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Amplified fragment length polymorphism analysis and genetic variation of the pinewood nematode *Bursaphelenchus xylophilus* in South Korea

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The pinewood nematode *Bursaphelenchus xylophilus* causes pine wilt disease and is a serious economic concern for the forest industry of South Korea. To achieve effective control with limited resources, it is necessary to clarify the transmission routes and mechanisms of dispersal of this organism. Highly polymorphic and easy-to-use molecular markers can be used for investigating this aspect. In this study, we evaluated the usefulness of amplified fragment length polymorphisms (AFLPs) for investigating the genetic variations of *B. xylophilus* and related individuals from China, Japan, and South Korea. The AFLP patterns obtained in our study were similar to the microsatellite patterns reported in a previous study; our AFLP patterns indicated high genetic variability and cryptic genetic structure, but did not indicate any peculiar geographic structure. Moreover, the genetic distances between individuals suggested that the Korean population was affected to a greater extent by the Chinese population than the Japanese population. Further, the gene flow among the related species appeared to be limited; however, there may be also the possibility of genetic introgression among species. These results confirm the usefulness of AFLPs for understanding the epidemiology of pine wilt disease, thereby contributing to the effective control of this disease.

Keywords: AFLPs; pinewood nematode; *Bursaphelenchus xylophilus*; pine wilt disease

Introduction

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934; Nickle 1970), causes pine wilt disease (Mamiya 1988) and is one of the main nematode species implicated in serious damage to the forest industry. *B. xylophilus* inhibits water transportation in pine trees so that they eventually wilt and die. The introduction of *B. xylophilus* to non-native regions has caused serious economic loss to these regions, including South Korea. In South Korea, *B. xylophilus* infection was first reported in 1988, and subsequently large forest areas have been ruined; considerable resources have therefore been committed to control pine wilt disease in this region (Shin and Han 2006). However, this organism can cause disease in previously uninfected regions every year, and the resulting damage has been steadily increasing every year. The transmission routes and mechanisms of dispersal of these organisms should be clarified to achieve effective control using limited resources. However, it is difficult to obtain this information because of the complicated life cycle of this species, which includes vectors such as cerambycid beetles and unrecognized human interventions. Therefore, molecular markers showing sufficient genetic polymorphism could be beneficial for understanding the epidemiology of this disease.

Microsatellites and amplified fragment length polymorphisms (AFLPs) might be good candidates for elucidating the epidemiology of the pine wilt disease owing to their high genetic variability and easy utility. Microsatellites exhibit polymorphism in the number of short tandem repeats and have been one of the most popular markers used for the evaluation of genetic variation in a population (Jarne and Lagoda 1996). Several polymorphic microsatellite loci have been identified in pinewood nematodes (Zhou et al. 2007; Jung et al. 2010). However, the initial development of microsatellite markers is expensive and their application might be limited for amplifying a large number of loci from small organisms such as nematodes (Fisher and Viney 1996; Jung et al. 2010). In contrast, AFLPs, which do not require any initial development process or prior information about the genome, are relatively cheap and can be used for analyzing a large number of loci at one time. However, AFLP markers cannot be used to analyze heterozygosity, which is determined on the basis of an unrealistic assumption that all the loci are in Hardy–Weinberg equilibrium. Recently, many statistical approaches have been applied to overcome

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this drawback of AFLP markers. Since AFLP markers are cheap and highly polymorphic, they are considered as promising molecular markers in population genetic studies (Bensch and Åkesson 2005; Meudt and Clarke 2007).

In this study, we used AFLP markers to examine the genetic structure of the pinewood nematode population by determining the genetic variability among individuals from South Korea, China, and Japan, and evaluated the usefulness of this marker for elucidating the epidemiology of pine wilt disease.

Materials and methods

Samples and DNA extraction

We selected 45 *B. xylophilus* individuals from 15 regional isolates sampled from South Korea, nine from three regional isolates from China (Nanjing, Sichuan, and Zhejiang), and nine from three strains (C14-5, S10, and T4) from Japan. Further, we sampled nine *Bursaphelenchus mucronatus* individuals from three regional isolates from South Korea, three from China, and three from Japan. *B. mucronatus* is native to Asia and is closely related to *B. xylophilus* (Kanzaki and Futai 2002). Detailed information about sampling locations and sample size is presented in Figure 1. The establishment of isolates and the culturing of *Bursaphelenchus* spp. individuals was performed according to the procedures described by Ayoub (1977).

AFLP genotyping

We performed AFLP genotyping according to the method described by Jung et al. (2006). A brief outline of the method is as follows. The total DNA of all individuals was cut using two different endonucleases (*Eco*RI and *Mse*I), and *Eco*RI and *Mse*I adaptors were ligated to the sticky ends of the restricted fragments. Then, the fragments were amplified through two steps of polymerase chain reaction (PCR) (pre- and selective amplifications). Eco-AGG/Mse-ACC primers were used for selective amplification, and the Eco-AGG primer was labeled with fluorescent dye, 6FAM. The PCR and ligation conditions followed those of Jung et al. (2006). The selectively amplified PCR fragments were analyzed using Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA), and the band size and genotypes were determined using GENEMAPPER 4.0 software (Applied Biosystems, Foster City, CA).

Data analyses

The redundant loci in the amplified fragments were identified, and, except for the first locus, all remaining loci were eliminated using AFLPOP software (Duchesne and Bernatchez 2002). The pairwise differences between individuals and pairwise F_{ST} values between isolates were calculated by using the Arlequin 3.11 program (Excoffier et al. 2005), and the latter were linearized with Reynold's transformation ($-\ln(1-F_{ST})$). The relationship among individuals and isolates was represented as

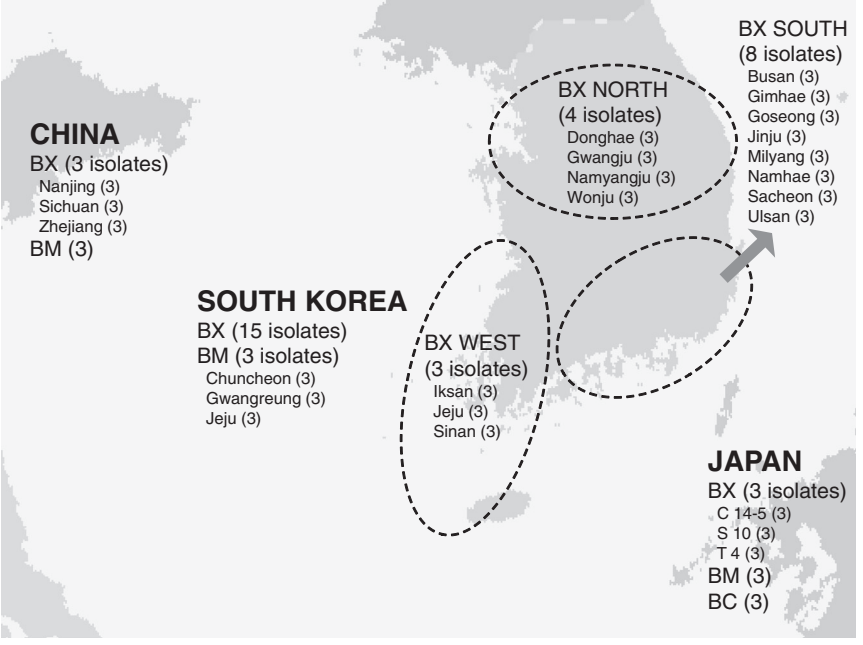

Figure 1. Map denoting sampling locations. BX, BM, and BC indicate *Bursaphelenchus xylophilus*, *B. mucronatus*, and *B. conicaudatus*, respectively. The numbers within parentheses are the sample sizes of each isolate.

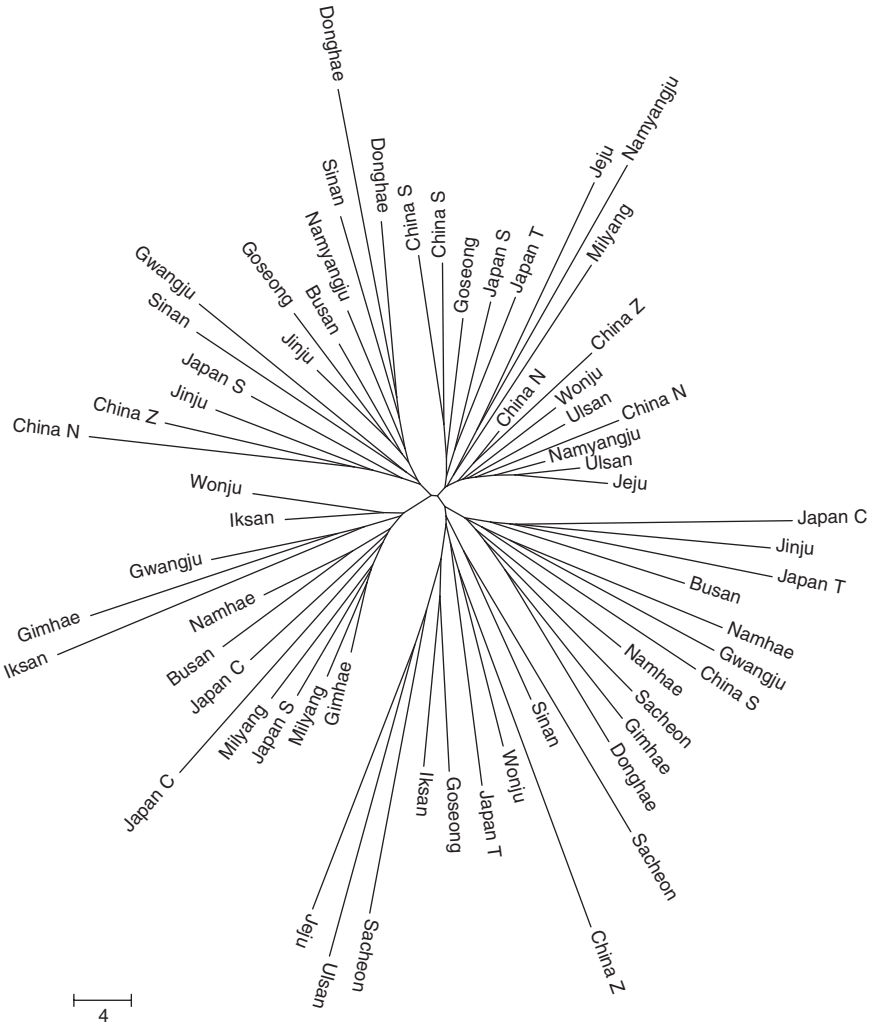

Figure 2. Neighbor-joining tree diagram showing the genetic relationship that was determined using pairwise differences among the genotypes of *Bursaphelenchus xylophilus* individuals collected from South Korea, China, and Japan. China N, China S, and China Z represent isolates from Nanjing, Sichuan, and Zhejiang, respectively. Japan C, Japan S, and Japan T represent Japanese isolates C14-5, S10, and T4, respectively. The bar indicates the difference in number of loci (number of bands).

set of pairwise values, which were determined using Molecular Evolutionary Genetics Analysis (MEGA) 4.0 software (Tamura et al. 2007), and by dendrograms, which were constructed using a neighbor-joining algorithm. Analysis of molecular variance (AMOVA) (Weir and Cockerham 1984) was determined using Arlequin 3.11 (Excoffier et al. 2005) for the following groups: (1) the three species groups (BX, BM, and BC); (2) *B. xylophilus* obtained from the three countries (South Korea, China, and Japan); (3) *B. mucronatus* obtained from the three countries; and (4) *B. xylophilus* from the three geographic regions named as north, south, and west, respectively, in South Korea (Figure 1). The number of populations (*K*) was determined from the genotypic data by using Structure 2.2 software (Falush et al. 2007). AFLP genotype data were transformed to the Structure format by using the R package of AFLPdata (Ehrich 2006). We did not use the available geographic location information, i.e., user-defined population information, and we selected the admixed model option for this analysis. The posterior density of *K* was analyzed using the prior distribution of *K* in the range 1–10. Markov chain Monte Carlo (MCMC) simulation was executed for each *K* with 10,000 steps of burn-in and 100,000 steps of Markov chain. Posterior probabilities of *K* were calculated using likelihood values of *K* according to the instructions in Structure 2.2.

Results

AFLP analysis revealed that the size of 208 loci was within the range 100–867 bp. The mean of pairwise differences among all the individuals in our study was 36.27 ± 15.94, and the average gene diversity over the

Table 1. Results of analysis of molecular variance (AMOVA).

Sources of variation	Variance components	Percentage of variation	*F* values
Bursaphelenchus xylophilus vs. *B. mucronatus* vs. *B. conicaudatus*			
Among groups	0.64285	3.49	0.03488**
Among isolates within groups	0.30582	1.66	0.01719
Within populations	17.48148	94.85	0.05147*
Korea vs. China vs. Japan (*B. xylophilus*)			
Among groups	0.18731	1.02	0.01022
Among isolates within groups	0.10958	0.60	0.00604
Within populations	18.03175	98.38	0.01620
Korea vs. China vs. Japan (*B. mucronatus*)			
Among groups	0.07407	0.46	0.00462
Among isolates within groups	0.57037	3.55	0.03571
Within populations	15.40000	95.98	0.04017
West vs. south vs. north (*B. xylophilus* in South Korea)			
Among groups	−0.01505	−0.08	−0.00083
Among isolates within groups	−0.00401	−0.02	−0.00022
Within populations	18.15556	100.11	−0.00105

*$0.001 \leq P < 0.05$; **$P < 0.001$.

loci was 0.174 ± 0.085. These two values for *B. xylophilus*, *B. mucronatus*, and *B. conicaudatus* were 36.45 ± 16.07 and 0.175 ± 0.086, 31.87 ± 17.74 and 0.153 ± 0.080, and 32.67 ± 19.88 and 0.157 ± 0.120, respectively. The results of our study indicated that the genetic variability among the species decreased in the following order: *B. xylophilus*, *B. mucronatus*, and *B. conicaudatus*. However, since all indices were within the tolerance range, the genetic variability among the species might be attributed to the differences in their sample size.

The genotypes of *B. xylophilus* individuals from the three countries were not clearly separated, as indicated in the neighbor-joining diagram, irrespective of their distinct regional origins (Figure 2). This finding indicates that the geographic structure of the population may be established neither among countries nor among regional isolates within South Korea, which conforms to the results of AMOVA [Korea vs. China vs. Japan (*B. xylophilus*) and west vs. south vs. north (*B. xylophilus* in South Korea)] (Table 1). The posterior probability of K was the highest ($K = 5$) from the analysis of population structure using Structure 2.2 (Figure 3). This number conformed roughly to the number of the main clades represented in the neighbor-joining diagram (Figure 2). These results suggest the existence of a cryptic genetic structure in the population of pinewood nematodes. Genetic structure among countries was not significant in *B. mucronatus,* either [Korea vs. China vs. Japan (*B. mucronatus*)] (Table 1).

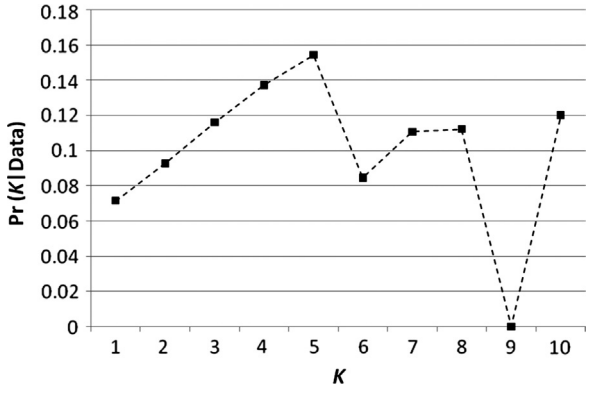

Figure 3. Posterior distribution of number of population (K) analyzed by Structure 2.2 (Falush et al. 2007).

AMOVA revealed a small (3.49%) but significant pattern of genetic variation among different species (*B. xylophilus* vs. *B. mucronatus* vs. *B. conicaudatus*; Table 1). The isolates of these three species were, on the whole, separated from each other in the neighbor-joining diagram which represented the genetic relationship among the isolates of the three species from the three countries (Figure 4). The isolates of *B. mucronatus*, however, were divided into two groups: group B and group C. The former contained isolates from Gwangreung and Chuncheon in South Korea and the latter contained isolates from Jeju in South Korea, from China and from Japan. Interestingly, the Japan T4 isolate of *B. xylophilus* belonged to the group B of *B. mucronatus*. The isolates from Namyangju and Sacheon of *B. xylophilus* in South Korea and the Japanese S10 isolate of *B. xylophilus* were positioned between the two aforementioned *B. mucronatus* groups. Group A contained only *B. xylophilus* isolates, which mainly included isolates from Korea, three from China,

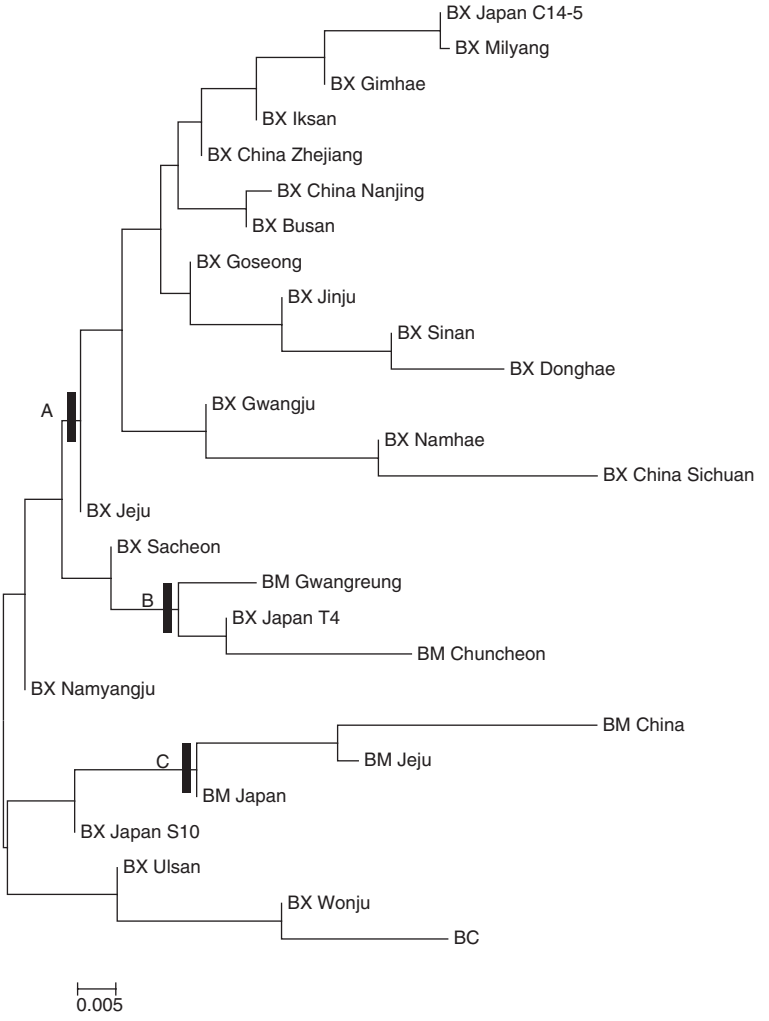

Figure 4. Neighbor-joining tree diagram indicating the genetic relationship among *Bursaphelenchus xylophilus* (BX), *B. mucronatus* (BM), and *B. conicaudatus* (BC) isolates obtained from South Korea, China, and Japan. This tree was generated using pairwise F_{ST} values, which were linearized through Reynold's transformation. The bar indicates the linearized F_{ST} value.

and one from Japan (C14-5), which indicate that the isolates from South Korea were genetically more related to those from China as compared to those from Japan.

Discussion

In this study, we found that AFLP and microsatellite markers showed similar patterns of genetic variation. The microsatellite markers revealed high allelic diversity (18–48 alleles/locus) and cryptic genetic structure ($K = 6$) (Jung et al. 2010). A high level of genetic variation observed in both types of markers might be primarily due to the continuous and/or large-scale introduction of individuals. This result is almost the same as that of the AFLP analysis of Chinese populations (Cheng et al. 2008). This inference was also supported by the results of AMOVA, which indicated low but significant genetic variance among the three related species and no remarkable geographic structure among the individuals of each species isolated from the three East Asian countries (Table 1). There is the possibility of genetic introgression because alleles from many loci must be shared between species, which is represented by the close relationship among isolates from different species as shown in Figure 4. A similar result (i.e. common occurrence of alleles between species) was observed in the analysis of microsatellite data (Jung et al. 2010).

Population genetics of pinewood nematodes in South Korea appear to be influenced more by cryptic genetic structure than by geographic structure. Microsatellite data also revealed a similar pattern (Jung et al. 2010). Although the K values of microsatellite data and AFLPs were slightly different ($K = 6$ and 5, respectively), it was certain that the population of pinewood

nematodes was highly structured in this region. The ecological properties of this species, such as a vector-mediated parasitic life cycle, may have contributed to its population structure with the condition of strong preventive measures to avoid the dispersal of these pinewood nematodes (Jung et al. 2010).

The population of Korean pinewood nematodes might be related to the Chinese and Japanese populations; further, more than the Japanese population, the Chinese population was closely related to the Korean population. The neighbor-joining diagram (Figure 2) and the results of the AMOVA tests (Table 1) revealed that the genetic variations in this region did not show any peculiar geographical structure. These results can be attributed to the high level of gene flow among these countries and/or the fact that the genetic variation of the populations in these countries is similar to that of the source population since its introduction. High gene flow might be the main factor in the generation of the geographic pattern of genetic variation just before the implementation of strict quarantine measures. The neighbor-joining analysis (Figure 4) revealed that group A, which included many isolates from South Korea, also included all Chinese isolates and only one Japanese isolate. This result suggests that the Chinese population had a stronger influence on the South Korean population as compared to the Japanese population. However, more data from these countries should be analyzed to confirm this inference.

AFLPs retain sufficient genetic variation; therefore, AFLP analysis can be used to infer the genetic relationship among species and among individuals of each species and to investigate the genetic structure of populations. With advantages such as cost-effectiveness, easy applicability, and a large number of polymorphisms, AFLPs can be considered as potential markers for elucidating the epidemiology of pine wilt disease, thereby contributing to the effective control of this disease.

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