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

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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 8 kb (~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 700,000 m^3 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 510,000 ha 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, 1000 km 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 Buhrer, 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 (*cyt*b) 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 *I* (*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 16 mm. Nematodes

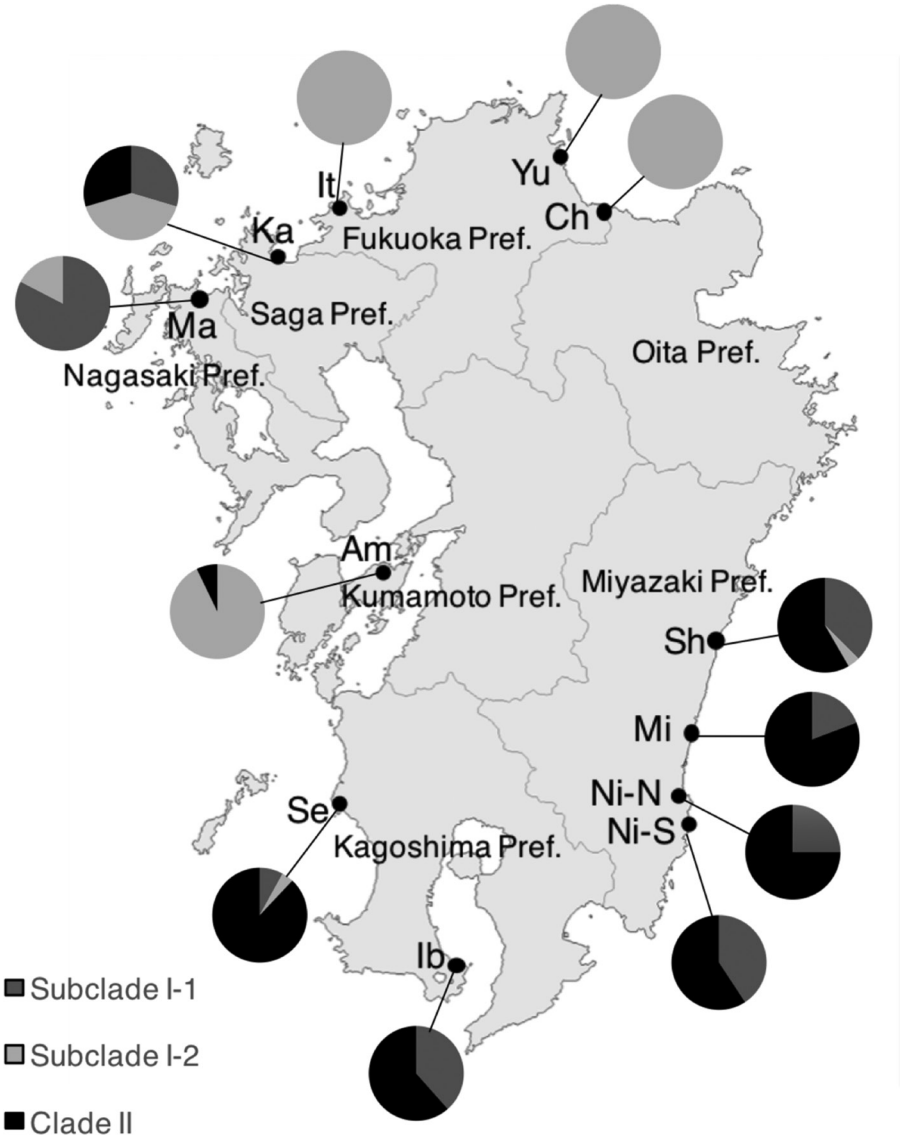

Figure 1: Location of the studied populations of *Bursaphelenchus xylophilus* (black dots) in Kyushu and the group of haplotype distribution of each population. The names of populations are written in code as indicated in Table 1. The classification of haplotypes is presented as in Figure 2.

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′-TCCTCCATTAAGAACTTTAGGGCAT-3′) and reverse (5′-TACAGTCAAAGCAATAGGACGAGA-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

[a]Number of individuals analyzed in each population. Total number of individuals analyzed = 285; [b]The number of trees in each population that became the source of the sequenced nematodes.

about 8 kb 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-µl PCR mixture [1× Gflex PCR Buffer (Takara), 0.43 µM 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 10 s and 68°C for 8 min. 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 extracted and were used for assembly into the mtDNA sequence (ca. 8 kb) 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 8,060 bp, corresponding to 54.5% of the PWN mitochondrial genome (ca. 14.8 kb), was sequenced. In the PWN mitochondrial genome, there is an extremely AT-rich and extensive non-coding region of about 1.7 kb (13,129-14,778 of GQ332424), while all mitochondrial genes are placed in the remaining 13.1 kb (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 51 bp, 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 8,060 bp 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/41 bp. Most of these SNPs (40 SNPs, 1 SNP/39 bp) were found in *nad5*, but *cox1* showed the highest average occurrence of SNPs (39 SNPs, 1 SNP/30 bp). Genes coding for NADH dehydrogenase subunits 1 and 6 (*nad1* and *nad6*) presented high number and average occurrence of SNPs (22 SNPs, 1 SNP/40 bp and 13 SNPs, 1 SNP/33 bp, 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/57 bp) and seven (1 SNP/60 bp) 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/99 bp). 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/223 bp). 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; 6-12 bp) 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 (*rrnS* and large subunit rRNA gene (*rrnL*)) found here contained six and seven SNPs, respectively, thereby showing a lower average occurrence of SNPs than the protein-coding genes (1 SNP/116 bp for *rrnS* and 1 SNP/134 bp for *rrnL*). The total length of the six non-coding regions identified here was 50 bp (1-20 bp 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 15 bp but contained two SNPs, thereby presenting an extremely high occurrence rate of SNPs (1 SNP/7.5 bp). 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 (Tv), 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.*

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

	trnC-trnM	7										
	trnM-trnD	20										
	trnD-trnG	3	–	–	1	1/3	1	0	–			
	nad3-nad5	1										
	rrnS-trnS	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

[a]The nucleotide sequence length for that gene or region; [b]The average occurrence of single nucleotide polymorphisms (SNPs) or insertions/deletions (Indels) in that specific part of the sequenced mitochondrial genome; [c]Ts, transitions; Tv, transversions; [d]Syn, synonymous substitution; Non-syn, non-synonymous substitution; [e]The total length contains 9 bp 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 that 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 Chikujo) 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 Hd above 0.8, which is considered high haplotype polymorphism, while populations from Sendai, Miyazaki, and Itoshima showed Hd below 0.4 and Nh 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 Chikujo) were similar to each other, and common haplotypes were also detected in four populations from southeastern Kyushu (Shintomi, Miyazaki, North Nichinan, and South Nichinan).

Phylogenic 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 phylogenic 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.

	Haplotype																														
Population	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	Nh[a]	Hd[b]
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	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	1	10	98	5	1	2	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	0.4	3.5	34.4	1.8	0.4	0.7	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. [a]Number of haplotypes detected in the population; [b]Haplotype diversity, Hd = $(1-\sum xi^2)\ n/(n-1)$, where xi is the frequency of the ith haplotype and n is the sample size (Nei and Tajima, 1981).

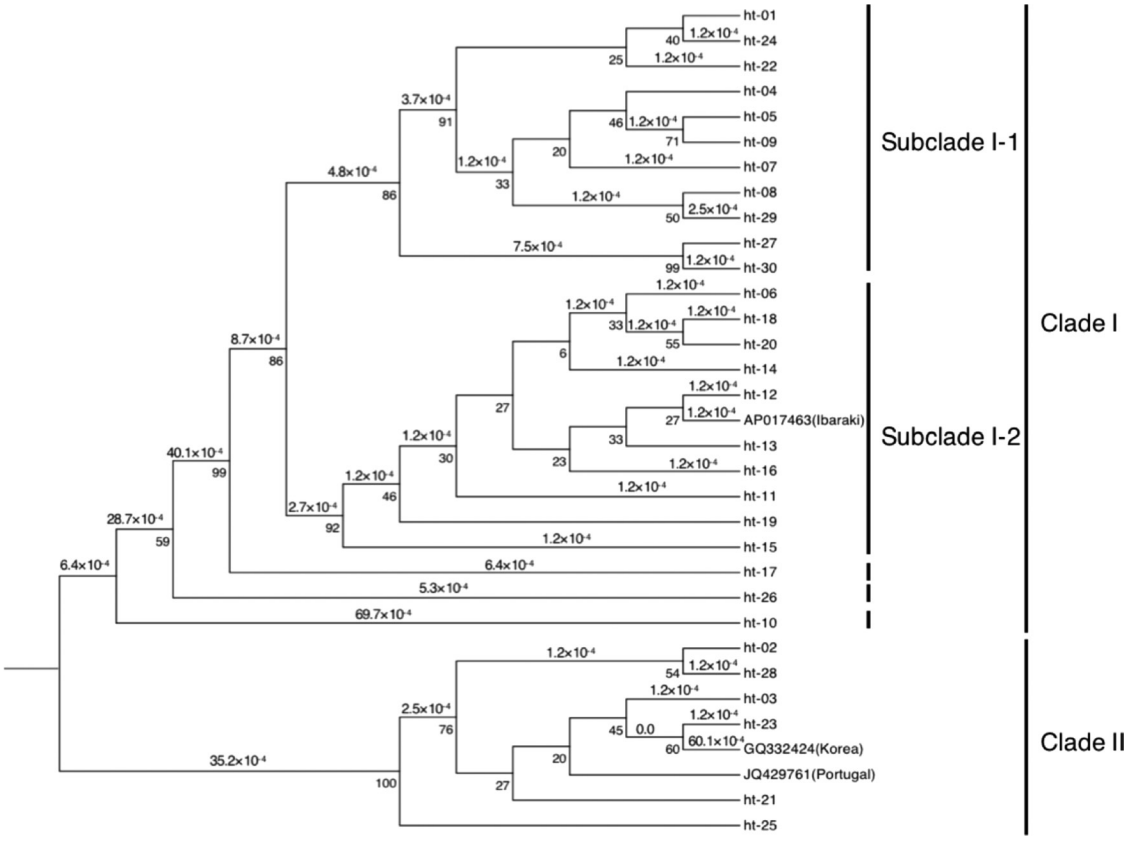

Figure 2: Phylogentic tree for 33 mitochondrial haplotypes of *Bursaphelenchus xylophilus* containing 30 from Kyushu and 3 from previous studies was inferred by using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model. Numbers under the branches represent support values from bootstrap 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 (Matsuura 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 8 kb, 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 (14,788 bp) or among the three Portuguese sequences (about 12 kb). 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*, *cyt*b, *nad5*, and *rrnS*) 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 *cyt*b 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 *rrnS* 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 *rrnS* 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.

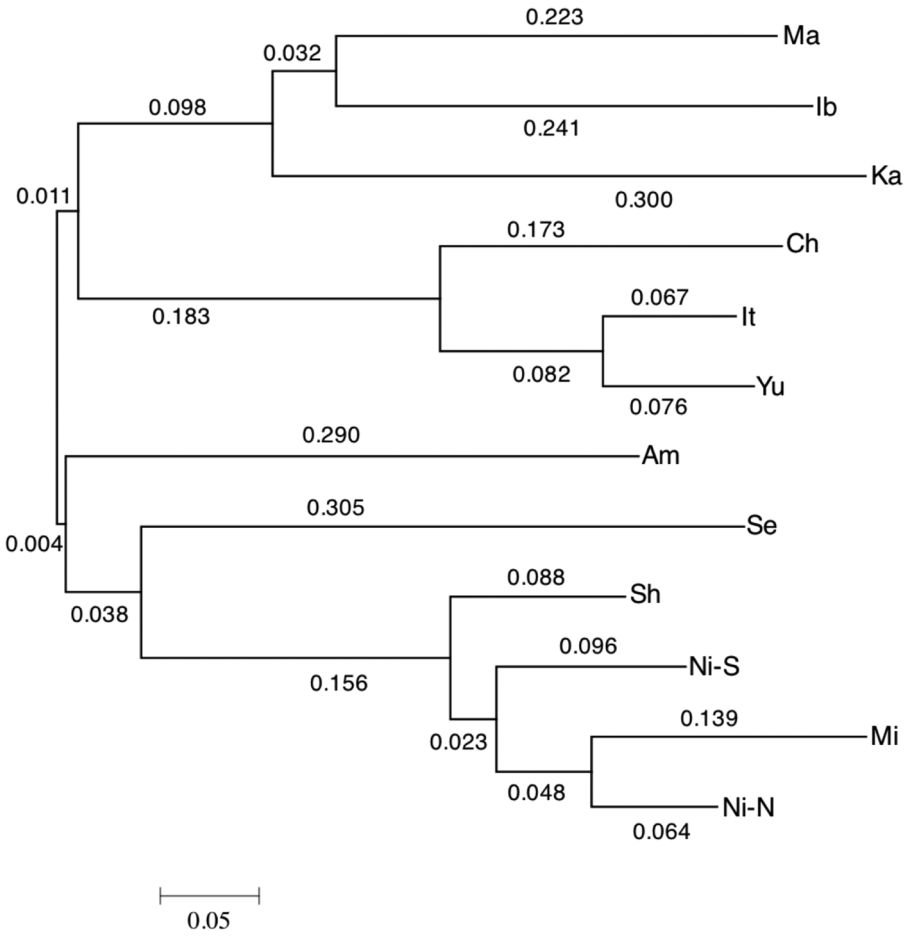

Figure 3: Neighbor-joining (NJ) tree diagram showing the genetic relationship that was determined using genetic distances of 12 populations. Numbers next to corresponding nodes indicate the branch lengths in the same units of the genetic distances that are computed based on the haplotype frequency for 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 (*Gst* = 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 Nm 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 Ds (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 *Gst* values for the three populations of northeastern Kyushu and four populations of southeastern Kyushu were 5.7% and 1.9%, and their Nm 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 Nm, 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 *Gst* and Nm for these seven populations were recalculated as 32.1% and 1.06, respectively. Because Nm 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, PWD 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 Ds 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.

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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	.	T	.	.
	231	638	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	T	.	.	.	.	.	.	.	.	.	.
	253	660	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	274	681	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	289	696	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	G	.	G	.	.
	292	699	T	C	C	.	.	C	.	.	.	C	C	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	.	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	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	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	.	T	.	.	T	.	.
	877	1284	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	919	1326	C	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	.	.	T	.	.
	934	1341	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
	940	1347	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cox1	961	1368	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
	1024	1431	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	1072	1479	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	1099	1506	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1116	1523	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
	1122	1529	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.
	1160		A	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	–	.	–	.	–	.	.	–	.	.	.	.	.
	1161		T	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	–	.	–	.	–	.	.	–	.	.	.	.	.
cox1-trnC	1162		A	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	–	.	–	.	–	.	.	–	.	.	.	.	.
	1163		A	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	–	.	–	.	–	.	.	–	.	.	.	.	.
	1166	1569	T	.	.	.	.	A	.	.	.	.	A	A	A	A	A	A	.	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1168	1571	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.
trnC	1216	1619	A	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	T	.	T	.	T	.	.	T	.	.	.	.	.
trnM	1233	1634	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
trnD-trnG	1364	1765	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1586	1987	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.	.	.	.
	1688	2089	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.
	1754	2155	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cox2	1825	2226	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.	.	.	.
	2015	2416	C	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.	.	.	.
	2021	2422	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	A	.	A	.	.	.	.	.
	2036	2437	C	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.	.	.	.
	2179	2579	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.	.	.	.
	2182	2582	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2324	2724	T	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.	.	.	.
rrnL	2328	2728	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2411	2812	T	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	A	.	A	A	.	.	.	.
	2450	2851	C	T	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	T	.	.	.	.
	2842	3242	T	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.	.	.	.

nad3	3152	3552	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3199	3599	A	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	3221	3621	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	G	.	G	.	G	.	.	G	.	.
	3231	3631	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	3242	3642	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	A	.	A	.	A	A	.	A	.	.
	3419	3819	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
nad5	3492	3892	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3507	3907	G	T	T	.	.	.	T	.	.	.	.	.	.	T	T	.	.	T	.	.	T	T	T	.	T	T	.	T	.	T
	3515	3915	A	.	.	.	.	T	.	.	.	.	T	T	T	T	.	T	.	T	.	T	.	.	.	.	.	.	.	.	.	.
	3516	3916	T	.	.	.	.	A	.	.	.	.	.	A	A	A	.	A	.	A	A	A	.	.	.	.	.	.	.	.	.	.
	3549	3949	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	C	.	.
	3643	4043	C	T	T	.	.	T	.	.	.	T	T	T	T	T	T	T	T	T	T	T	T	.	T	.	T	T	T	T	T	T
	3651	4051	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3706	4106	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	3810	4210	A	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	3864	4264	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	G	.	G	.	G	G	.	G	.	.
	3921	4321	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	3972	4372	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	A	.	A	.	.
	3975	4375	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3993	4393	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	4002	4402	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	4041	4441	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4140	4540	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
	4164	4564	A	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	4197	4597	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4235	4635	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4323	4723	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4329	4729	A	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	4374	4774	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4416	4816	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4449	4849	C	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	T	.	T	.	T	T	.	T	.	.
	4455	4855	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	A	.	A	.	.
	4514	4914	A	T	T	.	.	T	.	.	.	T	T	T	T	T	T	T	T	T	T	T	T	.	T	.	T	T	T	T	.	T
	4524	4924	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	A	.	A	.	A	A	.	A	.	.
	4587	4987	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.

nad5	4648	5048	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
	4653	5053	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	4716	5116	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4760	5160	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C
	4771	5171	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4776	5176	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	.	.	.
	4785	5185	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	4836	5236	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	.	.	T	.	.
	4937	5337	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	T
	4944	5344	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
	4962	5362	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
nad6	5238	5638	G	A	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
	5268	5668	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	5271	5671	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
	5293	5693	T	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
	5331	5731	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
	5373	5773	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	5388	5788	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5389	5789	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	5442	5842	A	.	.	.	.	.	.	.	.	.	G	G	G	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.
	5451	5851	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	5454	5854	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	A	.	.
	5545	5945	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5559	5959	T	.	.	C	C	.	C	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
nad4L	5651	6051	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	.	.	A	.	.
	5738	6138	G	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	5756	6156	C	T	T	.	.	T	.	.	.	T	T	T	T	T	T	T	T	T	T	T	T	.	T	.	T	.	.	T	.	.
	5768	6168	G	.	.	.	.	A	.	.	.	.	A	A	A	A	A	A	A	A	A	A	.	.	.	.	.	.	.	.	.	.
	5774	6174	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
rrnS	6028	6428	G	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	.	.	T	.	.
	6130	6529	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	6188	6588	G	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	.	.	T	.	.
	6343	6743	T	.	.	.	.	A	.	.	.	.	A	A	A	A	A	A	A	A	A	A	.	.	.	.	.	.	.	.	.	.
	6389	6789	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G
	6557	6956	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.

trnS	6665	7064	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
trnY	6748	7140	G	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6779	7171	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
	6802	7194	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	6814	7206	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	6847	7239	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6889	7281	G	A	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	A	.	A	.	.
	6934	7326	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6940	7332	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.
	6943	7335	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7009	7401	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7108	7500	C	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
nad1	7150	7542	A	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	7159	7551	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	A	A	.	A	.	.
	7189	7581	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7201	7593	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7306	7698	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	7309	7701	A	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	7359	7751	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7399	7791	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7435	7827	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	C	.	C	.	.
	7534	7926	A	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	7567	7959	C	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	7579	7971	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	T	T	.	T	.	.
	7760	8152	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7772	8164	T	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	7844	8236	T	C	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
atp6	7850	8242	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	C	.	.	C	.	.
	7865	8257	T	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	G	.	.	G	.	.
	7904	8296	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7985	8377	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

[a]The position of the SNPs and indels in the total sequence nucleotide in this study. [b]The position of the SNPs and indels in the reference sequence (GQ332424). Number indicates the position of the SNPs and indels in the total sequence nucleotide in this study; . indicates the same nucleotide with ht-01 at the polymorphic site; – indicates deletion. Blank indicates the position which was not exciting in the reference sequence.