	0.773	0.671	0.549	0.598	0.706	–	–	–	–	–	–
Shintomi (Sh)	0.560	0.771	0.693	0.703	0.703	0.465	–	–	–	–	–
Miyazaki (Mi)	0.570	0.916	0.752	0.714	0.714	0.752	0.347	–	–	–	–
North Nichinan (Ni-N)	0.549	0.809	0.703	0.693	0.693	0.673	0.144	0.203	–	–	–
South Nichinan (Ni-S)	0.560	0.752	0.693	0.703	0.703	0.652	0.223	0.347	0.144	–	–
Sendai (Se)	0.693	0.752	0.693	0.703	0.703	0.662	0.560	0.570	0.703	0.560	–
Ibusuki (Ib)	0.464	0.560	0.752	0.809	0.809	0.678	0.665	0.811	0.714	0.665	0.665

Mitogenome sequences (three complete and three incomplete) of six PWN isolates, one from Japan, two from Korea, and three from Portugal, have been reported so far. No variations were detected between the two Korean sequences (14,788bp) or among the three Portuguese sequences (about 12kb). However, 171 polymorphic sites containing 150 SNPs were confirmed among the sequences from Ibaraki, Korea, and Portugal, whereas sequences from Kyushu alone showed 158 SNPs.

Sequences of four PWN mitochondrial genes (*cox1*, *cytb*, *nad5*, and *rns*) have been revised to date. In *cox1*, 52 SNPs were found among 36 isolates from Japan, Korea, China, Portugal, Canada, USA, and Mexico. In total, 18 SNPs were identified in *cytb* sequences of 20 isolates obtained from Japan, Korea, China, Portugal, and USA, and 11 SNPs were observed in *nad5* sequences from 17 isolates derived from these five countries. In total, 13 polymorphic sites containing two SNPs and 11 indels were observed in *rns* sequences from six isolates. Except for the *cox1*, which has been sufficiently investigated on relatively large sample sizes, sequence diversity in mitochondrial genes is generally not high. However, the present study revealed 39 SNPs in *cox1*, 40 SNPs in *nad5*, and 6 SNPs in *rns* in sequences from the

Kyushu region alone, which can be considered a very limited area compared to the areas examined in the studies mentioned above.

Past genetic studies on PWN employed exclusively DNA extracted from one isolate. As an isolate consists of many nematodes, several genotypes are included and sequencing based on isolates masks many low-frequency variations. Therefore, it is presumed that only high-frequency variations could be treated as sequences of the isolate, which seriously underestimates variation. Sequencing based on the method employed here allowed reading sequences of individual mitogenomic sequences, thereby detecting rare alleles/haplotypes. A considerably large sample size (285 individuals) might also contribute to correctly estimate diversity. Unlike the worldwide PWN collections performed in previous studies, the present study targeted the Kyushu region only, and used a localized collection of PWN within an extremely small area. Nevertheless, it was evident that extremely high sequence diversity was preserved in the mitochondrial genome of PWN. Sequencing based on individuals instead of isolates is therefore a powerful approach for population genetic studies.

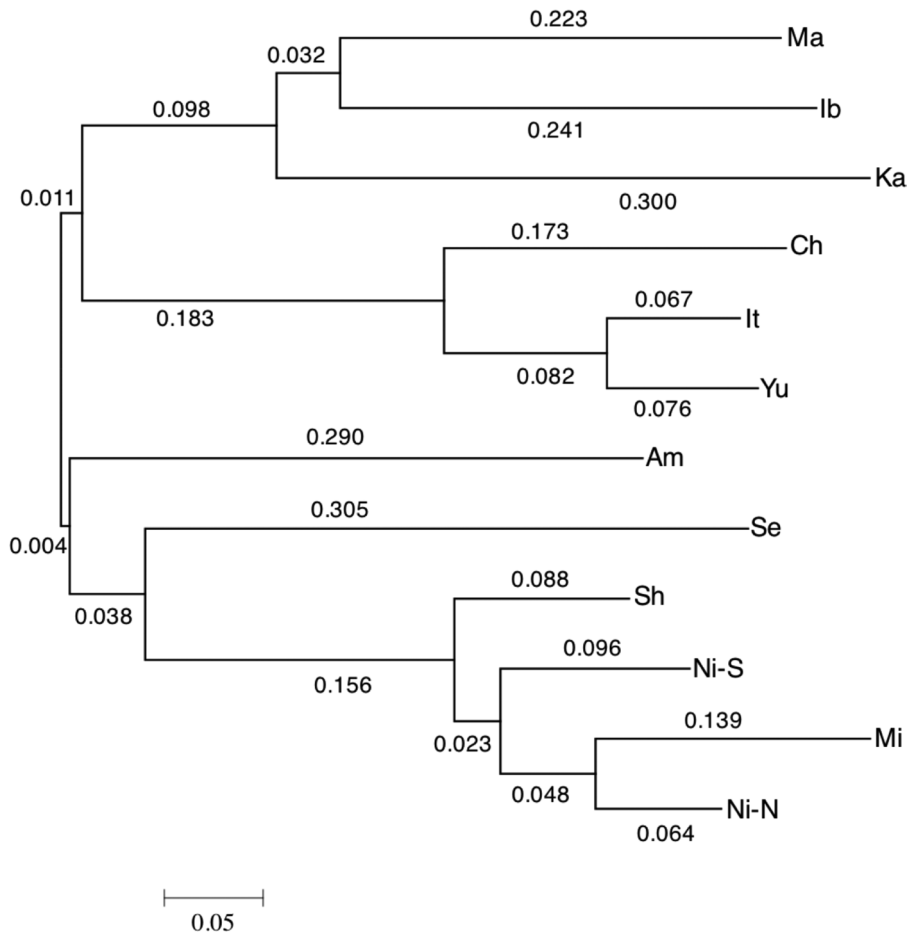


Figure 3: Neighbor-joining (NJ) tree diagram showing the genetic relationship that was determined using genetic distances of 12 populations. Numbers next to corresponding nodes indicate the branch lengths in the same units of the genetic distances that are computed based on the haplotype frequency for each population.

In the phylogenetic tree produced for haplotypes, 12 (ht-01, ht-04–14) out of the 14 haplotypes distributed in northern Kyushu belonged to Clade I. On the contrary, there was no evident trend in the haplotypes from southern Kyushu (Table 3 and Fig. 2). Sasebo (Nagasaki prefecture) was the first invasion point of PWN in Asia (Yano, 1913) from which PWN gradually spread to Shikoku and Honshu (Futai, 2008). It was then disseminated from Japan to Korea and China (Mamiya and Shoji, 2009). Furthermore, PWN populations appearing in Portugal in 1999 were assumed to have originated from East Asia (Metge and Burgermeister, 2006). Based on the method presented here, the propagation route of PWN could be further examined using mitochondrial haplotypes from invaded countries (Japan, Korea, China, and Portugal) and native countries (U.S.A and Canada).

Based on the past records of pine wilting and timber import and movement, Kishi (1988) estimated

three possible routes (Sasebo route, Nichinan route, and Aira route) through which PWN invaded and expanded into Kyushu. In the Sasebo route, PWN invaded from Sasebo, northwestern Kyushu in 1905, and extensively dispersed into the northern region of Kyushu. Because ht-13 was the major haplotype in the three populations from northeastern Kyushu (Itoshima, Yukuhashi, and Chikujo), this haplotype might characterize populations dispersed through the Sasebo route. Haplotype-13 was also relatively frequent in Amakusa in central western Kyushu, it was likely held with high frequency in the PWNs of the Sasebo route and then transmitted to northern Kyushu and Amakusa regions. According to Kishi (1988), pinewoods withered by PWD in Nagasaki prefecture were transferred to a pulp mill in Yatsushiro (Kumamoto prefecture), extending PWD to the central western region of Kyushu. A PWN group that irrupt-

ed from Nichinan in Miyazaki prefecture (Nichinan route) in 1939 expanded north and south along the east coast of Kyushu (Kishi, 1988). The PWN collections from four populations (Shintomi, Miyazaki, North Nichinan, and South Nichinan) in the coastal area of Miyazaki prefecture showed similar haplotype compositions, and ht-21, which presented an extremely high frequency, might be considered as the specific haplotype of the Nichinan route. The PWNs expanding through the Aira route (third route) invaded from Aira (Kagoshima prefecture) in 1942 and dispersed to the western region of Kagoshima prefecture (Sendai and Ibusuki) (Kishi, 1988). Although four haplotypes appeared in the Sendai population, most nematodes (84%) were ht-21. Because this haplotype might be considered specific to the Nichinan route and haplotype composition in Sendai was somewhat similar to that of populations dispersing through the Nichinan route, the Sendai population might have been recently influenced by the Nichinan route. However, the three low-frequency haplotypes (hts-24–26) that were not observed in other populations might persist as a vestige of the Aira route. The Ibusuki population, which was also regarded as dispersed through the Aira route, shared only one haplotype (ht-21) with Sendai and its frequency was quite low. The Ibusuki population, however, shared three haplotypes (hts-02–04) with Matsuura, northwestern Kyushu, which is geographically distant (Table 3 and Fig. 3), suggesting that artificial long-range migration might have occurred. Thus, in the region occupied by Aira route PWNs, profound changes in haplotype composition might be rapidly progressing by natural migration or artificial transfer from other nematode strains (Fig. 1).

Analysis of the genetic structure of the PWN population in Kyushu revealed high genetic differentiation among the 12 populations ($G_{st} = 0.331$). While 33.1% of the total genetic variability was due to variation among populations, 66.9% was due to variation within populations (Mouhaddab et al., 2015). The N_m among populations was nearly 1 (1.01) evidencing that PWN migration among populations had little effect on the genetic differentiation within the Kyushu region (Wright, 1951; Lowe et al., 2004). In the NJ tree of the 12 populations (Fig. 3) based on D_s (Table 4), the three populations within northeastern Kyushu (Itoshima, Yukuhashi, and Chikujo) and the four populations within southeastern Kyushu (Shintomi, Miyazaki, North Nichinan, and South Nichinan) formed cohesive clades. Average G_{st} values for the three populations of northeastern Kyushu and four populations of southeastern Kyushu were 5.7% and 1.9%, and their N_m values were 8.3 and 24.4, respectively. Thus, genetic differentiation among populations within

these areas was much smaller than within the whole Kyushu area. Additionally, due to the large value of N_m , it could be inferred that genetic differentiation within the referred local regions occurred due to genetic drift (Wright, 1951; Lowe et al., 2004).

When the three population in northeastern Kyushu and four populations in southeastern Kyushu were grouped as metapopulations and analyzed with the remaining five populations (Matsuura, Karatsu, Amakusa, Sendai, and Ibusuki), average G_{st} and N_m for these seven populations were recalculated as 32.1% and 1.06, respectively. Because N_m was nearly 1, the influence of gene flow on the genetic structure of those areas did not appear to be substantial (Wright, 1951; Lowe et al., 2004). In nature, PWN was expanded because PWN was propagated by vector insects. Due to the severe decline of the damaged pine forests in the past, the fragmentation of PWN populations has progressed and this geographical separation might have decreased the natural long-distance gene flow. Matsuura and Karatsu populations in the northwestern region and the Ibusuki population in the southernmost region formed a loose clade in the NJ tree (Fig. 3). Matsuura and Karatsu are geographically close, whereas Ibusuki is geographically distant from the other two (Fig. 1). As D_s among the three populations were not too large (Table 4), a long-range migration of PWN might have occurred by human activity.

According to Nose et al. (2009), the two populations of Ibusuki and Sendai in southwestern Kyushu were similarly affected by the PWN expanded from Aira in Kagoshima prefecture, as these two populations presented similar haplotype composition and shared a common dominant haplotype that was not very frequent in other areas. However, in the present study, the haplotype composition of the two populations was quite distinct. A total of 84% of the individuals in Sendai showed the haplotype (ht-21) appearing in the four populations (Shintomi, Miyazaki, North Nichinan, and South Nichinan) of the southeastern region. In Ibusuki, which is located in southernmost area of Kyushu, the only haplotype common to Sendai was ht-21, but its frequency was quite low.

Population size of nematodes invading an area seems to drastically decrease following the decline of the pine forest, and small populations are maintained or nearly extinguish if the pine forest is extensively destroyed. Therefore, through migration from other areas, the genetic composition of these populations will drastically change. In extremely small populations, drastic changes might occur by random drift (Miko et al., 2009), which might be responsible by the dynamics observed in the distribution of PWN strains in the Kyushu region. In the present study, PWN sampling

was performed in 2012–14, whereas that of Nose et al. (2009) was performed in 2006–07. Therefore, population composition changes seem to have occurred within a single decade. Because there is a distance limit for vector insects' natural travel, human activities might be heavily involved in genetic dynamics.

Investigations on PWN genetic variations have been conventionally concentrated on the nuclear genome. In recent years, the number of studies on the mtDNA of PWN gradually increased, although its utilization mainly involved comparing *Bursaphelenchus xylophilus* with close relative species (Pereira et al., 2013; Sultana et al., 2013; Moreira et al., 2014). In Nematode, it has been widely confirmed that there are intraspecific mutations in mitogenomes (Thomas and Wilson, 1991), and intraspecific mutations are also recognized in some genes of pinewood nematodes (Valadas et al., 2013). In the present research, intraspecific variation surveys were quantitatively evaluated by the mitochondrial genome scale.

In previous intraspecific variation studies, a few mitochondrial genes were used, and sample (isolate) sizes were small. Furthermore, there was no analysis of individual nematodes. All these facts hampered the detection of detailed intraspecific variation (Valadas et al., 2013). In the present study, we clarified the high diversity of PWN mitochondrial genome by using a wide range of nucleotide sequence information and sufficient number of individuals (285 individuals) that enabled genetic population analysis based on haplotype analysis. Furthermore, the present study evidenced that the haplotype analysis of a single mitochondrial genome sequence is a valid approach for several aspects of PWN studies, including population phylogenetic analysis, genetic diversity evaluation, artificial migration possibility, and invasion and expansion pathways' elucidation.

Acknowledgments

The authors thank Fumihiko Miyahara (Institute of Agricultural and Forest Resources, Fukuoka Agriculture and Forestry Research Ctr., Japan) and Koji Matsunaga (Kyushu Regional Breeding Office, Forest Tree Breeding Center, Forestry and Forest Products Research Institute, Japan) for providing the technology necessary to collect pinewood nematodes (PWNs) in the forest, and for isolating and rearing them in the laboratory. The authors are also grateful to the research staff at Saga Prefectural Forestry Exp. Stn., Nagasaki Agricultural and Forestry Exp. Stn., Amakusa forest owners' cooperative, Miyazaki Prefectural Forest Exp. Stn., and Kagoshima Prefecture Forest Exp. Stn., Japan, who supported PWNs sampling in forests damaged by these parasites.

References

- Abelleira, A., Picoaga, A., Mansilla, J. P., and Aguin, O. 2011. Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in North-western Spain. *Plant Disease* 39(6): 776.
- Cardoso, J. M. S., Fonseca, L., and Abrantes, I. 2012. Direct molecular detection of the pinewood nematode, *Bursaphelenchus xylophilus*, from pine wood, bark and insect vector. *European Journal of Plant Pathology* 133(2): 419–425.
- Cheng, X. Y., Cheng, F. X., Xu, R. M., and Xie, B. Y. 2008. Genetic variation in the invasive process of *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae) and its possible spread routes in China. *Heredity* 100(4): 356–365.
- Dwinell, L. D. 1997. The pinewood nematode: regulation and mitigation. *Annual Review of Phytopathology* 35(1): 153–166.
- Fonseca, L., Cardoso, J. M. S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M., and Abrantes, I. 2012. The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *Helminthologia* 49(2): 96–103.
- Futai, K. 2008. Pine wilt in Japan, from first incidence to the present. Pp. 5–6 in B. G. Zhao, K. Futai, J. R. Sutherland, and Y. Takeuchi, eds. Pine wilt disease. Tokyo: Springer.
- Gosset, W. S. 1908. The Probable Error of a Mean. *Biometrika* 6(1): 1–25.
- Hauge, X. Y., and Litt, M. 1993. A study of the origin of 'shadow bands' seen when typing dinucleotide repeat polymorphisms by the PCR. *Human Molecular Genetics* 2(4): 411–415.
- Hu, S. J., Ning, T., Fu, D. Y., Haack, R. A., Zhang, Z., Chen, D. D., Ma, X. Y., and Ye, H. 2013. Dispersal of the Japanese pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae), in mainland China as inferred from molecular data and associations to indices of human activity. *PLOS One* 8(2): e57568.
- Iwahori, H., and Futai, K. 1993. Lipid peroxidation and ion exudation of pine callus tissues inoculated with pinewood nematodes. *Japanese Journal of Nematology* 23(2): 79–89.
- Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K., and Futai, K. 1998. PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundamental and Applied Nematology* 21(6): 655–666.
- Jung, J., Han, H., Ryu, S. H., and Kim, W. 2010a. Microsatellite variation in the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle in South Korea. *Genes and Genomics* 32(2): 152–158.

- Jung, J., Han, H., Ryu, S. H., and Kim, W. 2010b. Amplified fragment length polymorphism analysis and genetic variation of the pinewood nematode *Bursaphelenchus xylophilus* in South Korea. *Animal Cells and Systems* 14(1): 31–36.
- Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16(2): 111–120.
- Kimura, M. 1983. The neutral theory of molecular evolution. New York, NY: Cambridge University Press.
- Kishi, Y. 1988. Pine wilt disease (*Matsu kui mushi*), (translation by authors). Tokyo: Thomas Company, (In Japanese).
- Kiyohara, T., and Tokushige, Y. 1971. Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *Journal of the Japanese Forest Society* 53(7): 210–218, (In Japanese).
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33(7): 1870–1874.
- Leal, I., Foord, B., Allen, E., Champion, C., Rott, M., and Green, M. 2013. Development of two reverse transcription-PCR methods to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood. *Forest Pathology* 43(2): 104–114.
- Librado, P., and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25(11): 1451–1452.
- Linnertz, C., Saunders, A. M., Lutz, M. W., Crenshaw, D. M., Grossman, I., Burns, D. K., Whitfield, K. E., Hauser, M. A., McCarthy, J. J., Ulmer, M., Rand Allingham, R., Welsh-Bohmer, K. A., Roses, A. D., and Chiba-Falek, O. 2012. Characterization of the poly-T variant in the TOMM40 gene in diverse populations. *PLOS One* 7(2): e30994.
- Lowe, A., Harris, S., and Ashton, P. 2004. Ecological genetics: design, analysis, and application. UK: Blackwell Publishing.
- Madani, M., Subbotin, S. A., Ward, L. J., Li, X., and De Boer, S. H. 2010. Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome *b* genes. *Canadian Journal of Plant Pathology* 32(2): 252–263.
- Mamiya, Y and Enda, N. 1979. *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and its biology and pathogenicity to pine trees. *Nematologica* 25(3): 353–361.
- Mamiya, Y., and Shoji, T. 2009. Pathogenicity of the pinewood nematode, *Bursaphelenchus xylophilus*, to Japanese larch, *Larix kaempferi*, seedlings. *Journal of Nematology* 41(2): 157–162.
- Metge, K., and Burgermeister, W. 2006. Intraspecific variation in isolates of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) revealed by ISSR and RAPD fingerprints. *Journal of Plant Diseases and Protection* 113(6): 275–282.
- Miko, I., LeJeune, L., Chial, H., Drovdic, C., Koopman, M., Nelson, S. C., Spivey, A., and Smith, R. 2009. The genetic variation in a population is caused by multiple factors. Pp. 125 in I. Miko. and I. LeJeune, eds. Essentials of genetics, Unit 5.4. Cambridge, MA: NPG Education.
- Moreira, C., Asch, B., Fonseca, L., Pereira-Castro, I., Silva, R., Azevedo, L., Mota, M., Abrantes, I., Amorim, A., and Pereira, F. 2014. The mitochondrial genome of the pinewood nematode (*Bursaphelenchus xylophilus*) lineage introduced in Europe. *Mitochondrial DNA* 25(6): 420–421.
- Mota, M. M., Braasch, H., Bravo, M. A., Penas, A. C., Burgermeister, W., Metge, K., and Sousa, E. 1999. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology: International Journal of Fundamental and Applied Nematological Research* 1(7): 727–734.
- Mouhaddab, J., Ait Aabd, N., Achtaq, H., Msanda, F., Zahidi, A., Filali-Maltouf, A., Ferradouss, A., EL Modafar, C., Nejmeddine, M., and EL Mousadik, A. 2015. Patterns of genetic diversity and structure at fine scale of an endangered Moroccan endemic tree (*Argania spinosa* L. Skeels) based on ISSR polymorphism. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 43(2): 528–535.
- Murray, V., Monchawin, C., and England, P. R. 1993. The determination of the sequences present in the shadow bands of a dinucleotide repeat PCR. *Nucleic Acids Research* 21(10): 2395–2398.
- Nei, M., and Tajima, F. 1981. DNA polymorphism detectable by restriction endonucleases. *Genetics* 97(1): 145–163.
- Nose, M., Shiraishi, S., Miyahara, F., Ohira, M., Matsunaga, K., Tobase, M., Koyama, T., and Yoshimoto, K. 2009. Ribosomal DNA haplotype distribution of *Bursaphelenchus xylophilus* in Kyushu and Okinawa islands, Japan. *Journal of Nematology* 41(3): 194–202.
- Pereira, F., Moreira, C., Fonseca, L., Van Asch, B., Mota, M., Abrantes, I., and Amorim, A. 2013. New insights into the phylogeny and worldwide dispersion of two closely related nematode species, *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*. *PLOS One* 8(2): e56288.
- Picard, D., Sempere, T., and Plantard, O. 2007. A northward colonisation of the Andes by the potato cyst nematode during geological times suggests multiple host-shifts from wild to cultivated potatoes. *Molecular Phylogenetics and Evolution* 42(2): 308–316.

- Plantard, O., Picard, D., Valette, S., Scurrah, M., Grenier, E., and Mugniery, D. 2008. Origin and genetic diversity of western European populations of the potato cyst nematode (*Globodera pallida*) inferred from mitochondrial sequences and microsatellite loci. *Molecular Ecology* 17(9): 2208–2218.
- Robertson, L., Arcos, S. C., Escuer, M., Merino, R. S., Esparrago, G., Abellera, A., and Navas, A. 2011. Incidence of the pinewood nematode *Bursaphelenchus xylophilus* Steiner & Buhner, 1934 (Nickle, 1970) in Spain. *Nematology: International Journal of Fundamental and Applied Nematological Research* 13(6): 755–757.
- Rodrigues, J. M. 2008. National eradication programme for the pinewood nematode. Pp. 5–44 in M. M. Mota and P. Vieira, eds. Pine wilt disease: A worldwide threat to forest ecosystems. Heidelberg, Germany: Springer.
- Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4): 406–425.
- Schrader, G., and Unger, J. G. 2003. Plant quarantine as a measure against invasive alien species: the framework of the international plant protection convention and the plant health regulations in the European Union. *Biological Invasions* 5(4): 357–364.
- Shi, J., Chen, F., Luo, Y. Q., Wang, Z., and Xie, B. Y. 2013. First isolation of pine wood nematode from *Pinus tabulaeformis* forests in China. *Forest Pathology* 43(1): 59–66.
- Steiner, G., and Buhner, E. M. 1934. *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. *Journal of Agricultural Research* 48(1): 949–951.
- Sultana, T., Han, H., and Park, J. K. 2013. Comparison of complete mitochondrial genomes of pine wilt nematode *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* (Nematoda: Aphelenchoidea) and development of a molecular tool for species identification. *Gene* 520(1): 39–46.
- Takemoto, S., and Futai, K. 2007. Polymorphism of Japanese isolates of the pinewood nematode, *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae), at heat-shock protein 70A locus and the field detection of polymorphic populations. *Applied Entomology and Zoology* 42(2): 247–253.
- Thomas, W. K., and Wilson, A. C. 1991. Mode and Tempo of Molecular Evolution in the Nematode *Caenorhabditis*: Cytochrome Oxidase II and Calmodulin Sequences. *Genetics* 128(2): 269–279.
- Valadas, V., Laranjo, M., Barbosa, P., Espada, M., Mota, M., and Oliveira, S. 2012. The pine wood nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods. *Nematology* 14(8): 899–911.
- Valadas, V., Laranjo, M., Mota, M., and Oliveira, S. 2013. Molecular characterization of Portuguese populations of the pinewood nematode *Bursaphelenchus xylophilus* using cytochrome *b* and cellulase genes. *Journal of Helminthology* 87(4): 457–466.
- Van der Veer, M., and De Vries, E. 2004. A single nucleotide polymorphism map of the mitochondrial genome of the parasitic nematode *Cooperia oncophora*. *Parasitology* 128(4): 421–431.
- Vieira, P., Burgermeister, W., Mota, M., Metge, K., and Silva, G. 2007. Lack of genetic variation of *Bursaphelenchus xylophilus* in Portugal revealed by RAPD-PCR analyses. *Journal of Nematology* 39(2): 118–126.
- Wright, S. 1951. The genetical structure of populations. *Annals of Eugenetics* 15(4): 323–354.
- Wu, J. 2004. Forest invasive species and management in China. *Science and Technology Review* 4: 41–44.
- Yang, B. J. 1995. The pine wood nematode in China. Pp. 207–210 in B. J. Yang, ed. International symposium on pine wilt disease caused by pine wood nematode. Beijing: Chinese Society of Forestry.
- Yano, S. 1913. Investigation on the cause of pine mortality in Nagasaki prefecture. *Sanrinkoho* 4(Suppl): 1–14.
- Ye, W. M., GiblinDavis, R. M., Braasch, H., Morris, K., and Thomas, W. K. 2007. Phylogenetic relationships among *Bursaphelenchus* species (Nematoda, Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* 43(3): 1185–1197.
- Yi, C. K., Byun, B. H., Park, J. D., Yang, S. I., and Chang, K. H. 1989. First finding of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhner) Nickle and its insect vector in Korea. Seoul: Research Reports of the Forestry Research Institute 38: 141–149.
- Yun, J. E., Kim, J., and Park, C. G. 2012. Rapid diagnosis of the infection of pine tree with pine wood nematode (*Bursaphelenchus xylophilus*) by use of host-tree volatiles. *Journal of Agricultural and Food Chemistry* 60(30): 7392–7397.
- Zhang, X. Y., and Luo, Y. Q. 2003. Major forest diseases and insect pests in China. Beijing: Chinese Forestry Publishing.
- Zhao, B. G. 2008. Pine wilt disease in China. Pp. 18–25 in B. G. Zhao, K. Futai, J. R. Sutherland, and Y. Takeuchi, eds. Pine wilt disease. Tokyo: Springer.
- 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(3): 304–310.

Supporting information

Table S1. Nucleotide variations at 160 polymorphic sites in the 30 haplotypes (Ht) detected in Kyushu (see text for details).

Table S1. Nucleotide variations at 160 polymorphic sites in the 30 haplotypes (Ht) detected in Kyushu.

Gene / Region	Nucleotide position ^a	Reference position ^b	Haplotype (Ht)																													
			01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
cox1	40	447	A	T	T	T	.	T	.	T	T	.	T	.	.	
	43	450	C	T	T	T	.	T	.	T	T	.	T	.	.	
	61	468	G	A	A	.	.	.	A	A	.	A	.	A	A	.	A	.	.	
	175	582	C	T	T	.	.	.	T	T	.	T	.	T	T	.	T	.	.	
	202	609	T	C	C	C	.	C	.	C	.	.	C	.	.	
	229	636	C	T	T	T	.	T	.	T	.	.	T	.	.	
	231	638	C	T	T	
	253	660	C	T	T	T	.	T	.	T	.	.	T	.	.	
	274	681	C	T	
	289	696	A	G	G	G	G	.	G	.	G	.	G	.	.	
	292	699	T	C	C	.	.	C	.	.	C	C	C	C	C	C	C	C	C	C	C	
	340	747	T	C	
	367	774	A	G	G	G	G	.	G	.	G	.	G	.	.	
	394	801	G	A	A	A	A	.	.	A	.	A	.	A	.	A	.	.	
	472	879	A	G	
	490	897	T	C	C	C	.	C	.	C	.	C	.	.	
	511	918	T	C	C	C	C	.	.	C	.	C	.	C	.	C	.	.	
	518	925	G	.	.	A	.	.	.	A	
	544	951	G	A	A	.	.	T	.	.	A	A	.	.	A	.	A	.	A	.	A	.	.		
	556	963	T	A	A	A	A	.	A	.	A	.	A	.	.	
628	1035	A	G		
634	1041	G	.	.	.	A	A	A	A	A	A	A	A	A	A	A	A	.	A			
682	1089	A	G	G	G	.	G	.	G	.	G	.	.		
703	1110	T	C	C	C	.	C	.	C	.	C	.	.		
724	1131	T		
796	1203	A	G	G	G	.	G	.	G	.	G	.	.		
823	1230	A	G	G	G	.	G	.	G	.	G	.	.		
829	1236	T	C		

868	1275	C	T	T	T	.	.	.	T	.	.	
877	1284	T	
919	1326	C	T	T	T	.	.	.	T	.	.	
934	1341	G	A	A	A	.	A	.	.	A	.	.	
940	1347	T	C	
961	1368	G	A	A	A	.	A	.	.	A	.	.	
1024	1431	A	G	G	G	.	G	.	.	G	.	.	.	
1072	1479	A	G	G	G	.	G	.	.	G	.	.	
1099	1506	A	C	
1116	1523	A	G	G	.	.
1122	1529	G	A	.	A
1160		A	-	-	-	.	.	.	-	.	.	.
1161		T	-	-	-	.	.	.	-
1162		A	-	-	-	.	.	.	-
1163		A	-	-	-	.	.	.	-
1166	1569	T	A	A	A	A
1168	1571	A	T	.	.	.
1216	1619	A	T	T	T	.	.	T	.	.	T
1233	1634	A	T
1364	1765	T	C
1586	1987	A	G	G	G	.	.	G	.	.	G	.
1688	2089	A	G	.	G
1754	2155	C	T
1825	2226	C	T	T	T	.	.	T
2015	2416	C	T	T	T	.	.	.	T
2021	2422	G	A	A	A	.	A	.	A	.	A	.	.
2036	2437	C	T	T	T	.	T	.	T	.	T	.	.
2179	2579	A	G	G	G	.	G	.	.	G	.	.	G	.
2182	2582	G	A
2324	2724	T	A	A	A	.	A	.	A
2328	2728	G	A
2411	2812	T	A	A	A	.	A	.	A	.	A	.	A
2450	2851	C	T	T	T	.	T	.	T	.	T	.	T	.
2842	3242	T	A	A	A	.	A	.	A	.	A	.	A	.

