

Article

Reforestation or Genetic Disturbance: A Case Study of *Pinus thunbergii* in the Iki-no-Matsubara Coastal Forest (Japan)

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Abstract: In the twentieth century, a substantial decline in *Pinus thunbergii* populations in Japan occurred due to the outbreak of pine wood nematode (PWN), *Bursaphelenchus xylophilus*. A PWN-*P. thunbergii* resistant trees-breeding project was developed in the 1980s to provide reforestation materials to minimize the pest damage within the population. Since climate change can also contribute to PWN outbreaks, an intensive reforestation plan instated without much consideration can impact on the genetic diversity of *P. thunbergii* populations. The usage and deployment of PWN-*P. thunbergii* resistant trees to a given site without genetic management can lead to a genetic disturbance. The Iki-no-Matsubara population was used as a model to design an approach for the deployment management. This research aimed to preserve local genetic diversity, genetic structure, and relatedness by developing a method for deploying Kyushu PWN-*P. thunbergii* resistant trees as reforestation-material plants into Iki-no-Matsubara. The local genotypes of the Iki-no-Matsubara population and the Kyushu PWN-*P. thunbergii* resistant trees were analyzed using six microsatellite markers. Genotype origins, relatedness, diversity, and structure of both were investigated and compared with the genetic results previously obtained for old populations of *P. thunbergii* throughout Japan. A sufficient number of Kyushu PWN-*P. thunbergii* resistant trees, as mother trees, within seed orchards and sufficient status number of the seedlings to deploy are needed when deploying the Kyushu PWN-*P. thunbergii* resistant trees as reforestation material planting into Iki-no-Matsubara population. This approach not only be used to preserve Iki-no-Matsubara population (genetic diversity, genetic structure, relatedness, and resilience of the forests) but can also be applied to minimize PWN damage. These results provide a baseline for further seed sourcing as well as develop genetic management strategies within *P. thunbergii* populations, including Kyushu PWN-*P. thunbergii* resistant trees.

Keywords: genetic conservation; genetic management; pine wood nematode; *Pinus thunbergii*; pine wood nematode-*Pinus thunbergii* resistant trees



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1. Introduction

In general, forests can be categorized based on their purpose as conservation forests, protected forests, production forests, and forests with specific functions such as mitigation or tourism [1]. Different management strategies are required to protect forests with multiple functions [2], such as in Indonesia [3], China and Germany [4], and recently genetic approach methods have been proposed for long-term management [5,6]. Forests today face numerous threats, including diseases and pests [7], human interference [8], loss of unique or

rare species and genetic resources [9,10], and loss of genetic diversity, which provides forest ecosystem resilience [11,12]. In Japan, climate change has led to changes in environmental conditions in, such as increased annual sunshine, temperature, and rainfall (precipitation); rapid sea-level rise at a rate of 3.2 mm/year from 1993–2010; and higher intensity and more frequent storm surges (27 tropical cyclones slightly above normal) [13,14]. Climate change is an unpredictable factor and one of the most serious threats to forest ecosystems [15].

By area, Japanese forests comprise almost 50% conifers; however, *Pinus thunbergii* accounts for only 1% of the conifer composition [16]. In the Kyushu area, the species has been planted in coastal areas since more than 400 years ago [17]. A characteristic of *P. thunbergii* is tolerance to extreme conditions such as high salinity, high temperature, and low precipitation. Moreover, as a pine forest, it provides protection to coastal areas, by reducing wind damage, inhibiting sand movement, and decreasing tsunami wave energy [18,19].

Severe outbreaks of *Bursaphelenchus xylophilus* (pine wood nematode; PWN) depleted *P. thunbergii* populations in Japan between the 1900s and 2000s. The spread of PWN in Japan is the most significant occurrences of pest-disease damage than another country. The individuals damaged by PWN reached its peak in 1979, exceeded 2.43 million m³; as of 2016, the damage was one-fifth of the peak volume [16]. Air temperatures significantly influence the growth of PWN [20,21]. From a forest pest/disease perspective, climate change can directly or indirectly affect forest dynamics, changing the way that host trees and pathogens interact [22]. The warming climate may provide conditions for further PWN outbreaks and damage in the future [23].

In 1978, a breeding project to develop a PWN-*P. thunbergii* resistant trees as a countermeasure against outbreaks [24] was established at Breeding Region Institutions in Japan. This breeding project was initiated to select surviving pine trees from heavily damaged forests in Southwestern Japan. In the case of *P. thunbergii*, 14,620 trees were selected as candidate, and after the artificial inoculation tests, 16 clones were certified as resistant trees [24,25]. Three rounds of breeding program, based on individual performance selection-trial, have been performed throughout Japan until 2018 with gradual changes in the methodology [26]. In each prefecture of Japan, seed orchards were designed based on these resistant trees breeding program and the seedlings were used as reforestation-material plant. To date, 211 PWN-*P. thunbergii* resistant trees from 71 forests had been developed [27]. The purpose of the *P. thunbergii* breeding project was to create PWN-resistant trees for use as reforestation materials to enhance old populations of *P. thunbergii* in Japan for mitigation functions. Before the existence of breeding project, artificial planting with natural seedling recruitment, as reforestation-material plants, had repeatedly performed to maintain the forest. Unfortunately, the mitigation functions have been given priority with little consideration of the seed sources or genetic impacts of artificial planting.

From a forest protection perspective, the deployment of PWN-*P. thunbergii* resistant trees at a given site would indeed protect forests against PWN infection, minimizing damage. However, deployment without proper genetic management could lead to a genetic disturbance within the population, such as genetic diversity loss and modification of the genetic structure, reduced adaptability to local environments, “gene swamping,” and increased homogeneity; thus, negatively impacting the population as a gene resource [7,10,28–30]. Therefore, genetic diversity management must be considered when implementing tree improvement-products such as PWN-*P. thunbergii* resistant trees [31]. Genetic management and silviculture are fundamental components of forest management systems that have the potential to affect one another [2]. Both strategies are important for preserving local genetic diversity and maintaining forest resilience against environmental changes [32] even to the ecosystem [33], especially in extreme environments such as coastal areas.

In Japan, genetic diversity as well as genetic management of *P. thunbergii* populations and PWN-*P. thunbergii* resistant trees topics have not been discussed. In this study, we developed a genetic management based on the current genetic informations (genetic diversity,

genetic structure, and relatedness) of a local pine forest, Iki-no-Matsubara, that has been repeatedly planted for mitigation functions under the situation of PWN damage is not yet under control. The origin of seedlings in this site were inferred based on their genetic relationships with neighboring *P. thunbergii* populations in Kyushu area and throughout Japan. In addition, we investigated the genetic diversity, genetic structure, and relatedness of Kyushu PWN-*P. thunbergii* resistant trees with *P. thunbergii* populations in Kyushu area. In this way, this study aimed to preserve the Iki-no-Matsubara *P. thunbergii* population (current genetic diversity, genetic structure, and relatedness) as genetic resources through the use of Kyushu PWN-*P. thunbergii* resistant trees with the possibility of genetic disturbance when deploying it into the site. The genetic knowledge obtained from this case study are expected to provide a baseline for further seed sourcing as well as develop genetic management strategies within *P. thunbergii* populations, including Kyushu PWN-*P. thunbergii* resistant trees.

2. Materials and Methods

2.1. Study Field

Total individual and diversity of *P. thunbergii* within the populations in Japan has been declined. Most of current *P. thunbergii* populations in Japan, including Iki-no-Matsubara, are an uneven-aged forest, because it has been replanted repeatedly to preserve the forest. There was no historical record of the origin of material-planted and genetic information.

The research area of Iki-no-Matsubara (33°34'52.8" N 130°17'59.7" E) was 12.56 hectares. A folktale claims that the forest was established in tribute to Empress Jingu for Silla conquest around 200 or 300 AD (Yamato periods) [34]. Iki-no-Matsubara is one of Japan's top 100 beautiful green pine forests [35]. It is not only an education forest that belongs to Kyushu University since 1922 but also as an urban forest and conservation forest with mitigation functions since Edo Era (1603–1868) or earlier [36]. Iki-no-Matsubara locates within Genkai Quasi-national Park, which under Nature Conservation Law based on Natural Park Act, designated by prefectural government as a conservation forest for mitigation functions [34]. Field survey, tree census, and measurement of the trees' diameter at Iki-no-Matsubara was conducted from January 2017 until June 2019. From tree census data, the diameter was classified into three classes: 1–30 cm DBH (Diameter at breast height) range, 31–60 cm DBH range, and 61–90 cm DBH range (Table 1). Then, measured the stumps to estimate the age based on the DBH class ranges [37]. Based on cross-dating dendrochronology observation of annual ring of the stump in different location within Iki-no-Matsubara [38], the oldest tree was estimated to be around 200 years old (Table 1). *P. thunbergii* was highly regarded by Japan's religion and culture and became Japan's cultural identity. Hence, it possible that domesticated and artificial regeneration has been conducted repeatedly by local people since 1500 BP [39].

Table 1. DBH class range based on stump wood and number of samples within the Iki-no-Matsubara population.

No	DBH Class Range (cm)	DBH Stump (cm)		Replication	Estimation of the Age (Years)			Sample (Trees)
		Min	Max		Min	Max	Mean	
1	1–30	4	29	9	12	36	23	109
2	31–60	32	51	9	32	190	84	108
3	61–90	65	85	7	170	195	178	52
Total:								269

2.2. DNA Analysis

A total of 269 mature leaves were collected from selected trees at Iki-no-Matsubara experimental research field representing each DBH ranges class (Table 1). Selected trees were chosen randomly which represented each DBH classification and research field. Genomic DNA was extracted from 50 mg of tissue per individual by using the cetyltrimethyl ammonium bromide (CTAB) method [40] with slightly modifications and a DNeasy Plant

Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. Simple Sequence Repeat (SSR) analysis was carried out by six markers, bcpt1075, bcpt1671, bcpt834, bcpt1823, bcpt2532, and bcpt1549 [18]. A total of 12 μL for PCR analysis was carried out by using 2 μL DNA elution, 1 μL of primer mix, DNase/RNase-free water, and 2 \times multiplex PCR kit by Qiagen (Qiagen Inc., USA). PCR reaction was carried out by Touchdown PCR [41]. PCR protocol began with denaturing 95 $^{\circ}\text{C}$ for 15 min, two step annealing: (1) 10 cycles of denaturation 94 $^{\circ}\text{C}$ (30 s), annealing 60 $^{\circ}\text{C}$ (90 s), annealing temperature was decreased by 0.5 $^{\circ}\text{C}$ per cycle until 55 $^{\circ}\text{C}$, and extension 72 $^{\circ}\text{C}$ (1 min); (2), 20 cycles of 94 $^{\circ}\text{C}$ (30 s), 55 $^{\circ}\text{C}$ (90 s), and 72 $^{\circ}\text{C}$ (1 min), and final extension 60 $^{\circ}\text{C}$ for 30 min. Then, 10 μL of DNA amplicon mixed with Genescan 500 Liz Size Standard and Hi-Di Formamide (Applied Biosystems Inc., Bedford, MA, USA) was electrophoresed by ABI PRISM 3730 Genetic Analyzer (Applied Biosystems Inc., USA). Genotype data was analyzed using Genemapper 4.0 software (Applied Biosystems Inc., USA).

2.3. Statistical Analysis

Genotype data of 42 old populations of *P. thunbergii* from Iwaizumi et al. (2018) and PWN-*P. thunbergii* resistant trees (Watanabe, unpublished data, see Appendix A Table A2), which have been selected based on three breeding programs, was analyzed with data from Iki-no-Matsubara. Old populations are remaining populations of *P. thunbergii* that had decline due to overbreak of PWN. PWN-*P. thunbergii* resistant trees are tree improvement products that have high PWN resistance, which managed by Japan Tree Breeding Institution office in each region (Tohoku, Kansai, Kanto, and Kyushu) except Hokkaido. GeneAlex version 6.503 [42] was used to measure genetic diversity, Hardy-Weinberg Equilibrium, private alleles, genetic differentiation pattern through by principal coordinates analysis (PCoA) among populations and investigated gene flow (Nm) for examining the relationship between genetic differentiation and number of migrants variable per generation at each locus. Allelic richness (AR) and F_{IS} (inbreeding coefficient) at each locus was calculated by Fstat version 2.9.3.2 software [43]. Structure 2.3.4 [44] was used to determine individual-based genetic structure assessment by Bayesian method with a simulation run 15 times replicated, K-set 1–6 for 30,000 iterations burn-in period, and 30,000 iterations LOCPRIOR model under admixture ancestral model. The optimum value of each cluster K and the ΔK value within the genetic structure was determined by Evanno method [45] then upload the results to structure harvester [46].

3. Results

3.1. Inference of Origin and Genetic Structure in Iki-no-Matsubara Based on DBH

Table 2 shows the genetic diversity in Iki-no-Matsubara. N_a values was ranged from 14 (bcpt1549) to 29 (bcpt2532), N_e value from 3.18 (bcpt1549) to 7.83 (bcpt2532), AR value from 5.75 (bcpt1549) to 11.49 (bcpt2532), H_O and H_E from 0.57 (bcpt2532) to 0.85 (bcpt1075), and 0.69 (bcpt1549) to 0.87 (bcpt2532), respectively. Lowest value on F_{IS} was -0.03 (bcpt1075) and highest was 0.35 (bcpt2532). Three markers, bcpt834, bcpt1823, and bcpt2532 showed deviation from Hardy-Weinberg equilibrium ($p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively).

The N_a , AR , H_o , and F_{IS} values for Iki-no-Matsubara were higher than those reported by Iwaizumi et al. (2018). Iki-no-Matsubara had more private alleles than another population within the Kyushu region and the presence private alleles in the same loci were none to be found in nearby populations in Kyushu area. Among 269 trees, 92 carried a total of 18 private alleles at four out of six loci (Table 3). Four trees were in the 61–90 cm DBH range, six trees were in the 31–60 cm DBH range, and the remaining were in the 1–30 cm DBH range (Appendix A Table A1).

Table 2. Genetic diversity of the Iki-no-Matsubara *P. thunbergii* population using six primer markers.

Locus	Size Range (bp)	N_a	N_e	AR	H_O	H_E	F_{IS}	HWE
bcpt1075	141–201	18	5.62	7.42	0.85	0.82	−0.03	ns
bcpt1671	162–225	22	5.79	8.25	0.82	0.83	0.01	ns
bcpt834	139–183	16	5.3	7.83	0.74	0.81	0.09	*
bcpt1823	128–169	15	5.71	7.51	0.62	0.82	0.25	***
bcpt2532	128–190	29	7.83	11.49	0.57	0.87	0.35	***
bcpt1549	93–130	14	3.18	5.75	0.67	0.69	0.03	ns
Mean		19	5.57	8.04	0.71	0.81	0.12	

N_a : number of allele, N_e : number of effective allele, AR: allelic richness, H_O : observed heterozygosity, H_E : expected heterozygosity, F_{IS} : inbreeding coefficient within the population, HWE: Hardy-Weinberg equilibrium (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 3. Private alleles in Iki-no-Matsubara with 42 old populations of *P. thunbergii* throughout Japan using six primers.

Pop	Locus	PA	Pop	Locus	PA	Pop	Locus	PA	Pop	Locus	PA
Fukiage	bcpt1075	1	Miyajima	bcpt1549	1	Oki	bcpt2532	1	Jusan	bcpt2532	1
Miyazaki	bcpt1075	1	Kubokawa	bcpt1075	2	Kaga	bcpt834	1	Wakinosawa	bcpt2532	1
Karatsu	bcpt1075	1	Imabari	bcpt2532	1	Komatsu	bcpt1549	1			
Iki-no-Matsubara (Fukuoka)	bcpt1671	3	Tsuda	bcpt1075	1		bcpt1075	1			
	bcpt834	2	Kaihu	bcpt834	1						
	bcpt1823	1		bcpt1823	1						
	bcpt2532	12	Suzuka	bcpt834	2						

The genetic structure of Iki-no-Matsubara showed two color patterns (Figure 1A), even on K2, K3, or K4 structure result (number 8 in Appendix A Figure A1). Further analysis of the spatial distribution of genetic structure at Iki-no-Matsubara showed that the blue pattern (blue color $\geq 55\%$) was dominantly observed on the east side of the research field (Appendix A Figure A2). In the 1–30 cm DBH range, 20 out of 109 trees showed the blue pattern, while in the 31–60 cm and 61–90 cm DBH range 12 out of 108 trees and no trees, respectively, showed the blue pattern.

Figure 1B shows the genetic structure among *P. thunbergii* populations throughout Japan. Iki-no-Matsubara was dominated by the yellow pattern, same with the other populations from the Kyushu area. Principle Coordinate Analysis (PCoA) showed that Iki-no-Matsubara was more similar to the Minami-Shimabara population than the Karatsu population, which is geographically closer to Iki-no-Matsubara. The Minami-Shimabara and Amakusa populations had the highest probability of taking part in the gene flow (Nm) into Iki-no-Matsubara, at 47.99% and 35.35%, respectively (Figure 2). Based on DBH class range (Figure 3) specifically, the similarity between the Minami-Shimabara and Iki-no-Matsubara 61–90 cm DBH, 31–60 cm DBH, and 1–30 cm DBH ranges were 14.18%, 36.93%, and 15.99%, respectively. More importantly, the relationship between the DBH ranges indicated that the 61–90 cm DBH range shared 53.44% of genetic similarity with the 31–60 cm range, and 6.51% with the 1–30 cm DBH range. This finding suggests that the origin of the young trees, 1–30 cm DBH range class, were not from the Iki-no-Matsubara population but another area.

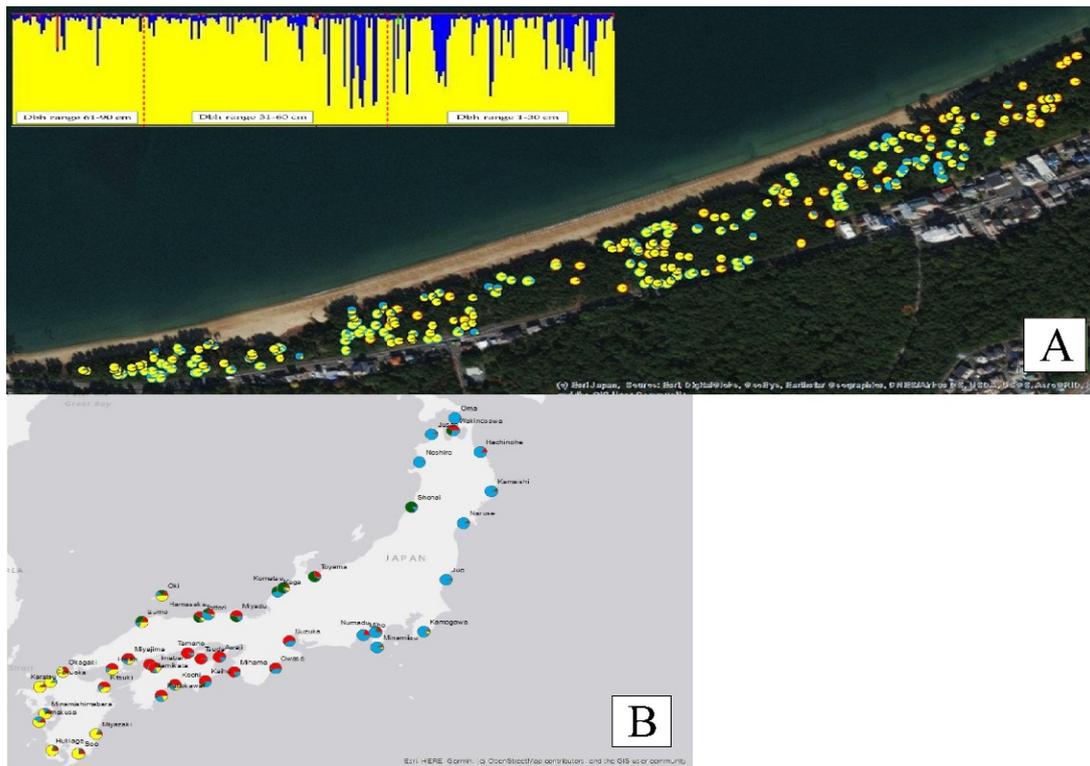


Figure 1. Spatial distribution of genetic structure at Iki-no-Matsubara (A); Iki-no-Matsubara with 42 old populations of *P. thunbergii* throughout Japan (B).

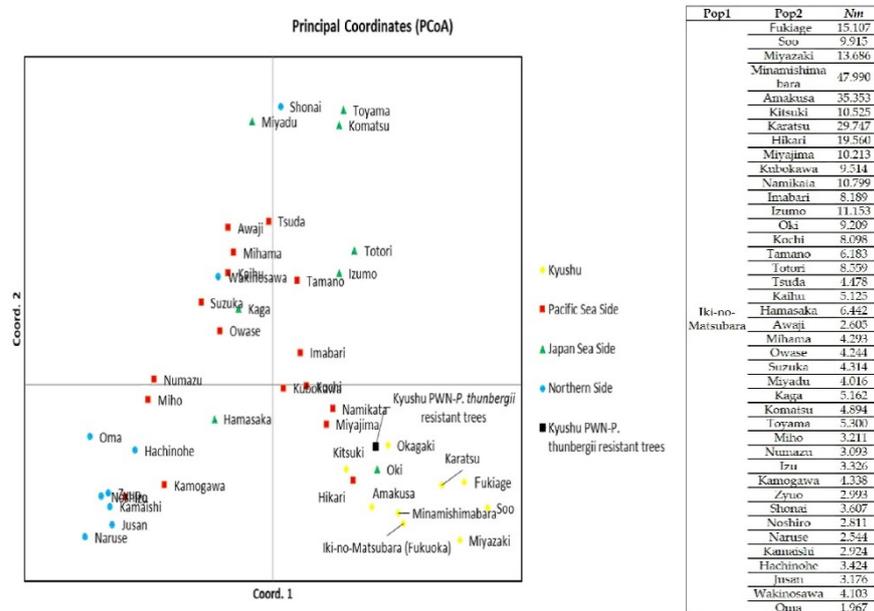


Figure 2. PCoA of Iki-no-Matsubara with other populations of *P. thunbergii* and the Kyushu PWN-*P. thunbergii* resistant trees with gene flow (Nm) between Iki-no-Matsubara and the other populations.

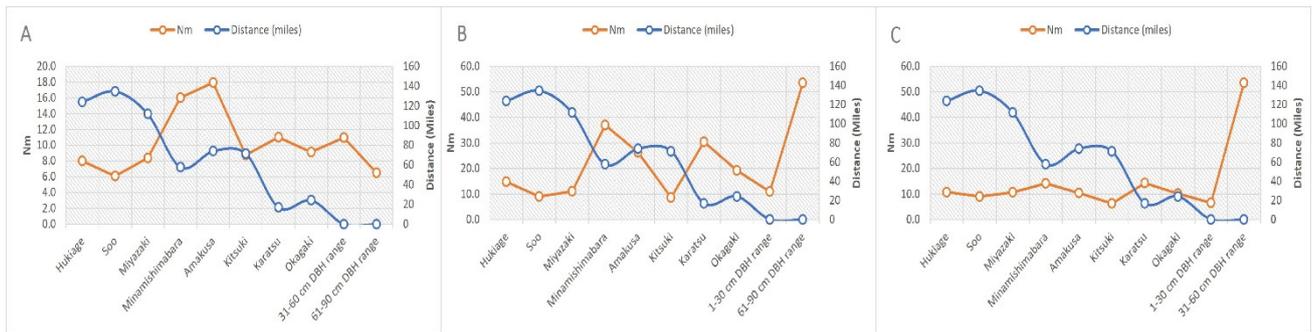


Figure 3. Relationship between gene flow (Nm) and distance with other populations in the Kyushu area on the following basis: (A) 1–30 cm DBH class range; (B) 31–60 cm DBH class range; (C) 61–90 cm DBH class range.

3.2. Genetic Diversity and Genetic Structure of PWN-*P. thunbergii* Resistant Trees

Since the 1990s, PWN-*P. thunbergii* resistant trees have been planted to enhance the old populations of *P. thunbergii*. Therefore, analyzing the local genotype of the Iki-no-Matsubara population (genetic diversity, genetic structure, and relatedness with other populations in Kyushu area) provides a baseline when deploying Kyushu PWN-*P. thunbergii* resistant trees. In general, the genetic structure of PWN-*P. thunbergii* resistant trees within each region (Figure 4) showed Kyushu (yellow color) and Kanto (green) PWN-*P. thunbergii* resistant trees had the most distinct genetic structure (dominated by region's structure pattern). In contrast, Tohoku and Kansai PWN-*P. thunbergii* resistant trees exhibited mixed patterns. The PCoA results show that the Kyushu PWN-*P. thunbergii* resistant trees are similar to the Okagaki populations (Figure 2). Some North Kyushu populations likely had a higher possibility of contributing to the gene flow than populations on the other side of Kyushu (see Appendix A Table A3). The genetic diversity of Kyushu PWN-*P. thunbergii* resistant trees was low compared with the mean genetic diversity of the *P. thunbergii* populations in entire Kyushu area (Table 4).

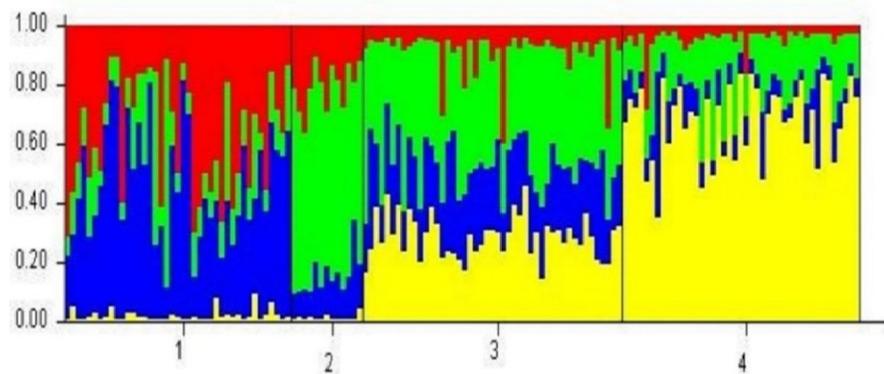


Figure 4. Genetic structure of PWN-*P. thunbergii* resistant trees on K4 (1) Tohoku region, (2) Kanto region, (3) Kansai region, and (4) Kyushu region.

Table 4. Genetic diversity of the Kyushu PWN-*P. thunbergii* resistant trees using six primer markers compared with the overall genetic diversity of populations in the Kyushu area.

Locus	N_a	N_e	AR	H_O	H_E	F_{IS}	HWE
bcpt1075	12	6.29	8.23	0.7	0.84	0.18	ns
bcpt1671	14	5.34	8.85	0.81	0.81	0.01	ns
bcpt834	9	3.88	6.05	0.67	0.74	0.1	ns
bcpt1823	9	3.77	6.14	0.51	0.73	0.31	***
bcpt2532	14	7.07	10.06	0.51	0.86	0.41	***
bcpt1549	7	2.86	4.84	0.63	0.65	0.05	ns
Mean	10.83	4.87	7.36	0.64	0.77	0.18	
Overall Populations in Kyushu Area	12.22	5.17	7.57	0.68	0.78	0.12	

N_a : number of allele, N_e : number of effective allele, AR: allelic richness, H_O : observed heterozygosity, H_E : expected heterozygosity, F_{IS} : inbreeding coefficient within the population, HWE: Hardy-Weinberg equilibrium (ns = not significant, *** $p < 0.001$).

4. Discussion

4.1. Inference of Origin and Genetic Structure in Iki-no-Matsubara based on DBH

Most *P. thunbergii* forests are located in coastal regions, including the Iki-no-Matsubara population. They have been expected for conservation area, especially to preserve mitigation functions such as reducing wind damage, inhibiting sand movement, and decreasing tsunami wave energy [19]. Before the existence of breeding project, artificial planting with natural seedling recruitment had repeatedly performed to maintain the forest.

In wind-pollinated conifers, the genetic diversity within the population has a tendency to be higher than that among populations. However, the genetic diversity within Iki-no-Matsubara was low in this study. Many *P. thunbergii* in Japan were damaged by the strong impact of PWN. After the 1980s, individuals with pest damage in the Iki-no-Matsubara population were removed and replanting has been continuously performed; however, the origin of seedlings were unknown. The number of private alleles was highest in Iki-no-Matsubara, and the presence of private alleles in the same loci were none to be found in the nearby populations in the Kyushu area. The lack of private alleles in a particular population within the Kyushu area is likely due to the small sample size compared to Iki-no-Matsubara [47]. The presence of private alleles in the Iki-no-Matsubara (Appendix A Table A1), interestingly, showed 31–60 cm DBH class range and 61–90 cm DBH class range shared on the same loci, while 1–30 cm DBH class range on different loci. Based on the structure analysis and PCoA results (Figure 2), we postulate that the Iki-no-Matsubara could be derived from the Kyushu area, especially the Minami-Shimabara or Amakusa population, which was farther from Iki-no-Matsubara than the Karatsu or Okagaki populations. In detail, 1–30 cm DBH class range was highly associated with Minami-shimabara and Amakusa. Meanwhile the 31–60 cm and 61–90 cm DBH class ranges displayed strong associated each other and the closest neighbour, Karatsu population (Figure 3). Such results, showed the recently planted the 1–30 cm DBH class range indicate that they were planted without considering genetic origin.

The genetic structure within the population was clearly divided into two patterns, and younger individual corresponding to DBH was remarkable. Furthermore, the genetic structure deviated to the area in the field. In more detail, some individuals exhibited the same pattern, yet different diameter class range (Figure 1A). The yellow patterns observed in Iki-no-Matsubara were common among populations in the Kyushu area (Figure 1B), while the blue pattern was not recognized in Karatsu nor Okagaki. There two possible explanations for this finding: (1) the materials planted in Iki-no-Matsubara were introduced from a different origin area, especially at DBH range 1–30 cm, which show dominantly blue color patterns; (2) Iki-no-Matsubara had more than two patterns of genetic structure

in the past, including the patterns observed in Karatsu and Okagaki, but the population was reduced as a result of a bottleneck [18]. The exact cause is still uncertain due to the lack of historical records regarding the artificially-planted materials and the *P. thunbergii* genetic structure of Iki-no-Matsubara in the past. It would be reasonable to assume that the origin of the seedling was not considered when new planting was performed after removing individuals damaged by pine wilt disease.

4.2. Genetic Management of *P. thunbergii* in Iki-no-Matsubara with Kyushu PWN-*P. thunbergii* Resistant Trees

From a forest protection viewpoint, artificially planting Kyushu PWN-*P. thunbergii* resistant trees to enhance Iki-no-Matsubara population and counter PWN infection still has its merits; however, the genetic aspects such as genetic diversity (avoid homogeneity), genetic structure, resilience of the forest, and relatedness with another populations must also be properly considered. Thus, there are two crucial points to consider: (1) how well the PWN-*P. thunbergii* resistant trees as seed-sourcing strategy and (2) genetic management within the population, including the PWN-*P. thunbergii* resistant trees.

In addition, there two aspects should be considered for the genetic management of PWN-*P. thunbergii* resistant trees as seed-source strategy: (1) How well the mother trees represent the genetic diversity and relatedness in the selected area? (2) A sufficient number of resistant trees should be sourced as mother trees? [48–51]. The mother trees will represent the genetic diversity, structure, and gene flow pattern of the population where it was taken [52,53]. The extent of gene flow among populations shows how alleles are shared (similarities) and play an important role in genetic differentiation among populations [54,55].

From the perspective of genetic structure, Kyushu PWN-*P. thunbergii* resistant trees were noticeably displayed a yellow pattern (Kyushu region's structure pattern) (Figure 4). However, from relatedness viewpoint, Kyushu PWN-*P. thunbergii* resistant trees were located in the middle between the Kyushu area and Pacific seaside area and shared similarity with the Okagaki populations (Figure 2). This may have occurred because the selected trees for Kyushu PWN-*P. thunbergii* resistant trees were not sufficiently balanced to represent all Kyushu area populations. In fact, among 43 Kyushu PWN-*P. thunbergii* resistant trees, ten were from Okagaki, and none were from Iki-no-Matsubara (Appendix A Table A2).

The sufficient number of mother trees, act as effective population size in seed orchard, must be examined first to manage the diversity and relatedness within Iki-no-Matsubara with other populations in Kyushu area [56]. The effective population size is a concept used to predict the ideal size of the population, considering that the genes transmitted to seeds will still possess the same level of genetic diversity after many generations [57]. However, this study case only provide the Iki-no-Matsubara, not of the entire *P. thunbergii* in Kyushu area. Thus, in the future, the breeding project of Kyushu PWN-*P. thunbergii* resistant trees need to develop a perspective based on genetic management according to the genetic characteristics in each local pine forest in the Kyushu area.

4.3. Kyushu PWN-*P. thunbergii* Resistant Trees Deployment Management as Part of Genetic Management

To maintain *P. thunbergii* population in Iki-no-Matsubara, both PWN resistance and genetic diversity must be considered as part of genetic management, which is PWN-*P. thunbergii* resistant trees deployment management. Only using clones (vegetative) or seeds of specific Kyushu PWN-*P. thunbergii* resistant trees as reforestation-material plants on a large scale repeatedly for long-terms would cause a genetic disturbance such as increased homogeneity, inbreeding depression, reduced genetic diversity and adaptability to local environments [30,31,58]; thus, negatively impacting the population as a gene resource. Therefore, it is necessary to determine the status numbers of Kyushu PWN-*P. thunbergii* resistant trees [59] using information from genetic analysis within the population by practice selective seed-cone harvesting to balance genetic gain and diversity [48,59] for the necessary reforestation. When considering genetic diversity in the next generation

and the status number of Kyushu PWN-*P. thunbergii* resistant trees, we can first refer to the local seed pool for reference, where at least 24 seedlings (generative) from each of the 30 mother trees will be needed to provide complete coverage for genetic diversity in the Iki-no-Matsubara population in the next generation [60]. Genetic diversity is defined as the genetic variation carried by individuals within a population as a part of their evolutionary path, providing a basis to form responses to environmental changes, as resilience of the forest [61].

Seedlings from a local seed pool or a neighbour population, such as Karatsu (geographically near of Iki-no-Matsubara), should be given priority. A seedling's adaptive potential from the local seed pool will have the optimal genotype because it has undergone many life cycles within the local environment over several generations. Proper seedling selection for planting is necessary to avoid maladaptation and improve the survival rate [62,63]. Furthermore, determining the origin of seedlings according to the Japan Forest Seeds and Seedlings Law 1939 [64] so that, at least, the structure pattern among the four areas shown in Figure 1B could be maintained. Subsequently, PWN-*P. thunbergii* resistant trees should be managed separately in each Japan Tree Breeding Institution office region. Using a non-local seed pool or non-local genetic pattern could lead to uncertain results in terms of adaptation and genetic differentiation among populations [30,65].

5. Conclusions

Declining *P. thunbergii* populations as a result of PWN outbreaks triggered to the consideration of genetic diversity management of the current populations for necessary genetic resources [18]. A forest with high genetic diversity provides a foundation for individuals to survive and adapt through evolution, especially when the forest has undergone human intervention [9,47,66,67]. Nevertheless, understanding the current genetic informations of Iki-no-Matsubara (genetic diversity, genetic structure, and relatedness) are essential for deploy Kyushu PWN-*P. thunbergii* resistant trees into the site, as part of genetic management. Genetic diversity (H_O) in Iki-no-Matsubara was 0.71 and dominated by yellow pattern from structure viewpoint. However, information based on DBH class range showed high relatedness with Minami-Shimabara and Amakusa, and there was a possibility that the origin of the materials that had been planted were not from the local seed pool was proposed, which was especially likely for the 1–30 cm DBH class range.

Additionally, the genetic structure of Kyushu PWN-resistant trees revealed a clear yellow genetic pattern. The Kyushu PWN-*P. thunbergii* resistant trees genetic diversity was lower than that of the overall population in the Kyushu area. An insufficient number of Kyushu PWN-*P. thunbergii* resistant trees unbalanced the gene flow, thus genetically to be found similar to the Okagaki population. A sufficient number of Kyushu PWN-*P. thunbergii* resistant trees, as mother trees, within seed orchards and sufficient status number of the seedlings need to be considered to safely deploy Kyushu PWN-*P. thunbergii* resistant trees as reforestation-material plants into Iki-no-Matsubara population. This approach can be used not only to preserve Iki-no-Matsubara population (genetic diversity, genetic structure, relatedness, and resilience of the forests) but can also be applied to minimize PWN damage. These results provide a baseline for further seed sourcing as well as develop genetic management strategies within *P. thunbergii* populations, including the PWN-*P. thunbergii* resistant trees.

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Appendix A

Table A1. Number of private alleles within Iki-no-Matsubara.

No	DBH Class Range	Number of Trees	Loci
1	1–30 cm	82	bcpt2532
		1	bcpt1823
2	31–60 cm	5	bcpt1671
		1	bcpt834
3	61–90 cm	1	bcpt1671
		4	bcpt834

Table A2. List of PWN-*P. thunbergii* resistant trees based on the region [68].

No	PWN- <i>P. thunbergii</i> Resistant Trees			
	Tohoku	Kansai	Kanto	Kyushu
1	Naruse39	Tanabe54	Odaka37	Shima64
2	Naruse72	Bizen143	Odaka203	Tsuyazaki50
3	Naruse6	Mitoyo103	Iwaki27	Karatsu1
4	Watari5	Namikata37	Osuga5	Karatsu4
5	Yamamoto82	Namikata73	Osuga6	Karatsu7
6	Yamamoto84	Misaki90	Osuga12	Karatsu9
7	Yamamoto90	Yoshida2	Osuga15	Karatsu11
8	Yuza27	Yasu37	Osuga23	Karatsu16
9	Yuza72	Tosashimizu63	Utchihara5	Karatsu17
10	Yuza33	Kumihama10	Tomiura7	Obama30
11	Yuza54	Kumihama21	Okazaki25	Oseto12
12	Yuza56	Kumihama109	Okazaki34	Kawaura8
13	Yuza58	Amino31	Okazaki35	Kawaura13
14	Yuza60	Amino43		Amakusa20
15	Yuza57	Tango47		Oita8
16	Yuza59	Tango50		Sadowara8
17	Yuza77	Tango51		Sadowara14
18	Murakami2	Tango58		Sadowara15
19	Murakami5	Tango60		Miyazaki20
20	Murakami11	Tango65		Sendai20
21	Murakami16	Tango69		Ei425
22	Murakami44	Tango71		Hiyoshi1
23	Murakami9	Totori7		Hiyoshi5
24	Murakami15	Totori13		Hukiage25
25	Nigata8	Iwami63		Okagaki1
26	Nigata40	Nisinosima142		Okagaki5

Table A2. Cont.

27	Nigata3	Komatsu99	Okagaki6
28	Aikawa27	Ota39	Okagaki8
29	Nagaoka15	Hamada6	Okagaki25
30	Nagaoka8	Hamada12	Okagaki29
31	Ozika151	Hamada24	Okagaki31
32	Sendai35	Hamada28	Okagaki32
33	Ishimaki251	Gotsu29	Okagaki35
34	Ishimaki260	Yunotsu52	Okagaki20
35	Ishimaki259	Hukube51	Munakata2
36	Atsumi43	Hukube54	Munakata4
37	Tsuruoka38	Hukube60	Munakata12
38	Tsuruoka44	Hukube61	Munakata19
39	Tsuruoka46	Hukube71	Shingu2
40	Zyoetsu1	Koryo60	Shingu5
41	Zyoetsu10	Koryo77	Shingu11
42		Kaga387	Shingu14
43		Kaga388	Shingu17
44		Kaga295	
45		Shiga396	
46		Tsuruga14	
47		Tsuruga15	

Table A3. Gene flow (Nm) of Kyushu PWN-*P. thunbergii* resistant trees with the populations within Kyushu area.

Pop1	Pop2	F _{ST}	Nm
Hukiage	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.020	12.308
Soo	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.033	7.349
Miyazaki	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.027	8.956
Minamishimabara	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.007	34.354
Amakusa	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.008	32.530
Kitsuki	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.016	15.698
Karatsu	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.008	32.144
Iki-no-Matsubara (Fukuoka)	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.010	25.961
Okagaki	Kyushu PWN- <i>P. thunbergii</i> resistant trees	0.010	24.077

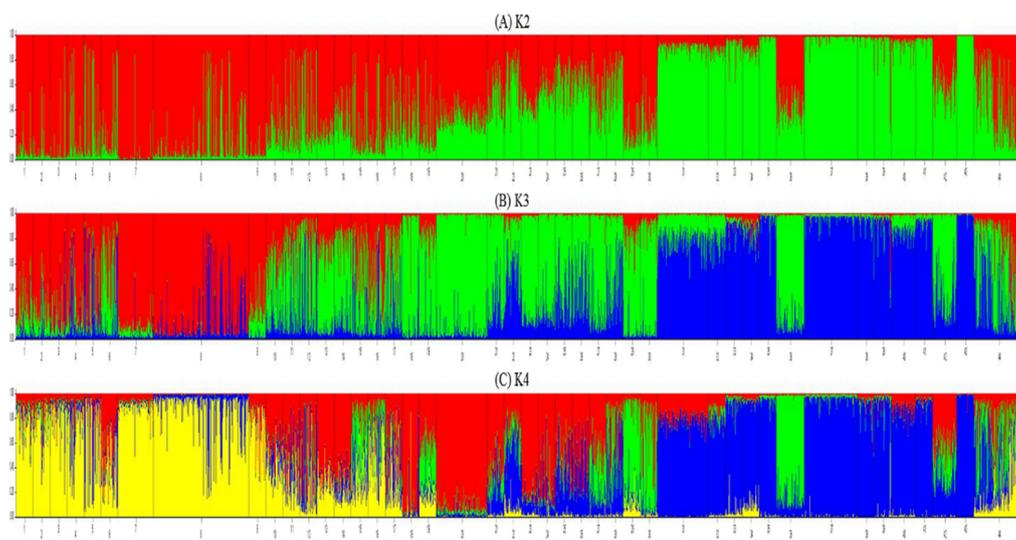


Figure A1. Genetic structure of Iki-no-Matsubara (Fukuoka) with 42 old populations of *P. thunbergii* on K2, K3, and K4 (From South-West (Left) to North-East (Right)).

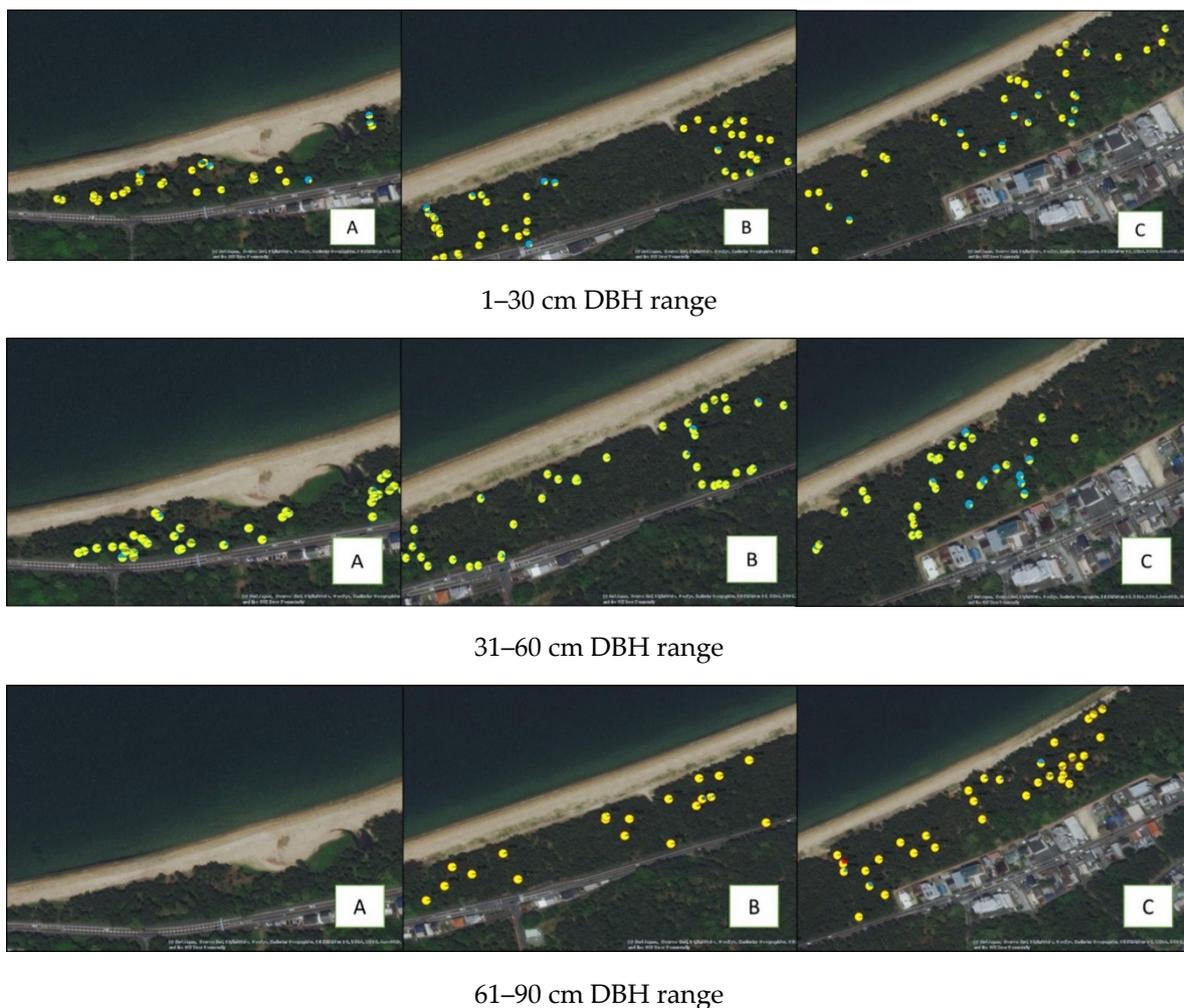


Figure A2. Spatial distribution of Iki-no-Matsubara genetic structure per DBH range (A) West side, (B) Central side, and (C) East side.

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