Eur J Plant Pathol (2009) 124:543–552
DOI 10.1007/s10658-009-9438-y

Relationships between morphological traits and resistance to pine wood nematode in two Japanese pines

Taro Yamanobe

Received: 20 September 2008 / Accepted: 29 January 2009 / Published online: 14 February 2009

Abstract It has been suggested that certain morphological traits of Japanese pines function as resistance factors against the pine wood nematode (PWN), *Bursaphelenchus xylophilus*. The aim of this study was to verify this concept, and to determine the relationships between traits and resistance at both the individual and family levels. Relationships between survival rate and morphological traits of *Pinus densiflora* and *Pinus thunbergii* were studied after inoculation with PWN. Morphometric parameters used were height, basal diameter of the axis, and the number of branches from the axis. Three indices of relationships were analysed: (1) among individuals within a family (survival difference between smaller and larger morphometric populations within each family); (2) among individuals (correlation between each morphometric average and survival rate in quintile populations of all subjects within each pine species based on the order of morphometric values); and (3) among families (correlation between each morphometric average and survival rate in the family). Both significant and non-significant differences were detected within a family, indicating a need for macro techniques. Among individuals, a thicker axis and more branches were correlated with increased survival, indicating that they are resistance factors. However, these correlations were not significant among different families. These results indicate that morphological traits cannot be used to evaluate resistance at a family level, even if they are significant at an individual level. Further studies on traits associated with resistance should be conducted at a family rather than an individual level, to breed for Japanese pines with resistance to PWN.

Keywords Diameter · Height · Number of branches · *Pinus densiflora* · *Pinus thunbergii* · Survival rate

T. Yamanobe (✉)
Kansai Breeding Office, Forest Tree Breeding Centre,
Forestry and Forest Products Research Institute,
Uetsukinaka 1043,
Shoo, Okayama 709-4335, Japan
e-mail: yamanobe@affrc.go.jp

Abbreviations

ANOVA	analysis of variance
Current-inoculation	inoculation of the current axis
FFPRI	Forestry and Forest Products Research Institute (Japan)
FTBC	Forest Tree Breeding Centre (in FFPRI)
KBO	Kansai Breeding Office (in FTBC)
PWD	pine wilt disease
PWN	pine wood nematode
SMSG	Shikoku Material Stock Garden (in KBO)
1y-inoculation	inoculation of the 1 year-old axis
2y-inoculation	inoculation of the 2 year-old axis

Introduction

Pine wilt disease (PWD) is the most serious tree disease in Japan, and has led to extensive damage of pine forests throughout the country (Mamiya 1988; Nakamura and Novotoy 1999; Forestry Agency of Japan 2007). The disease is caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Kiyohara and Tokushige 1971; Mamiya and Kiyohara 1972; Nickle et al. 1981). Since the detection of PWN in Portugal, the spread of PWD is an increasing concern in Europe (Mota et al. 1999; Futai and Sahashi 2006).

As part of a national programme to protect plantation pine forests, resistant clones were selected from stands after a PWD epidemic. Young grafted clones from these trees subsequently showed less damage after being inoculated with PWN (Fujimoto et al. 1989). These resistant lines consisted of 92 clones of Japanese red pine, *Pinus densiflora*, and 16 clones of Japanese black pine, *Pinus thunbergii*. Clonal seed orchards were established using these clones, and after inoculation with *B. xylophilus*, the survival rate of the young progeny seedlings from the selected clones was higher than that of non-selected pines (Toda and Kurinobu 2002). Therefore this selection technique should be successful in isolating a population with increased PWN resistance, at least in young material. Experiments aimed at selecting PWN-resistant pines are being conducted at the Forest Tree Breeding Centre (FTBC), Forestry and Forest Products Research Institute (FFPRI), and many prefectural institutes of forestry throughout Japan (Higasihara et al. 2007).

Recently, several studies have suggested that morphological traits (height, basal diameter of the axis, and the number of branches) of Japanese pines are associated with resistance to PWN at a family level (Toda et al. 1986; Toda and Fujimoto 1987; Sasaki et al. 2002; Kuroda 2004).[1] However, it is unclear whether these traits are truly relevant in predicting PWD survival rate, as there are many discrepancies among the various observations of morphological characteristics and PWN responses among tree populations (Toda et al. 1986; Toda and Fujimoto 1987; Sasaki et al. 2002; Kuroda 2004; Kuramoto et al. 2006; Table 1). These discrepancies may reflect the complexities of the relationships among traits, individuals, and families.

In the present study, the relationships between pine survival and morphological traits were examined from different perspectives: among individuals within a family, among individuals, and among families.

Materials and methods

Pinus densiflora experiment

PWN inoculation tests were carried out in the nursery of the Shikoku Material Stock Garden (SMSG), Kansai Breeding Office (KBO), FTBC-FFPRI, Kami, Kochi, Japan (33°36′ N, 133°41′ E). Temperature was recorded at 30-min intervals, and the mean was 28.0°C (range, 22.9–36.2°C). Total precipitation was 203 mm in the month following PWN inoculation (July 26–August 25, 2006).

The plant material consisted of 28 month-old seedlings obtained from five progenies of clones selected for resistance to PWN (Okada and Tsuda 1989; Takeuchi et al. 1989; Toda et al. 1989). Progenies were collected from one ramet of each clone. Each family was planted in a plot of 38–40 seedlings (25 seedlings m^{-2}) in a randomised block design with three replications. Plants were maintained in the nursery of SMSG-KBO-FTBC-FFPRI. All plots were divided into two subplots and assigned for inoculations—inoculation of the current axis (Current-inoculation) and inoculation of the 2 year-old axis (2y-inoculation) (Fig. 1).

The PWN inoculum was a Ka-4 isolate (strong virulence), cultured on the fungus *Botrytis cinerea* grown on autoclaved medium. *Botrytis cinerea* was cultured on a medium consisting of 20 g dried barley and 20 ml sucrose water (2%, *w*/*v*) in glass Petri dishes (9 cm width × 2 cm depth), cultured at 25°C in the dark. PWN were separated from the media using the Baermann funnel technique. The suspended population was adjusted to 10,000 nematodes per 0.1 ml aliquot (Yamanobe 2005). One aliquot was inoculated into a wound on each pine. Wounding was carried out by peeling the cortex and scratching the xylem using a fine saw. These methods followed normal practice at FTBC-FFPRI (Fujimoto et al. 1989; Toda 2004). The positions of wounds for

[1] Title was tentatively translated by author when reference only provided Japanese title.

Table 1 Previous reports on the relevance of morphological traits of seedlings to susceptibility to PWN

Morphological trait	Conclusion		Trend
	Relevance	Irrelevance	
Height	Toda and Fujimoto (1987)[a] Toda et al. (1986)[b]	Sasaki et al. (2002)[b]	Smaller seedlings tend to be PWD-susceptible
Basal diameter	Sasaki et al. (2002)[b]	Kuramoto et al. (2006)[b]	Thinner seedlings tend to be PWD-susceptible
Number of branches	Kuroda (2004)[a]		Lesser-branched subjects tend to be PWD-susceptible

[a] did not use any statistical test

[b] did not use family statistical test; Sasaki et al. (2002) used non-damaged rate whereas the other authors used survival rate

Current-inoculation and 2y-inoculation are shown in Fig. 1. Inoculations were carried out on 26 and 27 July 2006. Randomly selected plants (22 in total) from OI142, OI203, SA118 and SO39 were used as controls, and their 2 year-old axes were inoculated with water.

The measured variables were survival and three morphometric parameters; height, basal diameter of the axis, and the number of branches from the axis. Height and basal diameter were measured within 2 weeks of inoculation using a tape measure and a digital calliper. Survival and the number of branches were measured in mid-December 2006.

Pinus thunbergii experiment

The PWN inoculation test was carried out in the nursery of the KBO-FTBC-FFPRI, Shoo, Okayama, Japan (35°3′ N, 134°6′ E). Temperature was recorded at 30-min intervals, and mean temperature was 27.1°C (range, 18.7–37.9°C). Total precipitation was 82 mm in the month following PWN inoculation (21 July–20 August 2005). The plant material consisted of 28 month-old seedlings obtained from 15 families; 13 open-pollinated progenies from individuals selected as candidate PWN-resistant clones, selected from a coastal area in Shimane Prefecture in Japan (Table 2, selected by S. Tamaki and T. Yamanobe, KBO-FTBC-FFPRI, unpublished), and two artificially crossed progenies from PWN-resistant selections (Takeuchi et al. 1989, Toda et al. 1989). Each family was planted in a plot of 25–91 seedlings (25 seedlings m^{-2}), without replication, in the nursery. All plots were divided into two sub-plots and assigned for Current-inoculation and inoculation of 1 year-old axis (1y-inoculation), except for the two artificially crossed families (Fig. 1). In these two families, only Current-inoculation was carried out, to enable data sharing with other studies.

The Shimabara PWN isolate (strong virulence, somewhat weaker than Ka-4; Tamaki and Yamanobe

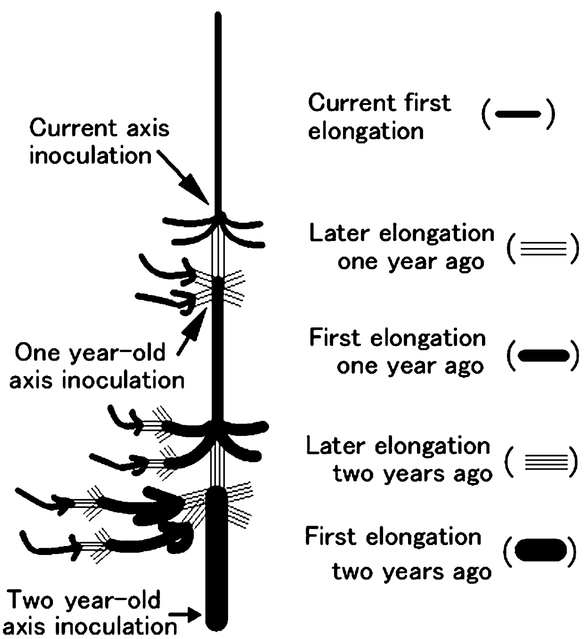

Fig. 1 Axis and verticillate branch formation in 28 month-old seedlings of *Pinus densiflora* and *P. thunbergii* observed at the end of July, and inoculation position. *Different lines* represent different elongation periods. Branch elongations at right-hand side are omitted for brevity. Some pines in temperate zones grow in a series of flashes/elongations during the growing season (Kozlowski and Pallardy 1997). *Pinus densiflora* and *P. thunbergii* seedlings usually formed four or fewer nodes of joints of verticillate branches and axis at the end of July, and occasionally lacked first and later flashes/elongations. Some adventitious branches formed from short shoots on the axis

Table 2 Morphometrics of seedling pines

Family	Family code	*n*	Height (cm)	Basal diam (mm)	Number of branches from axis
Pinus densiflora					
Niihama7	NI7	120	125.3±22.7	21.8±5.8	20.0±9.0
Ohita142	OI142	118	114.5±28.3	21.2±7.1	20.0±12.1
Ohita203	OI203	118	137.6±26.2	23.4±6.7	23.6±11.5
Saganoseki118	SA118	120	86.6±16.5	19.3±5.7	15.5±5.2
Soja39	SO39	119	125.8±32.2	22.7±6.8	20.4±10.2
Pinus thunbergii					
Misaki90 × Ohita8	MI90 × OI8	57	60.4±9.6	12.8±1.7	7.9±2.4
Namikata73 × Ohseto12	NA73 × OS12	25	63.2±10.2	13.7±2.1	10.6±3.1
Ohda3	OD3	91	64.6±10.5	13.5±2.0	10.7±2.9
Ohda4	OD4	91	72.1±11.3	14.1±1.9	10.3±3.4
Ohda8	OD8	84	64.7±12.5	14.2±2.2	10.5±2.6
Ohda9	OD9	90	63.8±12.4	12.1±2.1	9.8±2.5
Yunotsu2	Y2	90	70.0±10.5	14.6±1.8	9.0±2.9
Yunotsu3	Y3	88	68.2±13.7	12.9±2.5	9.6±2.7
Yunotsu4	Y4	90	64.5±8.8	12.8±1.7	10.3±3.0
Yunotsu6	Y6	91	71.8±11.7	13.3±2.0	8.5±2.4
Yunotsu7	Y7	91	62.8±11.1	13.0±1.8	9.2±2.5
Yunotsu8	Y8	90	52.3±7.4	11.3±1.6	9.7±2.8
Yunotsu10	Y10	68	50.8±10.6	11.0±2.0	9.6±3.3
Yunotsu11	Y11	91	53.0±9.3	11.2±1.9	9.0±2.6
Yunotsu12	Y12	89	53.6±8.5	12.1±1.8	10.5±2.8

All the values are mean ± SD

2006)[2] was used for inoculation. Other procedures were the same as described above for *P. densiflora*. Inoculations were carried out on 21 July 2005. Control plants (49 in total) were inoculated with water, at one or two per inoculation position per family. Morphometric values and survival of subjects were evaluated in 2005, as described above for *P. densiflora*.

Data and statistical analyses

The relationships between survival and three morphological traits were analysed among individuals within a family, among individuals, and among families. The following three indices were used in the analyses:

(1) Among individuals within a family [38 analyses per morphological trait (five families of *P. densiflora* plus 13 families of *P. thunbergii*) × two inoculation positions and two artificially crossed families of *P. thunbergii* in Current-inoculation]—all plants within a family per inoculation position were classified into smaller and larger populations by the median for each morphological trait. Plants in each population were further classified on the basis of survival (alive or dead). A 2×2 contingency table (smaller/larger morphometric population × alive/dead) was tested using Fisher's exact test.

(2) Among individuals (four analyses per morphological trait; two pine species × two inoculation positions)—all pine plants per inoculation position were classified into quintiles based on each morphological trait. Pearson's correlation between each morphometric average and survival rate in the quintile was analysed.

(3) Among families (four analyses per morphological trait; two pine species × two inoculation positions)—all plants per inoculation position were classified into each family. Pearson's correlation between each morphometric average and survival rate was analysed.

[2] Ibid.

For family comparison of all three morphometric variables and survival rate, a one-way analysis of variance (ANOVA) for a randomised block design was carried out for each inoculation position in *P. densiflora*. A positional comparison of survival rates between PWN inoculation sites by using family values was carried out. Differences between the two inoculation positions were tested by paired *t*-test in five families of *P. densiflora* using averages of three replications, and in 13 families of *P. thunbergii* using plot values. Fisher's exact test, Pearson's correlation, and the paired *t*-test were performed using Kyplot4.0 software (KyensLab, Inc., Tokyo, Japan). One way ANOVAs were performed using the GLM procedure in SAS version 9.1.3. Data for correlations, paired *t*-tests, and ANOVAs were logarithmically transformed morphometric values and arcsine square root transformed survival rates (Falconer and Mackay 1996). Survival rate was used in this study instead of mortality for consistency with previous reports on breeding for PWN-resistance (Kato et al. 1995, Toda and Kurinobu 2001, 2002).

Results

In *P. densiflora,* family average height was 86.6–137.6 cm, basal diam was 19.3–23.4 mm, and there were 15.5–23.6 branches. In *P. thunbergii*, family average height was 50.8–72.1 cm, basal diam was 11.0–14.6 mm, and there were 7.9–10.7 branches (Table 2). No damage was observed in the controls.

Among individuals within a family, there were some cases in which a small morphometric population showed reduced survival after PWN inoculation. Among the 38 analyses, significant differences in height and basal diam were detected in 13 analyses (34%) and in branch number in nine analyses (24%) (Table 3).

Among individuals, plants with thicker axis bases showed increased survival (r=0.87–0.99, P<0.057, Table 4). A greater number of branches was associated with increased survival rates in three of four analyses (r=0.89–0.96, P<0.05, Table 4), except for the 2y-inoculation in *P. densiflora* (r=0.71, P=0.18, Table 4). Height did not affect survival (r=0.45–0.84, P=0.08–0.45, Table 4) except in 2y-inoculation in *P. densiflora* (r=0.92, P<0.05, Table 4).

Among families, no morphological traits affected family survival rate. Coefficients of correlation were low to moderately positive or negative (r=−0.50 to 0.31, P=0.31–0.88, Table 4).

There were family differences in height in both inoculation position and survival rate after 2y-inoculation (ANOVA; F=4.5–16.0, P<0.05, Table 5) but not in the other dependent variables (ANOVA; F=1.2–2.9, P=0.09–0.39, Table 5).

Family survival rates were higher after Current-inoculation than those after 1y- or 2y-inoculation. In *P. densiflora,* survival after Current-inoculation was 61.7–86.7%, and 31.7–67.8% after 2y-inoculation (*d.f.*=4, t=5.5, P<0.05, Table 6). In *P. thunbergii*, 21.7–93.3% survival was observed after Current-inoculation and 0–54.5% after 1y-inoculation (*d.f.*=12, t=6.1, P<0.05, Table 6).

Discussion

Death of seedlings in the experimental treatments was due to PWN inoculation, as there was no damage detected in the controls.

The relationships between each morphological trait and survival should not be discussed with respect to differences among individuals within a family, because both significant and non-significant differences were detected (Table 3). Various conclusions have been made regarding the influence of morphological traits on disease resistance, and many discrepancies have been observed (Toda et al. 1986; Sasaki et al. 2002; Kuramoto et al. 2006). Such discrepancies are explained by the data in the present study.

The three morphological traits showed different levels of relevance among individuals. With respect to basal diameter, a thicker subject was predicted to survive better because all correlations using the quintile classification showed a P<0.06 value of significance in basal diameter (Table 4). Trees can survive as long as there is partial passage for xylem and phloem transport, even if almost no transport occurs (Kuroda 1999). Trees with a wider diameter at the base, therefore, may have a greater potential to retain functional passages than thinner trees. With respect to the number of branches, subjects with more branches would survive better as long as there are more branches below the inoculation position, because there were significant correlations using quintile

Table 3 Difference in survival rate between smaller (above) and larger (lower) population in the three morphometric values within each family

Family code	Height			Basal diam			Number of branches from axis		
	n	Survival rate (%)	*P*	*n*	Survival rate (%)	*P*	*n*	Survival rate (%)	*P*
Pinus densiflora—current axis inoculation									
NI7	31	45.2	0.00*	30	43.3	0.00*	30	53.3	0.10
	29	79.3		30	80.0		30	70.0	
OI142	30	60.0	0.00*	30	60.0	0.00*	32	62.5	0.02*
	29	89.7		29	89.7		27	88.9	
OI203	30	73.3	0.27	28	64.3	0.00*	33	81.8	0.34
	30	83.3		32	90.6		27	74.1	
SA118	28	75.0	0.00*	32	78.1	0.00*	31	77.4	0.03*
	32	96.9		28	96.4		29	96.6	
SO39	30	73.3	0.00*	29	69.0	0.00*	27	85.2	0.6
	30	96.7		31	100.0		33	84.8	
Pinus densiflora—2 year-old axis inoculation									
NI7	30	20.0	0.05*	31	19.4	0.03*	27	25.9	0.28
	30	43.3		29	44.8		33	36.4	
OI142	30	33.3	0.00*	29	24.1	0.00*	30	43.3	0.01*
	29	86.2		30	93.3		29	75.9	
OI203	30	23.3	0.03*	28	14.3	0.00*	30	30.0	0.23
	28	50.0		30	56.7		28	42.9	
SA118	31	38.7	0.03*	30	26.7	0.00*	30	46.7	0.30
	29	65.5		30	76.7		30	56.7	
SO39	30	53.3	0.02*	27	48.1	0.00*	31	61.3	0.20
	29	82.8		32	84.4		28	75.0	
Pinus thunbergii—current axis inoculation									
OD9	22	31.8	0.50	22	22.7	0.10	17	5.9	0.00*
	22	36.4		22	45.5		27	51.9	
Y2	22	13.6	0.03*	23	21.7	0.23	26	23.1	0.25
	23	43.5		22	36.4		19	36.8	
Y3	22	63.6	0.08	22	63.6	0.08	23	52.2	0.00*
	22	86.4		22	86.4		21	100.0	
Y4	22	63.6	0.62	22	50.0	0.06	21	38.1	0.00*
	22	63.6		22	77.3		23	87.0	
Y6	25	8.0	0.02*	23	17.4	0.36	15	6.7	0.09
	21	38.1		23	26.1		31	29.0	
Y7	24	12.5	0.05*	23	13.0	0.07	28	14.3	0.05*
	21	38.1		22	36.4		17	41.2	
Pinus thunbergii—1 year-old axis inoculation									
OD3	23	8.7	0.48	23	0.0	0.02*	17	5.9	0.37
	22	13.6		22	22.7		28	14.3	
OD8	21	42.9	0.26	21	19.0	0.03*	23	21.7	0.04*
	21	28.6		21	52.4		19	52.6	
OD9	23	8.7	0.04*	23	4.3	0.00*	20	15.0	0.27
	23	34.8		23	39.1		26	26.9	
Y7	22	13.6	0.40	23	8.7	0.12	22	4.5	0.03*
	24	20.8		23	26.1		24	29.2	

Above and below values are data of smaller and larger population in morphometric value divided by median. Family codes are shown in Table 2. No significant differences were detected in unshown families in *Pinus thunbergii*; OD3, OD4, OD8, Y8, Y10, Y11, Y12, NA73 × OS12, M90 × OI8 in current shoot inoculation; OD4, Y2, Y3, Y4, Y6, Y8, Y10, Y11, Y12, in 1 year-old shoot inoculation

*$P<0.05$ (significant difference in Fisher's exact tests of 2×2 contingency tables; alive/dead × smaller/larger morphometric population)

Table 4 Correlations between average morphometric values and survival rate in quintile classification based on the order of the morphometric values and in family classification

Morphometrics & pine		Inoculation position	Quintile				Family			
			Range of n in each quintile	Degrees of freedom	r	P	Range of n in each family	Degrees of freedom	r	P
Height	Pd	Cur	58–64	3	0.84	0.08	59–60	3	−0.37	0.53
	Pd	2 years	56–63	3	0.92	0.03*	58–60	3	−0.40	0.51
	Pt	Cur	122–138	3	0.45	0.45	25–57	13	−0.06	0.83
	Pt	1 year	114–126	3	0.48	0.41	34–46	11	−0.22	0.47
Basal diameter	Pd	Cur	50–72	3	0.99	0.00*	59–60	3	0.10	0.88
	Pd	2 years	55–63	3	0.99	0.00*	58–60	3	−0.47	0.43
	Pt	Cur	130–131	3	0.91	0.03*	25–57	13	0.10	0.73
	Pt	1 year	119–121	3	0.87	0.06	34–46	11	−0.17	0.59
Number of branches from axis	Pd	Cur	53–72	3	0.89	0.04*	59–60	3	−0.21	0.73
	Pd	2 years	50–74	3	0.71	0.18	58–60	3	−0.50	0.39
	Pt	Cur	79–219	3	0.96	0.01*	25–57	13	0.18	0.53
	Pt	1 year	73–151	3	0.95	0.01*	34–46	11	0.31	0.31

Pd and Pt are *Pinus densiflora* and *Pinus thunbergii*, respectively. Cur, 1 and 2 years are inoculation positions of current axis, 1 year-old axis and 2 year-old axis, respectively (shown in Fig. 1). Morphometric values were analysed after $\log(x+1)$ transformation. Survival rates were analysed after arcsine transformation

*$P<0.05$ (significant differences in correlation coefficients)

classification after Current- and 1y-inoculation, but not after 2y-inoculation (Table 4). These results are consistent with previous studies, in which the number of branches appeared to affect disease resistance. Numerous branches may present a barrier for PWN migration from the PWN inoculation site along the branch (Kuroda 2004), and more branches at the node might further limit PWN migration (Kawaguchi 2006).[3] In general, height did not affect survival of pines after PWN inoculation in this study. The quintile classifications showed that there were no significant correlations between height and survival, except after 2y-inoculation in *P. densiflora* (Table 4).

Although the various morphological traits were associated with resistance in individuals, all three morphological traits did not affect resistance against PWN at a family level. There were no significant correlations among families (Table 4). This was due to the fact that there were no family differences in basal diameter or number of branches, as indicated by ANOVA (Table 5), and few differences in the identical analyses of quintile correlations in height (Table 4). Therefore, family differences would affect survival rate of pines in the inoculation test, independent of the effects of basal diameter and the number of branches. These results suggest that morphological traits would not influence family evaluation as long as families of pine plants are grown under the same conditions.

PWNs must pass through the node of verticillate branches and the axis to cause death after Current-inoculation because exponential multiplication of nematodes throughout the entire tree occurs before the death of the tree (Kuroda et al. 1988). The node may limit PWN migration, as there was a higher survival rate among seedlings inoculated at higher positions in this study (Table 6). This finding is consistent with those of previous studies, which suggested that the node may have some function in limiting PWN migration after PWN inoculation (Kuroda 2004; Kawaguchi 2006). However, the effect of the node is a complicating factor in the family evaluation of Japanese pines for resistance to PWN because family differences of survival rate were detected only after 2y-inoculation but not after Current-inoculation (Table 5). The 2y-inoculation would be more suitable to evaluate hereditable

[3] Ibid.

Table 5 Analysis of variance (ANOVA) for family difference in morphometric values and survival rate

Factor	Source	Degrees of freedom	Current axis inoculation			2 year-old axis inoculation		
			Mean square	*F*	*P*	Mean square	*F*	*P*
Height	Replication	2	0.009			0.009		
	Family	4	0.020	16.0	0.00*	0.015	5.7	0.02*
	Error	8	0.001			0.003		
Basal diameter	Replication	2	0.042			0.046		
	Family	4	0.003	1.2	0.38	0.003	1.7	0.24
	Error	8	0.003			0.002		
Number of branches from axis	Replication	2	0.022			0.042		
	Family	4	0.010	2.9	0.09	0.008	1.5	0.29
	Error	8	0.004			0.005		
Survival rate	Replication	2	0.088			0.132		
	Family	4	0.044	1.8	0.23	0.075	4.5	0.03*
	Error	8	0.025			0.017		

Heights, basal diameters and numbers of branches from axis were analysed after log(x+1) transformation. Survival rates were analysed after arcsine transformation

*P<0.05 (significant difference)

Table 6 Family survival rate (%) in each inoculation position

Family code	Inoculation position		
	Current axis	1 year-old axis	2 year-old axis
Pinus densiflora			
NI7	61.7±24.7		31.7±5.8
OI142	74.6±13.0		59.5±26.2
OI203	78.3±2.9		37.0±20.8
SA118	86.7±7.6		51.7±22.5
SO39	85.0±18.0		67.8±12.5
Pinus thunbergii			
OD3	34.8	11.1	
OD4	37.8	6.5	
OD8	50.0	35.7	
OD9	34.1	21.7	
Y10	29.4	29.4	
Y11	40.0	19.6	
Y12	93.3	40.9	
Y2	28.9	4.4	
Y3	75.0	54.5	
Y4	63.6	21.7	
Y6	21.7	2.2	
Y7	24.4	17.4	
Y8	26.7	0.0	
MI90 & OI8	93.0		
NA73 & OS12	88.0		

Family codes are shown in Table 2. Values are mean ± SD calculated from survival rates in each of three blocks in *P. densiflora*. Inoculation onto 1 year-old axis in *P. thunbergii* and 2 year-old axis in *P. densiflora* show lower survival rates than onto current axis in respective pines (paired *t*-test using arcsine-transformed rate; *d.f.*=4, *t*=5.48, *P*=0.002 in *P. densiflora*; *d.f.*=12, *t*=6.05, *P*=0.000 in *P. thunbergii*) resistance, as the effect of the node is less in this inoculation than in the Current-inoculation.

In conclusion, there would be non-family (morphological), and family resistant factors to PWD, which works independently of each other, in the two Japanese pines. As morphological factors, wider diameter and more branches would work. Recently, a few new techniques for real-time tracing of PWN movement and pine tissue degradation by PWD have been developed (Fukuda et al. 2007; Komatsu et al. 2008). After the establishment of these techniques, the role of the above morphological factors may be rapidly revealed. On the other hand, the three morphological traits discussed in previous reports do not apply in a family context, even though they hold true at an individual level. The results of this study suggest that any traits associated with resistance should be assessed at a family level to breed for Japanese pines with resistance to PWN.

Acknowledgements I thank T. Aikawa, Tohoku Research Centre, FFPRI, for providing the Ka-4 isolate, M. Akiba, FFPRI, for supplying information about pathogenicity of the two nematode isolates, J. Nasu, Hokkaido Breeding Office, FTBC-FFPRI, for advice on SAS, and Dr Y. Ide, Graduate School of Agriculture and Life Science, The University of Tokyo, for reviewing an earlier draft of this manuscript. I also thank the staff of KBO-FTBC-FFPRI, especially S. Tamaki for providing Shimabara isolates, and Y. Fujiwara, Y. Kurahara, T. Hasebe, R. Hashimoto, K. Hayashi, K. Mizobuchi, T. Murayama, A. Tanaka, and Y. Unemoto for their assistance. SAS was performed through SCS MAFFIN provided by AFFRC, Ministry of Agriculture, Forestry and Fisheries, Japan.

References

Falconer, D. S., & Mackay, T. F. C. (1996). *Introduction to quantitative genetics (4th ed.)*. Harlow, UK: Pearson Education.

Forestry Agency of Japan (2007). Annual report on trends of forest and forestry in fiscal year 2006. Retrieved August 15, 2007, from http://www.maff.go.jp/hakusyo/rin/h18/html/data04.pdf. (in Japanese)

Fujimoto, Y., Toda, T., Nishimura, K., Yamate, H., & Fuyuno, S. (1989). *Breeding project on resistance to the pine-wood nematode*. *Bulletin of FTBC*, *7*, 1–84. (in Japanese with English summary)

Fukuda, K., Utsuzawa, S., & Sakaue, D. (2007). Disease development of pine wilt monitored MRI, AE and strain gauge. *The Japanese Forest Society Congress*, *118*, 69. (in Japanese)

Futai, K., & Sahashi, N. (2006). Perspectives for the studies of pine wilt disease in the 21st century. *Journal of the Japanese Forest Society*, *88*, 363. (in Japanese)

Higasihara, T., Yomogida, H., Konno, Y., Suda, K., Watanabe, K., Ito, S., et al. (2007). Selection of Japanese black pine (*P. thunbergii*) and red pine (*P. densiflora*) individuals resistant to pine wilt disease and result of screenings after artificial inoculation: the result of a breeding project for development of resistance to pine wilt disease in the Tohoku area (Iwate, Miyagi, Akita, Yamagata, Niigata, and Fukushima prefectures) from 1992 to 2005. *Bulletin of FTBC*, *23*, 319–413. (in Japanese with English summary)

Kato, K., Kawamura, K., & Ueki, C. (1995). Resistance to the pine wood nematode in seedlings obtained from resistant clonal seed orchards. *Japanese Journal of Nematology*, *25*, 52–55.

Kawaguchi, E. (2006). Relationship between pine wood nematode migration and the number of branch formation in *Pinus thunbergii*. *The Japanese Forest Society Congress*, *117*, 529. (in Japanese)

Kiyohara, T., & Tokushige, Y. (1971). Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *Journal of the Japanese Forest Society*, *53*, 210–218. (in Japanese with English summary)

Komatsu, M., Son, J., Matsushita, N., & Hogetsu, T. (2008). Fluorescein-labeled wheat germ agglutinin stains the pine wood nematode, *Bursaphelenchus xylophilus*. *Journal of Forest Research*, *13*, 132–136.

Kozlowski, T. T. & Pallardy, S. G. (1997). *Recurrently flushing shoots. In Physiology of woody plants, (2nd ed.)* (pp. 41–42). San Diego, CA: Academic.

Kuramoto, N., Ohira, M., Okamura, M., Taniguchi, T., Hiraoka, Y., Sato, S., et al. (2006). Inter-annual changing of the result of pine wood nematode inoculation test in the two crossing family of resistant Japanese black pine. *Kyushu Journal of Forest Research*, *59*, 239–240. (in Japanese)

Kuroda, K. (1999). Column 7—which factor decides dead or alive in tree? In K. Suzuki (Ed.), *Tree health* (pp. 81–82). Tokyo: Asakura Shoten. (in Japanese)

Kuroda, K. (2004). Inhibiting factor of symptom development in several Japanese red pine (*Pinus densiflora*) families selected as resistant to pine wilt. *Journal of Forest Research*, *9*, 217–224.

Kuroda, K., Yamada, T., Mineo, K., & Tamura, H. (1988). Effects of cavitation on the development of pine wilt disease caused by *Bursaphelenchus xylophilus*. *Annals of the Phytopathological Society of Japan*, *54*, 606–615.

Mamiya, Y. (1988). History of pine wilt disease in Japan. *Journal of Nematology*, *20*, 219–226.

Mamiya, Y., & Kiyohara, T. (1972). Description of *Bursaphelenchus lignicolus* n. sp. (Nematode: Aphelenchoidae) from pine wood and histopathology of nematode-infested trees. *Nematologica*, *18*, 120–124.

Mota, M. M., Braasch, H., Bravo, M. A., Penas, A. C., Burgermeister, W., Metge, K., et al. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology*, *1*, 727–734.

Nakamura, K., & Novotony, J. (1999). The use of Japanese experience with pine wilt disease control for forest protection against quarantine pests in Slovakia. *Lesnícky Časopis*, *45*, 397–407.

Nickle, W. R., Golden, A. M., Mamiya, Y., & Wergin, W. P. (1981). On the taxonomy and morphology of the pine wood nematode, *Bursaphelenchus xylophilus* (Stainer & Buhrer 1934) Nickle 1970. *Journal of Nematology*, *13*, 385–392.

Okada, S., & Tsuda, T. (1989). Selection of resistant pine trees to the pine-wood nematode in Kinki and Setonaikai. *Bulletin of FTBC*, *7*, 85–118. (in Japanese with English summary)

Sasaki, M., Hiraoka, Y., Okamura, M., Fujisawa, Y., & Akiba, M. (2002). Characteristics of pine wood nematode resistant Japanese black pine—the relation between resistance and characteristics of seedings. *The Japanese Forest Society Congress*, *113*, 649. (in Japanese)

Takeuchi, H., Handa, T., Ohguro, T., & Okamura, M. (1989). Selection of resistant pine trees to the pine-wood nematode in Shikoku. *Bulletin of FTBC*, *7*, 119–143. (in Japanese with English summary)

Tamaki, S., & Yamanobe, T. (2006). Study of selecting pressure control in breeding resistant Japanese pines against pine wilt disease. *The Japanese Forest Society Congress*, *117*, 504. (in Japanese)

Toda, T., & Fujimoto, Y. (1987). Resistance to the pine wood nematodes in *Pinus densiflora* (Kirishima-matsu). *The Japanese Forest Society Congress*, *98*, 261–262. (in Japanese)

Toda, T., Fujimoto, Y., Nishimura, K., Yamate, H., & Maeta, T. (1986). Resistance to the nematode in hybrid pines (*P. thunbergii* × *P. massoniana*). *Proceedings of Kyushu Branch Congress of Japanese Forest Society*, *39*, 67–68. (in Japanese)

Toda, T., Fijimoto, Y., Nishimura, K., Yamate, H., & Fuyuno, S. (1989). Selection of resistant pine tree to the pine-wood nematode in Kyushu. *Bulletin of FTBC*, *7*, 145–178. (in Japanese with English summary)

Toda, T., & Kurinobu, S. (2001). Genetic improvement in pine wilt disease in *Pinus thunbergii*: the effectiveness of pre-screening with artificial inoculation at the nursery. *Journal of Forest Research*, *6*, 197–201.

Toda, T., & Kurinobu, S. (2002). Realized genetic gains observed in progeny tolerance of selected red pine (*Pinus densiflora*) and black pine (*P. thunbergii*) to pine wilt disease. *Silvae Genetica*, *51*, 42–44.

Toda, T. (2004). Studies on the breeding for resistance to the pine wilt disease in *Pinus densiflora* and *P. thunbergii*. *Bulletin of FTBC*, *20*, 83–217. (in Japanese with English summary)

Yamanobe, T. (2005). Variation in the number of *Bursaphelenchus xylophilus* in aliquots of stock suspension artificially inoculated on to subject pines. *Nematology*, *7*, 623–630.