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RESEARCH ARTICLE

Microsatellite variation in the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle in South Korea

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Abstract

The pinewood nematode *Bursaphelenchus xylophilus* is the causative agent of pine wilt disease, which has caused heavy economic losses to the South Korean forest industry. In this study, we investigated the genetic variation among South Korean pinewood nematodes using newly developed microsatellite loci. In order to ensure sufficient templates for the amplification of multiple loci required for individual identification, we employed an amplifying step of restricted fragments during the microsatellite development procedure. We found atypical genetic patterns in this non-native pest species: high allelic diversity and population structure. The large number of alleles may be the result of continuous and/or large-scaled introduction, which apparently went unnoticed before the first official report of pine wilt disease in Korea in 1998, or may come from gene pools of closely related species through genetic introgression after hybridization. Ecological properties of this species, such as a vector-mediated life cycle, may have contributed to its population structure, which may be enhanced by governmental efforts to prevent dispersal of this disease. As a geographic population structure was not observed, geographic patterns of genetic variation appear to be more affected by anthropogenic mediation than by natural dispersion through vector insects. And genotypes of Korean populations were compared to genotypes found in neighboring countries such as China and Japan.

Keywords Microsatellites; Pinewood nematode; *Bursaphelenchus xylophilus*; Pine wilt disease; South Korea

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Introduction

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970, is the causative agent of pine wilt disease. This disease's progression may be mediated by cerambycid beetles, particularly *Monochamus* spp. (Mamiya, 1972). First reported in North America, the pinewood nematode gained notoriety in Japan for devastating pinewood forests in the early 1900s. Subsequently, this disease (or this species) was transmitted to other countries in Southeast Asia, including Taiwan (Tzean and Jan, 1985), China (Cheng, 1983), and South Korea (Yi et al., 1989), and it has recently been reported in Portugal (Mota et al., 1999). However, routes of introduction into these countries have not been clearly identified.

In South Korea, pine wilt disease was first identified in the Gumjung Mountain Region of Busan in 1988 (Yi et al., 1989). During the 20 years since then, the area affected by pine wilt disease has grown to approximately 7,800 ha over 57 cities. Pine wilt disease mainly affects red pine trees (*Pinus densiflora*) and black pine trees (*P. thunbergii*), which are the most abundant *Pinus* species in South Korea. Recently, it has been reported that this disease also affects Korean pine trees (*P. koraiensis*), which are found in the forest plantations of northern South Korea, around Gyeonggi and Gangwon provinces. Coniferous trees, including those of the genus *Pinus*, that are susceptible to pine wilt disease occupy about 35% of Korea's total forest area. As pine trees are important for both the natural resources they provide to the economy, and also for their cultural significance, the South Korean government has made

every endeavor to control this disease after the first infection was reported.

Most efforts for controlling pine wilt disease in South Korea, such as fumigation, injection of nematicides into tree trunks, and aerial spray of pesticides, focus on the prevention of disease transmission to other regions (Shin and Han, 2006). However, pine wilt disease has occurred sporadically every year in spite of these efforts. In order to effectively control this disease, we must understand its routes of transmission and related mechanisms of dispersal. Collection of information on pinewood nematodes is challenging, both because they may be dispersed via anthropogenic activities and also because their population dynamics are complex (Jones et al., 2008). Highly polymorphic molecular markers such as microsatellites could be helpful to elucidate the above questions regarding this species of pests.

Microsatellites exhibit extensive polymorphisms, and are easily genotyped by simple polymerase chain reactions (PCRs). These factors make microsatellites popular markers for population genetics and molecular ecology studies (Jarne and Lagoda, 1996). Moreover, if multiple microsatellite loci are analyzed, the results can be used to identify individuals. Hence, microsatellite analysis and tracking may be useful for understanding the epidemiology of pest species (Yu et al., 2001; Baliraine et al., 2003). Recently, Zhou et al. (2007) have reported that microsatellite markers may be useful for studying pine wilt disease. To meet this goal, however, adequate loci are needed for the identification of individuals, and a method must be designed to ensure sufficient amounts of template for multiple loci analysis, which is difficult to obtain from small organisms. In this study, we investigated genetic variation in pinewood nematodes from South Korea, and compared them with those of neighboring countries such as China and Japan by using newly developed microsatellite loci and a method, modified from part of the microsatellite development procedure, for the generation of sufficient amount of templates to amplify multiple loci.

Materials and Methods

Sampling and maintenance of isolates

We selected 68 individuals from 16 regional isolates of *B. xylophilus* sampled from Korea (Fig. 1), 8 individuals from 3 regional isolates from China (Nanjing, Sichuan, and Zhejiang), and 6 individuals from 3 regional isolates from Japan (C, S, and T) (Table 4). Ten individuals from isolates of *B. mucronatus* Mamiya and Enda, 1979, which were obtained from dead or dying trees and had little or no pathogenecity, were used for testing cross-species amplification. The nematodes were extracted from wood chips by Baermann's funnel method (Ayoub, 1977) and inoculated on a lawn of *Botrytis cinerea* cultured on potato dextrose agar. The plate was incubated in a chamber at 25℃ for 5 days. The cultures were subsequently maintained on *B. cinerea,* on steam-sterilized barleycorn, at 25℃ for 10 days, and then stored in a refrigerator at 6℃ until use.

Figure 1. Map denoting sampling locations in South Korea.

DNA extraction

The sample nematodes were picked up from *B. cinerea* culture plates and washed 3 times with sterile deionized water. A single female nematode was transferred to a 1.5-ml microcentrifuge tube containing a cell lysis buffer and proteinase K. The tube was then incubated in a water bath at 55℃ for 12 h. The remaining steps were performed by using the Qiagen DNeasy tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instruction, except that the final volume of DNA extract was made up to 20 ㎕ instead of 200 ㎕.

Microsatellite loci development

The nematodes were screened for the presence of microsatellite loci, and colony hybridization of microsatellite-containing clones was performed according to the procedure described by Jung et al. (2006, 2007). The DNAs of selected colonies were sequenced using the BigDye terminator ready reaction mix (Applied Biosystems, Foster City, CA) and each M13 primer. The DNA sequences were determined using the Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA) and were confirmed using Sequence Navigator software (version 1.1, PE, Applied Biosystems, Foster City, CA). Using

the PRIMER3 program (Rozen and Skaletsky, 2000), the DNA sequence obtained from each clone was used to design PCR primers for microsatellite region amplification. One of the primer pairs used to amplify each locus was labeled with a fluorescent dye, such as HEX, NED, or FAM.

Amplification of microsatellite loci from individual nematodes

In general, most nematodes are so small that the total DNA from an individual is inadequate for the collection of multiple loci's genotypes. This prohibits the performance of precise analyses, such as population assignment or individual identification (fingerprinting), which requires many polymorphic loci. However, in this study we used amplification of linker-attached fragments, which was originally used as an enrichment method for the development of microsatellite loci (Hammond et al., 1998), to generate enough templates for the genotyping of multiple loci. Hammond et al. (1998) used SAULA primers to enrich microsatellites, but this method can also be used to obtain sufficient templates for genotyping of multiple loci. The DNA of each nematode was treated with *Sau3*AI for 30 min, and the SAULA / B linkers were ligated to the sticky ends of fragments cut by restriction enzymes. These endonuclease-generated fragments were amplified, using SAULA primer, under the same conditions as those used in the first PCR. These fragments were then used as templates in microsatellite genotyping. In our study, the microsatellite loci were amplified under the following conditions: the reaction mixture (total volume, 25 ㎕) of each sample contained 0.2 ㎕ of template DNA, 0.5 ㎕ of each primer (10 μM), 0.5 ㎕ of dNTP solution (10 mM), 0.5 ㎕ of $MgCl_2$ (25 mM), 5 ㎕ of *Taq* buffer (5 ×), 0.2 ㎕ of *Taq* DNA polymerase (Promega, Madison, WI), and 18.1 ㎕ of distilled water (DW). PCR was performed on a GeneAmp 9700 unit, and included the following steps: initial denaturation at 94℃ for 5 min, 30 cycles of denaturation at 94℃ for 30 sec each, annealing at a specific temperature depending on the locus (Table 1) for 50 sec, elongation at 72℃ for 1 min, and final extension at 72℃ for 30 min. The PCR products were then analyzed using the Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA) and GeneMapper software (version 4.0, Applied Biosystems, Foster City, CA). Results of genotypes were checked again with eyes and genotypes, if ambiguous, were manually scored according to appendix S2 of Selkoe and Toonen (2006).

Analyses of the genotypic data

The observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using ARLEQUIN 3.11 (Excoffier et al., 2005), and the Hardy-Weinberg equilibrium (HWE) was tested by using GENEPOP 3.4 (Raymond and Rousset, 1995). Further, pairwise linkage disequilibrium was analyzed using contingency tables from Fisher's exact test with GENEPOP 3.4. Analysis of molecular variance (AMOVA) (Weir and Cockerham, 1984) was performed using ARLEQUIN 3.11, and the genetic variability of the hierarchical structure of the nematode isolates was assessed. Pairwise R_{ST} values, representing the genetic distances between the population pairs, were calculated using the ARLEQUIN 3.11. The associated distance matrix was used to construct a neighbor-joining (NJ) tree, showing the genetic relationships among the isolates, by using the MEGA 4.0.1 (Tamura et al., 2007). STRUCTURAMA (Huelsenbeck and Andolfatto, 2007) was used for Bayesian clustering analysis of populations, and assignments of in-

Table 1. Characteristics of the 7 microsatellite loci used to study the pinewood nematode, *Bursaphelenchus xylophilus*.

Locus (GenBank†)	Repeat Motif	Forward Primer / Reverse Primer	No. Alleles	Size Range	T_A‡ (℃)
BX1017 (FJ640843)	$(GT(/A))_{68}$	AGGGAGCTCGTGTGTGTGT GTCAAGCAGGCAGGCAGT	50 (48)	98−212	53
BX1063 (FJ640844)	$(GT)_{22}$	TGGAAGCTTGGGATCAGC GGGGTACGTGACAAGACCAT	20	104−158	53
BX1407 (FJ640845)	$(CA)_{71}$	GACCAGGCCACACACACTC AAGCTTGGGATGACAAGACG	42 (37)	127−267	55
BX2231 (FJ640846)	$(GT)_{92}$	TTCGCCACTCTAACCACTCA CCACCAACACCTCATCACAC	41 (35)	75−235	46
BX2260 (FJ640847)	$(GT)_7N_5(GT)_3N_{13}(GT)_{15}$	AAGACACAGGATGTAAACAGTCAGA AACGTTGCTGAACACACACA	35 (33)	100−178	45
BX2264 (FJ640848)	$(CAA(/G))_{40}$	AAGCTTGGGATCAGCAACAA ATCTGCCAGCAATGGTTCTT	22 (18)	139−229	45
BX2297 (FJ640849)	$(GTT)_{18}$	TCCAAGGTTTTATCCCAGCTT TGCAATAAAACGCAACTCTACAA	33 (29)	81−149	46

†GenBank accession number; ‡Annealing temperature.

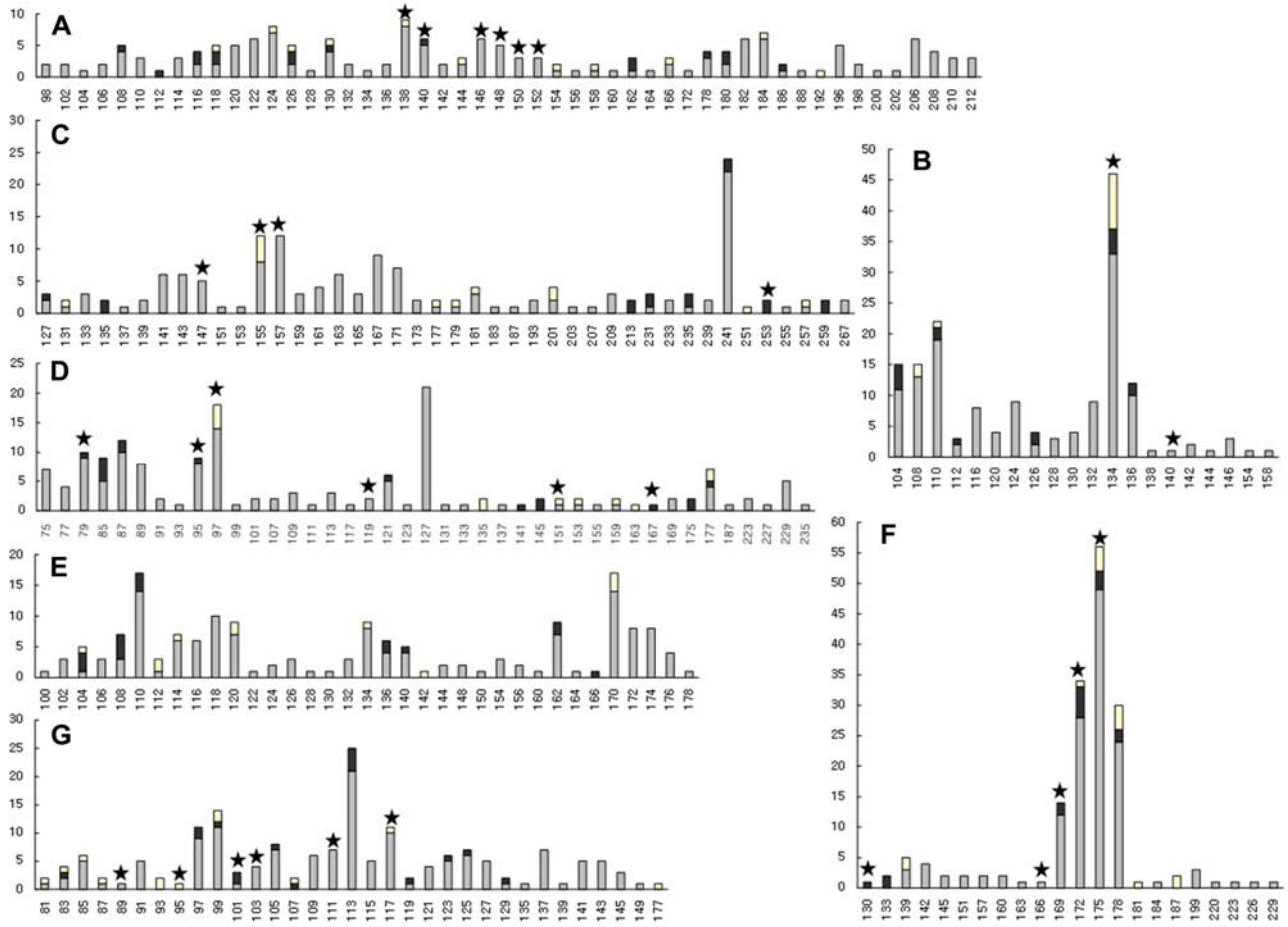

Figure 2. Alleles of each microsatellite locus of *Bursaphelenchus xylophilus*: BX1017 (A) BX1063 (B) BX1407 (C) BX2231 (D) BX2260 (E) BX2264 (F) and BX2297 (G). Numbers under the horizontal axis represent the allele size, and numbers to the left of the vertical axis represent the allele frequency. The alleles of the individuals from Korea, China, and Japan are represented as gray, black, and white bars, respectively. Stars above the bars represent that the alleles were also observed in *B. mucronatus*.

dividuals to these populations. For Bayesian clustering analysis, the number of generations and chains was set as 100,000 and 10, respectively, and the priors of number of populations and expected number of populations were randomly drawn from gamma distribution (shape parameter = 2.5 and scale parameter = 0.5).

Results

Development and characterization of polymorphic microsatellite loci

We screened the genomes of 180 clones for the identification of microsatellite loci, and we used flanking sequences to design primer sets for 42 of these clones. During test amplification, many loci were excluded due to lack of polymorphisms or difficulty in scoring; however, seven polymorphic microsatellite loci were eventually identified and characterized (Table 1). Hence, the linker-attached fragment amplification method may be successful in genotyping multiple loci of microsatellite (more than 10) from one individual. This method, therefore, can be used to study microsatellite genetic variations of organisms otherwise too tiny to amplify multiple loci.

Among the characterized microsatellites, BX2264 and BX2297 were trinucleotide repeats, while the others were dinucleotide repeats. The overall allelic diversity was high, ranging from 18 to 48 (Fig. 2 and Table 1), and the observed heterozygosities in Korean isolates varied from 0.47059 to 0.77941, and were significantly lower than the heterozygosity values expected under Hardy-Weinberg equilibrium (0.799–0.97658) (Table 2). Compared to the results found when pinewood nematode genotypes from Korea were pooled, the heterozygosities of each isolates, on the whole, fitted to the ex-

Table 2. Observed heterozygosities (H_O) and expected heterozygosities (H_E) under Hardy-Weinberg equilibrium in the population of pinewood nematodes from Korea, China, and Japan.

Loci	Population	Korea	China	Japan
	No. sample	68	8	6
BX1017	H_O	0.73529***	0.75000^{ns}	1.00000^{ns}
	H_E	0.97658	0.95833	1.00000
BX1063	H_O	0.47059***	0.25000***	0.16667^{ns}
	H_E	0.89477	0.92500	0.43939
BX1407	H_O	0.66667***	0.12500***	0.66667*
	H_E	0.94356	0.92500	0.89394
BX2231	H_O	0.50000***	0.50000**	0.33333***
	H_E	0.94298	0.92500	0.87879
BX2260	H_O	0.57353***	0.25000***	0.66667^{ns}
	H_E	0.95392	0.88333	0.92424
BX2264	H_O	0.77941***	0.87500^{ns}	0.66667^{ns}
	H_E	0.79946	0.85000	0.87879
BX2297	H_O	0.67647***	0.62500**	0.66667**
	H_E	0.94390	0.93333	0.96970

ns, non-significant; * $0.05 < P < 0.01$; ** $0.01 < P < 0.001$; *** $P < 0.001$.

pected ones (data not shown), which might be the result of random mating occurring within the culture dishes of each isolate. Non-random associations between loci were observed only in the BX1063 and BX2231 pair ($P = 0.049$). All the loci from *B. xylophilus* were amplified in *B. mucronatus,* and were polymorphic. Some alleles from *B. mucronatus* lay within the allelic size range of *B. xylophilus* (Fig. 2).

In spite of inadequate heterozygosity, high allelic diversity ensured that each individual in this study could be unambiguously identified, which underlines the usefulness of these markers in the epidemiological study of pine wilt disease.

Comparison with Chinese and Japanese specimens

Individuals from the Korean population were indistinguishable from those from the Japanese and Chinese populations in terms of their allelic and genotypic compositions. More loci seemed to be under the HWE in the populations of Japan and China than in those of Korea, which might be attributed to the smaller sample sizes taken from Japan and China. Though a few of alleles appeared only in Japan and China, most alleles were observed in all three countries, or in at least in two of them (Fig. 2). This suggests that pinewood nematode populations from these countries are closely related.

Population genetic structure

Hierarchical AMOVA results showed that most genetic variance lay within isolates (Table 3). Genetic variance among the Korean, Chinese and Japanese groups was significant but small (5.6%). The neighbor-joining tree, inferred from pairwise R_{ST} values, represents two groups of isolates: clade 1 and clade 2. Isolates from Chinese and Japanese populations belonged exclusively to the former and the latter clades, respectively (Fig. 3). Genetic variability between the two clades in South Korea was low but significant (Table 3). To investigate geographic genetic structure, we divided Korean isolates into three geographic groups (N, W and S) (Fig. 1), and then performed AMOVA on these groups. However, we could not find significant genetic variance among geographic groups (N vs. S vs. W) (Table 3). Bayesian clustering analysis demonstrates that the posterior estimate of number of populations is 6.7278 among individuals from 3 countries (Fig. 4). Based on the assignment of individuals into populations, the majority of Korean individuals belonged to population 2, and the majority of individuals from China and Japan belonged to populations 2 and 3 respectively (Table 4). We found a genetic

Table 3. Results of analysis of molecular variance (AMOVA).

Source of Variation	Variance Components	Percentage Variation	F-statistics
Korea vs. China vs. Japan			
Among Groups	0.05061	5.6	0.01561*
Among Isolates Within Groups	0.28157	25.3	0.08822**
Within Isolates	2.91005	69.1	0.10245**
Clade 1 vs. Clade 2 within Korea			
Among Groups	0.05438	1.69	0.01687**
Among Isolates Within Groups	0.24048	7.46	0.07587**
Within Isolates	2.92914	90.85	0.09146**
N vs.S vs.W within Korea			
Among Groups	0.00936	0.29	0.00293^{ns}
Among Isolates Within Groups	0.25787	8.07	0.08091**
Within Isolates	2.92914	91.64	0.08361**

ns, non-significant; * $0.05 < P < 0.01$; ** $P < 0.01$.

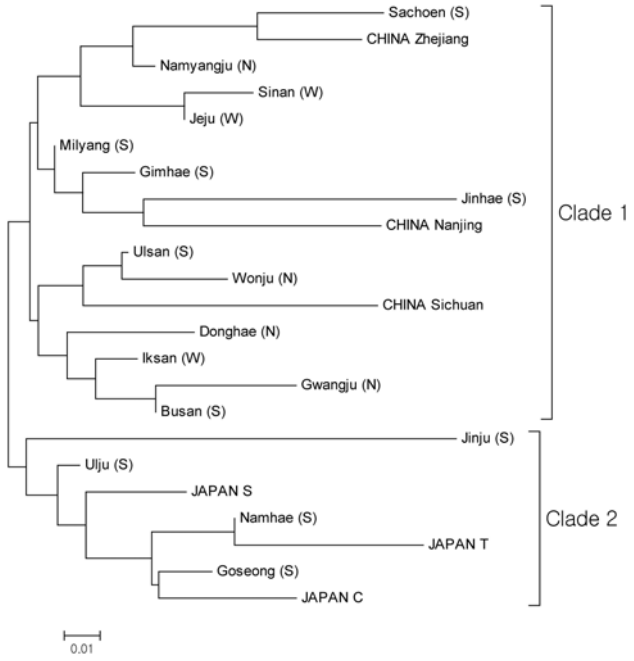

Figure 3. Neighbor-joining tree constructed using the pairwise R_{ST} values of the *Bursaphelenchus xylophilus* samples, indicating the genetic relationship between the isolates of *B. xylophilus*. Geographical groups in South Korea represented in Figure 1 are indicated within the parentheses.

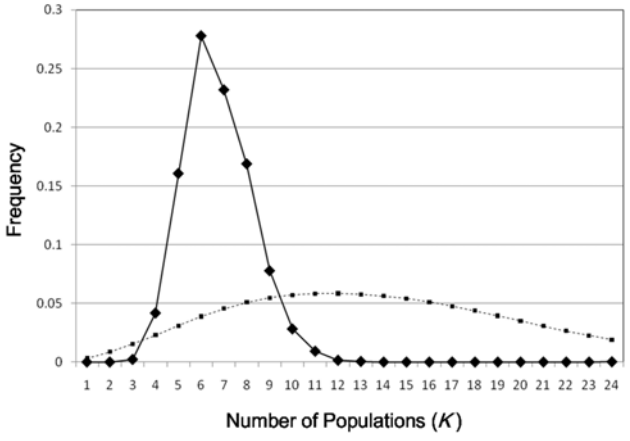

Figure 4. Posterior distribution of number of populations (K) using STRUCTURAMA (Huelsenbeck and Andolfatto, 2007). The dotted line indicates prior distribution and solid line represents posterior distribution of number of populations.

structure of pinewood nematodes in these countries, and a close relationship among the populations of these countries.

Discussion

Introduced populations, generally express less genetic diversity (allelic diversity) than their source populations because only a proportion of individuals could be introduced from source populations (Tsutsui et al., 2000; Sakai et al., 2001). Furthermore, introduced populations, if not genetically structured (i. e., in random mating states), can reach HWE within a few of generations, although they may initially demonstrate an inbreeding-associated excess homozygosity. Patterns of genetic variation, such as large numbers of alleles and genetic structures, observed in pinewood nematodes from South Korea, are not typical in introduced organisms.

The high allelic diversity observed in this study must be related to the large effective size of the population. One possible explanation is that this pest species has been introduced to this region continuously and/or on a large scale. The first occurrence of pinewood nematodes in South Korea was officially reported in 1988, and they have been strictly quarantined since then (Shin and Han, 2006); therefore, the chance of mass-introduction since that time is low. However, pine wilt disease occurred in South Korea's neighboring countries earlier: it occurred in Japan in 1905 (Mamiya, 1988; Takemoto and Futai, 2007) and in China in 1982 (Cheng, 1983). These countries have always maintained close commercial, cultural, and political ties with South Korea, and, therefore, the time and scale of introduction of pinewood nematodes might have

Table 4. Results of Bayesian clustering analysis using STRUCTURAMA (Huelsenbeck and Andolfatto, 2007).

Location	Number of sample	Assigned population					
		1	2	3	4	5	6
South Korea							
Gwangju	6	4	2				
Iksan	11	8	2	1			
Jinhae	4	4					
Namhae	6		3	3			
Gimhae	7	2	5				
Ulju	4	1	2	1			
Goseong	6	1	3	2			
Milyang	6		5	1			
Busan	2	1		1			
Jinju	2			1	1		
Ulsan	2		2				
Sacheon	2		1	1			
Sinan	2		2				
Jeju	2		2				
Donghae	2	1	1				
Namyangju	2		2				
Wonju	2		2				
Japan C	2			2			
Japan S	2	1		1			
Japan T	2		1	1			
China Nanjing	3	1	1			1	
China Sichuan	2		1				1
China Zhejiang	3	1	2				

been underestimated. Chinese researchers also suspected the large scale of introduction of this pest species into China from the results of the genetic variability of amplified fragment length polymorphism (AFLP) markers (Cheng et al., 2008).

Another possible explanation for high allelic diversity is genetic introgression of alleles from closely related species. Genetic introgression from related species through hybridization may play an important role in increasing genetic diversity of introduced organisms (Zidiana et al., 2009). Genetic introgression among *Bursaphelenchus* spp. has been reported (De Guiran and Bruguier, 1989), and it is highly probable that the gene pool of *B. xylophilus* in this region has been influenced by closely related species, including *B. mucronatus*, which dominates this region and shares habitats with *B. xylophilus* (Kanzaki and Futai, 2002; Ye et al., 2007). This could partially explain why alleles from most loci occurred commonly in both species, with the exception of BX2260 (Fig. 2). Though this finding may also have arisen due to homoplasy or the conservation of ancestral polymorphisms between species, we cannot exclude the possibility of genetic introgression.

Our results suggest that some factors have hampered random mating among isolates within South Korea. These factors seem to have led to population structure. Ecological properties of this pest, such as a parasitic life cycle, may contribute to this pattern. The genetic structure of populations of parasites such as pinewood nematodes is dependent on the parasite's life cycle (Barrett et al., 2008). *B. xylophilus* needs vector beetles to move into other pine trees (habitats). Due to vector-mediated dispersion, newly colonized isolates must have descended from its parental isolates. Strong quarantine activities might enhance the structure of pest populations by restricting gene flow. Once pine wilt disease occurs in South Korea, transmission of the disease to other region is thoroughly blocked by taking immediate and decisive measures such as the removal of infected trees and treatment of nematicides, which may strongly interrupt gene flow among neighboring isolates. The genetic structure of the South Korean pinewood nematode population seems to be unrelated to geography, according to the results of neighbor-joining analysis (Fig. 3), AMOVA (Table 3), and Bayesian clustering (Table 4). This pattern could be explained by anthropogenic activities such as unintentional movement of infected trees rather than natural dispersion via vector beetles.

Comparing pinewood nematode genotypes from South Korea with those from China and Japan, it is clear that regional isolates from these countries are closely related to each other. The Korean population of pinewood nematodes, therefore, may have been influenced by or introduced from populations of both countries. This conclusion has also been supported by the results found using AFLP, another type of genetic marker (Jung et al., 2010). Although, according to the neighbor-joining analysis, isolates from Korea demonstrate a closer relationship to those from China than those from Japan (Fig. 3), the sample size used in this study is too small to yield definitive results. Nevertheless, our results highlight the usefulness of microsatellite markers and the method for template preparation by amplification of multiple loci for the study of nematodes and other small organisms.

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