INTRODUCTION
The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, the causal agent of pine wilt disease (Kiyohara and Tokushige, 1971), is supposed to have originated in North America (de Guiran and Bruguir, 1989). Since 1905, when the incidence of this disease was first reported, the area damaged by this disease has continued to spread throughout Japan except for the northernmost prefectures, Aomori and Hokkaido. Pine wilt disease has also been severely destroying a huge extent of pine forests in other East Asian countries such as China and Korea, and has recently invaded Portugal (Mota et al., 1999), becoming a possible threat to European countries as an invading forest pest.

Among many PWN populations collected in Japan, several populations from different geographical origins are known to have only slight or no pathogenicity to host pine trees (e.g., Kiyohara and Bolla, 1990), and were generally called ‘avirulent’ isolates. Molecular phylogenetic study confirmed that the virulent and the avirulent isolates belong to different clades in a phylogenetic tree of *B. xylophilus* and related species (Iwahori et al., 1998), though no reproductive isolation has been developed between the two groups of isolates. The avirulent isolates have a lower potential to invade into the bark tissues of pine shoots (Asai, 2002), lower abilities to disperse (Ichihara et al., 2000) and propagate (Kiyohara and Bolla, 1990) within healthy pine trees. The number of the nematodes of the avirulent isolates carried by a vector beetle, *Monochamus alternatus* Hope, was far fewer than that of the virulent isolates (Aikawa et al., 2003b). Those features of the avirulent isolates must be disadvantageous to be an insect-dependent phytopathogenic nematode. To prove this assumption, however, we must examine selection forces that exert emphasis on respective isolates that have different features in their lifecycles.


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Abstract
A practical method for estimating the allele frequency in a population of the pinewood nematode, *Bursaphelenchus xylophilus*, was developed by employing the technique of PCR-RFLP image analysis, in which a relative quantity of DNA templates is determined. At first, nematode individuals of two isolates, a virulent (S10) and an avirulent isolate (C14-5), were mixed at various proportions, and then PCR-RFLP patterns of the heat-shock protein 70A (hsp70A) gene fragment were obtained from each mixture. Regression analysis between logarithmic values of relative intensity of isolate-specific bands and those of a relative number of individuals of the two strains showed a strong linear relationship. These regression lines were applied to pursue temporal changes in the frequency of the hsp70A alleles in several experimental populations. The changes observed in allele frequencies were well explained with an overdominance model.

Key words: Intraspecific competition; stabilizing selection; replacement series
To evaluate selection force, molecular markers could be powerful tools in the analysis of population genetics. In the case of B. xylophilus, some markers specific to each of virulent and avirulent isolates have been reported (e.g. Aikawa et al., 2003a). These markers enable us to evaluate selection forces exerting on such markers and/or neighboring genes. To apply such markers to various populations, a high-throughput analytical method is needed. For population genetic analyses of B. xylophilus, polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) markers of the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA (rDNA) (e.g. Aikawa et al., 2003a) and microsatellite markers (Enoki et al., 1999) have been utilized to genotype nematode individuals. However, both of these methods are unsuitable for high-throughput analysis because genotyping many individuals for estimations of allele frequency is laborious and time-consuming.

A convenient technique called PCR-RFLP image analysis has been used recently to estimate the relative quantity of DNA templates that were each derived from different species in their mixed samples. For the estimation, intensities of PCR-RFLP bands specific to each DNA template are compared. To avoid estimation bias caused by the different efficiencies of PCR amplification, primer sets should be carefully chosen so that the PCR amplicons are almost the same in length. This is one of the reasons why microsatellite markers, such as Enoki et al. (1999) developed, are unsuitable for this method of image analysis.

Until now, this method has been applied to evaluate inter-specific competition among several vesicular arbuscular (VA) fungi for colonizing plant roots (Oba et al., 2002) and to detect adulteration of spelt flour with wheat (von Büren et al., 2001). As for the nematode, this method was used recently to analyze temporal changes of the ratio of Meloidogyne incognita (Kofoid & White) Chitwood to M. arenaria (Neal) Chitwood in a mixed population (Iwahori and Sano, 2002).

In our present research, this PCR-RFLP image analysis was used as a simple and reliable method for population genetics, and the temporal changes in the frequencies of each allele that is specific to the virulent or to the avirulent isolates of B. xylophilus were estimated for mixed populations of the nematodes.

**MATERIALS AND METHODS**

**Nematode.** Two isolates of B. xylophilus, S10 (virulent isolate) and C14-5 (avirulent isolate) were used as representatives of the two phylogenetic groups, the virulent and the avirulent isolate group, of this nematode species. They have been vigorously used in a comparative experiment, which gives us meaningful knowledge about their ecological and physiological characteristics. The isolates have been cultured on a fungal mat of Botrytis cinerea Pers. grown on autoclaved barley grains, and maintained at 10°C.

**Preparation of DNA templates and PCR amplification.** DNA templates were prepared from S10, C14-5 and their mixtures following the method described by Iwahori et al. (1998). All PCR amplifications were performed in 12.5 μl reaction mixtures containing: 10 ng/μl of DNA template, 0.75 μl; 10 μM of each primer, 0.65 μl; 1.25 mM dNTP, 1.25 μl; Taq polymerase (New England Biolabs), 0.05 μl (0.25 U); 10× reaction buffer, 1.25 μl; autoclaved distilled water, 7.85 μl. Since clearly distinguishable banding patterns of the heat-shock protein 70 A (hsp70A) gene fragment had been obtained between S10 (two bands of ca. 270 and 200 bp) and C14-5 (three bands of ca. 200, 150 and 130 bp) in a preliminary PCR-RFLP analysis, we utilized this gene fragment for genotyping. We confirmed that several known virulent isolates (e.g. Ka4, T4 and S6-1) and an avirulent isolate (OKD1) were the same with S10 and C14-5, respectively, in banding pattern (unpublished data). We also successfully obtained a PCR-RFLP pattern of the ITS region, that differentiates virulent isolates from avirulent ones (e.g. Iwahori et al., 1998), however, irregular bands appeared in several cases. Therefore, we did not use the ITS region, but did use the hsp70A gene as a marker in the following experiment. We referred the hsp70A alleles specific to S10 and C14-5 as ‘S’ and ‘C’, respectively. To amplify these alleles, we used a primer set, hsp70f (5'-GAC ACC GAG CGT CTA ATC GGA G-3') and hsp70r (5'-GTA CCA CCA AGA TCG AAG-3') originally described in Beckenbach et al. (1992). The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 15 s, elongation at 72°C for 60 s, and concluded with a final elongation at
PCR-RFLP Image Analysis of the PWN

72°C for 10 min. All PCR amplifications were run on a thermocycler (TaKaRa Thermal Cycler personal TP240). Each 2.5 μl of the PCR products was resolved by electrophoresis using a 0.7% agarose gel in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-HCl, 20 mM acetic acid, 2 mM EDTA, pH 8.1) for 20 min at 100 V. The gels were stained with 2.5 mg/l ethidium bromide over 30 min and observed under UV light to confirm successful amplification.

Sequencing analysis. Purified PCR products served for sequencing the hsp70A gene fragments, and an ABI PRISM™ 310 Genetic Analyzer was used with a reaction kit (ABI PRISM™ Big Dye™ Terminator v1.1 Cycle Sequencing Kit). The PCR products were purified by recovering the hsp70A gene fragment after 1.0% agarose gel electrophoresis. Sequencing primers were the same as the ones used for PCR amplification (hsp70f and hsp70r). DNA sequence data were aligned and converted to amino acid sequences using a computer program GENETYX-MAC (ver. 11). For comparative purposes, sequence datum of the heat-shock protein 70A gene of Caenorhabditis elegans (Maupas) Dougherty from GenBank (Accession number M18540) was used as a reference.

RFLP analysis. Ten microliters of hsp70f/r-PCR products were immediately digested by adding 0.4 μl of AluI (New England Biolabs) and 1.2 μl of NEBuffer 2 (10×) (New England Biolabs), and incubating at 37°C overnight. Those digested PCR products were resolved by electrophoresis using 5% polyacrylamide gel in Tris-Borate-EDTA (TBE) buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 50 min at 100 V. The gels were stained with 2.5 mg/l ethidium bromide over 30 min and photographed under UV light.

Image analysis. The banding patterns were stored as image data and were processed to subtract background (NIH image ver. 1.62), and brightness was scanned over each of the lanes. From the resulting numerical data, the peak height and peak area of each band were calculated. In this procedure, unclear or short peaks were omitted.

‘Peak height ratio’ was determined by dividing the peak height of the S10-specific band by total peak height of the two bands specific to C14-5. ‘Peak area ratio’ was determined by dividing the peak area of the S10-specific band by the total peak area of the two C14-5-specific bands. Both of these two indices reflected an abundance of S-type allele.

Construction of the standard lines. Standard mixtures each containing S10 and C14-5 at various proportions (S10 proportion = 100, 99, 90, 70, 50, 30, 10, 1 or 0%), 30,000 individuals in total, were prepared. DNA templates were obtained from each of the mixtures according to the procedure of Iwahori et al. (1998). Then, the PCR-RFLP patterns of the hsp70A gene fragment were obtained, and served for the calculation of the peak height ratio and peak area ratio. The ‘allele frequency ratio’ of each mixture was determined by dividing the number of S10 by that of C14-5, both contained in the mixture. A linear regression analysis between the natural logarithm of the allele frequency ratio and the natural logarithm of the peak height ratio or peak area ratio was performed. Resulting regression lines were used as standard curves to estimate the frequency of the hsp70A alleles in the following experiment.

Temporal changes in the allele frequency in the mixed culture of two Bursaphelenchus xylophilus isolates. A 500 μl suspension containing c.a. 10,000 second-stage juveniles of S10 and/or C14-5 were pipetted onto the mycelia of B. cinerea grown in a 50-ml conical flask. Each cultural line started at various proportions of S10 (i.e., 100, 99, 90, 70, 50, 30, 10, 1 or 0% of initial population). These lines were maintained at 20°C with subculturing once a month. In these experiments, 1-mo-old mycelia of B. cinerea grown on barley grain medium (unthreshed barley grain 10 ml; tap water 10 ml, autoclaved at 121°C for 20 min) in 50-ml conical flasks served as the food source for the nematode.

Nematode populations were sampled from each of the lines 20, 30, 40, 50, 60, 90, 150 and 270 d after the commencement of the mixed culture. A DNA template was obtained from each of the samples, and PCR-RFLP image analysis was carried out with the standard curves to estimate the frequency of the S-type allele (specific to S10) in each population sampled.

Estimation of selection coefficients. Supposing that the generation interval of B. xylophilus at 20°C is 6 d (Mamiya, 1975), and the marker hsp70A gene has a single locus, the relative fitness of SS homozygote and CC homozygote were estimated. For this purpose, a regression analysis was applied following Kimura (1960).

The frequency of the S-type allele at the poly-
morphic equilibrium is expressed as $\hat{x}$, and is given from following formula:

$$\hat{x} = \frac{S_C}{S_S + S_C}$$  \hspace{1cm} (1)

where $S_S$ and $S_C$ are the selection coefficients of $SS$ and $CC$ homozygote, and relative fitness of these two genotypes against the $SC$ heterozygote is expressed as $(1-S_S)$ and $(1-S_C)$, respectively.

When the selection coefficients are small enough, the relationship between the frequency of $S$-type allele, $x$ and the number of generation, $t$ is approximately expressed as:

$$\frac{dx}{dt} = x(1-x)(S_S - (S_S + S_C)x).$$  \hspace{1cm} (2)

A solution of this differential equation can be rewritten under the condition (1) as:

$$\ln x + \frac{\hat{x}}{1-\hat{x}}\ln(1-x) - \frac{1}{1-\hat{x}}\ln|\hat{x} - x| = S_C t + S_C t_0.$$  \hspace{1cm} (3)

Given $\hat{x}$, the left side is calculable with observed $x$. The slope of the regression line between the left side calculation and the number of generation $t$ in the right side is an estimate of $S_C$, the selection coefficient of $CC$ homozygote. $S_S$, the selection coefficient of $SS$ homozygote, is calculable from $S_C$ under condition (1).

**RESULTS**

**DNA sequence of the isolate specific markers**

The strain-specific marker hsp70A gene was partially sequenced (ca. 430 bps) for both C14-5 (avirulent isolate) and S10 (virulent isolate). Each nucleotide sequence dataset for C14-5 and S10 was deposited in the DNA Databank of Japan under the accession numbers AB211292 and AB211293, respectively. Alignment of the two sequences showed a difference in just one base (Fig. 1). This difference causes a difference in digestibility with $Alu$ I, and thus leads to the different banding patterns in PCR-RFLP analysis. However, these two sequences of the hsp70A gene fragment are synonymous at the amino acid level.

**Regression analysis**

The mixtures of S10 and C14-5 containing various proportions of S10 (i.e., 100, 99, 90, 70, 50, 30, 10, 1 or 0%) generated the PCR-RFLP patterns shown in Fig. 2. Two bands of ca. 270 and 200 bp can be seen on the lane for 100% S10, and three bands of ca. 200, 150 and 130 bp can be seen on the lane for 0% S10 (i.e., 100% C14-5). We denote these bands by first, second, third and fourth band, respectively, in order of the fragment size. As the proportion of S10 decreased, the intensity of the first band (specific to S10) became weaker and that of the third and fourth bands (both specific to C14-5) became stronger. The regression analysis performed between the peak height ratio and allele
frequency ratio generated a formula; ln(y)\/H11005 \(0.139\)/H11001 \(0.727\) ln(x), where \(r^2\)/H11005 \(0.943\) (Fig. 3A), and that between the peak area ratio and the allele frequency ratio generated another; ln(y)/H11005 \(0.082\)/H11001 \(0.800\) ln(x), where \(r^2\)/H11005 \(0.949\) (Fig. 3B).

Temporal changes in the allele frequency of the marker hsp70A gene in mixed cultures of the virulent and avirulent isolates

Figure 4 shows the temporal changes in the % frequency of \(S\)-type allele. Regardless of the initial proportion of S10 to C14-5, the frequency of \(S\)-type allele in the S10/C14-5 mixtures converged to ca. 70% with the lapse of time. This indicates that a stabilizing selection force acted on the hsp70A gene. Here, Fig. 5 shows the result of the regression analysis for the estimation of relative fitness of \(CC\) homozygote, where the generation interval of \(B. xylophilus\) was supposed to be 6 d (Mamiya, 1975), the marker locus to be single, and the frequency of the \(S\)-type allele at the polymorphic
equilibrium to be 71% (overdominance model). All regression lines have almost the same slopes except for a few cases, suggesting that the selection coefficient of CC homozygote was constant irrespective of the initial proportion of S10 in the mixed cultures. Relative fitness against SC heterozygote was estimated as 0.90–0.91 for SS homozygote and as 0.76–0.79 for CC homozygote. Similar results were obtained when we performed an exploratory curve fitting with the least-square method, in which the selection coefficients, $S_c$ and $S_s$, were varied to calculate frequencies of S-type allele in each discrete generation and to compare these theoretical frequencies with the actual ones (data not shown).

**DISCUSSION**

The results of the sequencing analysis of the hsp70A gene fragment demonstrated a single-nucleotide variation between the avirulent and virulent isolates of *B. xylophilus* which leads two PCR-RFLP patterns, either specific to each isolate. However, triplet codes obtained from the sequences were synonymous. Aikawa et al. (2003a) inoculated 100 specimens of each of two *B. xylophilus* isolates, OKD1 (avirulent isolate) and Ka4 (virulent isolate), on the same plate, and reported that three types of PCR-RFLP pattern (i.e., virulent-virulent homozygote, virulent-avirulent hetero and avirulent-avirulent homozygote type) were found for the ITS region in the ratio of 1 : 2 : 1 on day 25 after the inoculation. When the initial S10/C14-5 ratio was adjusted to 1 : 1, in the present research, however, the frequency of the virulent-type (S-type) allele of the hsp70A gene was 90–96% from 20 to 30 d after the inoculation, though the isolates utilized and the region examined in our experiment were different from those of Aikawa et al. (2003a).

Quantitative competitive PCR is one of the prevailing methods to estimate DNA amount. This method employs an internal standard shorter or longer than the target sequence, which easily gives quantification bias caused by the different efficiency of PCR amplification. In the modified method, PCR-RFLP image analysis is based on comparing the intensities of the PCR-RFLP bands specific to each of the target organisms to estimate the relative quantity of each DNA template derived from the different target organisms. So, the estimations are hardly biased a different efficiency in amplification because the length of the PCR amplicon is almost the same among the target organisms. In our case, several known virulent isolates (e.g., Ka4, T4 and S6-1) and an avirulent isolate (OKD1) were the same as S10 and C14-5, respectively, in terms of the length of the PCR amplicon and their RFLP patterns (unpublished data). So, the marker, hsp70A, may be applicable not only to the experimental system with S10 and C14-5, but also to those with other virulent and avirulent isolates.

The present study shows that the PCR-RFLP image analysis is applicable to population genetic analysis of a nematode species, applying this method to estimate gene frequencies in nematode populations. To estimate gene frequencies in a nematode population, labor consuming procedures have routinely been required so far, where many nematodes must be genotyped individually. In contrast, the present method is unique and effective, as the DNA template extracted from each population reliably serves for estimation of the allele frequency in that population. The DNA templates, once extracted and purified, could serve for any loci to be examined in PCR-RFLP image analysis. In fact, standard lines for another PCR-RFLP marker, the ITS region of rDNA, were successfully generated by PCR-RFLP image analysis with the same templates used in the present study, though irregular bands appeared in several cases (data not shown). This method is rather simple and does not require the special reagents or equipment used in microsatellite studies (e.g. Enoki et al., 1999), and is potentially applicable not only to nematodes, but to other microorganisms also. Therefore, we propose that this is a valuable method for high-throughput estimation of the allele frequency of PCR-RFLP markers for many loci and in many populations of microorganisms, although the genotypes of the specimens cannot be determined.

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