# Genetic management of Pinus thunbergii forest in Japan

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# GENETIC MANAGEMENT OF *Pinus thunbergii* FOREST IN JAPAN

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## ABBREVIATIONS

PWN : Pine Wilt Nematode

PPT : Pine Wilt Nematode-*Pinus thunbergii* resistant trees

DBH : Diameter at breast height

EST-SSR : Expressed sequence tag-simple sequence repeat

G-SSR : Genomic-simple sequence repeat

DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid

Gst : Proportion of genetic diversity among populations

PCR : Polymerase chain reaction

NGS : Next generation sequence

AD : Anno domini

BP : Before present

CTAB : Cetyltrimethylammonium bromide

BLAST : Basic local alignment search tool

## Summary

*Pinus thunbergii* is a famous tree species and highly related to Japanese activities. Most of *P. thunbergii* forest can be found near of the Shinto shrine. It used as integral part of the Shinto shrine and has been artificially planted since the Edo period (1603-1868) mainly as mitigation forest at coastal areas. However, *P. thunbergii* populations have been greatly declined due to pine wilt nematode (PWN) by *Bursaphelenchus xylophilus* since 1970. Hence, preference to planting a PPTs, which is highly resistant to PWN, have surged to mitigate the damage and ensure the survival of the forests. In fact, PPTs are genetically improved varieties from tree improvement program and may have narrow genetic diversity. On the other hand, tree planting programs usually have not considered aspect of genetic diversity as element of forest management until now. In addition, the traces of genetic disturbance within the gene flow of old populations of *P. thunbergii*, ambiguity, and mixed pattern in some populations of *P. thunbergii,* which prevent further population genetic studies of the species. Therefore, inappropriate deployment of PPTs will lead to further genetic disturbances within/between populations, such as loss of genetic diversity and altered genetic structure, reduced adaptability to local environments, “gene swamping,” and increased homogeneity; thus, negatively impacting the population as a gene resource. In order to safely deploy a PPTs into the populations as well as preserving current genetic diversity within/between the populations, it is necessary to identify, measure, and analyze current genetic informations within/between the populations. The use and development of appropriate genetic marker as a vital importance to provide in population genetic studies of *P. thunbergii*, especially in genetic management aspect.

In this studies, a local population (Iki-no-Matsubara) was investigated using six genomic SSR markers, and then compared the genetic diversity, similarities, and genetic structure to other populations (within the same region) and PPT Kyushu cultivar population. The genetic structure of the Iki-no-Matsubara population differed along with change of the tree age, especially between older and younger individuals. This suggested that the old individuals represent the original genetic structure of the Iki-no-Matsubara population, while the younger individuals were introduced neither from origins site nor neighbour site.

Furthermore, PPT Kyushu cultivars showed difficulty in covering current genetic diversity in Iki-no-Matsubara. The results implied that non-conserved gene based of seeds transfer or PPTs introduction can has serious consequences for local gene conservation.

Next, we attempted to develop EST (Expressed Sequence Tag)-SSR markers, which have different characteristics from genomic SSRs, in order to obtain more detailed information. The EST-SSR markers are important markers to elaborate the genetic diversity and genetic structure of *P. thunbergii* populations. A total of 10 pairs EST-SSR markers were successfully developed from EST information of *P. thunbergia*, and then, applied to 21 *P. thunbergii* populations throughout Japan. The analysis of EST-SSR markers showed different results from genomic SSRs. Based on EST-SSR markers results, the genetic structure of *P. thunbergii* populations was divided into two clusters. This results suggested that seed transfer between the two clusters should be prohibited.

In order to use PPTs as reforestation materials within the region, it is necessary to prepare a sufficient number of PPTs as mother trees in the seed orchard and a sufficient number of seedlings to deploy. In some *P. thunbergii* populations, the seedlings have been introduced from relatively close area. As a result, most of the remaining *P. thunbergii* forests today reflect the unique genetic structure. If the genetic diversity of PPT cultivar from original site does not cover current genetic diversity in the target area, then the seedlings from distant area could be introduced as long as the seed transfer zones match. Otherwise, it may disturb the genetic structure specific to that region. This studies showed that the historical background of afforestation in Japan needs to be taken into account for the maintenance of the genetic resources of *P. thunbergii*. In addition, it is necessary to re-evaluate the existing regulations and planting program for *P. thunbergii* afforestation including seed transfer policy in Japan. These results will contribute to the maintenance of genetic resources of *P. thunbergii*.

# CHAPTER 1. GENERAL INTRODUCTION

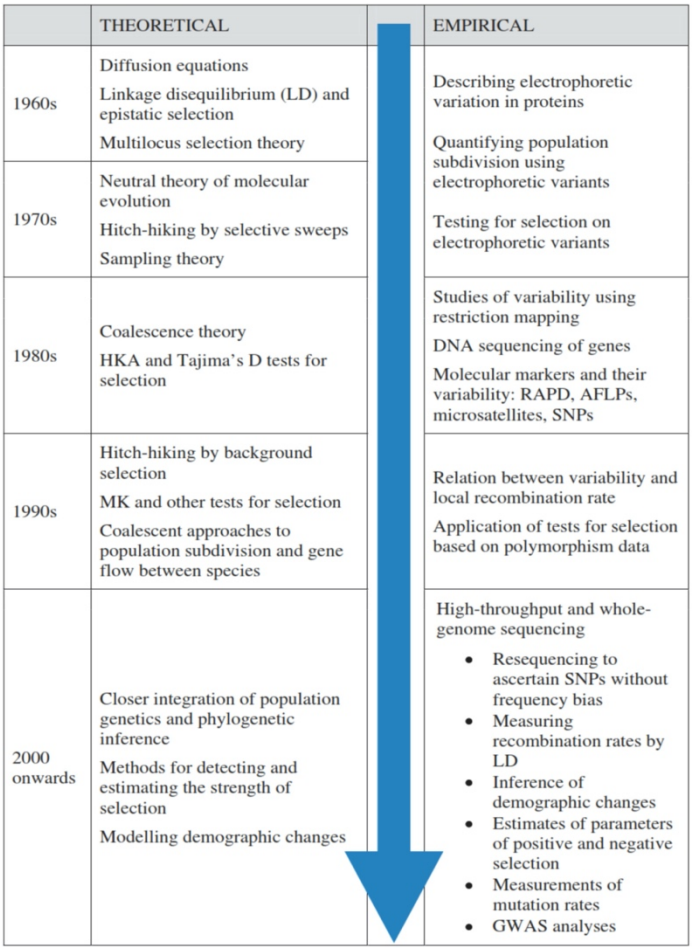
## 1.1. Literature Review

### *1.1.1. Population Genetics History*

The existence of population genetics is all thank to Darwin-evolution and natural selection theory and Mendelian-inheritance theory (Crow, 1987; Okasha, 2016), but neither was recognized until early nineteenth century. Population genetics field study began to get attention around early nineteenth century through paper published by Hardy and Weinberg in 1908. Nevertheless, it became the first basis for population genetics. The aspects within population genetic are 1) the fact that allele frequencies do not change in the immigration, mutation, random fluctuation, which genetically affect to fitness of the species, 2) the fact that in the presence of random mating, the diploid genetics array is given by the square of gametic array in binomial term, and 3) the environment aspect is taking into account in principle of multiple loci and multiple alleles (Crow, 1987). Later, in the 1920s, statistical genetics proposed by Fisher, Haldane, and Wright had major impact on population genetics. Even though their discoveries were overlapped each other, their results showed a bounding of both Darwin-evolution theory and Mendelian-inheritance theory. In addition, those results were a major step to understand of evolutionary process within population genetics (Crow, 1987; Okasha, 2016).

The greatest change of population genetics era was around 1960s where empirical study of evolution at genetic level began to the surface. Kimura's neutral theory has kept population genetics research more focusing on molecular evolution studies to the present (Crow, 1987). In 1966, the first attempt to quantify the genetic variability on protein sequences by using gel electrophoresis was introduced. Then, around 1980s, molecular markers were invented, and experiments were conducted using DNA sequences as material to study molecular variation (Charlesworth and Charlesworth, 2017). Population genetics is a study of understanding genetic composition within species populations regarding their adaptability, evolution, and demographic history over generation (Griffiths et al., 2015; Milgroom, 2015). Its goal is to determine how the genes are used to explain the creation,

maintenance and distribution process of individuals within the populations (Provine, 1971). Through time and space, evolution occurs. Such organisms diverge from the same common ancestor into different organisms that are related to each other. All of the information is in the gene. Then, depending on the experimental materials and an appropriate molecular marker, the genetic information can be revealed. Recently, population genetic studies have had a significant impact on genetic management. By using an appropriate genetic marker, the genetic information of a population can be the basis to implementing genetic management. Major advancement in population genetic studies in general is shown in Figure 1.1.



| | THEORETICAL | EMPIRICAL |
|---|---|---|
| 1960s | Diffusion equations<br>Linkage disequilibrium (LD) and epistatic selection<br>Multilocus selection theory | Describing electrophoretic variation in proteins<br>Quantifying population subdivision using electrophoretic variants<br>Testing for selection on electrophoretic variants |
| 1970s | Neutral theory of molecular evolution<br>Hitch-hiking by selective sweeps<br>Sampling theory | |
| 1980s | Coalescence theory<br>HKA and Tajima's D tests for selection | Studies of variability using restriction mapping<br>DNA sequencing of genes<br>Molecular markers and their variability: RAPD, AFLPs, microsatellites, SNPs |
| 1990s | Hitch-hiking by background selection<br>MK and other tests for selection<br>Coalescent approaches to population subdivision and gene flow between species | Relation between variability and local recombination rate<br>Application of tests for selection based on polymorphism data |
| 2000 onwards | Closer integration of population genetics and phylogenetic inference<br>Methods for detecting and estimating the strength of selection<br>Modelling demographic changes | High-throughput and whole-genome sequencing<br>• Resequencing to ascertain SNPs without frequency bias<br>• Measuring recombination rates by LD<br>• Inference of demographic changes<br>• Estimates of parameters of positive and negative selection<br>• Measurements of mutation rates<br>• GWAS analyses |

**Figure 1.1.** Timeline of major advance in population genetics field study (Charlesworth and Charlesworth, 2017).

### *1.1.2. Management of Genetic Diversity and Silvicultural Approaches*

Reforestation, artificial regeneration, land rehabilitation, and enrichment are silvicultural approaches that target critical land or deforestation area. The purpose is to manage the forest for sustainable forest management. However, the approaches usually consider only conservation of the biodiversity in species level (Ratnam et al., 2014). Disregarding genetic composition aspect of the seedlings as material-replanting will lead a genetic disturbance in the future (Ledig, 1988). Thus, a forest management without considering aspect of gene conservation has significant impact on the genetic diversity within the area. Genetic variation plays a key role as forest resilience against dynamic climate change, pest-disease, even other potential harmful.

There are three threats to biodiversity at the DNA level: 1) extinction, which resulting in irreversible loss of the genes; 2) hybridization/introduced-individuals, which become threats to the individuals in local site (gene swamping); and 3) decreasing genetic variants within the population (Kenchington and Heino, 2002). Genetic diversity is one of parameters within genetic composition for population genetic studies and one of indicators to measure the adaptability of a population against dynamic climate change or other potential harmful (such as pest and disease, human interference, etc). Therefore, studying genetic composition is an essential aspect within genetic management of the species populations (Jamieson et al., 2008).

The goal of genetic management is to maintain the availability of genetic variation so that it can develop and evolve through both natural processes and direct or indirect human influence, because the loss of genetic variation within a population can increase the risk to the extinction (Palmberg-Lerche,1999; Meuwissen et al., 2020). In practically, the exact methodology to manage genetic diversity of the species depends on the current genetic diversity status of the species within/between the populations, time-scale of concern, the objective and scope of the program, institutional concern, and the economic (Palmberg-Lerche,1999). Essentially, when considering about silvicultural approaches, we need to consider the genetic management and the forest as genetic resource. Especially, the

manager as forest owner shall consider several aspects to uptake of adaptive measure to that matter into a policy decision-making process for the sake of genetic management (Vicenti et al., 2020).

### *1.1.3. Molecular Marker*

The recent advances in molecular genetic studies provide methods to explain evolution and ecological process through time and space. Genetic or molecular markers are sequences of DNA or protein, which usually located in known location on the chromosome and specifically associated with specific trait. The bulk of variation within DNA sequence can be observed thoroughly with a level of accuracy by using some genetic markers (Duran et al., 2009; Al-samarai and Al-kazaz, 2015). There are two categories of molecular marker 1) protein-based marker and 2) DNA-based marker. The only difference between protein-based and DNA-based marker only lies on the character of material which is used as marker. Proteins are more related to a gene that may only be expressed on one tissue due to environmental influence, while DNA directly reflects the genotype and located in either a coding region or noncoding region of every chromosome (Mondini et al., 2009). However, the usability and choice of markers depend on the ability of the researcher, material/focus studies of interest, and availability of the tools for DNA analysis. Furthermore, the development of molecular markers has only been applied to limited species due to relative high cost. Up to date, several DNA marker systems have been developed (Figure 1.2) and the resolution and usability of the markers have considerably been improved with advancement of PCR (polymerase chain reaction), nucleic acid hybridization, and DNA sequencing tools (Grover and Sharma, 2014).

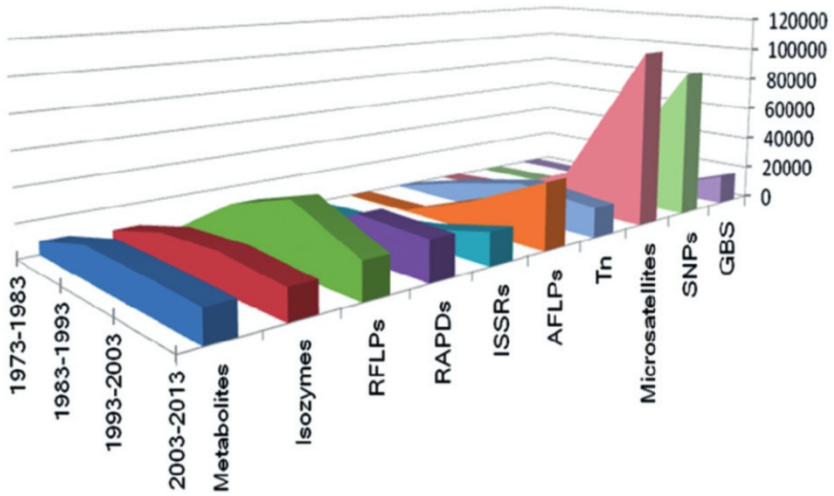



**Figure 1.2.** Popularity of molecular marker system in last 40 years. Right panel shown total number of citations each marker system on google scholar (Grover and Sharma, 2014).

*1.1.3.1. Genomic SSR (Microsatellite) and EST-SSR (Expressed Sequence Tag)*

Microsatellite or SSR (simple sequence repeat) is genomic-based and co-dominant marker. This marker is frequently used in population genetic studies to assess genetic variation within/between populations (Moore et al., 2005; Vigouroux et al., 2005). SSRs are located at both noncoding and coding region and have tandem repeat motif of one to six nucleotides. It is not only widely distributed throughout the genome (Beckmann and Weber, 1992), but also in chloroplast (Provan et al., 2001) and mitochondria as well (Rajendrakumar et al., 2007). In general, the development of SSR markers (Figure 1.3) involves of the following steps: 1) knowledge of target sequence, where SSRs occur, 2) designs of the oligonucleotide (primers) complementary, 3) validation of the candidate primers by PCR or electrophoresis, and 4) confirming of the polymorphism of the primers by screening (Mason, 2015). In the recent development of SSR markers, NGS (Next Generation Sequencing) is used to obtain the transcriptome of target species from public database to shorten the time for marker development (Zalapa et al., 2012). However, the transcriptome data which deposited within public database is limited. Thus, it is usually difficult to obtain sufficient sequence data to make a marker for our target species of

interest (Zhan et al., 2009) and the PCR amplification success rate is low (Hollenbeck et al., 2012).

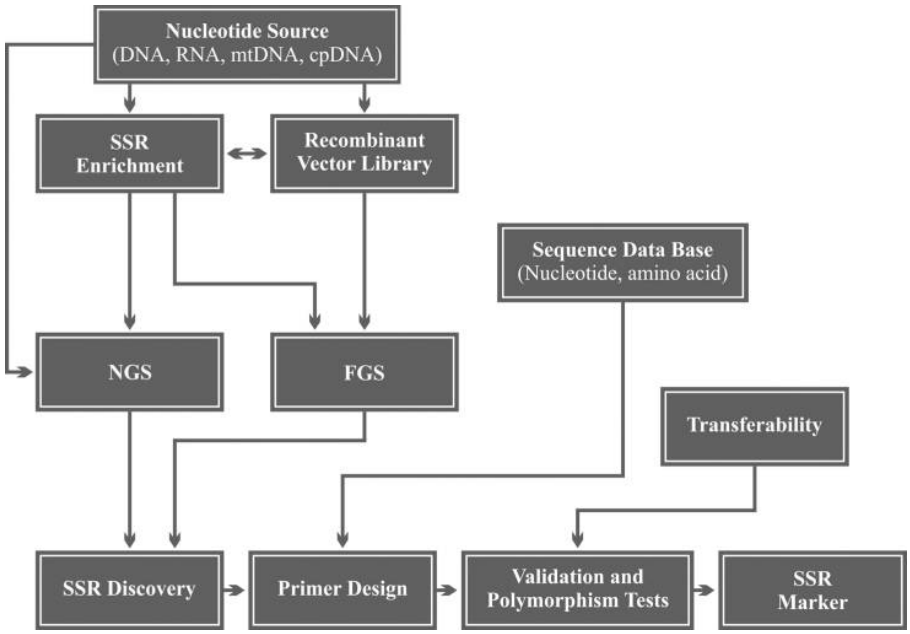



**Figure 1.3.** Workflow of SSRs development (Vieira et al., 2016).

SSRs can be divided into genomic SSR and EST (Espressed Sequence Tag)-SSR according to origins of the DNA material (Hou et al., 2017). Genomic SSRs are high mutation rate-DNA type, occurrence within gene region is low, and they exhibit high level of polymorphisms. Hence, genomic SSRs have been preferred for population genetic studies and identification of closely related genotype due to their high degree of variability (Vieira et al., 2016). The development of genomic SSRs is time and cost consuming, therefore the screening and analysis of the marker usually depend on closely related species (Hou et al., 2017). In addition, high informative genomic SSRs output often lead a misunderstanding regarding interpretation of heterozygosity (Zimmerman et al., 2020), which consequently obscures divergence among subpopulation (Coates et al., 2009). EST-SSRs are gene-based marker. In general, the development of EST-SSRs is low-cost and rapidly because the transcriptome data are obtained from NGS. EST-SSRs have low polymorphisms level, fewer null alleles, and a low mutation rate of DNA type. Furthermore,

EST-SSRs are more concerned to gene related of DNA region and basically can be found on the related genera due to common component of the gene. Hence, it is expected to be well conserved (Varshney et al., 2005) and has high transfer ability to other species (Xu et al., 2004). The interest of using EST-SSRs for detecting polymorphism in population genetic studies is due to the gene highly related to phenotypic trait (Holton et al., 2002). Remarkably, genomic SSRs and EST-SSRs work are different in assessing the genetic diversity in population genetic studies. However, combination of both markers can reveal more details about micro-evolution of the target species (Parthiban et al., 2018).

### *1.1.4. Pinus thunbergii*

*Pinus thunbergii* is an evergreen and fast growing species of pine (Park et al., 2015). It is commonly known as Japanese black pine, native in Japan, Korea, and China. Especially in Japan, the species is widely distributed throughout the country except in Hokkaido and Okinawa (Kudoh, 1985). *P. thunbergii* dominated from the coast to about 1,000 m elevation (Richardson, 2000). The species is tolerant to drought, summer heat, and high salinity condition. According to previous studies about population genetic studies in old population of *P. thunbergii* by Iwaizumi et al. (2018), standardized Gst (genetic differentiation among populations) was higher than other Japanese pine species, but lower compared to another species (*Quercus aliena* G′st: 0.369; Jose-Maldia et al., 2017). Genetic differentiation among populations within conifer species normally tends to be low value because of wind-pollinated. Based on those character, at least the populations in the same region should be shown the same pattern. In reality, however, there are ambiguities in the gene flow among old populations of *P. thunbergii* throughout Japan (especially on mainland of Japan such as Chugoku, Shikoku, and Kansai regions), indicating traces of genetic disturbance. In addition, *P. thunbergii* seeds transfer policy implied that the seeds transfer zone was divided into two area (Sea of Japan side and Pacific Ocean side). The seed transfer policy only mentioned about the movement of the seeds from Sea of Japan side to Pacific Ocean side is permitted, while the other way is prohibited. Then, the seeds transfer is allowed as long as within the permitted area (MAFF, 1971).

*P. thunbergii* is highly valued for the wood-strongness and rugged form for house-material construction. The extant structural woods from Muromachi period (14$^{th}$ centuries) have been proven to be *P. thunbergii* wood (Farjon, 1984). *P. thunbergii* has been planted along the coast mainly as mitigation forest to stabilize shorelines and serve as a windbreak (Suwa, 2013). Historically, *P. thunbergii* was highly related to Japanese activities. It has been closely associated with religion (Shintoism) and Japanese social-history, making it a cultural identity of Japan (Omura, 2004). Japanese believed in gods and considered *P. thunbergii* as symbol of longevity. *P. thunbergii* forest is believed as one of the places where the god of the polestar resides or dwells in. Therefore, most of *P. thunbergii* forest in Japan can be found near of the shrine. Forest as a part of Shinto shrine have been considerably influenced by Japanese activities as well (Ono, 1962; Omura, 2004). The practice of planting *P. thunbergii* when establishing a shrine has been a tradition for generations (Totman, 1989). This can be understood from the evidence of the corresponding traces and timing when the virgin forests replaced by pine trees forests along with appeared newly shrines in Japan (Omura, 2004).

### 1.2. Research Objectives

The main objective of this studies is to manage genetic diversity of *P. thunbergii* populations as forest genetic resources. Then, I discuss about the current problems (from a genetic viewpoint) within/among populations and PPT cultivated population as well and how to introduce the genetic management into *P. thunbergii* populations. Specific objectives are:

1) To obtain current genetic information (genetic structure, the similarities, and the origins of the seedlings), in this case Iki-no-Matsubara, as a local population, which designed to be a role model.
2) To understand genetic information (genetic diversity, genetic structure, and the similarities to *P. thunbergii* populations) of PPTs, especially from Kyushu cultivar
3) To re-evaluate seed resources management and its implementation
4) To design an approach method for genetic management *P. thunbergii* populations

5) To re-evaluate existing seed transfer policy

### 1.3. Structure of Dissertation

This thesis dissertation is organized into five chapters (Figure 1.4) in total as follows. **Chapter 1** represents general introduction about literature review, objectives of this studies, and the connectivity among chapters (framework) in this studies. It details (1) history of population genetic studies, (2) the definition and importance of genetic management and its implementation through silvicultural approaches, (3) definition, function, and development of molecular marker up to date, especially genomic SSR and EST-SSR, and (4) information about *Pinus thunbergii* and its connectivity with Japan's history.

**Chapter 2** presents a role model based on a local population "Iki-no-Matsubara" to approach genetic management of *P. thunbergii* populations. For this purpose, the genetic diversity, genetic structure and the similarities of the Iki-no-Matsubara as a local population were identified and compared with the results obtained from other populations and PPT Kyushu cultivar. Based on the comparison, approaches for genetic management were discussed.

In **chapter 3**, gene-based marker, the EST-SSR markers have been developed. The EST-SSR markers were screened from the private database of NGS. Several selection, screening, and trial had been conducted to develop EST-SSR primer markers. The successfully developed EST-SSR primer markers were tested on 96 samples of "Iki-no-Matsubara" to determine their ability to reveal genetic information.

**Chapter 4** shows the impact of the use of genomic SSR and EST-SSR markers to better understand the population genetic studies of *P. thunbergii* populations. Through the analysis of the genetic diversity and genetic structure of 21 populations of *P. thunbergii*, the characteristics, functions and advantages of both markers were discussed. Furthermore, the relationships between the loci and the effects of natural selection, regional differentiation were examined based on neutrality tests (Ewens-Watterson test), bottleneck tests, and linkage disequilibrium tests. Finally, the existing seed transfer policies were re-evaluated by introducing both markers.

**Chapter 5** provides a conclusion based on the results of the study, as well as my opinions and recommendations for this studies.

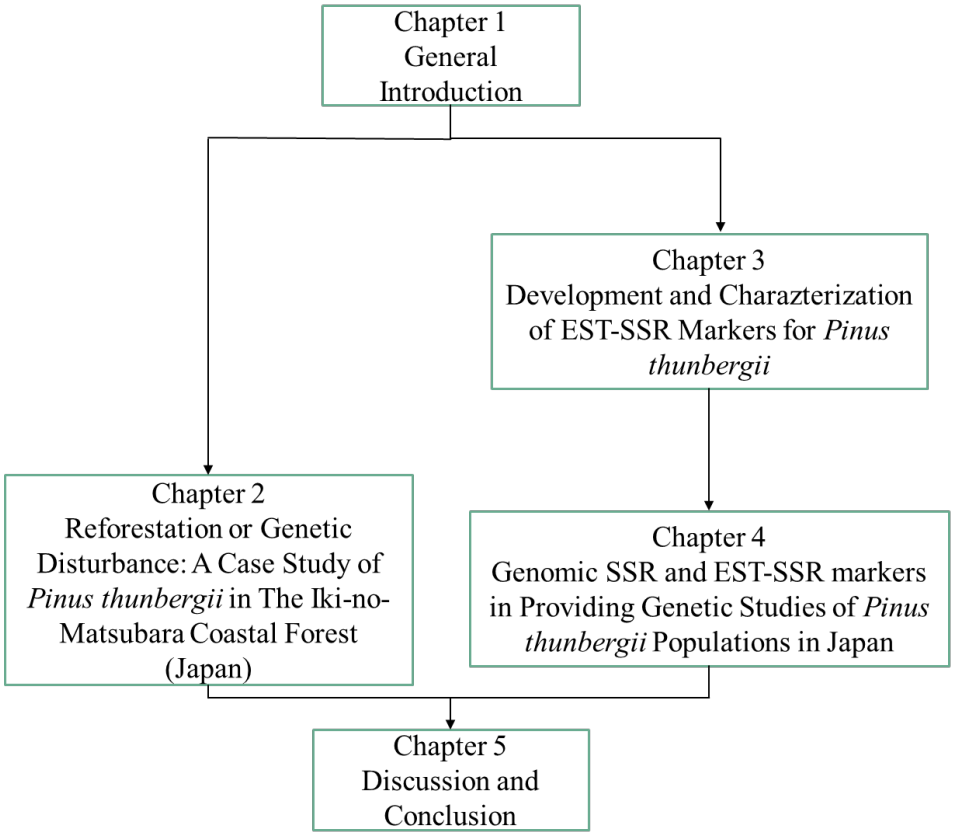



**Figure 1.4.** Framework of thesis organization

# CHAPTER 2. REFORESTATION OR GENETIC DISTURBANCE: A CASE STUDY OF *Pinus thunbergii* in THE IKI-NO-MATSUBARA COASTAL FOREST (JAPAN)

## 2.1. 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 (Fuhrer, 2000). Different management strategies are required to protect forests with multiple functions (Krott, 2005), such as in Indonesia (Santika et al., 2015), China and Germany (Benz et al., 2020), and recently genetic approach methods have been proposed for long-term management (Aravanopoulos, 2018; Ratnam et al., 2014). Forests today face numerous threats, including diseases and pests (Tubby and Webber, 2010), human interference (Millner-Gulland, 2012), loss of unique or rare species and genetic resources (Ledig, 1988; Aravanopoulos, 2016), and loss of genetic diversity, which provides forest ecosystem resilience (Rajora and Pluhar, 2003; White et al., 2007). 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) (Japan Meteorological Agency, 2018; Ministry of the Environment et al., 2018). Climate change is an unpredictable factor and one of the most serious threats to forest ecosystems (Sturrock et al., 2011).

By area, Japanese planted forests comprise almost 41%; however, *P. thunbergii* accounts include in 8% of the pine forest compositions (Forestry Agency, 2019). In the Kyushu area, the species has been planted in coastal areas since more than 400 years ago (MAFF, 2020). 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 (Iwaizumi et al., 2018; Suwa, 2013).

Severe outbreaks of *Burshaphelencus 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 $m^3$; as of 2016, the damage was one-fifth of the peak volume (Forestry Agency, 2019). Air temperatures significantly influence the growth of PWN (Ichihara et al., 2000; Yamaguchi et al., 2019). From a forest pest/disease perspective, climate change can directly or indirectly affect forest dynamics, changing the way that host trees and pathogens interact (Linnakoski et al., 2019). The warming climate may provide conditions for further PWN outbreaks and damage in the future (Hirata et al., 2017).

In 1978, a breeding project to develop PPTs as a countermeasure against outbreaks (Fujimoto et al., 1989) was established at breeding region institutions, Forestry and Forest Products Research Institute (FFPRI), 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 (Fujimoto et al., 1989; Kurinobu, 2008). Three rounds of breeding program, based on individual performance selection-trial, have been performed throughout Japan until 2018 with gradual changes in the methodology (Matsunaga and Watanabe, 2018). 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, 144 PPTs had been developed (FFPRI, 2019). The purpose of the *P. thunbergii* breeding project was to create PPTs 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 PPTs at a given site would indeed protect forests against PWN infection, minimizing damage. However, deployment

without proper genetic management can 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 (Ledig, 1988; Tubby and Webber, 2010; Aitken and Whitlock, 2013; Icgen et al., 2006; Konnert et al., 2015). Therefore, genetic diversity management must be considered when implementing tree improvement-products such as PPTs (Ingvarsson and Dahlberg, 2019). Genetic management and silviculture are fundamental components of forest management systems that have the potential to affect one another (Krott, 2005). Both strategies are important for preserving local genetic diversity and maintaining forest resilience against environmental changes (Kavaliauskas et al., 2018) even to the ecosystem (Bailey et al., 2009), especially in extreme environments such as coastal areas.

In Japan, genetic diversity as well as genetic management of *P. thunbergii* populations and PPT topics have not been discussed. In this study, we developed a genetic management based on the current genetic information (genetic diversity, genetic structure, and similarities) of a local pine forest, Iki-no-Matsubara, that has been repeatedly planted for mitigation functions under the situation where PWN damage has not yet been under control. The origins 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 similarities of PPT Kyushu cultivar 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 similarities) as genetic resources throughs the use of PPT Kyushu cultivar with the possibility of genetic disturbance when deploying it into the site. The genetic knowledges 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 PPT Kyushu cultivar.

## 2.2. Materials and Methods

### *2.2.1. Study Field*

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 origins 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) (Fukuoka City, 2016). Iki-no-Matsubara is one of Japan's top 100 beautiful green pine forests (Awano, 2015). 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 (Park et al., 2003). 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 (Fukuoka City, 2016). 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 range, 31–60 cm DBH range, and 61-90 cm DBH range (Table 2.1). Then, measured the stumps to estimate the age based on the DBH class ranges (Studhalter et al., 1963). Based on cross-dating dendrochronology observation of annual ring of the stump in different location within Iki-no-Matsubara (Pilcher, 1963), the oldest tree was estimated to be around 200 years old (Table 2.1). *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 (Omura, 2004).

**Table 2.1.** DBH class range based on stump wood 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 |

*2.2.2. 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 2.1). 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 (Fukatsu et al., 2005) with slightly modifications and a DNeasy Plant Kit (Qiagen Inc., USA) following the manufacturer's protocol. SSR analysis was carried out by six markers, bcpt1075, bcpt1671, bcpt834, bcpt1823, bcpt2532, and bcpt1549 (Iwaizumi et al., 2018). 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 2x multiplex PCR kit by Qiagen (Qiagen Inc., USA). PCR reaction was carried out by Touchdown PCR (Korbie and Mattick, 2008). PCR protocol began with denaturing 95 °C for 15 min, two step annealing: (1) 10 cycles of denaturation 94 °C (30 s), annealing 60 °C (90 s), annealing temperature was decreased by 0.5 °C per cycle until 55 °C, and extension 72 °C (1 min); (2), 20 cycles of 94 °C (30 s), 55 °C (90 s), and 72 °C (1 min), and final extension 60 °C for 30 min. Then, 10 µL of DNA amplicon mixed with Genescan 500 Liz Size Standard and Hi-Di Formamide (Applied Biosystems Inc., 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).

*2.2.3. Statistical Analysis*

Genotype data of 42 old populations of *P. thunbergii* from Iwaizumi et al. (2018) and PPTs (FFPRI, 2019, see Appendix Table 2), 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. PPTs are tree improvement products that have high PWN resistance, which have been managed by FFPRI regional office in each region (Tohoku, Kansai, Kanto, and Kyushu) except Hokkaido. GeneAlex version 6.503 (Peakall and Smouse, 2012) 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 (Goudet, 2001). Structure 2.3.4 (Pritchard et al., 2000) was used to determine individual-based genetic structure assessment by Bayesian method with a simulation run 15 times replicated, K-set 1-6 for 30000 iterations burn-in period, and 30,000 iterations LOCPRIOR model under admixture ancestral model. The optimum value of each cluster *K* and the $\Delta K$ value within the genetic structure was determined by Evanno method (evanno et al., 2005) then upload the results to structure harvester (Earl and von Hodlt, 2012).

**2.3. Results**

*2.3.1. Inference of Origins and Genetic Structure in Iki-no-Matsubara Based on DBH*

Table 2.2 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).

**Table 2.2.** Genetic diversity of the Iki-no-Matsubara *P. thunbergii* population based on 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, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The $N_a$, $AR$, $Ho$, 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 2.3). 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 Table 1).

**Table 2.3.** 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 | | bcpt1549 | 1 | | | |
| | | | | | | Komatsu | | | | | |
| | bcpt1671 | 3 | Tsuda | bcpt1075 | 1 | | bcpt1075 | 1 | | | |
| | bcpt834 | 2 | | bcpt834 | 1 | | | | | | |
| Iki-no-Matsubara (Fukuoka) | bcpt1823 | 1 | Kaihu | bcpt1823 | 1 | | | | | | |
| | bcpt2532 | 12 | Suzuka | bcpt834 | 2 | | | | | | |

The genetic structure of Iki-no-Matsubara showed two color patterns (Figure 2.1A), even on K2, K3, or K4 structure result (number 8 in Appendix Figure 1). Further analysis of the spatial distribution of genetic structure at Iki-no-Matsubara showed that the blue pattern (blue color ≥ 55%) was dominantly observed on the east side of the research field (Appendix Figure 2). 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.

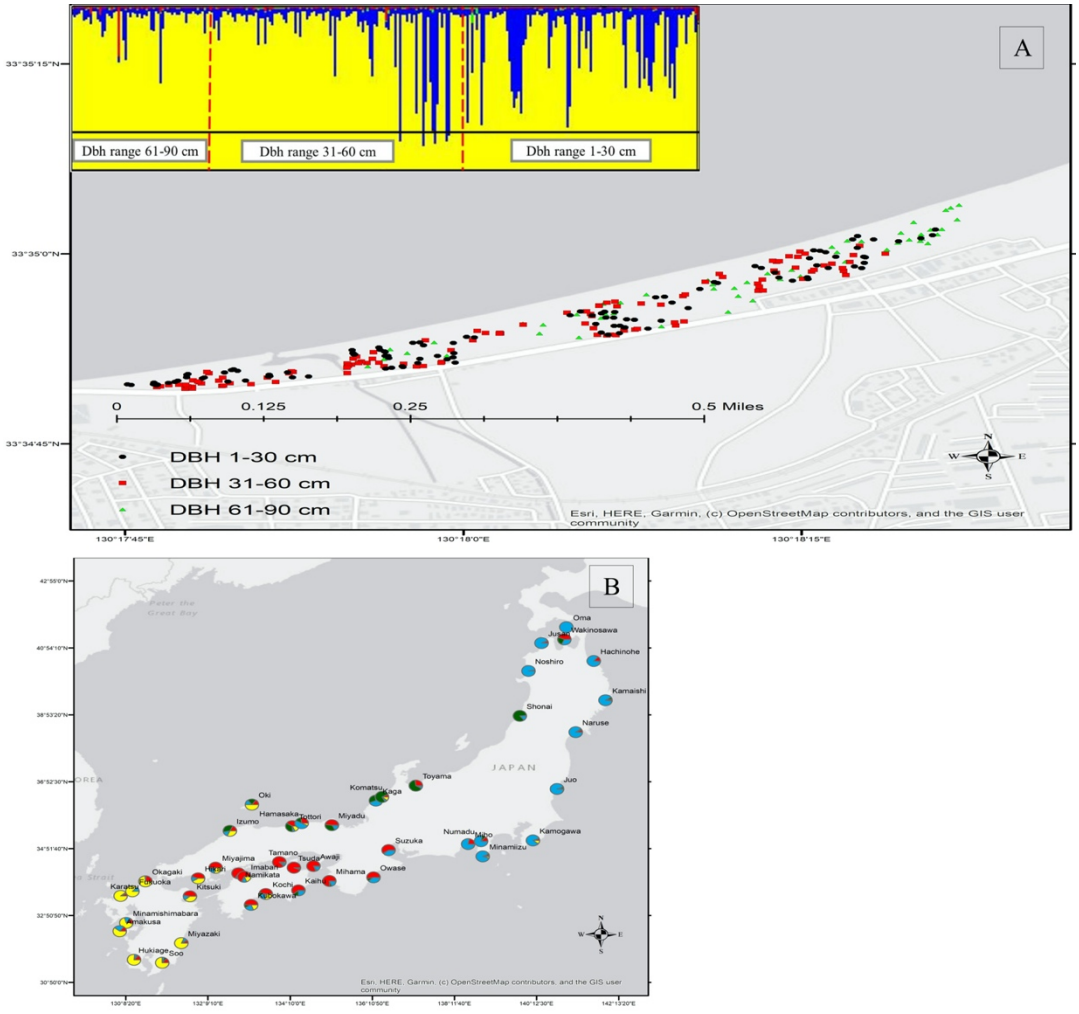
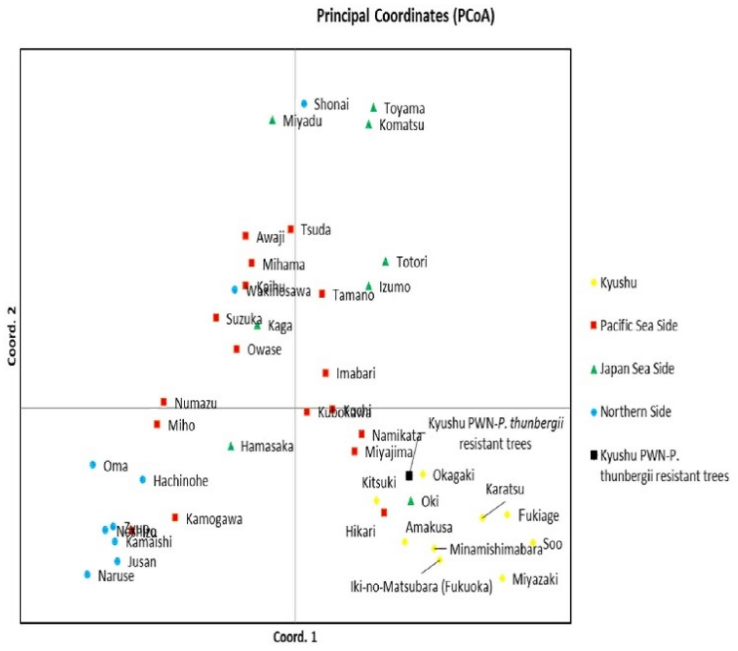



**Figure 2.1.** Genetic structure in Iki-no-Matsubara based on DBH (**A**); Iki-no-Matsubara with 42 old populations of *P. thunbergii* throughout Japan (Iwaizumi et al., 2018)(**B**).

Figure 2.1B 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 2.2). Based on DBH class range (Figure 2.3) specifically, the similarity between the Minami-Shimabara and Iki-no-Mastubara, 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 origins of the young trees, 1–30 cm DBH range class, were not from the Iki-no-Matsubara population but another area.




| Pop1 | Pop2 | *Nm* |
|---|---|---|
| Iki-no-Matsubara | Fukiage | 15.107 |
| | Soo | 9.915 |
| | Miyazaki | 13.686 |
| | Minamishimabara | 47.990 |
| | Amakusa | 35.353 |
| | Kitsuki | 10.525 |
| | Karatsu | 29.747 |
| | Hikari | 19.560 |
| | Miyajima | 10.213 |
| | Kubokawa | 9.514 |
| | Namikata | 10.799 |
| | Imabari | 8.189 |
| | Izumo | 11.153 |
| | Oki | 9.209 |
| | Kochi | 8.098 |
| | Tamano | 6.183 |
| | Totori | 8.559 |
| | Tsuda | 4.478 |
| | Kaihu | 5.125 |
| | Hamasaka | 6.442 |
| | Awaji | 2.605 |
| | Mihama | 4.293 |
| | Owase | 4.244 |
| | Suzuka | 4.314 |
| | Miyadu | 4.016 |
| | Kaga | 5.162 |
| | Komatsu | 4.894 |
| | Toyama | 5.300 |
| | Miho | 3.211 |
| | Numazu | 3.093 |
| | Izu | 3.326 |
| | Kamogawa | 4.338 |
| | Zyuo | 2.993 |
| | Shonai | 3.607 |
| | Noshiro | 2.811 |
| | Naruse | 2.544 |
| | Kamaishi | 2.924 |
| | Hachinohe | 3.424 |
| | Jusan | 3.176 |
| | Wakinosawa | 4.103 |
| | Oma | 1.967 |

**Figure 2.2.** PCoA and gene flow (*Nm*) of Iki-no-Matsubara with other populations of *P. thunbergii* and PPT Kyushu cultivar.

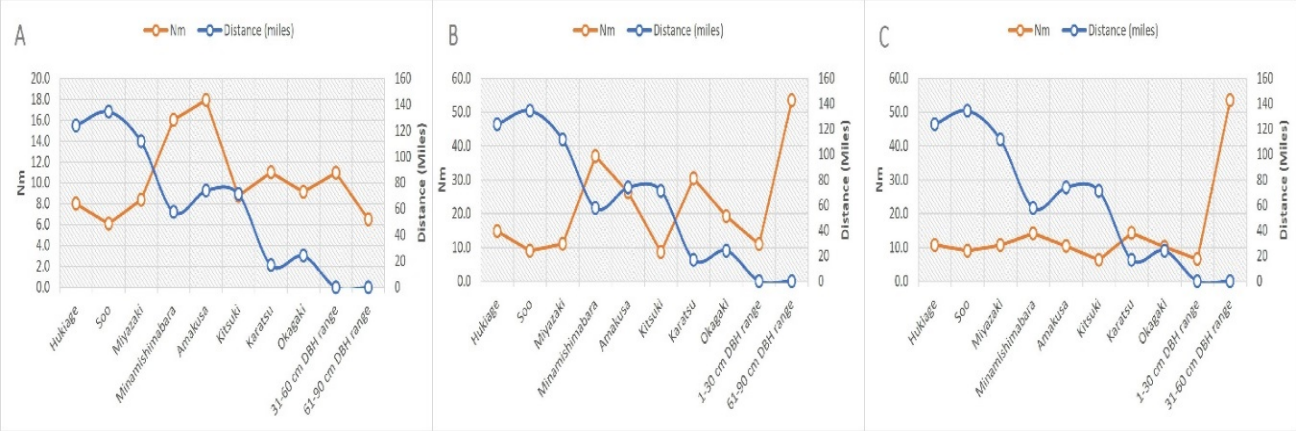



**Figure 2.3.** Relationship between gene flow (*Nm*) and distance with other populations in the Kyushu area on the following basis: (**A**) 1–30 cm DBH class range; (**B**) 31–60 cm DBH class range; (**C**) 61–90 cm DBH class range.

*2.3.2. Genetic Diversity and Genetic Structure of PPTs*

Since the 1990s, PPTs 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 similarities with other populations in Kyushu area) provides a baseline when deploying PPT Kyushu cultivar. In general, the genetic structure of PPTs within each region (Figure 2.4) showed PPT Kyushu and Kanto cultivars had the most distinct genetic structure (dominated by region's structure pattern). In contrast, PPT Tohoku and Kansai cultivars exhibited mixed patterns. The PCoA results show that the PPT Kyushu cultivar are similar to the Okagaki populations (Figure 2.2). 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 Table 3). The genetic diversity of PPT Kyushu cultivar were low compared with the mean genetic diversity of the *P. thunbergii* populations in entire Kyushu area (Table 2.4).

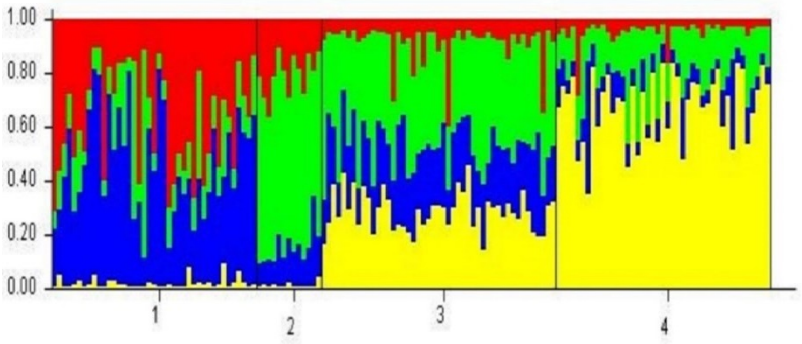



**Figure 2.4.** Genetic structure of PPTs at K = 4 (1) Tohoku region, (2) Kanto region, (3) Kansai region, and (4) Kyushu region.

**Table 2.4.** Genetic diversity of the PPT Kyushu cultivar 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, FIS: inbreeding coefficient within the population, HWE: Hardy-Weinberg equilibrium (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

## 2.4. Discussion

### *2.4.1. Inference of Origins 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 (Suwa, 2013). 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. The genetic diversity within Iki-no-Matsubara was high 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 origins 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 (Welt et al., 2015). The presence of private alleles in the Iki-no-Matsubara (Appendix Table 1), 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 2.2), we postulate that the Iki-no-Matsubara can 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 2.3). Such results showed the recently planted the 1–30 cm DBH class range indicate that they were planted without considering genetic origins.

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 2.1A). The yellow patterns observed in Iki-no-Matsubara were common among populations in the Kyushu area (Figure 2.1B), 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 origins 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 (Iwaizumi et al., 2018). 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 origins of the seedling was not considered when new planting was performed after removing individuals damaged by pine wilt disease.

### *2.4.2. Genetic Management of P. thunbergii in Iki-no-Matsubara with PPT Kyushu cultivar*

From a forest protection viewpoint, artificially planting PPT Kyushu cultivar 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, forest resilience, and similarities with another populations must also be properly considered. Thus, there are two crucial points to consider: (1) how well PPTs seed-sourcing strategy and (2) genetic management within the population, including the PPTs.

In addition, there are two aspects should be considered for point number 1: (1) How well the mother trees represent the genetic diversity and similarities in the selected area? (2) A sufficient number of resistant trees should be sourced as mother trees? (Kang et al., 2001; David et al., 2003; Funda et al., 2009; Funda, 2012). The mother trees will represent the genetic diversity, structure, and gene flow pattern of the population where it was taken (Kitzmiller, 1990; Wheeler and Jech, 1992). The extent of gene flow among populations shows how alleles are shared (similarities) and play an important role in genetic differentiation among populations (Slatkin, 1987; Stefenon et al., 2008).

From the perspective of genetic structure, PPT Kyushu cultivar were noticeably displayed a yellow pattern (Kyushu region's structure pattern) (Figure 2.4). However, from similarities viewpoint, PPT Kyushu cultivar were located in the middle between the Kyushu area and Pacific Ocean side area and shared similarity with the Okagaki

populations (Figure 2.2). This may have occurred because the selected trees for PPT Kyushu cultivar were not sufficiently balanced to represent all Kyushu area populations. In fact, among 43 PPT Kyushu cultivar, ten were from Okagaki, and none were from Iki-no-Matsubara (Appendix Table 2).

The sufficient number of mother trees, act as effective population size in seed orchard, must be examined first to manage the diversity and similarities within Iki-no-Matsubara with other populations in Kyushu area (Lindgren and Mullin, 1998). 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 (Crow and Kimura, 2009). However, this study was conducted only for the Iki-no-Matsubara, and not for the entire *P. thunbergii* in Kyushu area. Thus, in the future, the breeding project of PPT Kyushu cultivar need to develop a perspective based on genetic management according to the genetic characteristics in each local pine forest in the Kyushu area.

### *2.4.3. PPT Kyushu cultivar 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 PPTs deployment management. Only using clones (vegetative) or seeds of specific PPT Kyushu cultivar 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 (Konnert et al., 2015; Ingvarsson and Wu, 2018; Dahlberg, 2019), which negatively affect the population as a gene resource. Therefore, it is necessary to determine the status numbers of PPT Kyushu cultivar as mother trees (Wu, 2018), because it will directly affect number of seedlings to deploy into given site. Practice selective seed-cone harvesting to balancing genetic gain and genetic diversity (Lindgren and El-Kassaby, 1989; Funda et al., 2009) can be used as approach method to solve the matter about insufficiency in status number of PPT Kyushu cultivar for the necessary reforestation. When considering genetic diversity in the next generation and

the status number of PPT Kyushu cultivar, we can first refer to the local seed pool for reference, where at least 30 mother trees and each mother tree is 24 seedlings. Then a total of 720 seedlings are needed to provide complete coverage for genetic diversity in the Iki-no-Matsubara population in the next generation (Iwaizumi et al., 2020). 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 (Jost et al., 2018).

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 (Johnson et al., 2004; O'Brien and Krauss, 2010). Furthermore, the origins of the seedlings should be determined according to the Japan Forest Seeds and Seedlings Law 1939 (Nagamitsu, 2015) so that, at least, the structure pattern among the four areas shown in Figure 4B can be maintained. Subsequently, PPTs should be managed separately in each FFPRI regional office. Using a non-local seed pool or non-local genetic pattern can lead to uncertain results in terms of adaptation and genetic differentiation among populations (Adams and Burczyk, 2000; Konnert et al., 2015).

## 2.5. 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 (Iwaizumi et al., 2018). 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 (Hughes et al., 2008; Jump et al., 2009; Welt et al., 2015; Aravanopoulos, 2016). Nevertheless, understanding the current genetic information of Iki-no-Matsubara (genetic diversity, genetic structure, and similarities) are essential for deploy PPT Kyushu cultivar 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 similarities with Minami-Shimabara and Amakusa, and there was a possibility that the origins 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 PPT Kyushu cultivar was distinctly dominated by yellow genetic pattern. The PPT Kyushu cultivar genetic diversity was lower than that of the overall population in the Kyushu area. An insufficient number of PPT Kyushu cultivar can lead to an imbalance in gene flow and, in some cases, the planting of PPT Kyushu cultivar may indicate that the genetic structure in any population may be similar to that of the Okagaki population. A sufficient number of PPT Kyushu cultivar, as mother trees, within seed orchards and sufficient status number of the seedlings need to be considered to safely deploy PPT Kyushu cultivar as reforestation-material plants including Iki-no-Matsubara population. This approach can be used not only to preserve Iki-no-Matsubara population (genetic diversity, genetic structure, similarities, 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 PPTs.

# CHAPTER 3. DEVELOPMENT AND CHARACTERIZATION OF EST-SSR MARKERS FOR *Pinus thunbergii*

## 3.1. Introduction

The previous population genetic studies of *P. thunbergii* showed that the seedlings origins were basically from relatively close regions (Iwaizumi et al., 2018; Mukasyaf et al., 2021a). However, for some regions, the seedlings origins are unknown. Thus, novel DNA markers are needed to understand genetic divergence and examine the genetic origins of *P, thunbergii* populations. One of purposes using genetic markers is to provide insight into genetic composition within/between populations. Genomic SSR markers are useful for population genetic analysis, but the identification and the development of genomic SSR are labor-intensive, expensive, and time-consuming (Abdelkrim et al., 2009; Csencsics et al., 2010). Hence, one solution to those problems is using genomic resources based on next-generation sequencing to develop gene-based EST-SSR markers (Ellis and Burke, 2007).

EST-SSRs are a co-dominant DNA marker and a gene-based derivative from the genomic SSR marker. EST-SSRs are widely distributed in both coding and non-coding regions (Morgante et al., 2002) and can be developed cheaply and rapidly (Ellis and Burke, 2007). EST-SSRs have less polymorphism level, fewer null alleles than genomic SSR (Sullivan et al., 2013), and a low mutation rate (Zhang et al., 2019). Furthermore, EST-SSR markers development can help the preservations of population genetic resources (Du et al., 2017). Even though EST-SSR markers of *P. thunbergii* had been developed by Hirao et al. (2019), the focus was on the study of genetic linkage maps to identify quantitative trait loci (QTL), and the repeat number of EST-SSRs developed was small. This study's objectives were to (1) develop EST-SSR markers from a *P. thunbergii* transcriptome using next-generation sequencing, (2) evaluate the EST-SSR markers through genetic diversity analysis of an old *P. thunbergii* population in Iki-no-Matsubara.

## 3.2. Material and Methods

### *3.2.1. RNA Isolation and Library Creation*

*P. thunbergii* ESTs were collected using RNA from the trunk of two seedlings, which were two years old. Total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific). Total RNA quality was verified using Agilent 2100 Bioanalyzer (Agilent) with RNA integrity number (Schroeder et al., 2006). A library was prepared by TruSeq RNA Sample Prep Kit v2 (Illumina) according to the manufacturer's recommended protocol.

### *3.2.2. Identification of EST-SSRs, Primer Design, and Sequencing Validation*

The sequences were obtained using the Miseq platform and a Miseq Reagent Kit v3 (Illumina), resulting in pair-end reads of 300 bps. Adaptor sequences and low-quality sequences in the obtained NGS data were removed using Trimmomatic software (Bolger et al., 2014), and a de novo assembly was performed using Trinity software (Grabherr et al., 2011, Haas et al., 2013). MISA software was used to detect SSR for the assembled contig (Thiel et al., 2003). Sequences containing flanking regions of 200 base pairs before and after the SSRs motif were compared, and overlapping regions were removed. The selected sequences were used to design primer pair using Primer3.0 version 0.4.0 software (Untergasser, et. al., 2012).

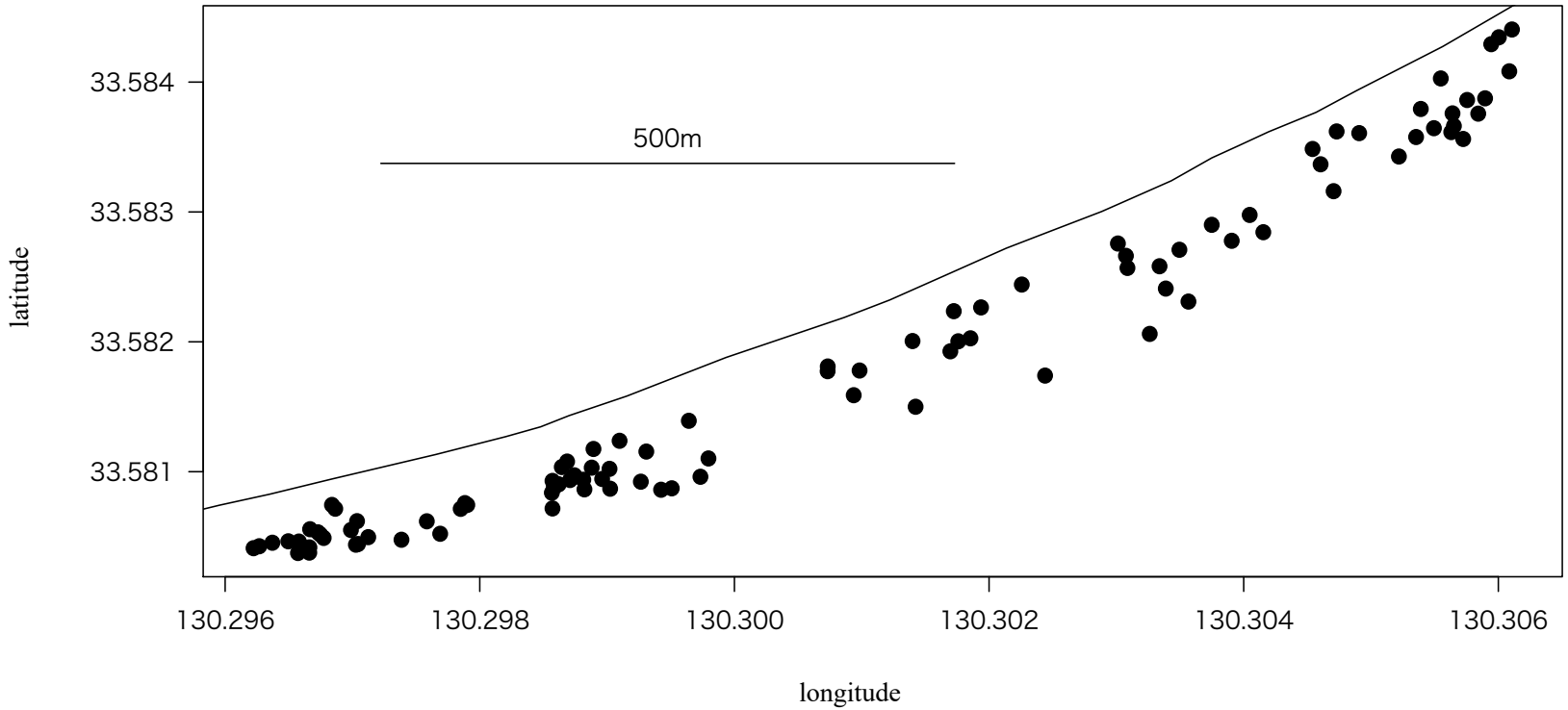



**Figure 3.1**. A map of the individuals sampled at Iki-no-matsubara.

*3.2.3. DNA Analysis and Statistic Analysis*

In order to evaluate the developed EST-SSR markers, 96 individuals in Iki-no-Matsubara, Fukuoka Prefecture, Japan (33°34 52.8 "N 130°17 59.7 "E) were used (Figure 3.1). These individuals were selected from those used by Mukasyaf et al. (2021a), which revealed the genetic structure of Iki-no-Matsubara using genomic SSR markers, especially those with tree diameters greater than 50 cm and estimated ages greater than 75 years. DNA isolation and genotyping procedures were the same as in chapter 2 with slight modifications to the PCR amplification and annealing temperatures on touchdown PCR. Genotype data was obtained using Genemapper 4.0 software (Chatterji and Pachter, 2006)

Polymorphism information content (PIC) was analyzed using Cervus 3.0.7 software (Kalinowski, et al., 2007). The number of alleles per locus ($N_a$), expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), probability of two unrelated individuals (PI) and two siblings (PI-Sib), deviation from Hardy-Weinberg equilibrium (HWE), private alleles, and genetic differentiation pattern using a principal coordinates analysis (PCoA) among individuals was constructed in GenAlEx 6.5 (Peakall and Smouse, 2012).

**3.3. Results and Discussions**

Approximately 6.2 Gbps of NGS data were obtained and assembled into 165,852 contigs (average length: 774.94 bps, N50: 1,264 bps) using the Miseq platform. Of these, 18,524 SSR regions were detected in 14,804 contigs. Table 3.1 shows the motifs with more than 12 repeats for mono-nucleotide motifs, more than six repeats for di-nucleotide motifs, more than four repeats for tri-nucleotide motifs, and more than three repeats for tetra- to hexa-nucleotide motifs. For the di- and tri-nucleotide motifs (total 8513 contigs), 34 flanking regions were selected for primer design. Finally, 12 primers were designed after removing duplicated regions.

**Table 3.1**. Distributions of motifs and repeat numbers of EST-SSR loci extracted from a *Pinus thunbergii* transcriptome database

| motif | repeats number | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| mono | - | - | - | - | - | - | - | - | - | 243 | 145 | 87 | 39 | 26 | 27 | 6 | 8 | 10 | 7 | 0 | 1 | 2 |
| di | - | - | - | 387 | 167 | 115 | 70 | 61 | 25 | 43 | 3 | 8 | 0 | 4 | 5 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| tri | - | 6036 | 1112 | 320 | 97 | 33 | 4 | 9 | 3 | 2 | 0 | 1 | 0 | 3 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 0 |
| tetra | 3699 | 253 | 30 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| penta | 967 | 100 | 12 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| hexa | 1512 | 220 | 14 | 17 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3.2.** Characteristics of 10 EST-SSR markers developed for *Pinus thunbergii*

| Locus | Primer sequence (5'3') | Repeat Motif | Allele range (bp) | registered accesion no. | Blastx hit description | E-value | GenBank accesion no. |
|---|---|---|---|---|---|---|---|
| Pt_ESTSSR_10048 | F CCTCATCCTTCATCTGTGGGG<br>R ACTGACATAGAGCTTGGGTGC | (CTC)7 | 234-243 | LC633345 | Unknown [*Picea sitchensis*] | 2.00E-52 | ABK22494.1 |
| Pt_ESTSSR_33385 | F CTTGTCAATGCTGGGCTGTTC<br>R CATTACCCGAGTGACCAACCA | (AGA)7 | 228 | LC633346 | HD-zip protein HDZ32 [*Pinus taeda*] | 4.00E-20 | ABG73246.1 |
| Pt_ESTSSR_36750 | F TGCAGATGAGGAGGCTGACTA<br>R CACACTCAACTGCAACACCTC | (GAA)7 | 173-184 | LC633347 | Hypothetical protein CL192Contig1_03, partial [*Pinus taeda*] | 2.00E-28 | AEX12956.1 |
| Pt_ESTSSR_38962 | F CTGGGCGAGAATTCTGAGTGT<br>R GCGATCCTTGTGAAGCCAATG | (CGC)7 | 230-242 | LC633348 | Unknown [*Picea sitchensis*] | 5.00E-22 | ABK25752.1 |
| Pt_ESTSSR_34351 | F GAGTGTGGAGGTGGAGATAAGG<br>R GAATGAATACAGTCGCGAGAGC | (GCA)8 | 233-248 | LC633349 | - | - | - |
| Pt_ESTSSR_34853 | F CTGCTGGTTCTAGTTCTACTTCTGG<br>R ACCATATGCAGTAAGTCATCTGTGA | (TGA)8 | 249-261 | LC633350 | - | - | - |
| Pt_ESTSSR_27622 | F GCTTTTAGAATTTCTGGTTTGCAT<br>R TCCTGATGTCTTCTGAATGGTAAA | (TG)14 | 242-255 | LC633351 | - | - | - |
| Pt_ESTSSR_24700 | F AAACACACTGATGAATGGGATG<br>R ATCCTTTTACCCTGGGGATG | (TG)12 | 248-270 | LC633352 | - | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pt_ESTSSR_22572 | F GCCTATATGCTTCGCCAACAG<br>R AGGGAGAGGTGACATTGCATG | (CAG)7 | 158-164 | LC633353 | - | - | - |
| Pt_ESTSSR_36847 | F ATTGGAGTGGAGGAGGAGGAA<br>R GATCATTGGTACCTCGTCCCC | (TGG)7 | 164-185 | LC633354 | RRM_1 domain-containing protein/zf-RanBP domain-containing protein [*Cephalotus follicularis*] | 1.00E-28 | GAV87544.1 |

Of these 12 primers designed, amplification of two primers (Pt_ESTSSR_33286, accession no. LC633355; Pt_ESTSSR_32254, accession no. LC633356) were not clear and excluded from this study. Therefore, the total number of new EST-SSR markers developed in this study were ten. As a result of BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990), five markers were annotated, while the remaining five markers had no similar genes in the database (Table 3.2). Also, the di-nucleotide motifs of the EST-SSR markers showed higher polymorphism than the tri-nucleotide motifs.

The number of alleles at each locus for the new markers ranged from one to six (Table 3.3). The observed heterozygous alleles ranged from 0.000 to 0.707, and the expected heterozygosity ranged from 0.000 to 0.720. Only one marker, Pt_ESTSSR_33385, was monomorphic. The mean number of alleles observed heterozygosity and expected heterozygosity were 3.6, 0.299, and 0334, respectively. Such results suggest that the polymorphism of EST-SSR markers were lower than our previous study (Mukasyaf et al., 2021a), which investigated 269 pine trees using six genomic SSR markers and found the mean number of alleles, observed heterozygosity, and expected heterozygosity to be 19, 0.71, and 0.81, respectively. PIC value ranged from 0.000 to 0.669, with a mean PIC value 0.30. According to the PIC information score by Botstein et al. (1980), the present studies had four loci with a high information score (PIC > 0.5). One of the ten loci possessed a moderate information score (0.5 > PIC > 0.25). The remaining loci had low information score (0 < PIC < 0.25).

EST-SSR markers can be developed with less labor, and they may provide different genetic structures from genomic SSRs because EST-SSRs are gene-based, as expected from the genes' evolutionary rate and non-neutral evolution (Varshney et al., 2005; Nagaraj et al., 2007; Khimoun et al., 2017; Manco et al., 2020). The results of PCoA analysis derived from EST SSR and genomic SSR were slightly different. (Figure 3.2A and Figure 3.2B). Therefore, the EST-SSR markers developed in this study, when used in combination with the previous genomic SSR markers, will contribute to a more detailed understanding of genetic diversity and genetic structure of *P. thunbergii* populations.

**Table 3.3**. Genetic diversity statistics of the 96 individuals in a *Pinus thunbergii* population using 10 EST-SSR markers.

| Locus/Parameter | N | $N_a$ | $H_O$ | $H_E$ | $F_{IS}$ | HWE | PIC | PI | PI-Sib |
|---|---|---|---|---|---|---|---|---|---|
| Pt_ESTSSR_10048 | 96 | 4 | 0.500 | 0.578 | 0.135 | ns | 0.515 | 0.241 | 0.521 |
| Pt_ESTSSR_33385 | 96 | 1 | 0.000 | 0.000 | 1.000 | monomorpic | 0.000 | 1.000 | 1.000 |
| Pt_ESTSSR_36750 | 96 | 5 | 0.208 | 0.19 | -0.094 | ns | 0.179 | 0.667 | 0.822 |
| Pt_ESTSSR_38962 | 96 | 3 | 0.302 | 0.344 | 0.122 | ns | 0.288 | 0.486 | 0.699 |
| Pt_ESTSSR_34351 | 81 | 5 | 0.543 | 0.555 | 0.021 | * | 0.505 | 0.248 | 0.535 |
| Pt_ESTSSR_34853 | 93 | 3 | 0.183 | 0.203 | 0.097 | ns | 0.186 | 0.653 | 0.812 |
| Pt_ESTSSR_27622 | 92 | 6 | 0.707 | 0.701 | -0.007 | ns | 0.655 | 0.136 | 0.433 |
| Pt_ESTSSR_24700 | 83 | 5 | 0.518 | 0.720 | 0.280 | * | 0.699 | 0.130 | 0.423 |
| Pt_ESTSSR_22572 | 95 | 2 | 0.000 | 0.021 | 1.000 | *** | 0.021 | 0.959 | 0.979 |
| Pt_ESTSSR_36847 | 95 | 2 | 0.032 | 0.031 | -0.016 | ns | 0.031 | 0.939 | 0.969 |
| Mean | 92.3 | 3.6 | 0.299 | 0.334 | 0.254 | | 0.304 | 0.546 | 0.719 |

$N_a$ : number of allele, $H_O$ : observed heterozygosity, $H_E$ : expected heterozygosity, $F_{IS}$ : inbreeding coefficient within the population, PIC : polymorphism information content; PI : probability of identity for two unrelated individuals, PI-Sibs : probability of identity for two siblings, HWE : Hardy-Weinberg equilibrium (ns : not significant, *p<0.05, **p<0.01, ***p<0.001)

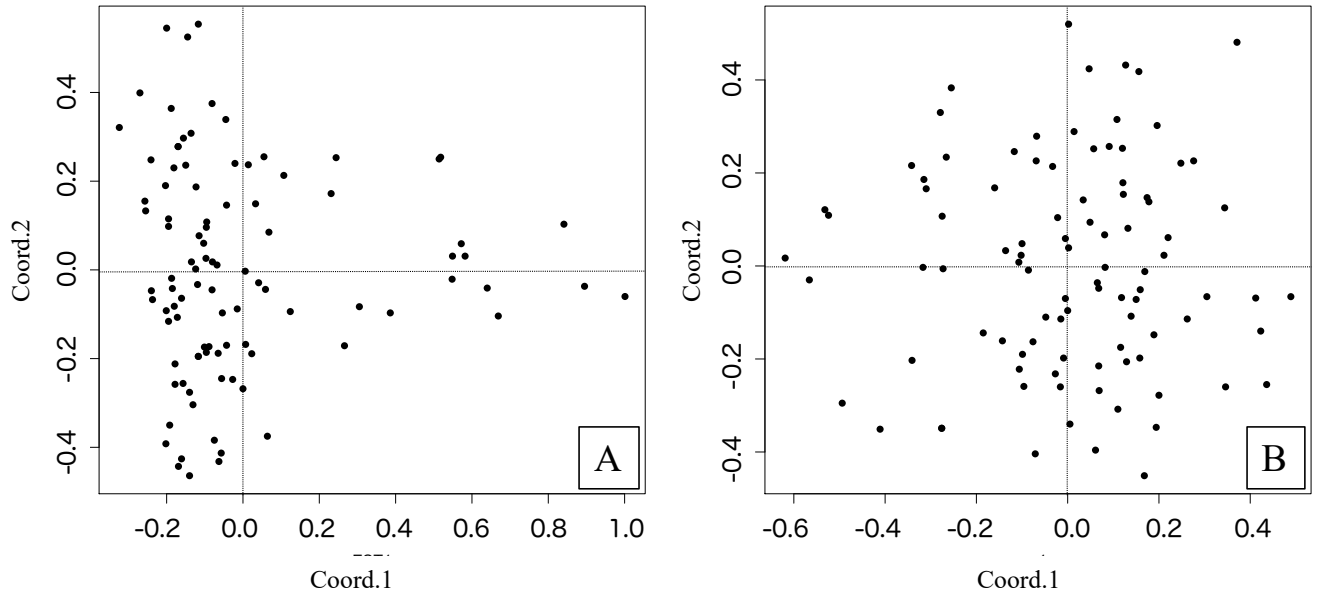



**Figure 3.2.** PCoA of the 96 individuals based on A) EST-SSR, B) Genomic SSR.

# CHAPTER 4. GENOMIC SSR AND EST-SSR MARKERS IN PROVIDING GENETIC STUDIES OF *Pinus thunbergii* POPULATIONS IN JAPAN

## 4.1. Introduction

Each population has different degree of variation (distribution of alleles, frequency of alleles, number of alleles, genetic diversity, genetic structure, genetic differentiation, etc) within their gene pools. Various alleles present at each locus through generations within/among population are represent its degree of polymorphism (Kimura, 1983) and become one of the causes for the presence of genetic variation (Griffiths et al., 2015). In literally, the capacity and the usefulness of genetic variation affects population genetics (Olson-Manning et al., 2012). In addition, distribution of allele frequencies among populations can give understanding of gene flow, mutation, genetic drift (Hartl and Clark, 1997; Port and El-Kassaby, 2014), and even local adaptation (Wit and Palumbi, 2013) through generations. Therefore, understanding allelic divergence and adaptability are fundamental aspect in population genetics. Observing the phenotype through field experiments has long history in population genetic studies. However, field experiments are time-consuming, expensive, only estimate measurable traits, and must be based on the individual (González-Martínez et al., 2006).

On other hand, the use of molecular markers provide insight about genetic variation by measuring DNA variations that reside within both non-coding and coding regions. (Port and El-Kassaby, 2014). Nowadays studies about spatial genetic variation among populations has proven as useful tool for understanding dynamics of population genetics as well (Berens et al., 2014). Nucleus, mitochondria, chloroplast (plastid), etc are subcellular organelles which contains of genes. All subcellular organelles are interconnected each other (Han, 2008) as molecular networks. In addition, they have tendency to retain their own genetic systems (as regulation of gene expression) (Allen, 2015). DNA can transfer between those subcellular organelles as a part of molecular evolutionary process (Raman, et al., 2019). In this case, molecular networks itself can represent adaptation, evolution, and mutation of the species in a population. Nevertheless, the appropriate choice of molecular marker can provide understanding of those process (Grünwald and Goss, 2011). In addition, no one has mentioned that population genetic

studies can be assessed by limited or specific genetic marker. So far molecular networks itself still complicated. Each genetic marker works to reveal its biological function and evolution according to its characteristic.

Combination of different type molecular markers has been used to deepen understanding about population genetic studies (Coates et al., 2009; Port and El-Kassaby, 2014; Hodel et al., 2017; Borrell et al., 2018). The choice of genetic markers depends on the problems to be addressed such as number of loci/markers, reproducibility, degree of polymorphism (Lowe et al., 2004), and the objectives of focus study (Hu et al., 2011; Parthiban et al., 2018; Zimmerman et al., 2020). Among molecular marker, genomic SSRs (microsatellite) have been widely used in genetic studies of forest trees species. Genomic SSRs are codominant markers commonly located in non-coding region (Zane et al., 2002) and high mutation rate-DNA type, so SSRs are reproducibility, abundance, and extensively coverage of the genome (Sorkheh et al., 2016; Parthiban et al., 2018). On the other hand, EST-SSRs are usually provide information of gene expression (Nagaraj et al., 2007). Hence, EST-SSR markers have been used for the purpose of easy transferability to other species and phylogenetic in population genetics studies (Xu et al., 2004; Meyer et al., 2017; Fan et al., 2019; Li et al., 2020). However, for such evolutionary genetic studies, EST-SSRs are receiving little attention (Ellis et al., 2006). In fact, EST-SSRs may give insight of adaptive and evolutionary genetic studies (Li et al., 2004). EST-SSRs are well conserved and they contain of common components of genes (Varshney et al., 2005). So, it can efficiently identify of diverse parents (Parthiban et al., 2018).

Genomic SSRs contain non-repetitive of DNA, (Morgante et al., 2002), efficiently used to broad base-grouping the genotypes (Parthiban et al., 2018) and high informative output. As consequence, this marker may also lead to misinterpretation of heterozygosity through homoplasy or a mutation not from common ancestor (Zimmerman et al., 2020). It may end up to erode the divergence among subpopulation (Coates et al., 2009). EST-SSRs have been used to study genetic variation of forest tree species (*Picea likiangensis*, Cheng et al., 2013; *Cinnamomum camphora*, Zhong et al., 2017; *Liquidambar formosana*, Chen et al., 2020). Genomic SSRs and EST-SSRs work differently in assessing the genetic diversity because they are located in different genome (Wen et al., 2010). Thus, combination of both genomic SSR and EST-SSR markers can efficiently identify the

change in the gene during domestication (explain the genetic variation in the population) (*Jatropha curcas*, Wen et al., 2010; Cucumber, Hu et al., 2011; Sugarcane, Parthiban et al., 2018).

Up to date, studies about genetic variation of *P. thunbergii* populations in Japan have been reported by Miyata and Ubukata (1993) using isozyme, chloroplast (Shiraishi and Watanabe, 1995), genomic SSR (Iwaizumi et al., 2018; Mukasyaf et al., 2021a), and EST-SSR on a local population (Chapter3; Mukasyaf et al., 2021b). EST-SSR markers have not been used for population genetic studies of *P. thunbergii* in Japan. Thus, comparative and efficiency value of both markers have not been found. In addition, Iwaizumi et al. (2018) suggested that there was ambiguity and mixed pattern in some populations, especially between Sea of Japan side and Pacific Ocean side. Hence, population genetic studies of *P. thunbergii* cannot progress further. The objectives in this studies were: 1) to understanding genetic variation of 21 *P. thunbergii* populations, especially between Japan Sea and Pacific Ocean side by using EST-SSR markers, 2) to present the genetic parameters and interpretation of relative advantage of EST-SSR markers system studies compared to genomic SSR, and 3) to implement the results of both markers into genetic management for *P. thunbergii* populations.

### 4.2. Material and Methods

A total 672 samples obtained from 21 *P. thunbergii* populations (Figure 4.1) were used in this study. The samples were the same as those used by Iwaizumi et al. (2018). In this study, we used seven EST-SSR markers (Pt_ESTSSR_36750, Pt_ESTSSR_38962, Pt_ESTSSR_34351, Pt_ESTSSR_27622, Pt_ESTSSR_24700, Pt_ESTSSR_34853, and Pt_ESTSSR_33385) developed in Chapter 3 were used. DNA genotyping procedures of EST-SSR markers were the same as in our previous studies (Chapter 2; Mukasyaf et al., 2021b). Genomic SSRs genotyping data were the same as in Chapter 1 (Mukasyaf et al., 2021a). Genotyping data from both markers were analyzed, compared, and integrated. GenAlex version 6.503 (Peakall and Smouse, 2012) was used to measure number of allele ($N_a$), number of effective allele ($N_e$), observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), allele frequency spectrum, and inbreeding coefficient within population ($F_{IS}$). Polymorphism Index Content (PIC), Hardy-Weinberg deviation, and *p*-

value of linkage disequilibrium of the loci estimated by Fstat version 2.9.3.2 (Goudet, 2001). The polymorphism percentage of the loci within the population and pairwise population genetic distance ($R_{ST}$) (Slatkin, 1995) assessed by using Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). Then, pairwise population genetic distance ($R_{ST}$) was visualized by neighbor-joining tree method (Saitou and Nei, 1987) by using Mega X version 10.2.4 (Kumar et al., 2018). Bottleneck version 1.2.02 (Piry et al., 1999) was used to evaluate loci that may have occurred a bottleneck in the population. After that, linkage disequilibrium of the loci and Ewens-Watterson test executed to testing a neutrality of the loci by using Popgene32 version 1.32 (Yeh and Boyle, 1997). Structure 2.3.4 (Pritchard et al., 2000) was used to determine individual-based genetic structure assessment by Bayesian method with a simulation run 10 times replicated, K-set 1-10 for 50000 iterations burn-in period, and 50,000 iterations replication after burning under admixture ancestral with using the sampling locations as prior (LOCPRIOR) model.

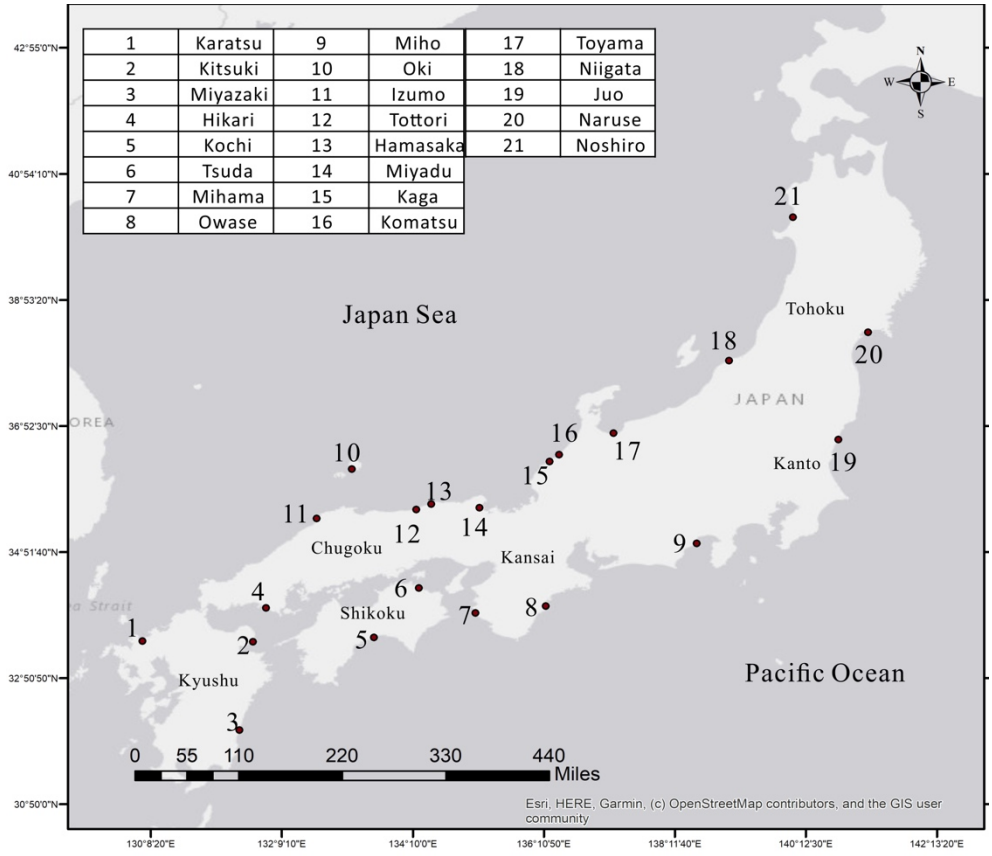



**Figure 4.1.** Location of 21 *P. thunbergii* populations.

## 4.3. Results

### *4.3.1. Genetic Diversity Parameter on Microsatellite Markers*

Genetic diversity parameter of seven EST-SSR markers showed that number of allele ($N_a$) of the populations ranged from 3.000 (Mihama, Niigata, and Hamasaka) to 5.286 (Miyadu), and observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$) of the populations were 0.231-0.463 and 0.331-0.458, respectively (Table 4.1). Kyushu region, Izumo and Naruse had the highest polymorphism percentage (85.7%), while the lowest was Juo (14.7%). As for $N_a$, $H_O$, and $H_E$ value, the lowest value was Pt_ESTSSR_33385 (1.05, 0.001, and 0.001, respectively). Pt_ESTSSR_24700 had the highest value in number of alleles (5.52 alleles) and Pt_ESTSSR_34351 exhibited the highest value in observed heterozygosity (0.588) and expected heterozygosity (0.597) (Table 4.1). Locus Pt_ESTSSR_33385 showed minus value on inbreeding coefficient (-0.016). Polymorphism information content (PIC) of 21 populations showed on Appendix Table 5. The present studies had two loci with a high information score (PIC > 0.5). two of six loci possessed a moderate information score (0.5 > PIC > 0.25). The remaining loci had low information score (0 < PIC < 0.25) (Botstein et al., 1980). Bottleneck analysis of EST-SSR markers (Table 4.1) on *p*-value of Wilcoxon sign rank test (SMM and TPM) showed no sign of bottlenecks event within the populations. However, bottleneck analysis of genomic SSR (Appendix Table 5) exhibited that bottleneck did occur in 13 populations (SMM) and 10 populations (TPM). When the neutrality of the loci test was evaluated, Obs. F values of the loci in both markers were within lower value (L95) and upper value (U95) limits of neutrality test for the loci, suggesting all loci for both markers were neutral and can utilized in population genetic studies (Table 4.2).

**Table 4.1.** Genetic diversity statistics of 21 *P. thunbergii* population using 7 EST-SSR markers.

| No | Pop | $N_a$ | $N_e$ | $H_O$ | $H_E$ | $F_{IS}$ | *p*-Wilcoxon Sign Rank Test | | | % polymorphism loci |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | IAM | SMM | TPM | |
| 1 | Karatsu | 3.143 | 1.842 | 0.356 | 0.360 | 0.016 | 0.622 | 0.236 | 0.554 | 85.7 |
| 2 | Kitsuki | 3.857 | 2.393 | 0.463 | 0.458 | -0.025 | 0.241 | 0.396 | 0.598 | 85.7 |
| 3 | Miyazaki | 3.286 | 2.205 | 0.362 | 0.402 | 0.046 | 0.125 | 0.551 | 0.562 | 85.7 |
| 4 | Hikari | 3.286 | 2.119 | 0.364 | 0.410 | 0.088 | 0.118 | 0.239 | 0.554 | 28.6 |
| 5 | Kochi | 3.286 | 1.923 | 0.251 | 0.352 | 0.243 | 0.372 | 0.251 | 0.551 | 42.9 |
| 6 | Tsuda | 3.429 | 2.057 | 0.369 | 0.364 | -0.031 | 0.035 | 0.432 | 0.433 | 71.4 |
| 7 | Mihama | 3.000 | 1.863 | 0.281 | 0.337 | 0.125 | 0.199 | 0.422 | 0.419 | 71.4 |
| 8 | Owase | 4.429 | 2.364 | 0.289 | 0.378 | 0.177 | 0.221 | 0.138 | 0.140 | 28.6 |
| 9 | Miho | 3.714 | 2.089 | 0.257 | 0.358 | 0.405 | 0.552 | 0.406 | 0.396 | 42.9 |
| 10 | Oki | 3.429 | 1.883 | 0.272 | 0.362 | 0.174 | 0.350 | 0.590 | 0.578 | 42.9 |
| 11 | Izumo | 4.143 | 2.438 | 0.281 | 0.373 | 0.172 | 0.382 | 0.251 | 0.537 | 85.7 |
| 12 | Tottori | 3.429 | 1.990 | 0.292 | 0.373 | 0.237 | 0.400 | 0.214 | 0.207 | 57.1 |
| 13 | Hamasaka | 3.000 | 1.821 | 0.263 | 0.328 | 0.123 | 0.203 | 0.416 | 0.406 | 42.9 |
| 14 | Miyadu | 5.286 | 2.351 | 0.323 | 0.412 | 0.251 | 0.254 | 0.052 | 0.060 | 42.9 |
| 15 | Kaga | 3.286 | 2.090 | 0.358 | 0.385 | 0.051 | 0.102 | 0.272 | 0.281 | 71.4 |
| 16 | Komatsu | 4.286 | 2.461 | 0.288 | 0.406 | 0.398 | 0.386 | 0.249 | 0.556 | 71.4 |
| 17 | Toyama | 3.286 | 2.005 | 0.330 | 0.376 | 0.046 | 0.369 | 0.239 | 0.564 | 71.4 |
| 18 | Niigata | 3.000 | 1.865 | 0.347 | 0.367 | 0.054 | 0.102 | 0.285 | 0.275 | 71.4 |
| 19 | Juo | 3.857 | 1.706 | 0.231 | 0.331 | 0.283 | 0.359 | 0.090 | 0.095 | 14.3 |
| 20 | Naruse | 4.000 | 1.927 | 0.304 | 0.353 | 0.063 | 0.279 | 0.218 | 0.213 | 85.7 |
| 21 | Noshiro | 4.000 | 2.313 | 0.304 | 0.368 | 0.140 | 0.241 | 0.108 | 0.105 | 71.4 |
| | Mean | 3.639 | 2.081 | 0.314 | 0.374 | 0.145 | 0.281 | 0.288 | 0.385 | 60.5 |

$N_a$ : number of allele, $N_e$ : number of effective allele, $H_O$ : observed heterozygosity, $H_E$ : expected heterozygosity, $F_{IS}$ : inbreeding coefficient within the population, Wilcoxon Sign Rank Test : IAM (Infinite Allele Model), SMM (Stepwise Mutation Model, and TPM (Two Phase Mutation Model)

**Table 4.2.** The Ewens-Watterson test for neutrality of the markers in 21 *P. thunbergii* populations.

| Marker | Locus | Obs F | SE | L95 | U95 |
|---|---|---|---|---|---|
| EST-SSR | Pt_ESTSSR_36750 | 0.951 | 0.039 | 0.304 | 0.961 |
| | Pt_ESTSSR_38962 | 0.767 | 0.037 | 0.309 | 0.968 |
| | Pt_ESTSSR_33385 | 0.999 | 0.028 | 0.506 | 0.999 |
| | Pt_ESTSSR_34351 | 0.207 | 0.017 | 0.180 | 0.677 |
| | Pt_ESTSSR_27622 | 0.197 | 0.027 | 0.212 | 0.827 |
| | Pt_ESTSSR_24700 | 0.256 | 0.016 | 0.154 | 0.627 |
| | Pt_ESTSSR_34853 | 0.284 | 0.010 | 0.134 | 0.545 |
| Genomic SSR | bcpt1075 | 0.181 | 0.007 | 0.112 | 0.044 |
| | bcpt1671 | 0.119 | 0.006 | 0.109 | 0.039 |
| | bcpt834 | 0.243 | 0.013 | 0.142 | 0.587 |
| | bcpt1823 | 0.149 | 0.010 | 0.133 | 0.527 |
| | bcpt2532 | 0.085 | 0.002 | 0.077 | 0.266 |
| | bcpt1549 | 0.270 | 0.010 | 0.136 | 0.532 |

Obs. F: Observed sum of the squared of allelic frequency; L95, U95: The 95% confidence interval upper and lower limit; SE: Standard error for observed F were calculated using 1000 simulated sample.

Linkage disequilibrium (LD) for pair loci within and between markers were tested to understand which pair loci may linked to bottleneck even in the populations. The results of linkage disequilibrium based on EST-SSR markers (Table 4.3) indicated that the loci were not linked to each other. In addition, the value of $D_{ST}^2$ and $D'_{IS}^2$ were lower than $D_{IS}^2$ and $D'_{ST}^2$ (Table 4.3). On the other hand, linkage disequilibrium among genomic SSR markers suggests that some loci were linked, e.g: bcpt1075 linked with three loci, bcpt1671 with two loci, and bcpt834 only one locus (Appendix Table 6). Bcpt1671 and bcpt834 were also linked with EST-SSR marker.

**Table 4.3.** Linkage disequilibrium of EST-SSR markers in 21 *P. thunbergii* populations.

| Locus A | Locus B | $D_{IT}^2$ | $D_{IS}^2$ | $D'_{IS}^2$ | $D_{ST}^2$ | $D'_{ST}^2$ | *p*-LD |
|---|---|---|---|---|---|---|---|
| Pt_ESTSSR_36750 | Pt_ESTSSR_38962 | 0.0302 | 0.0000 | 0.0301 | 0.0301 | 0.0000 | ns |
| | Pt_ESTSSR_33385 | 0.0009 | 0.0000 | 0.0009 | 0.0009 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.1879 | 0.0002 | 0.1878 | 0.1873 | 0.0000 | ns |
| | Pt_ESTSSR_27622 | 0.2113 | 0.0003 | 0.2112 | 0.2117 | 0.0000 | ns |
| | Pt_ESTSSR_24700 | 0.0435 | 0.0005 | 0.0434 | 0.0440 | 0.0000 | ns |
| | Pt_ESTSSR_34853 | 0.2170 | 0.0003 | 0.2169 | 0.2164 | 0.0000 | ns |
| Pt_ESTSSR_38962 | Pt_ESTSSR_33385 | 0.0279 | 0.0000 | 0.0279 | 0.0279 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.1696 | 0.0010 | 0.1678 | 0.1695 | 0.0017 | ns |
| | Pt_ESTSSR_27622 | 0.1926 | 0.0014 | 0.1913 | 0.1904 | 0.0013 | ns |
| | Pt_ESTSSR_24700 | 0.0471 | 0.0017 | 0.0466 | 0.0459 | 0.0005 | ns |
| | Pt_ESTSSR_34853 | 0.1874 | 0.0006 | 0.1855 | 0.1874 | 0.0018 | ns |
| Pt_ESTSSR_33385 | Pt_ESTSSR_34351 | 0.1972 | 0.0000 | 0.1971 | 0.1972 | 0.0000 | ns |
| | Pt_ESTSSR_27622 | 0.2220 | 0.0000 | 0.2222 | 0.2222 | 0.0000 | ns |
| | Pt_ESTSSR_24700 | 0.0482 | 0.0000 | 0.0482 | 0.0481 | 0.0000 | ns |
| | Pt_ESTSSR_34853 | 0.2283 | 0.0000 | 0.2283 | 0.2283 | 0.0000 | ns |
| Pt_ESTSSR_34351 | Pt_ESTSSR_27622 | 0.1331 | 0.0025 | 0.0964 | 0.1303 | 0.0367 | ns |
| | Pt_ESTSSR_24700 | 0.0750 | 0.0063 | 0.0733 | 0.0685 | 0.0017 | ns |
| | Pt_ESTSSR_34853 | 0.1396 | 0.0026 | 0.1028 | 0.1365 | 0.0368 | ns |
| Pt_ESTSSR_27622 | Pt_ESTSSR_24700 | 0.0819 | 0.0065 | 0.0800 | 0.0752 | 0.0019 | ns |
| | Pt_ESTSSR_34853 | 0.1415 | 0.0021 | 0.0995 | 0.1395 | 0.0419 | ns |
| Pt_ESTSSR_24700 | Pt_ESTSSR_34853 | 0.0871 | 0.0042 | 0.0850 | 0.0820 | 0.0021 | ns |

$D'_{ST}^2$ : variance of disequilibrium of total population; $D_{ST}^2$ : variance of correlation of the gene of two loci of different gametes within a population to total population; $D'_{IS}^2$ : variance of correlation of two loci of one gamete in a population; $D_{IS}^2$ : expected variance of linkage disequilibrium within a population; $D_{IT}^2$ : total variance of disequilibrium of total population; *p*-LD : *p* value of linkage disequilibrium of two loci (ns = not significant, **p*<0.05, ***p*<0.01, ****p*<0.001)

### *4.3.2. Genetic Differentiation on Microsatellite Markers*

The AMOVA results (Table 4.4) implied that the diversity within population was greater than between populations. This diversity of *P. thunbergii* populations are fully compatible with characteristic of outbreed forest tree species such as conifer (Prunier et al., 2012). Phylogenetic tree based on neighbor-joining method based on genomic SSR and

EST-SSR markers were showed on Figure 4.2. Both genomic SSR and EST-SSR markers exhibited that *P. thunbergii* diverged into two clusters. However, the phylogenetic tree constructed based on genomic SSR showed that degree of differentiation between two clusters was low and the populations seem to be closely related to each other. In contrast, in the EST-SSR phylogenetic tree, the two clusters distinctly had high degree of differentiation around 0.28-0.30 (Figure 4.2B).

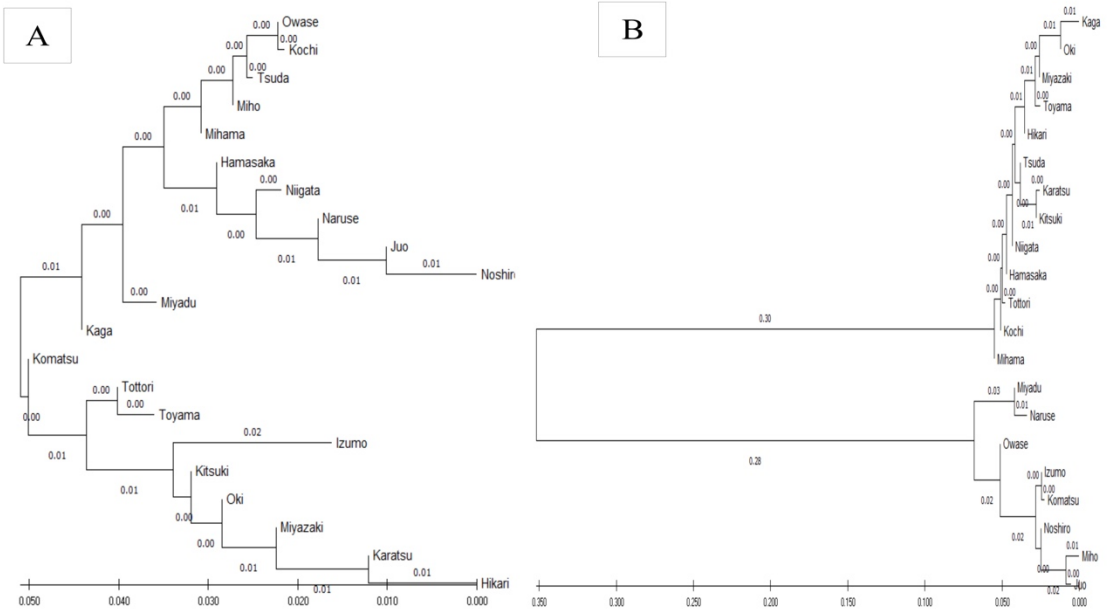



**Figure 4.2.** Neighbor-joining method of phylogenetic tree based on $R_{ST}$ genetic distance on A) Genomic SSR; B) EST-SSR.

Genetic structure analysis between the genomic SSR and the EST-SSR markers were showed distinguishable results. There was ambiguity and mixed pattern of genetic structure in some populations (especially on the Sea of Japan side) based on genomic SSR markers results (Figure 4.3B). However, *P. thunbergii* populations distinctly showed divided into two clusters by using EST-SSR markers. Especially Izumo, Miyadu, and Komatsu had shown different cluster than the remaining population on the Sea of Japan side. The genetic structure based on EST-SSR markers (Figure 4.3B) also suggested the existence of a geographic cline, but the pattern was more distinct than that of genomic SSRs.

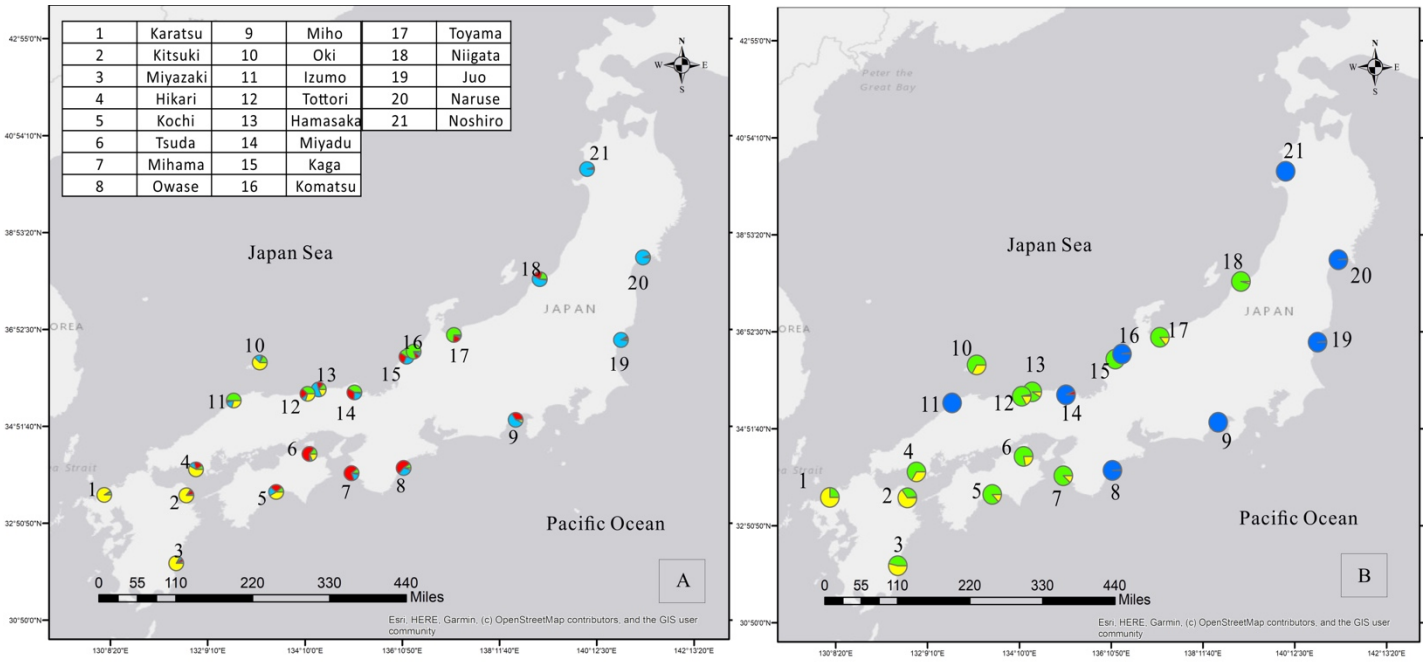



**Figure 4.3.** Genetic structure of 21 *P. thunbergii* populations at K = 4 based on A) Genomic SSR (Iwaizumi et al., 2018); B) EST-SSR.

The results of neighbor-joining method based on combination from both markers was showed in Figure 4.4, while genetic structure analysis results in Figure 4.5. The phylogenetic tree indicated that the relationship between the populations were divided into two clusters as well as EST-SSR markers. The phylogenetic tree results was supported by the result of genetic structure as well. Thus, it concluded that *P. thunbergii* populations in Japan can be divided into two ancestral populations, which is mainly in Kyushu and Tohoku. Central Japan, from Hokuriku to Shikoku, showed a complex structure and may have weaker genetic structure than another area. Discrepancies genetic pattern (in the same region but had different genetic pattern of populations) and non-origins pattern (such as Izumo, Miyadu, and Komatsu) were the reasons.


**Figure 4.4.** Neighbor-joining tree of combination markers based on $R_{ST}$ genetic distance of 21 *P. thunbergii* populations.

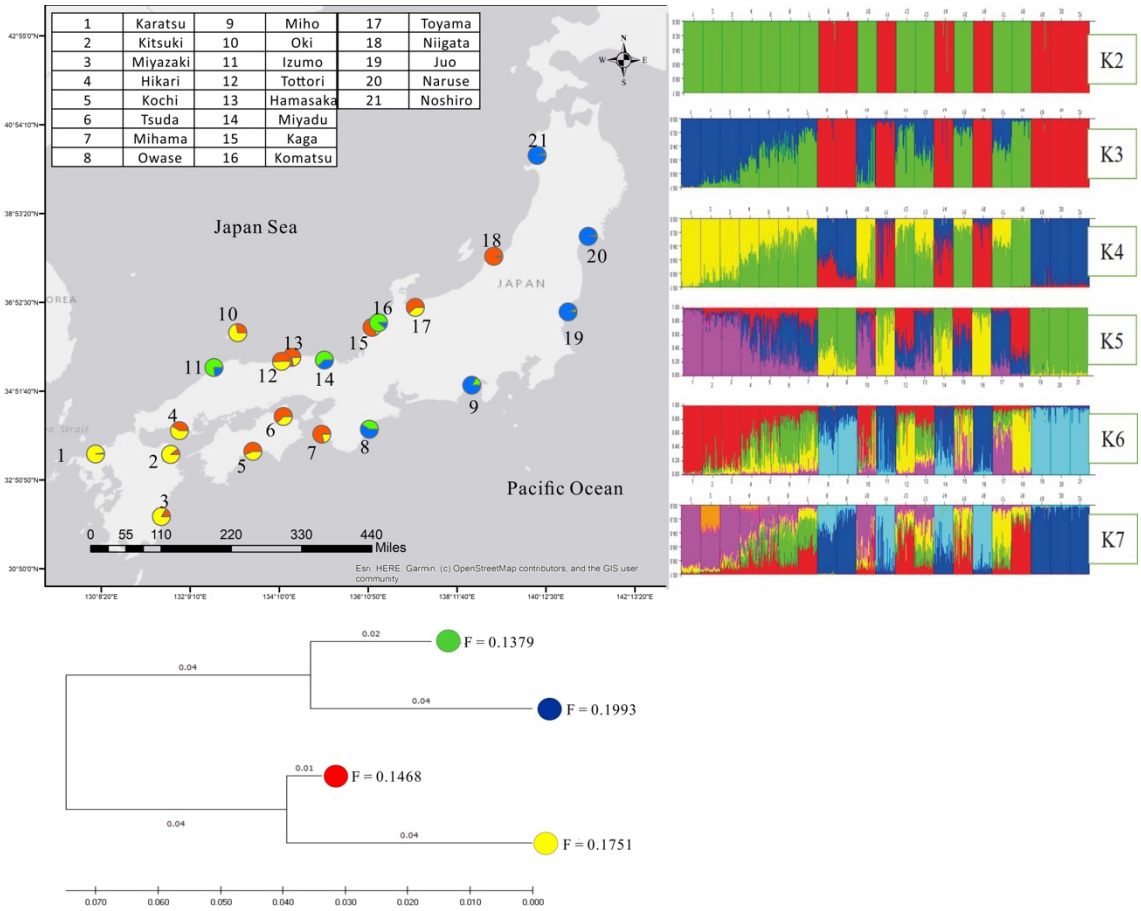



**Figure 4.5.** Genetic structure of 21 *P. thunbergii* populations at K = 4 based on combination markers.

**Table 4.4.** AMOVA of 21 *P. thunbergii* populations based on combination markers.

| Source of variation | Degree of freedom | Sum of square | Variance component | Percentage of variation |
|---|---|---|---|---|
| Among clusters | 1 | 68242.48 | 106.201 | 23.03 |
| Among populations within clusters | 19 | 17496.7 | 8.502 | 1.84 |
| Among individuals withins populations | 651 | 245239.03 | 30.296 | 6.57 |
| Within populations | 672 | 212432 | 316.119 | 68.55 |
| Total | 1343 | 543410.2 | 461.119 | |

## 4.4. Discussion

### *4.4.1. Genetic Diversity Parameter on Microsatellite Markers*

In general, genetic diversity is a key to understand population size, mating system, and geographic distribution, which always change through time (White et al., 2007). However, genetic diversity itself is affected by molecular network activities (such as mutation, recombinant rate, gene density, chromosomal region, chromosomal associated, etc) as it represents genome variation at population level (Casillas and Barbadilla, 2017). The higher genetic diversity within a population will affect success rates level to adapt against the environment (Gadissa et al., 2018). The mean of genetic diversity ($H_E$) of EST-SSR and Genomic SSR markers (Appendix Table 5) showed 0.373 and 0.775, which were on lower level and moderate level, respectively. The genetic parameter results of 21 *P. thunbergii* populations generated by EST-SSRs and genomic SSRs were different. The low genetic parameter of EST-SSRs than genomic SSRs (Appendix Table 5) met our expectation. EST-SSRs are gene (transcribed region) derived of marker, thus, to be found less polymorphic than genomic SSR (Thiel et al., 2003; Varshney et al., 2005).

A bottleneck was detected in Tsuda (Table 4.1, $p<0.05$) according to IAM (Infinite Allele Method). However, the appropriate bottleneck analysis among Wilcoxon sign rank test for microsatellite marker is TPM (Two Phase Mutaion Model) and SMM (Stepwise Mutation Model). However, TPM is considered to be the most fit model regarding to

reveal heterozygosity (Luikart et al., 1998; Piry et al., 1999). The bottleneck results (SMM and TPM) based on EST-SSR markers (Table 4.1) indicated that any bottleneck events did not occur in 21 *P. thunbergii* populations. Different results were obtained when using genomic SSR markers (Appendix Table 4). Using TPM as the reference, 13 of 21 *P. thunbergii* populations (almost all of the populations with Sea of Japan side) undergoes mutation-drift equilibrium. A bottleneck event did not always become a bad momentum for the population itself, because bottleneck usually related to demographic history of the populations to their growth process (loss and recovery) through time. Even though it is not followed by increasing the variation of the genomic, the variety and degree of allele frequencies greatly changed (Cammen et al., 2018). The possibilities of bottleneck occurring in genomic SSR markers may reflect in the selection that be advantageous for the populations to growth as genomic SSRs are rapid and high mutation rate type of DNA characteristic marker (Sorkheh et al., 2016). The bottleneck event in some populations based on genomic SSR markers may be related to linkage disequilibrium of pair loci, because LD reflects history of natural selection, genetic drift, bottleneck event, inbreeding, inversion, and gene conversion in each genome region (Slatkin, 2008). LD of genomic SSR markers showed three loci (bcpt1075, bcpt1671, and bcpt834) and linked with 1-2 loci (Appendix Table 6). These three loci may have relationship to a bottleneck which occurred in a particular population (Slatkin, 2008) as 8 *P. thunbergii* population showed occurred a bottleneck (Appendix Table 4).

LD of EST-SSR markers indicated that the loci were not linked to each other (Table 4.3), but linked with genomic SSR markers (Appendix Table 6). Such results are common within expressed sequence tag of marker, because pine genomes are complex and contain of multi-gene families (Temesgen et al., 2001). This suggested that LD between both markers may have relationship especially to dynamic of allele frequencies on Pt_ESTSSR_38962 and Pt_ESTSSR_24700. LD between both markers may hold a key role to understand adaptive genetic polymorphism which caused by heterogeneity of the environment (phenotypic plasticity). Adaptive genetic polymorphism is dynamics of alleles polymorphism which will remain stable or gradually lead to adaptive change (in allelic value or phenotypic) (Svardal et al., 2015). EST-SSR markers were exhibited three patterns (Appendix Figure 3), while genomic SSR only showed the same pattern in all of

the loci (Appendix Figure 4). Pattern no. 1 showed monomorphic on Pt_ESTSSR_36750, Pt_ESTSSR_38962, and Pt_ESTSSR_33385 according to definition of genetic polymorphisms by Cavalli-Sforza and Bodmer (1971). Pattern no. 2, Pt_ESTSSR_24700 exhibited the same results with genomic SSR loci. Pattern no. 3, Pt_ESTSSR_34853, Pt_ESTSSR_34351, and Pt_ESTSSR_27622 showed that the populations were divided into two clusters. The declined allele frequencies within Kyushu region (e.g: on locus Pt_ESTSSR_38962) may from: 1) changes in certain environment conditions (Mattei et al., 2009) or 2) genetic hitchhiking (Smith and Haigh, 1974), because loci on genes are well known as targets for selection by genetic hitchhiking (Ford, 2002).

The ratio between $D_{ST}^2 < D_{IS}^2$ and $D'_{IS}^2 < D'_{ST}^2$ are used to estimate the factor that mainly responsible for the value of LD in a population, which is due to natural selection or local differentiation (Ohta, 1982; Milankov et al., 2004). All pair loci in each marker indicated that natural selection held no responsible of allelic association. Then, the main factor is due to stochastic process such as random process, genetic drift (bottleneck is cause type of genetic drift) (Ohta, 1982a, 1982b). Neutrality test was assessed because microsatellite marker can become subject of natural selection (Hardy et al., 2003). Ewens-Watterson test for both markers showed that all loci were neutral and none of them related to some selection trait (Table 4.2). It may make a sense that neutral loci hold responsibility for genetic hitchhiking event. The genetic hitchhiking usually highly related to favourable alleles (Kim and Stephan, 2000) which have high contribute to the population (Grivet et al., 2009; Lachance et al., 2018). The genetic hitchhiking means that genes are close to each other. Its mechanism through operating (linked) with specific allele at another locus which as represent of co-inherintace network process (Perbal, 2015; Portin and Wilkins, 2017). These process can affect to degree of differentiation among populations (during recombination or separation process) and the possibility change of allele frequencies (Kumar et al., 2009).

### *4.4.2. Genetic Differentiation on Microsatellite Markers*

$R_{ST}$ is a genetic distance among populations which take account of mutation (Rousset, 1996), migration (Estoup and Angers, 1998), and the difference in number of repeat that affects allele size, which is fitted to microsatellite data (Holsinger and Weir,

2009). Phylogenetic trees were constructed by using neighbour-joining tree method based on $R_{ST}$ distance matrix of 21 *P. thunbergii* populations (Figure 4.2). Genomic SSR markers formed two clusters, but their degree of differentiation was ambiguous, whereas EST-SSR markers showed a distinguishable pattern between the clusters. The EST-SSR markers showed two clusters with high degree of differentiation between them, which we called West-South Ward cluster (Kyushu, Shikoku and Japan Sea side) and East-North Ward cluster (Pacific Ocean side and Tohoku). This result may infer that two cluster are closely linked to important genes and significantly associated with phenotypic traits of *P. thunbergii* (Lu et al., 2013; Bai et al., 2016; Ukoskit et al., 2019). In the past, the south-western and north-eastern of the Japanese archipelago were separated by mountainous terrain and acted as a wall-like constitute for the passage of plant species that disperse seed/pollen due to climatic fluctuation (Tsukada, 1983). Therefore, it is possible that the impact of past geographical structure in the Japanese archipelago was involved in the process.

The genetic structure results by EST-SSR markers indicated that the ancestral cluster in the Kyushu region has been mutated over time. On the other hand, the differences in genetic structure observed in the mainland (from Chugoku until Tohoku) were distinguishable. It is suggested that there may be a completely different ancestral population from the one presumed to be centered in the Kyushu region. The most interesting point from genetic structure by EST-SSR markers is Izumo, Miyadu, and Komatsu. The three populations showed the same pattern as East-North Ward cluster. This means that Izumo, Miyazu, and Komatsu may originate from one of the populations in the East-North Ward cluster in the past. The pattern of EST-SSR makers within Izumo, Miyadu, and Komatsu were perfectly fitted with EST-SSRs characteristic described. The genes are well conserved and recognized through times, even if they were brought from another population which located in different cluster (Varshney et al., 2005). In addition, the results observed in Izumo, Miyadu, and Komatsu distinctly proved that individuals may had been introduced anthropogenically in the past as already pointed out by Iwaizumi et al. (2018) and Mukasyaf et al. (2021a). Although genomic SSR markers had already revealed large-scale transfer in some areas, the present results also revealed seedling transfer in areas that had not been previously identified. The results of this study will

provide valuable information on the establishment process of *P. thunbergii* forests in Japan. But, misleading interpretation of genetic variability in the populations due to high informative of genomic SSRs cannot be ignored. Thus, combination of both markers was conducted as confirmation. Combination of both markers showed interesting results than in the results from each marker. Genetic structure results based on combination of both markers implied that weat-south ward and east-north ward had the possibility an adaptation process in each

### *4.4.3. Implication to Genetic Management of P. thunbergii*

Genetic management in each species depends on the species circumstances. In addition, it may have a chance to strengthen the forest resilience against the environment as well (Vinceti et al., 2020). Thus, an actual research-based strategy is considerable as a way to approach to design of genetic management (Dyke, 2008). Undoubtedly *P. thunbergii* populations throughout Japan cannot be separated from human interference aspect. As it wrote down in the history, *P. thunbergii* artificial planting or domestication activities in Japan have been conducted approximately from 1500 BP (Omura, 2004), thus it affects the genetic pattern of the populations throughout Japan (Iwaizumi et al., 2018). In this studies, those statements may seems fitted with the genetic structure results based on EST-SSR markers (Figure 4.3B), especially on the Sea of Japan side. Along with purpose to mitigate the PWN damage within the populations by using PPTs as reforestation-planting material, those become a crucial reason in order to do genetic management on *P. thunbergii* (Mukasyaf et al., 2021a). Main goal of genetic management on *P. thunbergii* populations itself is to maintain not only current genetic composition, but also the sustainability through time by applying a suitable strategy based on actual research (Mukasyaf et al., 2021a).

Genomic SSR and EST-SSR markers, which were used in this study, were developed from *P. thunbergii* as target species. Genomic SSR markers had used in previous studies and it was successfully exhibited historical seed use, clustering, and its application to genetic management of *P. thunbergii* population throughout Japan (Iwaizumi et al., 2018; Mukasyaf et al., 2021a). However, EST-SSR markers were newly developed (Mukasyaf et al., 2021b). The cluster of *P. thunbergii* populations based on genomic SSR suggested

limitation seed transfer among 4 cluster regions to prevent genetic disturbance based on adaptability to each environment. The cluster of *P. thunbergii* populations based on EST-SSR implied a demography history of *P. thunbergii* in Japan especially on Izumo, Miyadu, and Komatsu populations (see genetic structure Figure 4.3B). Thus, it suggested an act to prevent seed transfer activities between West-South Ward cluster and East-North Ward cluster further and to control seeds transfer among the populations especially in West-South Ward cluster. Those suggestions shall become a consideration to re-evaluate the existing rule or program activities regarding planting/reforestation and seed transfer of *P. thunbergii* in Japan.

### 4.5. Conclusion

In Japan, *P. thunbergii* population cannot be separated from human interference aspect. As written in the history of Japan, artificial planting and domestication of *P. thunbergii* have been conducted since 1500 BP. This species became their identity and was highly related to their socio-religion (Omura, 2004). Population genetic studies of *P. thunbergii* populations throughout Japan offered a great information for genetic management matter of those species in the future. By using both genomic SSR and EST-SSR markers, several purposes can be achieved. According to our previous study, genomic SSR markers had been used to identify seed transfer patterns, origins, and clustering based on their adaptability to the environment (Iwaizumi et al., 2018; Mukasyaf et al., 2021a). In addition, Genomic SSR markers found out that bottleneck occurred in some populations. It may be related to LD pair loci within the marker. In general, genetic diversity of EST-SSR markers were lower than that of genomic SSR markers. All loci of EST-SSR markers were neutral and not linked to each other, but linked with genomic SSR markers. Its capability to associate with another locus in genomic SSR markers (as complexity of molecular network activities) may have caused a genetic hitchhiking (Smith and Haigh, 1974) and lead to adaptive bottleneck event within the population (Grivet et al., 2009; Lachance et al., 2018). Genetic differentiation based on EST-SSR markers significantly differed from genomic SSR markers, meaning that two ancestral clusters (Sea of Japan cluster and Pacific Ocean cluster) were distinguished. Thus, the clusters based on EST-SSR markers suggest a phylogeographic history of *P. thunbergii* in Japan

and the need to prevent further seed transfer between West-South Ward cluster and East-North Ward cluster. These suggestions shall become a consideration to re-evaluate the existing rule or program activities regarding reforestation and seed transfer of *P. thunbergii* in Japan

# CHAPTER 5. GENERAL DISSCUSSION AND CONCLUSION

The main objective of this studies is to evaluate genetic diversity of *P. thunbergii* populations as forest genetic resources, to discuss about the problems in current conditions (in genetic terms) within/among populations and PPT cultivars population as well, and to examine how genetic management can be carried out. Close relationships between forests and development human society cannot be denied. In Japan, *P. thunbergii* highly related to social-religion in Japan history. Most of *P. thunbergii* forest can be found near of Shinto shrine (Omura, 2004). The forest is used an integral part of Shinto shrines (Ono, 1962). Shinto believers, who believe to the gods and the gods dwelling in many kind of places, including *P. thunbergii* tree. Additionally, the forests near the shrine are managed by the shrine and the forest management concept itself is different each other (Nilzén, 2021). Unfortunately, the mindset of many peoples still believe traditional conservation (let the nature take its course and forbidden a human intervention) is the best way to manage the forest, as is the case around the shrine. Tree planting programs usually focus solely on biodiversity at the species level. However, the fact about three levels of biodiversity such as ecosystems, species, and genetic cannot be denied either. When all levels of biodiversity are present, the ecosystem itself is balanced in all aspects (Verma, 2016). Ecosystems are based on environmental factors that encourage the emergence of genetic variation in each place, leading to adaptation and evolution through time and space. Therefore, forest genetic management must take into account to maintain variability in genetic level within the species.

Basically, gene can be divided into two types in terms of molecular genetics: neutral gene and non-neutral gene. In simple way, neutral genes are affected by stochastic processes and do not directly affect the fitness, while non-neutral genes are affected by selective and stochastic processes. (Holderegger et al., 2005; Kirk and Freeland, 2011; Rice et al., 2011). Nevertheless, the selection of appropriate genetic markers play a role for that purposes. In this studies, genomic SSR markers (neutral genetic marker) and EST-SSR markers (non-neutral genetic marker) were successfully revealed the genetic information from *P. thunbergii* populations.

This studies found an evidence regarding that the origins for young trees within local populations were different from those for the old trees based on genomic SSR markers

results (Figure 2.1A). The origins of young trees were neither from study site nor neighbour site, but a slightly far away to the Central of Kyushu. Normally, the seedlings from original site should be prioritized to be used as replanting-material or at least from neighbour site because of the adaptability to the environment site. By using the seedlings from non-origins site, it has two probabilities 1) adapt well in the site or 2) can not adapt in the site. In addition, if we use superior trees as material-plating, then it has a chance to become dominant within the given site. Genetic diversity results of PPT Kyushu cultivar suggested that how importance to manage the diversity in seed orchard as well, especially when we want to deploy PPTs into *P. thunbergii* populations. The introduction of PPTs into a given site in this matter need to consider the balance between genetic gain and genetic diversity. The matter about balancing between genetic gain and genetic diversity within seed orchard emerged since the last of nineteenth centuries. Multitude methods had been introduced as the solution such as linear by Lindgren and Matheson (1986), optimization the deployment of the parents (Lindgren et al., 2009), selective seed cone harvesting (Lindgren and El-Kassaby, 1989), or optimization seed crop genetic gain given a certain value of *Ne* while collectivelly considering parental female, male fecundity, co-ancestry among parents, inbreeding, and germination success rate (Funda, 2012). However, the methods are only applied to one given site without considering the similarities among populations.

Common seed orchard and tree improvement-seed orchard have different management. The application of genetic consideration in common seed orchard management is not complicated than in tree improvement-seed orchard. The solution for common seed orchard is collecting the seeds from origins site to preserve genetic diversity and to increase survivability of the seedlings within the population. While in tree improvement-seed orchard (if it relate to PWN), we have not only considered about genetic, but how deep to do PWN inoculation trial for seeds selection, seed-sources strategy, and design plot of the seed orchard as well. According to the results, PPT Kyushu cultivar are highly similar to Okagaki (Figure 2.2). Furthermore, genetic diversity of PPT Kyushu cultivar showed lower than that of local population (see Table 2.4 for PPT Kyushu cultivar genetic diversity; see Table 2.2 for local population genetic diversity). If we ignore this fact and attempt to plant the PPT Kyushu cultivar into local population, then genetic structure of

local population become more complex and may be close to Okagaki in the future. Thus, in order to cover the diversity within the local population, a sufficient number of seedlings of PPT Kyushu cultivar are needed as reforestation material. As remainder, this solution will only cover the local population, not all populations in Kyushu region. In addition, the similarities among the populations are not included as well. The divergence among *P. thunbergii* populations will be close to zero in the future if we are repeatedly planting PPT Kyushu cultivar in everywhere within Kyushu region in large scale. The deployment should be limited or only in prioritized populations. Then, the next questions are how deep to conduct PWN inoculation trial, what planting design to use within seed orchard, and what kind of seed-collecting method to use in order to obtain enough mother trees to produce the seedlings as planting-materials while balancing between genetic gain aspect and genetic diversity aspect. In my solution, I prefer to establish a provenant trial design-like seed orchard even though it will labour and might need some time and space. However, it will cover all populations for a longterms. Summary of the elements of consideration in genetic management and the outcome for the target population are showed in Table 5.1.

**Table 5.1.** Elements of consideration in genetic management of *P. thunbergii* forests

| No | Element of Consideration | Difficulty Level | Note | Outcome |
|---|---|---|---|---|
| 1 | Genetic Diversity | Standard | ➢ Only considering genetic diversity (*Ho*).<br>➢ The seedling from different origins or area will allowed to be planted.<br>➢ It has a possibility to lead a change in genetic composition, genetic structure, and the similarities among the populations. | Greatly change |
| 2 | Genetic Structure | Slightly High | ➢ Considering genetic diversity (*Ho*) and genetic structure.<br>➢ The seedlings from different origins or area still allowed<br>➢ It has a possibility to lead a change in genetic composition within the population and the similarity among the populations | Rather change |
| 3 | Similarities among populations | Rather High | ➢ Elements no. 1-3 are considered as important point.<br>➢ The seedlings from target population or neighbour population are prioritized over non-neighbour population. | Slightly change |
| 4 | Local genetics potential | High | ➢ All elements (1-4) are considered as important point. | Close to previous condition |

Although the genetic structure of some populations based on genomic SSR markers (Figure 4.3A) were undefinable, the EST-SSR marker results distinctly showed distinguished pattern among populations. Afterall, *P. thunbergii* populations were divided into two clusters. The results based on EST-SSR markers really were unexpected in this studies, but it was no surprising because west-south ward and east-north ward are separated by mountains, which act as barrier for the plants migration through pollen-dispersal (Tsukada, 1983). The differences between two clusters had proved with degree of differentiation between both clusters (Figure 4.2). It was consideberaly high value of genetic distance, ranging from 0.28-0.30, but the phenotypic differences between both major clusters were still uncertain yet and might need further studies. The similar results were showed by Kimura et al. (2014) in *Cryptomeria japonica* as well. In my opinions, the Sea of Japan side may be a place with more shrines and once had became the center of civilization in Japan than the Pacific Ocean side in the past. The relationship with the feudal lords as well may also play a major key in *P. thunbergii* distribution among populations. The rationale for such ideas is; 1) The EST-SSRs genetic structure results in three populations (Izumo, Miyadu, and Komatsu) within Sea of Japan side (Figure 4.5B), were exhibited different genetic structure than other populations in the neighbour area. Furthermore, the oldest Shinto shrine is located on Izumo, even more than Ise Grand Shrine. 2) Niigata population, which is close to Northern Japan, had the same genetic structure as the populations in Honshu and Shikoku areas. 3) It is possible that Kyoto, which was capital city of Japan at the past, was part of “silk road” of *P. thunbergii* distribution connecting between Pacific Ocean side and Sea of Japan side. This is suggested by the genetic structure of Mihama, Owase, and Miyadu populations. The genetic structure in those three populations based on genomic SSR markers (Figure 4.3A) were dominated by the same pattern, yet actually Mihama had different origins based on EST-SSR markers results (see Figure 4.3B). EST-SSRs results for Miyadu and Owase showed the same pattern (Figure 4.3B).

It may be a wrong choice to condemn the history for all of this outcome. Even if my studies are right, there is a need to accept the movement of seeds and seedlings as a result of the local wisdom of the time. The important thing is from now on what we should do as forest managers in response to this result for the future. In this studies, the combination of

two molecular markers (genomic SSRs and EST SSRs) implied an interesting results. *P. thunbergii* populations based on genomic SSR markers seems to be divided into four clusters, while EST-SSR markers implied two major clusters. The genetic structure results of *P. thunbergii* populations based on combination of both markers implied two clusters and a possibility that an adaptation process occurred in each cluster. Although its adaptation process has not been proven yet, results from my studies suggest that the populations of *P. thunbergii* throughout Japan can be classified into four basic clusters based on the two clusters (Figure 4.5). These clusters are thought to be the result of evolution to the local climate over generations.

In order to use PPTs as reforestation materials in the region, it is necessary to prepare a sufficient number of PPTs mother trees in the seed orchard and a sufficient number of seedlings to deploy. In some *P. thunbergii* populations, the seedlings have been introduced from relatively close area. As a results, most of the remaining *P. thunbergii* forests today reflect the unique genetic structure. If the genetic diversity of PPT cultivar from original site does not cover current genetic diversity in target area, seedlings from distant area could be introduced if seed transfer zones match. Otherwise, it may disturb the genetic structure specific to that region. This study showed that the historical background of afforestation in Japan needs to be taken into account for the maintenance of the genetic resources of *P. thunbergii*. In addition, it is necessary to re-evaluate the existing regulations and planting program for *P. thunbergii* afforestation including seed transfer policy in Japan. These results will contribute to the maintenance of genetic resources of *P. thunbergii*.

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# APPENDIX

**Appendix Table 1.** 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 |

**Appendix Table 2.** List of PPTs based on the region (FFPRI, 2019).

| No | PWN-*P. thunbergii* 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 |

| | | | |
|---|---|---|---|
| 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 | |

**Appendix Table 3.** Gene flow (*Nm*) of Kyushu PWN-*P. thunbergii* resistant trees with the populations within Kyushu area.

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

**Appendix Table 4.** Genetic diversity parameter of 21 *P. thunbergii* population using 6 genomic SSR markers

| No | Pop | $N_a$ | $N_e$ | $H_O$ | $H_E$ | $F_{IS}$ | *p*-Wicoxon Sign Rank Test | | | % polymorphism loci |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | IAM | SMM | TPM | |
| 1 | Karatsu | 8.000 | 4.559 | 0.708 | 0.772 | 0.079 | 0.044 | 0.189 | 0.185 | 83.3 |
| 2 | Kitsuki | 10.000 | 5.081 | 0.698 | 0.778 | 0.103 | 0.539 | 0.043 | 0.042 | 66.7 |
| 3 | Miyazaki | 10.000 | 5.051 | 0.695 | 0.761 | 0.081 | 0.544 | 0.193 | 0.052 | 66.7 |
| 4 | Hikari | 10.833 | 5.747 | 0.682 | 0.810 | 0.150 | 0.238 | 0.050 | 0.048 | 100.0 |
| 5 | Kochi | 10.333 | 5.565 | 0.656 | 0.783 | 0.156 | 0.046 | 0.047 | 0.043 | 33.3 |
| 6 | Tsuda | 11.167 | 6.334 | 0.706 | 0.809 | 0.125 | 0.236 | 0.045 | 0.052 | 100.0 |
| 7 | Mihama | 12.500 | 6.368 | 0.677 | 0.811 | 0.163 | 0.549 | 0.047 | 0.185 | 100.0 |
| 8 | Owase | 10.500 | 5.420 | 0.619 | 0.770 | 0.197 | 0.546 | 0.049 | 0.045 | 33.3 |
| 9 | Miho | 9.500 | 4.532 | 0.679 | 0.754 | 0.097 | 0.537 | 0.043 | 0.047 | 83.3 |
| 10 | Oki | 8.333 | 4.906 | 0.687 | 0.778 | 0.111 | 0.234 | 0.473 | 0.524 | 83.3 |
| 11 | Izumo | 9.833 | 5.374 | 0.729 | 0.800 | 0.087 | 0.051 | 0.049 | 0.196 | 100.0 |
| 12 | Tottori | 9.667 | 5.680 | 0.691 | 0.799 | 0.132 | 0.047 | 0.199 | 0.199 | 100.0 |
| 13 | Hamasaka | 10.333 | 5.783 | 0.655 | 0.802 | 0.177 | 0.242 | 0.045 | 0.509 | 83.3 |
| 14 | Miyadu | 10.000 | 5.265 | 0.648 | 0.783 | 0.164 | 0.047 | 0.181 | 0.198 | 100.0 |
| 15 | Kaga | 9.500 | 4.679 | 0.561 | 0.770 | 0.274 | 0.552 | 0.045 | 0.045 | 50.0 |
| 16 | Niigata | 9.500 | 5.237 | 0.708 | 0.784 | 0.097 | 0.050 | 0.050 | 0.192 | 100.0 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 17 | Toyama | 8.833 | 4.953 | 0.683 | 0.777 | 0.117 | 0.044 | 0.198 | 0.188 | 83.3 |
| 18 | Komatsu | 10.333 | 4.410 | 0.617 | 0.747 | 0.178 | 0.528 | 0.005 | 0.005 | 100.0 |
| 19 | Juo | 8.167 | 4.733 | 0.667 | 0.757 | 0.121 | 0.046 | 0.175 | 0.196 | 50.0 |
| 20 | Noshiro | 9.000 | 4.418 | 0.688 | 0.734 | 0.059 | 0.555 | 0.209 | 0.192 | 66.7 |
| 21 | Naruse | 8.333 | 3.794 | 0.590 | 0.702 | 0.159 | 0.545 | 0.047 | 0.047 | 100.0 |
| | Mean | 9.746 | 5.138 | 0.669 | 0.775 | 0.135 | 0.296 | 0.113 | 0.152 | 80.2 |

$N_a$ : number of allele, $N_e$ : number of effective allele, $H_O$ : observed heterozygosity, $H_E$ : expected heterozygosity, $F_{IS}$ : inbreeding coefficient within the population, Wilcoxon Sign Rank Test : IAM (Infinite Allele Model), SMM (Stepwise Mutation Model, and TPM (Two Phase Mutation Model)

**Appendix Table 5.** Comparison genetic diversity parameter of 21 *P. thunbergii* populations between genomic SSR and EST-SSR

| | Locus | $N_a$ | $N_e$ | $H_O$ | $H_E$ | $F_{IS}$ | PIC | HWE |
|---|---|---|---|---|---|---|---|---|
| G-SSR | bcpt1075 | 9.19 | 4.84 | 0.715 | 0.778 | 0.08 | 0.798 | *** |
| | bcpt1671 | 10.81 | 6.51 | 0.812 | 0.841 | 0.035 | 0.87 | *** |
| | bcpt834 | 7.67 | 3.45 | 0.642 | 0.699 | 0.077 | 0.725 | *** |
| | bcpt1823 | 9.14 | 5.16 | 0.56 | 0.793 | 0.294 | 0.835 | *** |
| | bcpt2532 | 14.48 | 7.56 | 0.683 | 0.86 | 0.207 | 0.909 | *** |
| | bcpt1549 | 7.19 | 3.31 | 0.602 | 0.682 | 0.115 | 0.698 | *** |
| | Mean | 9.74666667 | 5.1383333 | 0.669 | 0.7755 | 0.1346667 | 0.8058333 | |
| EST-SSR | Pt_ESTSSR_36750 | 1.95 | 1.05 | 0.041 | 0.048 | 0.126 | 0.041 | ND |
| | Pt_ESTSSR_38962 | 2.43 | 1.34 | 0.188 | 0.202 | 0.105 | 0.192 | *** |
| | Pt_ESTSSR_33385 | 1.05 | 1 | 0.001 | 0.001 | -0.016 | 0.002 | ND |
| | Pt_ESTSSR_34351 | 5.29 | 2.55 | 0.588 | 0.597 | 0.017 | 0.589 | *** |
| | Pt_ESTSSR_27622 | 4.33 | 2.63 | 0.541 | 0.581 | 0.084 | 0.541 | *** |
| | Pt_ESTSSR_24700 | 5.52 | 3.47 | 0.38 | 0.694 | 0.46 | 0.385 | *** |
| | Pt_ESTSSR_34853 | 4.91 | 2.52 | 0.456 | 0.493 | 0.06 | 0.455 | *** |
| | Mean | 3.64 | 2.08 | 0.313571 | 0.373714 | 0.119429 | 0.315 | |

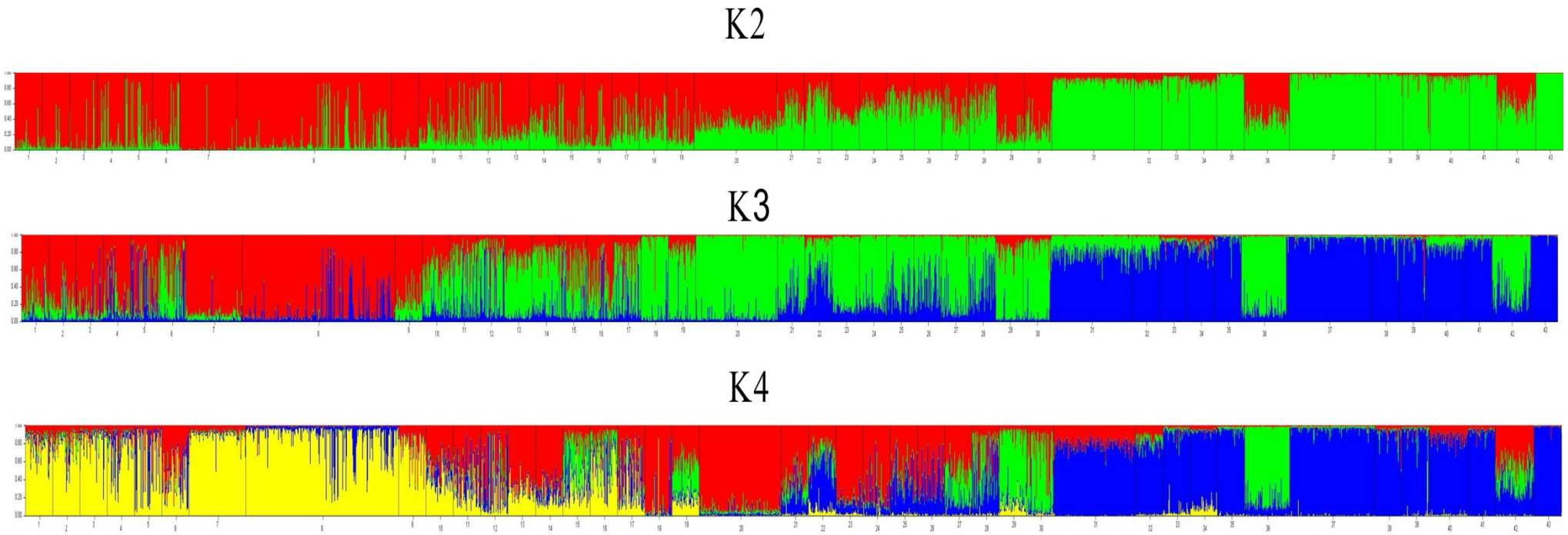
$N_a$ : number of allele, $N_e$ : number of effective allele, $H_O$ : observed heterozygosity, $H_E$ : expected heterozygosity, $F_{IS}$ : inbreeding coefficient within the population, HWE : Hardy-Weinberg equilibrium (nd= not defined, ns = not significant, *$p$<0.05, **$p$<0.01, ***$p$<0.001)

**Appendix Table 6.** Linkage disequilibrium between genomic SSR and EST-SSR marker of 21 *P. thunbergii* populations

| Locus A | Locus B | $D_{IT}^2$ | $D_{IS}^2$ | $D'_{IS}^2$ | $D_{ST}^2$ | $D'_{ST}^2$ | *p*-LD |
|---|---|---|---|---|---|---|---|
| bcpt1075 | bcpt1671 | 0.0198 | 0.0063 | 0.0193 | 0.0135 | 0.0005 | ns |
| | bcpt834 | 0.0283 | 0.0050 | 0.0274 | 0.0227 | 0.0009 | * |
| | bcpt1823 | 0.0271 | 0.0078 | 0.0263 | 0.0194 | 0.0008 | * |
| | bcpt2532 | 0.0246 | 0.0080 | 0.0236 | 0.0163 | 0.0010 | * |
| | bcpt1549 | 0.0278 | 0.0049 | 0.0270 | 0.0223 | 0.0007 | ns |
| | Pt_ESTSSR_36750 | 0.0412 | 0.0004 | 0.0411 | 0.0408 | 0.0000 | ns |
| | Pt_ESTSSR_38962 | 0.0408 | 0.0016 | 0.0405 | 0.0405 | 0.0003 | ns |
| | Pt_ESTSSR_33385 | 0.0413 | 0.0000 | 0.0413 | 0.4130 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.0575 | 0.0037 | 0.0565 | 0.0538 | 0.0010 | ns |
| | Pt_ESTSSR_27622 | 0.0650 | 0.0050 | 0.0641 | 0.0603 | 0.0008 | ns |
| | Pt_ESTSSR_24700 | 0.0286 | 0.0076 | 0.0281 | 0.0214 | 0.0005 | ns |
| | Pt_ESTSSR_34853 | 0.0643 | 0.0042 | 0.0629 | 0.0592 | 0.0014 | ns |
| bcpt1671 | bcpt834 | 0.0259 | 0.0058 | 0.0250 | 0.0192 | 0.0008 | ** |
| | bcpt1823 | 0.0273 | 0.0073 | 0.0228 | 0.0157 | 0.0009 | ns |
| | bcpt2532 | 0.0201 | 0.0075 | 0.0195 | 0.0128 | 0.0006 | ns |
| | bcpt1549 | 0.0251 | 0.0058 | 0.0242 | 0.0189 | 0.0008 | ** |
| | Pt_ESTSSR_36750 | 0.0390 | 0.0004 | 0.0390 | 0.0387 | 0.0000 | ns |
| | Pt_ESTSSR_38962 | 0.0370 | 0.0014 | 0.0365 | 0.0353 | 0.0004 | ns |
| | Pt_ESTSSR_33385 | 0.0406 | 0.0000 | 0.0406 | 0.0408 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.0437 | 0.0043 | 0.0426 | 0.0397 | 0.0010 | ns |
| | Pt_ESTSSR_27622 | 0.0478 | 0.0043 | 0.0467 | 0.0438 | 0.0010 | ns |
| | Pt_ESTSSR_24700 | 0.0271 | 0.0087 | 0.0262 | 0.0182 | 0.0008 | * |
| | Pt_ESTSSR_34853 | 0.0521 | 0.0035 | 0.0507 | 0.0476 | 0.0014 | ns |
| bcpt834 | bcpt1823 | 0.0361 | 0.0076 | 0.0346 | 0.0267 | 0.0015 | ns |
| | bcpt2532 | 0.0294 | 0.0071 | 0.0281 | 0.0221 | 0.0013 | ** |
| | bcpt1549 | 0.0379 | 0.0058 | 0.0370 | 0.0302 | 0.0009 | ns |
| | Pt_ESTSSR_36750 | 0.0555 | 0.0004 | 0.0555 | 0.0560 | 0.0000 | ns |
| | Pt_ESTSSR_38962 | 0.0554 | 0.0010 | 0.0544 | 0.0552 | 0.0010 | ** |
| | Pt_ESTSSR_33385 | 0.0594 | 0.0000 | 0.0593 | 0.0592 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.0756 | 0.0042 | 0.0720 | 0.0724 | 0.0035 | ns |
| | Pt_ESTSSR_27622 | 0.0838 | 0.0048 | 0.0803 | 0.0805 | 0.0034 | ns |
| | Pt_ESTSSR_24700 | 0.0380 | 0.0073 | 0.0370 | 0.0295 | 0.0010 | ns |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pt_ESTSSR_34853 | 0.0890 | 0.0032 | 0.0844 | 0.0844 | 0.0045 | ns |
| bcpt1823 | bcpt2532 | 0.0278 | 0.0095 | 0.0261 | 0.0174 | 0.0016 | ns |
| | bcpt1549 | 0.0331 | 0.0080 | 0.0322 | 0.0259 | 0.0008 | ns |
| | Pt__ESTSSR_36750 | 0.0563 | 0.0004 | 0.0563 | 0.0562 | 0.0000 | ns |
| | Pt_ESTSSR_38962 | 0.0540 | 0.0015 | 0.0537 | 0.0529 | 0.0002 | ns |
| | Pt_ESTSSR_33385 | 0.0585 | 0.0000 | 0.0584 | 0.0585 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.0609 | 0.0059 | 0.0585 | 0.0536 | 0.0024 | ns |
| | Pt_ESTSSR_27622 | 0.0660 | 0.0058 | 0.0641 | 0.0599 | 0.0018 | ns |
| | Pt_ESTSSR_24700 | 0.0332 | 0.0104 | 0.0323 | 0.0245 | 0.0009 | ns |
| | Pt_ESTSSR_34853 | 0.0645 | 0.0045 | 0.0621 | 0.0589 | 0.0024 | ns |
| bcpt2532 | bcpt1549 | 0.0291 | 0.0079 | 0.0285 | 0.0217 | 0.0006 | ns |
| | Pt_ESTSSR_36750 | 0.0526 | 0.0004 | 0.0526 | 0.0519 | 0.0000 | ns |
| | Pt_ESTSSR_38962 | 0.0494 | 0.0018 | 0.0489 | 0.0481 | 0.0005 | ns |
| | Pt_ESTSSR_33385 | 0.0556 | 0.0000 | 0.0556 | 0.0556 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.0448 | 0.0053 | 0.0433 | 0.0401 | 0.0014 | ns |
| | Pt_ESTSSR_27622 | 0.0498 | 0.0067 | 0.0485 | 0.0429 | 0.0012 | ns |
| | Pt_ESTSSR_24700 | 0.0309 | 0.0096 | 0.0298 | 0.0219 | 0.0009 | ns |
| | Pt_ESTSSR_34853 | 0.0519 | 0.0049 | 0.0499 | 0.0470 | 0.0020 | ns |
| bcpt1549 | Pt_ESTSSR_36750 | 0.0473 | 0.0004 | 0.0472 | 0.0459 | 0.0000 | ns |
| | Pt_ESTSSR_38962 | 0.0496 | 0.0009 | 0.0495 | 0.0475 | 0.0001 | ns |
| | Pt_ESTSSR_33385 | 0.0483 | 0.0000 | 0.0483 | 0.0482 | 0.0000 | ns |
| | Pt_ESTSSR_34351 | 0.0776 | 0.0032 | 0.0767 | 0.0734 | 0.0008 | ns |
| | Pt_ESTSSR_27622 | 0.0850 | 0.0041 | 0.0840 | 0.0808 | 0.0010 | ns |
| | Pt_ESTSSR_24700 | 0.0346 | 0.0068 | 0.0340 | 0.0285 | 0.0005 | ns |
| | Pt_ESTSSR_34853 | 0.0882 | 0.0049 | 0.0837 | 0.0847 | 0.0009 | ns |

$D'_{ST}{}^2$ : variance of disequilibrium of total population; $D_{ST}{}^2$ : variance of correlation of the gene of two loci of different gametes within a population to total population; $D'_{IS}{}^2$ : variance of correlation of two loci of one gamete in a population; $D_{IS}{}^2$ : expected variance of linkage disequilibrium within a population; $D_{IT}{}^2$ : total variance of disequilibrium of total population; *p*-LD : p value of linkage disequilibrium of two loci (ns = not significant, *P<0.05, **P<0.01, ***P<0.001)




**Appendix Figure 1.** 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**)).

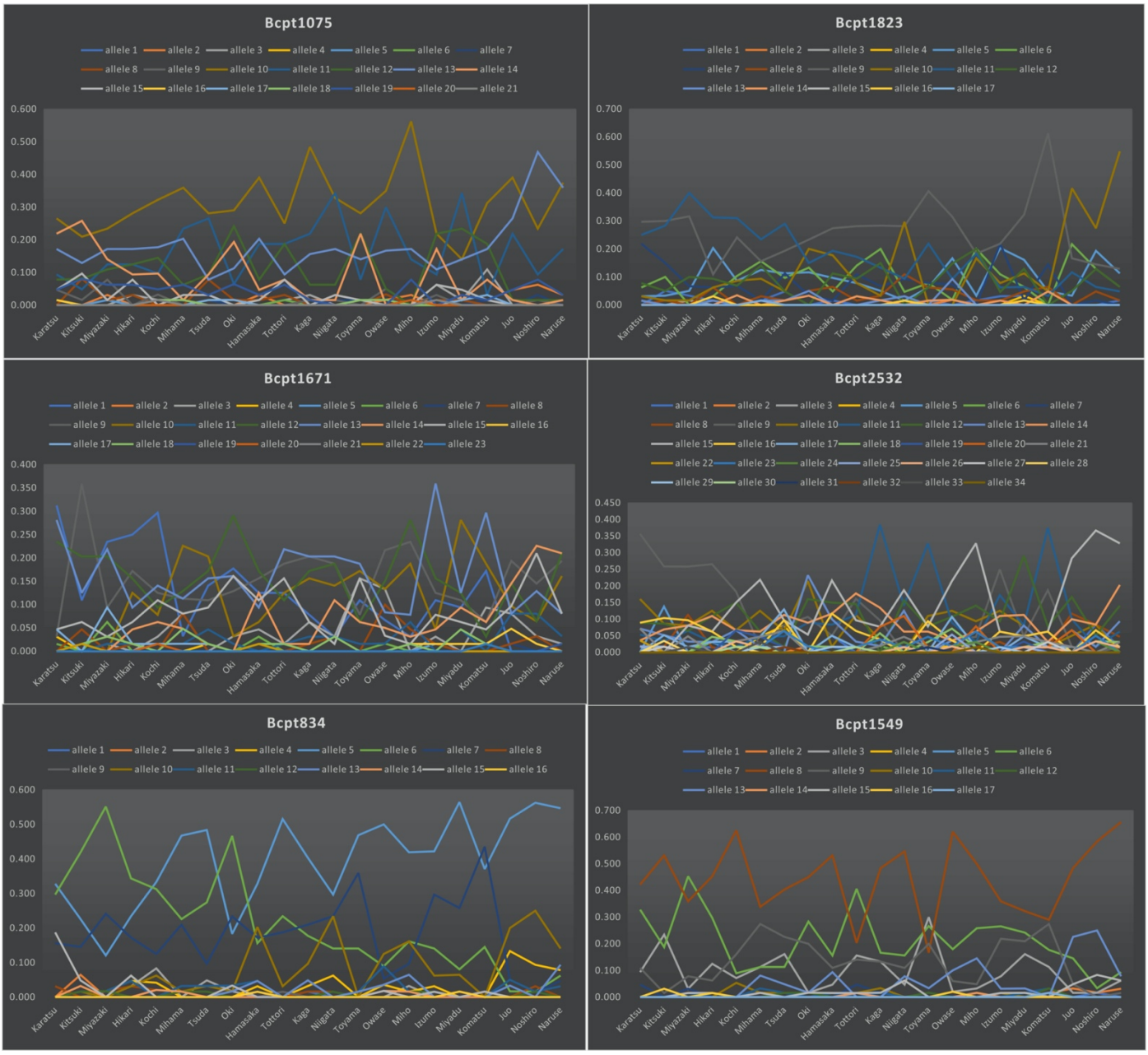

1–30 cm DBH range


31–60 cm DBH range


61–90 cm DBH range

**Appendix Figure 2.** Spatial distribution of Iki-no-Matsubara genetic structure per DBH range (**A**) West side, (**B**) Central side, and (**C**) East side.


**Appendix Figure 3**. Dynamics of allele frequencies 21 *P. thunbergii* populations by using EST-SSR markers. (From South-West (**Left**) to North-East (**Right**)).




**Appendix Figure 4**. Dynamics of allele frequencies 21 *P. thunbergii* populations by using 6 genomic SSR. (From South-West (**Left**) to North-East (**Right**)).