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# Do Seedlings Derived from Pinewood Nematode-Resistant *Pinus thunbergii* Parl. Clones Selected in Southwestern Region Perform Well in Northern Regions in Japan? Inferences from Nursery Inoculation Tests

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**Abstract:** Background and Objectives: To determine whether the progeny of pinewood nematode-resistant *Pinus thunbergii* Parl. clones selected in the southwestern region of Japan could be successful in reforestation in the northern region, we investigated the magnitude of the genotype–environment interaction effect on the resistance against *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle in *P. thunbergii*. Materials and Methods: We inoculated *P. thunbergii* seedlings of six full-sib families, with various resistance levels, with *B. xylophilus* in nurseries at three experimental sites in the northern and southern regions of Japan. All parental clones of the tested families originated from southwestern Japan, and selection of parental clones for resistance was performed in the same region. Sound rates after nematode inoculation were calculated, and survival analysis, correlation analysis and variance component analysis were performed. Results and Conclusions: Families with high sound rate in the southern region also showed a high sound rate in the northern region. In almost all cases, Spearman's correlation coefficients for sound rates were more than 0.698 among sites. The variance component of the interaction between site and family was small compared to that of site and family separately. Thus, we conclude that the resistant clones selected in the southern region would retain their genetic resistance in the northern regions.

**Keywords:** pine wilt disease; *Bursaphelenchus xylophilus*; genotype by environment interaction; Japanese black pine; variance component

## 1. Introduction

Japanese black pine *Pinus thunbergii* Parl. is one of the major forestry species in Japan. Pine seedlings have been planted across a wide coastal area of Japan, from the northern part of Honshu island to the southern part of Kyushu island, to protect land and houses against strong winds and sand movement inland [1]. After the invasion of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, from North America to Kyushu island in the early 20th century causing pine wilt disease (PWD) in *P. thunbergii* forests, the disease has spread to the northern part of Japan [2–4]. Currently, the disease has been reported in all the prefectures of Japan except Hokkaido, the northern most prefecture [5]. From a global perspective, PWD in East Asia (Japan, South Korea, China and Taiwan) has now spread to southwestern Europe (Portugal and Spain) [4–9], and there is a risk that the disease will spread to neighboring countries [10,11].

To combat PWD, a national resistance breeding program of *Pinus densiflora* Sieb. et Zucc. (Japanese red pine) and *P. thunbergii* was started in southwestern Japan in 1978 as a part of an integrated pest management. In the program, 92 *P. densiflora* and 16 *P. thunbergii* resistant clones were selected [12]. The selected clones were propagated by grafting and used in PWD-resistant seed orchards. As PWD spread into the eastern and northern parts of Japan, supplemental resistance breeding programs were started in the Tohoku and Kanto regions [13,14]. Although many resistant clones were selected and resistant seed orchards were established in the eastern and northern regions in the programs, these eastern and northern orchards included resistant clones selected in the southern region of Japan to supplement shortages of resistant clones in the surrounding regions. Japan has a large geographic extension from north to south, with a highly variable climate. Until now, the genetic capability of resistant clones selected in southern Japan, or their progeny, in the northern regions of Japan has not been examined.

Species, provenance and family variation in resistance or susceptibility to pinewood nematode has been reported in artificial inoculation experiments using graftings, half- or full-sib families of pine species, and studies have shown the relatively high heritability of resistance or susceptibility in *P. thunbergii*, *P. densiflora* and *Pinus pinaster* Ait. (maritime pine) [15–20]. On the other hand, environmental factors also affect PWD development in infected trees. High air temperature, dry soil conditions and low-light intensity promote disease development, shorten the time until death and increase mortality [21–23]. However, there is limited knowledge of the effect of the interaction between genotype by environmental factors (G × E) on resistance. A previous study, based on a six-year *B. xylophilus* inoculation experiment using open-pollinated families of *P. thunbergii*, showed that the family-by-year effect for resistance level is smaller than the family effect [16]. In *P. pinaster*, a G × E interaction was reported based on a greenhouse inoculation test using seedlings from six provenances [18].

Resistance breeding against invasive pests generally begins at the site of the pest introduction. When there is a risk of pest expansion to neighboring regions with different climates, and if the clones or gene pool selected by resistance breeding display resistance in other regions with different climates, those genetic resources and breeding materials can be used in pest control strategies in other regions. In recent years, PWD has invaded southwestern Europe, and resistance breeding of *P. pinaster* has begun in Portugal and Spain [19,20]. In Japan, the first PWD outbreak occurred in Nagasaki, Kyushu in the southwestern region, and resistance breeding began in the southwestern region.

Here, to clarify if the progeny of southern resistant clones retain their genetic resistance to PWD in the northern region of Japan, the seedlings of six *P. thunbergii* families with various resistance levels were inoculated with an isolate of *B. xylophilus* at three sites with different climates. Then, the external symptom was assessed and analyzed.

## 2. Materials and Methods

### 2.1. Experimental Sites

Nurseries in the Tohoku Regional Breeding Office (TBO), Forest Tree Breeding Center (FTBC), and Kyushu Regional Breeding Office (KYBO) were used as the three sites for the experiment (Figure 1).

TBO (39°49′4.8″ N, 141°8′13.2″ E) is located in Iwate Prefecture in the Tohoku region, in the northern part of Honshu island. FTBC (36°41′31.2″° N, 140°41′24″ E) is located in Ibaraki Prefecture in the Kanto region, central Honshu island. KYBO (32°52′51.6″ N, 130°44′9.6″ E) is located in Kumamoto Prefecture in the Kyushu region, Kyushu island. The distance between TBO and KYBO is about 1200 km. The climate around TBO is cool and the monthly average temperature in winter is below 0 °C (Figure 2) [24]. On the other hand, the climate around KYBO is warm and the monthly average temperature in summer exceeds 25 °C. The climate around FTBC is intermediate between the two; in summer, the temperature is close to that of TBO, and in winter it is close to that of KYBO. Precipitation in June and July is high as it is the rainy season around KYBO.

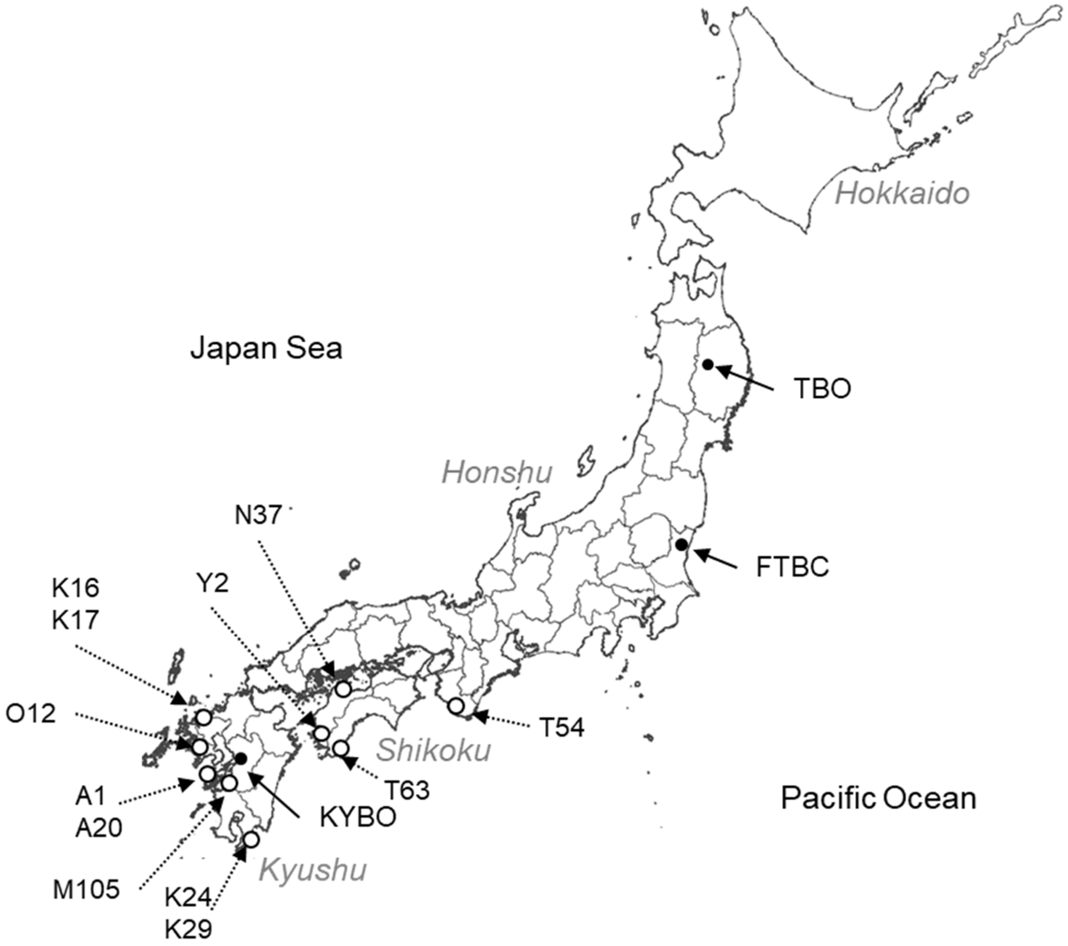

**Figure 1.** The experimental sites and the origin of materials used in this study. Filled circles indicate the three experimental sites, Tohoku Regional Breeding Office (TBO), Forest Tree Breeding Center (FTBC) and Kyushu Regional Breeding Office (KYBO). Open circles indicate the origins of 12 parental clones crossed to produce full-sib families used in this study. Italicize letters indicate the names of the four main islands of Japan. This map was created from the blank map published Geospatial Information Authority of Japan. Clone name abbreviations: Amakusa20 (A20), Namikata37 (N37), Yoshida2 (Y2), Tanabe54 (T54), Kimotuki24 (K24), Minamatasho105 (M105), Karatsu17 (K17), Karatsu16 (K16), Tosashimizu63 (T63), Oseto12 (O12), Kimotsuki29 (K29), and Amakusa1 (A1).

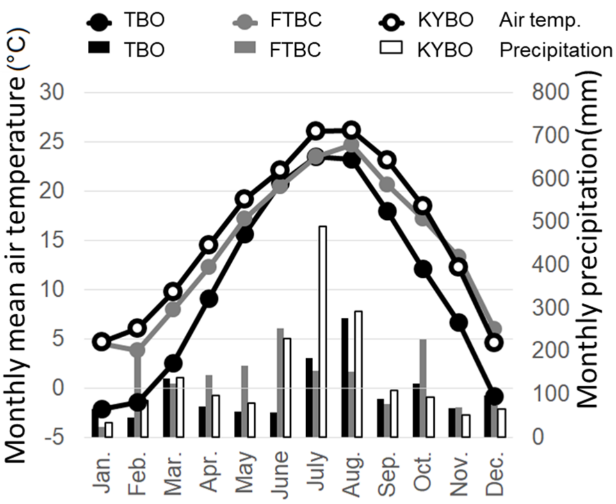

**Figure 2.** Monthly mean air temperature and monthly precipitation of three experimental sites in 2014. Data was obtained from the AMeDAS (Automated Meteorological Data Acquisition System) of Japan Meteorological Agency. The values for TBO, FTBC, and KYBO sites were measured at Morioka, Hitachi, and Kikuchi observatories, which are close to the experimental sites.

### 2.2. Pine Seedlings

Six *P. thunbergii* full-sib families produced by artificial crossing were used in the experiment (Table 1). In order to include pine trees with a large variation in their resistance to PWD, we used six PWD-resistant clones with high or intermediate resistance (Amakusa20 (A20), Namikata37 (N37), Yoshida2 (Y2), Karatsu17 (K17), Karatsu16 (K16), and Tosashimizu63 (T63)), two PWN-resistant clones with relatively low resistance (Tanabe54 (T54) and Oseto12 (O12)), and four plus-tree clones (Kimotuki24 (K24), Kimotsuki29 (K29), Minamatasho105 (M105), and Amakusa1 (A1)). The resistant clones were selected using *B. xylophilus* artificial inoculation tests and plus-tree clones were selected by phenotypically superior evaluation for growth and stem form. All parental clones originated in southwestern Japan (Figure 1) and were propagated by grafting and stored in KYBO. The inoculation test for resistant clone selection was also performed in southwestern Japan. The resistance levels of eight parental resistant clones, based on the nematode inoculation test using their open-pollinated families, have already been reported [25,26]. The resistance rank of parental clones is described in Table 1. The other four plus-tree clones were not selected for their resistance and the resistance levels of their open-pollinated or crossed families were low, based on preliminary inoculation tests.

**Table 1.** Details of the *Pinus thunbergii* full-sib families used in this study.

| Mother | | Father | | No. of Seedlings, Height (Mean ± SD) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Clone (Abbreviation) | Rank* | Clone (Abbreviation) | Rank* | TBO | | FTBC | | KYBO | |
| Amakusa20 (A20) | 3/13 | Karatsu17 (K17) | 1/13 | 39 | 31.9 ± 7.2 a | 70 | 30.1 ± 4.0 b | 92 | 26.6 ± 5.4 b |
| Namikata37 (N37) | 3/16 | Karatsu16 (K16) | 5/13 | 42 | 30.2 ± 7.6 ab | 69 | 32.7 ± 5.2 a | 77 | 30.9 ± 5.1 a |
| Yoshida2 (Y2) | 4/16 | Tosashimizu63 (T63) | 6/16 | 39 | 29.7 ± 6.5 ab | 71 | 26.6 ± 3.9 d | 52 | 21.1 ± 3.0 c |
| Tanabe54 (T54) | 10/16 | Oseto12 (O12) | 14/16 | 25 | 22.5 ± 6.0 c | 68 | 25.4 ± 4.6 e | 76 | 15.2 ± 4.3 d |
| Kimotsuki24 (K24) | - | Kimotsuki29 (K29) | - | 22 | 25.8 ± 7.0 bc | 70 | 28.5 ± 5.3 c | 58 | 19.7 ± 4.0 c |
| Minamatasho105 (M105) | - | Amakusa1 (A1) | - | 33 | 32.7 ± 7.5 a | 71 | 26.7 ± 5.2 d | 72 | 20.6 ± 4.5 c |
| Total | | | | 200 | 29.4 ± 7.7 | 419 | 28.3 ± 5.3 | 427 | 22.7 ± 7.0 |

Rank* shows the rank of clone resistance based on the progeny test by Matsunaga et al, [26]. The denominator and the numerator indicate the number of resistant clones evaluated at the same time and the clone rank among them, respectively. -: no evaluation. Means followed by a common letter are not significantly different at 5% level of significance.

One hundred seeds belonging to each of the six families were sown in the TBO, FTBC and KYBO nurseries in the spring of 2013. The following spring, the seedlings of each family were transplanted to another location in the nursery, with a random block design of family with two replicates at 20 cm × 20 cm spacing in FTBC and KYBO. In the TBO nursery, our preliminary test results showed that 1.5-year-old seedlings were not large enough for use in the inoculation experiment. In the present study, seedlings were not transplanted in the TBO nursery; instead, 2–3 seeds were sown at 20 cm × 20 cm spacing, and extra seedlings were removed to ensure only one remained in each 20 cm × 20 cm grid. Prior to inoculation, there were 200, 419, and 427 seedlings in the TBO, FTBC, and KYBO sites, respectively (Table 1). The height of each seedling was measured during the week prior to inoculation.

### 2.3. Nematode Inoculation and Symptom Observation

For inoculation, an isolate of *B. xylophilus* (Ka4) obtained from dead *P. densiflora* in Ibaraki Prefecture in 1999 [27] and sub-cultured in the laboratory at FTBC was used. After an incubation of approximately 10 d on *Botrytis cinerea* Pers., a fungus, on barley grains, the nematodes were separated from the media using the Baermann funnel method. The nematode suspension was adjusted to 200,000 nematodes/mL of water. Nematode incubation and suspension adjustment were conducted at each site.

Nematode inoculation was conducted on 1 July 2014 in all sites. A 5 cm length of seedling stem was peeled with a sharp knife at approximately 5–10 cm above the ground, and the wound was scratched with small sow before inoculation with 50 µL of suspension containing 10,000 nematodes using a micropipette.

The inoculated seedlings were observed weekly and external symptoms were classified into three categories (0: no symptoms, 1: browning of needles on one or more branches, 2: browning of all needles). Seedlings with an external symptom level of 1 were considered as diseased, and the seedlings with a level of 2 as dead. Subsequently, sound seedling rate and survival rate were calculated as follows:

$$\text{Sound seedling rate} = \text{No. of seedlings in symptom class 0/No. of inoculated seedlings}$$
$$\text{Survival rate} = \text{No. of seedlings in symptom class 0 and 1/No. of inoculated seedlings}$$

The sound seedling rate was the rate of seedlings without external symptoms, and focused on the seedlings with higher resistant level. On the other hand, the survival rate was the rate of surviving seedlings that included not only sound seedlings but also diseased and partially dead ones. From the viewpoint of preventive counteracts, we used the sound seedling rate as a major indicator and the survival rate as the supplemental result, as we considered that no symptoms were more important than surviving. Observations were carried out for 10 weeks after inoculation (WAI); however, in TBO the 4-week and 8-week survey was not conducted.

### 2.4. Statistical Analysis

R version 4.0.0 [28] was used for all statistical analyses. Seedling height was analyzed with a linear mixed model using the lmer function of the lme4 package [29] to determine the size variation of seedlings. In the model, mean height of each replicate of each of the six families from the three sites was calculated and used as the response variable; while family, site and their interaction were used as explanatory variables with fixed effects and replication within site was used as an explanatory variable with random effects. As model selection based on the AIC value with the function dredge in the MuMIn package [30] selected the model with an interaction between family and site (Table S1), we separated the data of each site and conducted multiple comparisons among families using the glht function in the multcomp package [31].

To compare the disease development process among sites and families, we conducted a two-step survival analysis using Kaplan–Meier estimators. For comparison among sites, Kaplan–Meier estimators were calculated for sound rate of seedlings and log-rank test with Bonferroni-adjusted $p$ values was applied for multiple comparisons among sites. Since the composition ratio of the six families did not differ significantly by site (Chi-square test, $X^2$-value: 15.56, *d. f.*: 10, $p$-value: 0.1130), no weighting was applied to the number of seedlings for each family in each site. Then, to compare the disease development process among families within sites, Kaplan–Meier estimators were calculated and the log-rank test with Bonferroni-adjusted *p* values was also applied. For the survival analysis, the functions survfit and survdiff in the survival package [32] were used.

Pairwise Spearman's correlation coefficients among sites were calculated to compare the order of resistance level among the six families.

To compare the relative effects of the site, family and their interaction on the variance of sound seedling rate, variance components of the factors were estimated using generalized linear mixed models with the glmer function of the lme4 package, with family assumed as a binomial error structure and logit link function [29]. Number of diseased seedlings (symptom class 1 and 2) and number of sound seedlings (symptom class 0) in a family in each replicate in a site was used as the response variable, and site, family, their interaction and replicate within each site were used as explanatory variables with random effects. Mean seedling height was added to the model as an explanatory variable with fixed effects. This analysis was applied to the data for 5, 6, 7, 9 and 10 WAI only, because no data was collected weeks 4 and 8 in TBO and there were few diseased seedlings before 3 WAI in FTBC.

Survival analysis, calculation of Spearman's correlation coefficient and variance component analysis were also conducted on survival rate.

## 3. Results

### 3.1. Seedling Height

Overall, mean seedling height was 26.4 cm across the three sites. Mean height across the six families at sites TBO, FTBC and KYBO were 29.4, 28.3, and 22.7 cm, respectively (Table 1). A model including the interaction between site and family was selected as the best model for seedling height (Table S1). After separating the data according to site, the model selected for each site included the family component. Multiple comparisons showed that seedling height significantly varied among families in all sites (Table 1). The height of T54 × O12 was always significantly lower than that of the other families. M105 × A1 was the tallest family in TBO, but was the fourth tallest family in FTBC and KYBO.

### 3.2. Sound Seedling Rate

The Kaplan–Meier estimators for sound seedling rates showed that the disease developmental process varied among the three sites (Figure 3). Diseased seedlings were first observed at 2 WAI at TBO and KYBO, and one week later at FTBC. The sound seedling rate sharply decreased until 6, 5 and 4 WAI in TBO, FTBC and KYBO, respectively, and then decreased more gradually. Total sound seedling rate at 10 WAI across the three sites was 0.27 ± 0.45 (mean ± SD) and 0.24 ± 0.43, 0.50 ± 0.50, and 0.07 ± 0.25 in TBO, FTBC, and KYBO, respectively. Pairwise log-rank tests showed that the survival curves of the three sites significantly differed from each other ($X^2$: 97.7, *d.f.*: 1, *p*: <0.001 for TBO vs. FTBC; $X^2$: 60.9, *d.f.*: 1, *p*: <0.001 for TBO vs. KYBO; $X^2$: 399, *d.f.*: 1, *p*: <0.001 for FTBC vs. KYBO).

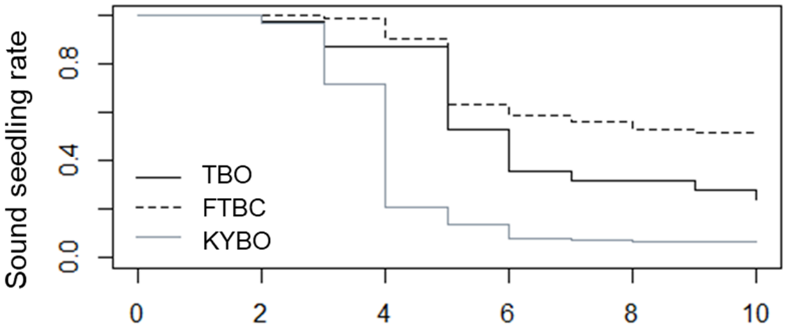

**Figure 3.** Kaplan–Meier estimator for sound seedling rate of six *Pinus thunbergii* full-sib families inoculated with *Bursaphelenchus xylophilus* in the three experimental sites. Black, dashed and gray lines indicate TBO, FTBC, and KYBO respectively.

The Kaplan–Meier estimators showed that the disease developmental process varied among families in all sites (Figure 4). Diseased seedlings were observed at 2 WAI in three (T54 × O12, K24 × K29, and M105 × A1) of the six families at TBO and in four (Y2 × T63, T54 × O12, K24 × K29, and M105 × A1) of the six families at KYBO (Table S2). At FTBC, disease development in inoculated seedlings appeared at 3 WAI in two families (T54 × O12 and K24 × K29). Pairwise log-rank tests showed that families derived from high- and intermediate-resistance parental clones had a significantly lower risk of disease development than the families derived from low-resistance and plus-tree parental clones (Figure 4). The curves of the disease development process were more clearly divergent among families in FTBC and TBO than in KYBO.

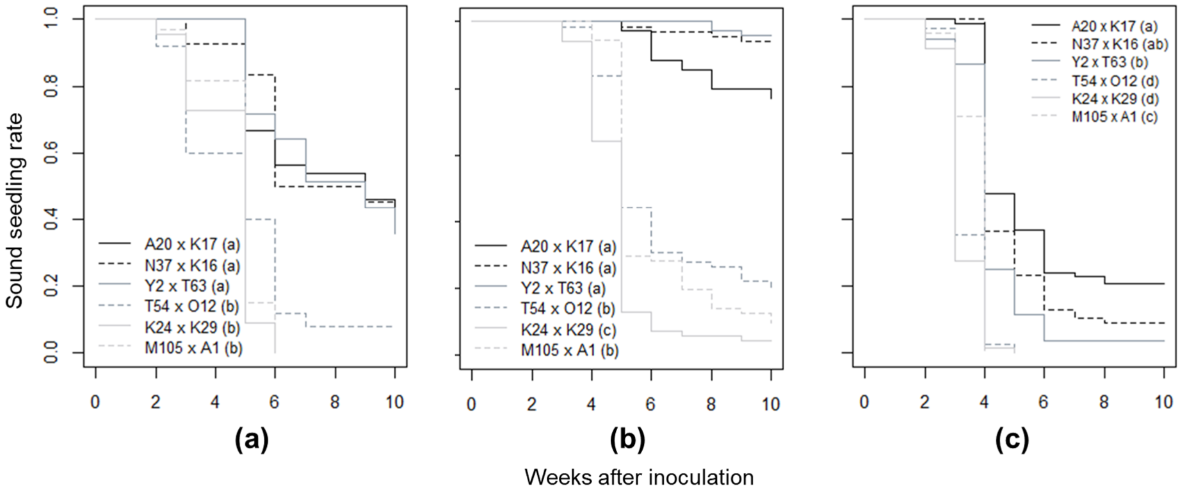

**Figure 4.** Kaplan–Meier estimator for sound seedling rate of six *Pinus thunbergii* full-sib families inoculated with *Bursaphelenchus xylophilus* in each of the three experimental sites. (**a**) TBO; (**b**) FTBC; and (**c**) KYBO. See Table 1 for abbreviated names of pine families. Family names followed by a common letter are not significantly different at the 5% level of significance.

Spearman correlation coefficients for sound rate were higher than 0.698 at three or more WAI in each pair of sites (Table 2). At 2 WAI, when disease development was in the initial phase, the coefficient was relatively low: 0.400 between TBO and KYBO.

**Table 2.** Spearman's correlation coefficient for sound seedling rate of *Pinus thunbergii* inoculated with *Bursaphelenchus xylophilus*.

| Week | Spearman's Correlation Coefficient ($p$-Value) | | |
|---|---|---|---|
| | TBO vs. FTBC | TBO vs. KYBO | FTBC vs. KYBO |
| 2 | | 0.400 (0.419) | |
| 3 | 0.789 (0.058) | 0.754 (0.103) | 0.845 (0.033) |
| 4 | | | 0.759 (0.058) |
| 5 | 0.943 (0.003) | 0.759 (0.058) | 0.698 (0.136) |
| 6 | 0.928 (0.017) | 0.770 (0.058) | 0.698 (0.136) |
| 7 | 0.812 (0.058) | 0.893 (0.017) | 0.698 (0.136) |
| 8 | | | 0.698 (0.136) |
| 9 | 0.754 (0.103) | 0.955 (0.003) | 0.698 (0.136) |
| 10 | 0.812 (0.058) | 0.893 (0.017) | 0.698 (0.136) |

Correlation coefficients were calculated for data after the occurrence of disease development in seedlings.

For the variance components from 5–10 WAI, the family component consistently occupied the largest proportion (Figure 5, Table S3). The proportion of the variance component of the interaction between site and family was consistently small compared to that of both site and family separately.

Variance component proportions of replication within site were consistently very small (less than 1% in all examined weeks).

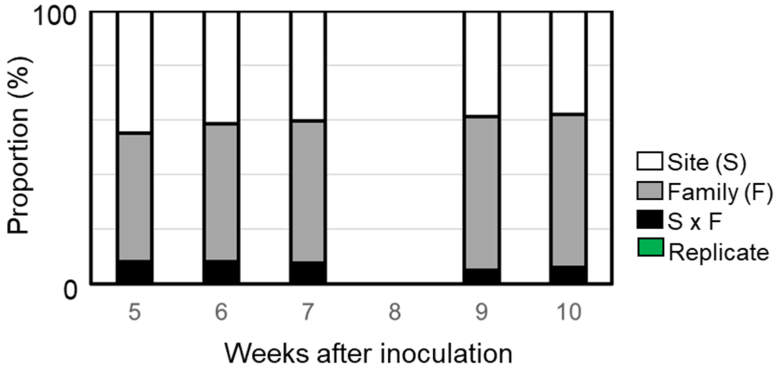

**Figure 5.** Proportion of variance components of site, family, their interaction, and replication within site for sound seedling rate of *Pinus thunbergii* after inoculation with *Bursaphelenchus xylophilus*. Variance component analysis was not conducted for 8 weeks after inoculation due to missing data in TBO.

Survival rate data was similar to that of the sound seedlings rate. All survival rate results are shown in supplemental figures and tables (survival analysis among sites: Figure S1, survival analysis among families: Figure S2, Correlation: Table S4, and variance component analysis: Figure S3 and Table S5).

## 4. Discussion

In this study, we inoculated six *P. thunbergii* families with variable resistance to PWD with a *B. xylophilus* isolate in nurseries at three different sites in the northern and southern regions of the Japanese archipelago. Consequently, families with higher sound seedling rate in the KYBO site exhibited higher sound seedling rate in the TBO and FTBC sites. Spearman correlation coefficients for family sound seedling rate among sites were relatively high and positive. Moreover, variance component analyses revealed only a small contribution of the interaction between site and family to total variance in sound seedling rate. These results show that the *P. thunbergii* seedlings obtained from the selected resistant clones with high resistance level in southern Japan may retain their high resistance in northern Japan.

Previous studies have described the G × E interaction of resistance or susceptibility of pine seedlings to *B. xylophilus*. A six-year inoculation experiment using the 16 half-sib families of resistant *P. thunbergii* clones showed that the variance component of the interaction between year and family was less than one half of the family variance component [16]. G × E interactions in the susceptibility to *B. xylophilus* was reported in *P. pinaster*, based on greenhouse inoculation tests using seedlings derived from six provenances [18]. Although the magnitude of the effect of the interaction was not clearly described in the paper, seedlings from a particular provenance may exhibit some degree of interaction. The results of the previous studies and the present study suggest that the effect of the G × E interaction of resistance to *B. xylophilus* could be small in the half-sib or full-sib families of pine seedlings, although certain genetic groups may be more sensitive to the ambient environment.

In this study, the sound seedling rate was highest in FTBC, followed by TBO and KYBO. TBO is located northward of FTBC, with a cooler climate (specifically, climatological standard normal of the average monthly temperature in July is 21.8 °C for TBO and 22.8 °C for FTBC). Since low temperature suppresses the progress of PWD development [33,34], we expected that the sound seedling rate of TBO would be the highest, but based on the inoculation test results of TBO this was not the case. Close examination of the meteorological data in the experimental year, 2014, revealed that the average temperature in July was 23.5 °C in both TBO and FTBC, and the average temperature during the week after inoculation was 22.8 °C in TBO and 21.6 °C in FTBC. The low sound seedling rate in TBO may have been affected by the slightly higher temperature just after inoculation.

By March 2020, 54 first-generation and 40 second-generation PWD-resistant *P. thunbergii* clones had been selected in southwestern Japan. If progeny of the resistant clones selected in the southern region were to be planted in the northern region, the following factors should be considered: growth, snow-resistance, reproductive traits of clones, administrative seed transfer zones [35] and genetic structure of *P. thunbergii* throughout Japan [36]. However, the present study focused on the most important factor, which is the resistance to pinewood nematode. Using the most- and least-resistant seedlings available from southern *P. thunbergii* clones, we showed that the possibility of southern high-resistance clones could be used in the eastern and northern regions of Japan. Introduction of the southern resistant clones into the production population in eastern and northern regions could enable the promotion of resistance breeding programs in those regions. Conversely, the possibility of utilizing northern resistant clones in the southern region should also be considered.

## 5. Conclusions

Inoculation tests for six *P. thunbergii* families with different resistance levels were carried out using an isolate of *B. xylophilus* at three sites with different climates. We indicated that the resistant rank of the families was relatively stable regardless of different climates among three sites. The results obtained in this study suggest that the resistant *P. thunbergii* clones selected in the southern region of Japan may relatively perform their high genetic resistance well in the northern region of Japan.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1999-4907/11/9/955/s1, Figure S1: Kaplan–Meier estimator for survival rate of seedlings of six *P. thunbergii* full-sib families inoculated with *B. xylophilus* in three experimental sites, Figure S2: Kaplan–Meier estimator for survival rate of six *P. thunbergii* full-sib families inoculated with *B. xylophilus* in each of three experimental site, Figure S3:Propertion of variance components of site, family, their interaction and replication within site for survival rate of *P. thunbergii* after inoculation of *B. xylophilus*, Table S1: Model selection table for mean height of *P. thunbergii* seedlings, Table S2: Time trend in mean sound seedling rate and survival rate of seedlings of six *P. thunbergii* families inoculated with *B. xylophilus* in three experimental sites, Table S3: Estimated variance components for sound seedling rate of *P. thunbergii* after inoculation of *B. xylophilus*, Table S4: Spearman's correlation coefficients for survival rate of *P. thunbergii* seedlings inoculated with *B. xylophilus*, Table S5: Estimated variance components for survival rate of *P. thunbergii* seedlings inoculated with *B. xylophilus*.

**Author Contributions:** Conceptualization, K.M.; materials management and investigation, T.I., M.O., T.Y., M.G.I, M.M., K.I., M.K.; materials production, K.M., M.K.; methodology, T.H.; data compilation and analysis, T.I, K.M.; writing—original draft preparation, K.M., T.I.; writing—review and editing, T.Y., T.H., M.O., M.G.I., M.M., M.K., M.T. and A.W.; project administration, T.H., M.T. and A.W. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Forest Tree Breeding Center (Japan). *Statistics and States of Implementation in Forest Tree Breeding Projects in Japan*; Forest Tree Breeding Center: Hitachi, Japan, 2020; pp. 68–69.
2. Kiyohara, T.; Tokushige, Y. Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *J. Jpn. For. Soc.* **1971**, *53*, 105–114. (In Japanese with English Summary)
3. Mamiya, Y. History of pine wilt disease in Japan. *J. Nematol.* **1988**, *20*, 219–226. [PubMed]
4. Kazuyoshi, F. Pine wilt in Japan from first incidence to the present. In *Pine Wilt Disease*; Zhao, B.G., Futai, K., Sutherland, J.R., Takeuchi, Y., Eds.; Springer: New York, NY, USA, 2008; pp. 5–12.
5. Nakajima, G.; Iki, T.; Yamanobe, T.; Nakamura, K.; Aikawa, T. Spatial and temporal distribution of *Bursaphelenchus xylophilus* inoculated in grafts of a resistant clone of *Pinus thunbergii*. *J. For. Res.* **2019**, *24*, 93–99. [CrossRef]

6. Mota, M.M.; Braasch, H.; Bravo, M.A.; Penas, A.C.; Burgermeister, W.; Metge, K.; Sousa, E. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* **1999**, *1*, 727–734. [CrossRef]
7. Abelleira, A.; Picoaga, A.; Mansilla, J.P.; Aguin, O. Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in northwestern Spain. *Plant Dis.* **2011**, *95*, 776. [CrossRef] [PubMed]
8. Robertson, L.; Cobacho Arcos, S.; Escuer, M.; Santiago Merino, R.; Esparrago, G.; Abelleira, A.; Navas, A. Incidence of the pinewood nematode *Bursaphelenchus xylophlius* Steiner & Buhrer, 1934 (Nickle, 1970) in Spain. *Nematology* **2011**, *13*, 755–757. [CrossRef]
9. Inácio, M.L.; Nóbrega, F.; Vieira, P.; Bonifácio, L.; Naves, P.; Sousa, E.; Mota, M. First detection of *Bursaphelenchus xylophilus* associated with *Pinus nigra* in Portugal and in Europe. *For. Path.* **2015**, *45*, 235–238. [CrossRef]
10. Vicente, C.; Espada, M.; Vieira, P.; Mota, M. Pine Wilt Disease: A threat to European forestry. *Eur. J. Plant Pathol.* **2012**, *133*, 89–99. [CrossRef]
11. Hirata, A.; Nakamura, K.; Nakao, K.; Kominami, Y.; Tanaka, N.; Ohashi, H.; Takano, K.T.; Takeuchi, W.; Matsui, T. Potential distribution of pine wilt disease under future climate change scenarios. *PLoS ONE* **2017**, *12*, e0182837. [CrossRef]
12. Fujimoto, Y.; Toda, T.; Nishimura, K.; Yamane, H.; Fuyuno, S. Breeding project on resistance to the pine-wood nematode an outline of the research and the achievement of the project for ten year. *Bull. For. Tree Breed. Inst.* **1989**, *7*, 1–84.
13. Kurinobu, S. Current status of resistance breeding of Japanese pine species to pine wilt disease. *Forest Sci. Technol.* **2008**, *4*, 51–57. [CrossRef]
14. Nose, M.; Shiraishi, S. Breeding for resistance to pine wilt disease. In *Pine Wilt Disease*; Zhao, B.G., Futai, K., Sutherland, J.R., Takeuchi, Y., Eds.; Springer: New York, NY, USA, 2008; pp. 334–350. [CrossRef]
15. Futai, K. Furuno Tooshu The variety of resistances among pine-species to pinewood nematode, Bursaphelenchus lignicolus. *Bull. Kyoto Univ. For.* **1979**, *51*, 23–36, (In Japanese with English Summary).
16. Toda, T.; Kurinobu, S. Genetic improvement in pine wilt disease resistance in *Pinus thunbergii*: The effectiveness of pre-screening with an artificial inoculation at the nursery. *J. For. Res.* **2001**, *6*, 197–201. [CrossRef]
17. Iwaizumi, M.G.; Tamaki, S.; Isoda, K.; Kubota, M. Evaluation of combining ability for pine wood nematode-resistance based on half-diallele lines of *Pinus densiflora* resistant clones. *For. Genet. For. Breed* **2019**, *8*, 121–130. [CrossRef]
18. Menéndez-Gutiérrez, M.; Alonso, M.; Toval, G.; Díaz, R. Variation in pinewood nematode susceptibility among *Pinus pinaster* Ait. provenances from the Iberian Peninsula and France. *Ann. For. Sci.* **2017**, *74*, 76. [CrossRef]
19. Menéndez-Gutiérrez, M.; Alonso, M.; Toval, G.; Díaz, R. Testing of selected *Pinus pinaster* half-sib families for tolerance to pinewood nematode (*Bursaphelenchus xylophilus*). *Forestry* **2018**, *91*, 38–48. [CrossRef]
20. Carrasquinho, I.; Lisboa, A.; Inácio, M.L.; Gonçalves, E. Genetic variation in susceptibility to pine wilt disease of maritime pine (*Pinus pinaster* Aiton) half-sib families. *Ann. For. Sci.* **2018**, *73*, 45–67. [CrossRef]
21. Kiyohara, T. Effect of temperature on the disease incidence of pine seedlings inoculated with *Bursaphelenchus lignicolus*. *Forest Soc. Ann. Trans. Meet. Jpn.* **1973**, *84*, 334–335. (In Japanese)
22. Kazuo, S.; Tomoya, K. Influence of water stress on development of pine wilting disease caused by *Bursaphelenchus lignicolus*. *Eur. J. For. Pathol.* **1978**, *8*, 97–107. [CrossRef]
23. Shigeru, K. Effect of light intensity on the development of pine wilt disease. *Can. J. Bot.* **1989**, *67*, 1861–1864. [CrossRef]
24. Japan Meteorological Agency. 2020 Climate Statistics. Available online: http://www.data.jma.go.jp/obd/stats/etrn/index.php (accessed on 26 February 2020).
25. Toda, T. Studies on the breeding for resistance to the pine wilt disease in *Pinus densiflora* and *P. thunbergii*. *Boll. Tree Breed. Cent.* **2004**, *20*, 83–217.
26. Matsunaga, K.; Ohira, M.; Kuramoto, N.; Kurahara, Y.; Hoshi, H.; Yamada, H.; Koyama, T.; Miyazato, M. Evaluation of pinewood-nematode resistance in open pollinated seedlings from additionally selected *Pinus thunbergii* clones Kyushu. *J. For. Res.* **2011**, *64*, 84–86.
27. Aikawa, T.; Kikuchi, T.; Kosaka, H. Demonstration of interbreeding between virulent and avirulent populations of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) by PCR-RFLP method. *Appl. Entomol. Zool.* ***2003***, 38, *565–569*. [CrossRef]

28. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020.
29. Bates, D.; Mächler, M.; Bolker, B.M.; Walker, S.C. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **2015**, *67*, 1–48. [CrossRef]
30. Barton, K. MuMIn: Multi-Model Inference. R Package Version 1.43.17 2020. Available online: https://cran.r-project.org/web/packages/MuMIn/index.html (accessed on 22 May 2020).
31. Hothorn, T.; Bretz, F.; Westfall, P. Simultaneous inference in general parametric models. *Biometr. J.* **2008**, *50*, 346–363. [CrossRef]
32. Therneau, M. A Package for Survival Analysis in R. R Package Version 3.2-3. 2020. Available online: https://CRAN.R-project.org/package=survival (accessed on 22 May 2020).
33. Ichihara, Y.; Fukuda, K.; Suzuki, K. Early symptom development and histological changes associated with migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. *Plant Dis.* **2000**, *84*, 675–680. [CrossRef]
34. Yamaguchi, R.; Matsunaga, K.; Watanabe, A. Influence of temperature on pine wilt disease progression in *Pinus thunbergii* seedlings. *Eur. J. Plant Pathol.* **2020**, *156*, 581–590. [CrossRef]
35. Masahiro, K. Breeding district and seed transfer zone. *For. Genet. Tree Breed.* **2015**, *4*, 12–15. (In Japanese)
36. Iwaizumi, M.G.; Miyata, S.; Hirao, T.; Tamura, M.; Watanabe, A. Historical seed use and transfer affects geographic specificity in genetic diversity and structure of old planted *Pinus thunbergii* populations. *For. Ecol. Manag.* **2018**, *408*, 211–219. [CrossRef]