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Phenotypic and genotypic traits of recombinant inbred lines of pine wood nematode, *Bursaphelenchus xylophilus*

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ABSTRACT

Pine wood nematode, *Bursaphelenchus xylophilus*, exhibits a wide range of intraspecific variation in several biological traits. Among them virulence (degree of pathogenicity), reproductive ability and boarding ability on the vector beetle are important pathogenicity-related traits, although their molecular basis has not been determined. In this study we generated a set of recombinant inbred lines (RILs) of *B. xylophilus* from two inbred lines, F7 and P9, which greatly differ in the degree of pathogenicity. In addition, we conducted bioassays to estimate above-mentioned three traits in the newly obtained 17 RILs and two parental inbred lines. As a result, RILs showed various virulences and reproductions along a continuum and two distinct transmission abilities. This indicates that virulence and reproduction may be quantitative, polygenic trait, while transmission ability is a qualitative trait which is controlled by a single or few genes.

1. INTRODUCTION

Pine wilt is a disease of pine caused by the pine wood nematode, *Bursaphelenchus xylophilus*, transmitted by vector beetle of the genus *Monochamus*. In recent years, molecular biological approach have been vigorously conducted for comprehension of disease mechanism (e.g. Jones *et al.* 2008), although the pathogenic factor has not yet determined. In this study we applied a classical genetics to address this matter by using newly conducted recombinant inbred lines (RILs) of *B. xylophilus*.

2. MATERIALS AND METHODS

2.1. Construction of RILs

Two parental lines, a virulent inbred strain 'P9' and an avirulent inbred strain 'F7' of *B. xylophilus* (Shinya *et al.* 2012), served consecutive full-sib mating (brother-sister mating) to yield a set of RILs. One unmated virgin female of one strain was transferred into a breeding plate containing one adult male of the other strain (P9 female for F7 male, and vice versa) to let them cross. Unmated adult nematodes of F1 generation thus obtained served crossing to obtain the F2 generation. Each couple of nematodes of F2 generation was used as ancestral RIL for subsequent full-sib mating that was repeated 20 times.

2.2. SSR marker-based characterization of RILs

Genomic data of *B. xylophilus* (sequence data ver1.2.) downloaded from the GeneDB website (http://www.genedb.org/Homepage) was used to find out the repeated sequences and for each SSR a unique pair of primers were designed. For genotyping a set of RILs, PCR amplification of candidate SSR markers was carried out using genomic DNA of them as template. PCR amplicons were then separated by electrophoresis and compared.

2.3. Reproductive ability of RILs on grey mould

Bursaphelenchus xylophilus of 19 test population including 17 RILs and 2 progenitors, the virulent P9 and avirulent F7, was examined for reproduction. The food source fungus *Botrytis cinerea* was initially cultured on PDA medium in a Petri dish. A nematode suspension containing 100 individuals was inoculated on the fungal mat and incubated at 25ºC. Twelve days after inoculation nematodes were extracted and counted under a stereomicroscope.

2.4. Estimation of Virulence of RILs against Japanese Black Pine Seedlings by Inoculation Test

To determine the virulence of each of the RIL populations of *B. xylophilus* 3-month-old seedlings of a highly susceptible Japanese black pine, *Pinus thunbergii*, served as experimental host plants to be challenged. After making a lengthwise slit that reached cambium on main stem, nematode suspension containing 500 individuals was inoculated into the incision. Twenty seedlings were challenged with each test population and 20 other seedlings were inoculated with an equal volume of distilled water as control. This experiment was repeated 4 times. Seedlings inoculated were incubated for 2 months with weekly health checks.

2.5. Boarding Ability of RILs on Vector Beetle

To create a culture vessel, barley and woodchip of Japanese red pine (*Pinus densiflora*) were added in this order to a glass tube and plugged. The tube was inoculated with the

blue-stain fungus (*Ophiostoma minus*). After incubation, 100 *B. xylophilus* individuals of each test population was inoculated to the tube and incubated for another 2 weeks. Finally a larva of *Monochamus alternatus*, obtsined from naturally-infected pine trees, was introduced to the tube, incubated and monitored at the same hour every day. Eclosed beetle was taken from the tube and nematodes were extracted and counted both from the beetle and from the medium in the tube.

3. RESULTS AND DISCUSSION

3.1. Construction and SSR marker-based characterization of RILs

Using two separate inbred lines of *B. xylophilus*, P9 and F7, a set of 17 RILs derived from 17 couples of F_2 generation has been generated. Among them was eight RILs descended from F7 female and P9 male, and nine RILs descended from P9 female and F7 male. A search of the genomic data of *B. xylophilus* permitted 16 SSRs primer design in different scaffolds. These SSRs PCR-amplified with unique primer pairs showed polymorphism across the two progenitors, i.e. P9 and F7, and they were therefore used in genotyping of RILs as SSR marker. As a result, 17 RILs showed unique genotype different from each other with high degree of homozygosity ranging from 0.88 to 1.00 (0.99 in average).

3.2. Reproductive Ability of RILs on Grey Mould

Change in the number of *B. xylophilus* grown on the fungus is shown in Figure 1. Reproductive ability shown by the newly obtained RILs were intermediate between those shown by the two progenitors; that is, no RIL showed a significantly larger or a significantly smaller population than P9 or F7, respectively. Thus the resultant RIL populations showed a continuously varying distribution of reproductive ability, which can be explained by quantitative inheritance controlled by polygene.

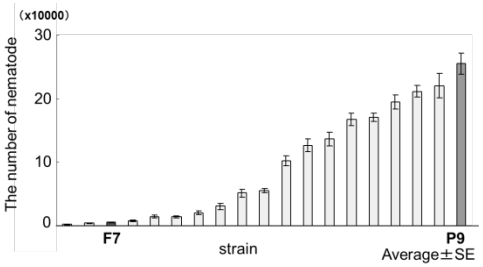

Figure 1. The number of each RIL population grown on *B. cinerea* for 12 days.

3.3. Estimation of Virulence of RILs against Japanese Black Pine Seedlings by Inoculation Test

Figure 2 shows increase of the number of dead *P. thunbergii* seedlings after inoculation of *B. xylophilus*. The seedling mortality, which indicates virulence of the *B. xylophilus* isolate inoculated, widely varied, from 0% to 58%. The progenitor F7 caused 1% mortality, while the other progenitor P9 caused 49% mortality. Distribution of mortality

resulted from the 17 RIL populations varied along a continuum between those from the progenitors, with two exceptions. This suggests that virulence of *B. xylophilus* is a quantitative trait to which a combination of several genes contributes.

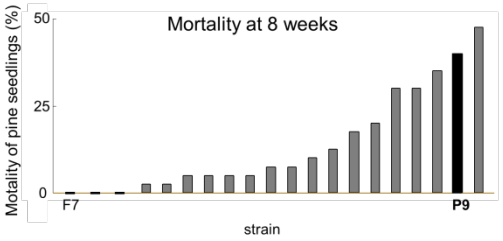

Figure 2. Mortality of pine seedlings after inoculation with each of the RIL populations

3.4. Boarding Ability of RILs on Vector Beetle

Data obtained by coculture of *B. xylophilus* with its vector beetle is summarized in Figure 3. Three RILs were omitted since no nematode offspring was extracted from either of beetle body and medium in culture vessels. All RILs showed a largely similar fluctuation pattern in number of total nematodes recovered from the beetle body and medium decreased with time. Multiple comparisons demonstrated that the progenitor P9 had a significantly higher value than the other progenitor F7. RILs were divided into two groups; one gave extremely low scores in the similar manner to F7, and the other generated a large number of boarded nematode that got aboard in the similar manner to P9. Thus the pattern of inheritance for boarding ability seems monogenic, suggesting that this trait is influenced by one or few genes with a major effect.

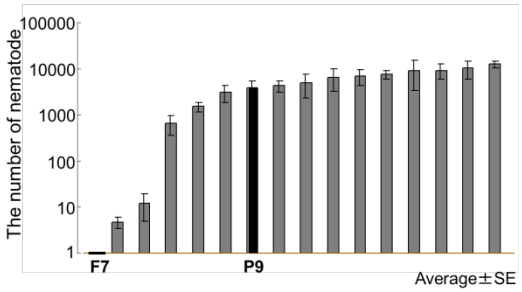

Figure 3. the number of Jiv nematode boarding on *M. alternatus* in each of RILs populations

4. ACKNOWLEDGEMENTS

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