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Biogeographic patterns of genetic diversity of *Pinus thunbergii* populations across Japan

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ABSTRACT: This study examines the genetic diversity of Pinus thunbergii populations across various biogeographic regions in Japan, with a focus on the influence of geographic and environmental factors on genetic variation. Using seven EST-SSR markers, the objective was to analyze the influence of geographic factors, gene flow, and historical events on population structure. The research also compared the utility of EST-SSR markers with six genomic SSR (G-SSR) markers, revealing significant differences between them. EST-SSR markers showed lower genetic diversity compared to G-SSR, which could indicate higher inbreeding or reduced gene flow due to their location within genes. Differences in allele numbers, observed and expected heterozygosity, and inbreeding coefficients indicated substantial genetic variations among the studied *P. thunbergii* populations. Additionally, the comparison between marker types reinforces the importance of methodological choice in genetic diversity assessments. This research provides valuable insights into the genetic diversity patterns of *P. thunbergii* populations, highlighting the crucial role of markers and geographic regions in understanding the dynamics of genetic variation, with important implications for biogeography and species conservation.
Keywords: biogeography; EST-SSR marker; genetic diversity.

Padrões biogeográficos da diversidade genética: um estudo de caso das populações de *Pinus thunbergii* no Japão

RESUMO: Este estudo investiga a diversidade genética das populações de *Pinus thunbergii* em diferentes regiões biogeográficas do Japão, focando no impacto dos fatores geográficos e ambientais sobre a variação genética. Utilizando sete marcadores EST-SSR, o objetivo foi analisar a influência de fatores geográficos, fluxo gênico e eventos históricos na estrutura genética das populações. A pesquisa também comparou a utilidade dos marcadores EST-SSR com seis marcadores genômicos SSR (G-SSR), revelando diferenças significativas entre eles. Os marcadores EST-SSR apresentaram menor diversidade genética em relação aos G-SSR, o que pode indicar maior endogamia ou menor fluxo gênico devido à sua localização dentro de genes. Diferenças no número de alelos, heterozigosidade observada e esperada, bem como no coeficiente de endogamia, indicaram variações genéticas substanciais entre as populações de *P. thunbergii* estudadas. Além disso, a comparação entre os tipos de marcadores reforça a importância da escolha metodológica nas avaliações de diversidade genética. Esta pesquisa oferece insights valiosos sobre os padrões de diversidade genética das populações de *P. thunbergii*, destacando o papel crucial dos marcadores e das regiões geográficas para entender as dinâmicas de variação genética, com implicações importantes para a biogeografia e a conservação da espécie.
Palavras-chave: biogeografia; marcador EST-SSR; diversidade genética.

1. INTRODUCTION

In biogeography studies, the spatial distribution of species across various geographical scales is of paramount importance. Biogeography examines how species, ecosystems, and biodiversity are influenced by environmental factors such as geography, climate, topography, and human activities (Torres-Romero; Olalla-Tárraga, 2015), as well as by historical events like migration and isolation. Genetic diversity, as one aspect of biodiversity, plays a crucial role in the resilience and adaptability of species to shifting ecological conditions (LOMONILO, 2020).

Within biogeography, genetic diversity is influenced by biological factors and geographic elements, which contribute to population structure and species distribution across landscapes. Geographic barriers, such as mountains, rivers, and climate zones, can create isolated populations, leading to genetic differentiation through allopatric speciation and genetic drift. This makes biogeography essential in understanding how species evolve and adapt across different regions (MAIGRET et al., 2020).

By examining genetic diversity in the context of biogeographical divisions, researchers can uncover patterns of species dispersal, adaptation, and the influence of geographic isolation on genetic variation. Understanding these patterns is critical for biodiversity conservation,

particularly in the face of environmental changes. Genetic variation manifests genetic diversity at the individual level, which cannot be seen through a morphological view (PECK et al., 2015). Preserving genetic diversity within species becomes paramount, as it is essential for the long-term health and survival of ecosystems and endangered species (DEWOODY et al., 2021). Nevertheless, these interdependencies have been extensively studied in the fields of conservation genetics and ecological genomics, shedding light on how genetic variation influences the intricate web of life on our planet and guiding efforts to protect (such as molecular mechanism related to the disease: Sutrisna; Aisyah, 2011; Sidiq et al., 2020) and sustain it.

Every population exhibits varying levels of genetic variation within its gene pool, including allele distribution, allele frequency, allele count, genetic diversity, genetic structure, and genetic differentiation, among others (SUSANTO; MASHUDI, 2018). Different alleles at each locus across generations within or among populations represent their level of genetic polymorphism (FOROUD; KOLLER, 2008). It contributes to genetic diversity (NWOSISI et al., 2019). In essence, the extent and utility of genetic variation impact population genetics. Hence, comprehending allelic differences and adaptability constitutes a fundamental aspect of population genetics (BAMBA et al., 2019). The historical approach in population genetic research has involved the observation of phenotypes through field experiments. Nevertheless, field experiments are labor-intensive and costly, provide estimations for only measurable characteristics, and necessitate a focus on individual subjects (CHEN, 2015).

Utilizing molecular markers offers insights into genetic variation by assessing DNA variations in non-coding and coding regions (Port and El-Kassaby, 2014). In contemporary research, examining spatial genetic variation among populations has emerged as a valuable tool for comprehending the biodiversity dynamics at the genetic level within species (Berens et al., 2014). The nucleus, mitochondria, chloroplasts (plastids), and other subcellular organelles house genetic material, which are interconnected (KRUPINSKA et al., 2020). In this instance, molecular networks alone can signify a species' adaptation, evolution, and mutation within a population. However, carefully selecting a molecular marker can enhance our understanding of these processes (GRÜNWALD; GOSS, 2011). By examining genetic markers within species populations across distinct geographic regions, we can understand how geographic features such as mountain ranges, rivers, or climate gradients affect species' evolution and diversity.

Among molecular markers, EST-SSRs (Expressed Sequence Tag - Simple Sequence Repeats) are commonly used to gain insights into gene expression patterns (SAHU et al., 2012). Consequently, EST-SSR markers have found extensive use due to their ease of transferability to other species and their relevance in phylogenetic and population genetics studies (MEYER et al., 2017; FAN et al., 2019; LI et al., 2020). Indeed, EST-SSR markers can also offer valuable insights into adaptive and evolutionary genetic studies. Their ability to reflect gene expression patterns and their transferability to various species make them versatile for investigating genetic adaptations and evolutionary processes (JIANG et al., 2020). EST-SSRs are often well-conserved and encompass common gene components. As a result, they can efficiently aid in identifying diverse genetic parents or individuals within a population (PARTHIBAN et al., 2018).

This study aims to investigate the biogeographical patterns of genetic diversity within species distributed across various regions, focusing on how geographic and environmental factors influence this variation. Through molecular markers and genetic analysis, this research aims to map the genetic diversity of species in relation to their geographical distribution and assess the implications for conservation strategies across diverse ecological zones.

2. MATERIAL AND METHODS

In this study, 672 samples were collected and used for analysis from 21 different *P. thunbergii* populations (Figure 1).

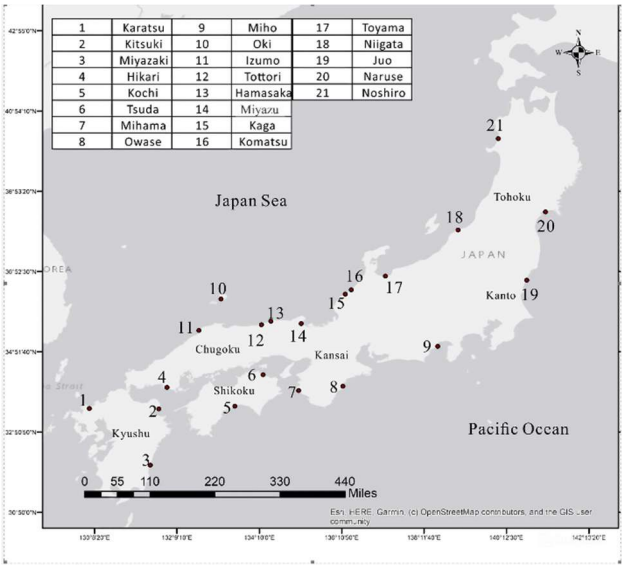

Figure 1. Location of 21 *P. thunbergii* populations (Source: Mukasyaf, 2024).

Figura 1. Localização de 21 populações de P. thunbergii (Fonte: Mukasyaf, 2024).

In this study, DNA analysis was carried out using seven specific 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. The DNA genotyping procedures for these EST-SSR markers followed the protocols outlined in previous studies (Mukasyaf et al., 2021b) conducted by the research team.

Data from the six EST-SSR markers were analyzed to assess genetic diversity and population structure. Additionally, data from six genomic SSR markers (bcpt1075, bcpt1671, bcpt834, bcpt1823, bcpt2532, and bcpt1549) were included for comparison within the 21 *P. thunbergii* populations (MUKASYAF et al., 2021a).

Various genetic parameters were calculated using GenAlEx version 6.503, which included the number of alleles (Na), the number of effective alleles (Ne), observed heterozygosity (HO), expected heterozygosity (H_E), and the inbreeding coefficient within populations (F_{IS}). These analyses aimed to provide insights into the genetic makeup and diversity of the *P. thunbergii* populations under investigation, focusing on the utility of the EST-SSR markers compared to six genomic SSR markers by Mukasyaf et al. (2021a).

The polymorphism percentage of the loci within the population and the Analysis of Molecular Variance (AMOVA) were evaluated using Arlequin version 3.5.2.2. This software is commonly used for population genetic analysis to assess genetic variation and the partitioning of genetic diversity within and among populations. Both analyses are valuable in understanding the genetic structure and relationships of populations in evolutionary biology, conservation genetics, and population genetics studies. Bottleneck version 1.2.02 was employed to assess the possibility of a bottleneck event in the population. This software is designed to detect changes in the effective population size over time, which can indicate historical events where populations may have experienced a significant size reduction, known as a population bottleneck.

3. RESULTS

3.1. Genetic Parameters

Table 1 presents a set of genetic diversity parameters for 21 populations, with data on observed (G-SSR) and estimated (EST-SSR) values for several key metrics: population size (Pop), number of alleles (Na), the effective number of alleles (Ne), observed heterozygosity (HO), expected heterozygosity (HE), and the inbreeding coefficient (FIS).Regarding the number of alleles (Na), populations like Miyadu, Owase, and Izumo show relatively higher values, with Owase reaching a peak of 4.429 in the G-SSR data. In contrast, the EST-SSR values generally remain lower, indicating possible discrepancies in allele richness under observed versus estimated conditions. The effective number of alleles (Ne) ranges from 3.794 to 6.368, suggesting moderate genetic diversity, with Mihama showing the highest value, implying that this population may have a more diverse gene pool than others like Karatsu or Naruse.

The observed heterozygosity (HO) values are relatively consistent across populations, with most values ranging between 0.56 and 0.73, indicating moderate levels of genetic variation within populations. However, the estimated heterozygosity (HE) values are generally lower than HO, suggesting a potential deficit in heterozygotes, possibly due to inbreeding or sampling bias. This is further highlighted by the inbreeding coefficient (FIS), where positive values indicate inbreeding. Populations like Kochi and Mihama show relatively higher FIS values (0.243 and 0.125), which may reflect higher levels of inbreeding in these groups. Conversely, negative FIS values, such as in Kitsuki (-0.025) and Tsuda (-0.031), suggest an excess of heterozygotes in these populations, possibly due to outcrossing or other factors promoting genetic diversity.

Overall, the 21 populations exhibit moderate levels of genetic diversity, with some variation in allele numbers and heterozygosity and varying degrees of inbreeding.

Table 1. Genetic parameters of 21 *P. thunbergii* populations using 7 EST-SSR markers.
Tabela 1. Parâmetros genéticos de 21 populações de *P. thunbergii* usando 7 marcadores EST-SSR.

No	Pop	*Na*		*Ne*		H_O		H_E		F_{IS}	
		G-SSR	EST-SSR	G-SSR	EST-SSR	G-SSR	EST-SSR	G-SSR	EST-SSR	G-SSR	EST-SSR
1	Karatsu	8.000	3.143	4.559	1.842	0.708	0.356	0.772	0.360	0.079	0.016
2	Kitsuki	10.000	3.857	5.081	2.393	0.698	0.463	0.778	0.458	0.103	-0.025
3	Miyazaki	10.000	3.286	5.051	2.205	0.695	0.362	0.761	0.402	0.081	0.046
4	Hikari	10.833	3.286	5.747	2.119	0.682	0.364	0.810	0.410	0.150	0.088
5	Kochi	10.333	3.286	5.565	1.923	0.656	0.251	0.783	0.352	0.156	0.243
6	Tsuda	11.167	3.429	6.334	2.057	0.706	0.369	0.809	0.364	0.125	-0.031
7	Mihama	12.500	3.000	6.368	1.863	0.677	0.281	0.811	0.337	0.163	0.125
8	Owase	10.500	4.429	5.420	2.364	0.619	0.289	0.770	0.378	0.197	0.177
9	Miho	9.500	3.714	4.532	2.089	0.679	0.257	0.754	0.358	0.097	0.405
10	Oki	8.333	3.429	4.906	1.883	0.687	0.272	0.778	0.362	0.111	0.174
11	Izumo	9.833	4.143	5.374	2.438	0.729	0.281	0.800	0.373	0.087	0.172
12	Tottori	9.667	3.429	5.680	1.990	0.691	0.292	0.799	0.373	0.132	0.237
13	Hamasaka	10.333	3.000	5.783	1.821	0.655	0.263	0.802	0.328	0.177	0.123
14	Miyadu	10.000	5.286	5.265	2.351	0.648	0.323	0.783	0.412	0.164	0.251
15	Kaga	9.500	3.286	4.679	2.090	0.561	0.358	0.770	0.385	0.274	0.051
16	Komatsu	10.333	4.286	4.410	2.461	0.617	0.288	0.747	0.406	0.178	0.398
17	Toyama	8.833	3.286	4.953	2.005	0.683	0.330	0.777	0.376	0.117	0.046
18	Niigata	9.500	3.000	5.237	1.865	0.708	0.347	0.784	0.367	0.097	0.054
19	Juo	8.167	3.857	4.733	1.706	0.667	0.231	0.757	0.331	0.121	0.283
20	Noshiro	9.000	4.000	4.418	2.313	0.688	0.304	0.734	0.368	0.059	0.140
21	Naruse	8.333	4.000	3.794	1.927	0.590	0.304	0.702	0.353	0.159	0.063
	Mean	9.746	3.639	5.138	2.081	0.669	0.314	0.775	0.374	0.135	0.145

Note: Na: number of alleles; Ne: number of effective alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient within the population.
Nota: Na: número de alelos; Ne: número de alelos efetivos; HO: heterozigosidade observada; HE: heterozigosidade esperada; FIS: coeficiente de endogamia dentro da população.

3.2. Analysis of Molecular Variance (AMOVA)

Table 2 shows the results of a molecular analysis of variance (AMOVA), which partitions genetic variation into different hierarchical levels. The analysis reveals that there are three sources of variation: among groups, among populations within groups, and among populations. First, the variation among groups accounts for 8.15% of the total genetic variation, with a sum of squares of 140.277 and a variance component of 0.09705. The P-value for this variation is less than 0.05, indicating that the differences among the groups are statistically significant. Next, the variation among populations within groups explains 20.65% of the total

variation, with a sum of squares of 282.054 and a variance component of 0.24599. This shows that there is considerable genetic differentiation within the groups themselves.

Finally, the greatest source of variation comes from within populations, contributing 71.2% of the total variation. The sum of squares for this component is 1122.188, with a variance component of 0.84821. This indicates that most genetic diversity is found within individual populations, reflecting high intra-population variation.

Table 2. The Results of the AMOVA (molecular analysis of variants) of *P. thunbergii* 21 populations.
Tabela 2. Resultados da AMOVA (análise molecular de variantes) de 21 populações de *P. thunbergii*.

Source of Variation	Degree of Freedom	Sum of Squares	Variance Components	Percentage of Variation	P value
Among groups	3	140.277	0.09705 a	8.15	< 0.05
Among populations within	17	282.054	0.24599 b	20.65	
Within populations	1323	1122.188	0.84821 c	71.2	
Total	1343	1544.519			

3.3. Wilcoxon Sign Rank and Polymorphism Loci

The Wilcoxon Sign Rank Test results are shown for three models: IAM (Infinite Allele Model), SMM (Stepwise Mutation Model), and TPM (Two-Phase Mutation Model), with varying values across the populations. The mean Wilcoxon Sign Rank Test values for IAM, SMM, and TPM are 0.281, 0.288, and 0.385, respectively. These figures indicate that the IAM and SMM models have similar mean values, while the TPM model shows a higher mean, suggesting greater significance under TPM for the populations tested. In the analysis of *P. thunbergii* populations using EST-SSR markers, it's notable that a bottleneck event (Table 3, $p<0.05$) was detected in the Tsuda population when employing the Infinite Allele Model (IAM).

Table 3. Wilcoxon sign rank test and percentage of polymorphism loci of *P. thunbergii* 21 populations.
Tabela 3. Teste de classificação de sinais de Wilcoxon e porcentagem de loci de polimorfismo de 21 populações de *P. thunbergii*.

No	Pop	*p-Wilcoxon Sign Rank Test*			% polymorphism loci
		IAM	SMM	TPM	
1	Karatsu	0.622	0.236	0.554	85.7
2	Kitsuki	0.241	0.396	0.598	85.7
3	Miyazaki	0.125	0.551	0.562	85.7
4	Hikari	0.118	0.239	0.554	28.6
5	Kochi	0.372	0.251	0.551	42.9
6	Tsuda	0.035	0.432	0.433	71.4
7	Mihama	0.199	0.422	0.419	71.4
8	Owase	0.221	0.138	0.140	28.6
9	Miho	0.552	0.406	0.396	42.9
10	Oki	0.350	0.590	0.578	42.9
11	Izumo	0.382	0.251	0.537	85.7
12	Tottori	0.400	0.214	0.207	57.1
13	Hamasaka	0.203	0.416	0.406	42.9
14	Miyadu	0.254	0.052	0.060	42.9
15	Kaga	0.102	0.272	0.281	71.4
16	Niigata	0.102	0.285	0.275	71.4
17	Toyama	0.369	0.239	0.564	71.4
18	Komatsu	0.386	0.249	0.556	71.4
19	Juo	0.359	0.090	0.095	14.3
20	Noshiro	0.241	0.108	0.105	71.4
21	Naruse	0.279	0.218	0.213	85.7
	Mean	0.281	0.288	0.385	60.5

Note: Wilcoxon Sign Rank Test: IAM (Infinite Allele Model), SMM (Stepwise Mutation Model, and TPM (Two PhaseMutation Model).
Nota: Teste de classificação de sinais de Wilcoxon: IAM (modelo de alelo infinito), SMM (modelo de mutação gradual) e TPM (modelo de mutação de duas fases).

The data reveal considerable variation across the populations in terms of the percentage of polymorphic loci, ranging from 14.3% in Juo to 85.7% in several populations (Karatsu, Kitsuki, Miyazaki, Izumo, and Naruse). The mean percentage of polymorphism loci across all populations is 60.5%, indicating a generally high level of genetic diversity within the populations. Populations such as Hikari, Owase, and Juo exhibit lower levels of polymorphism (28.6% or below), which could suggest limited genetic variation in these locations.

4. DISCUSSION

4.1. Genetic Parameters

The genetic diversity observed in species across different biogeographic zones highlights geography's significant role in shaping biodiversity. For example, populations in geographically isolated regions often show higher levels of genetic differentiation than populations in more connected regions. This is evident in the genetic parameters such as allele frequencies and heterozygosity levels, which vary significantly across populations depending on their

geographic location. Higher genetic diversity within a population will enhance the ability to adapt to changing environments, reflecting increased chances of success (GADISSA et al., 2018). The genetic diversity parameters for seven EST-SSR markers offer a comprehensive view of the genetic makeup of 21 *P. thunbergii* populations in Japan (Table 1).

Genetic parameters reveal significant biogeographic patterns in the study of 21 populations of *P. thunbergii* across various regions of Japan. It shows geographically distinct groups and exhibits varying levels of genetic variation. There are four groups: group 1 represents the Kyushu region; Group 2 exhibited Shikoku, the South part of Kansai, and the South part of Chubu region; Group 3 referred Chugoku, the North part of Kansai, and the North part of Chubu region; and Group 4 hinted Kanto and Tohoku regions (MUKASYAF et al., 2021a). Each group represents genetically distinct groups within the population, demonstrating how humans can affect the distribution pattern of genetics that fosters unique allelic compositions. The results of the four groups seem like populations situated in geographically isolated regions, but they are dominated by humans from the past (MUKASYAF, 2024). Because of nature, geographic isolation may occur when it is separated by mountains, rivers, or climate barriers, resulting in distinct genetic profiles within species populations.

The number of alleles (Na) ranged from 3.000 in populations such as Mihama, Niigata, and Hamasaka to a higher 5.286 in the Miyadu population. This variation signifies distinct genetic profiles among these populations. Observed heterozygosity (H_O) and expected heterozygosity (H_E) displayed ranges from 0.231 to 0.463 and 0.331 to 0.458, respectively, underscoring the differences in genetic diversity within these populations. Furthermore, individual marker analysis revealed specific characteristics; ESTSSR_33385 displayed the lowest values for Na, H_O, and H_E, suggesting reduced genetic diversity, while ESTSSR_24700 had the highest number of alleles (5.52 alleles), and ESTSSR_34351 showed the highest values for observed and expected heterozygosity (0.588 and 0.597, respectively). Notably, ESTSSR_33385 exhibited a negative inbreeding coefficient (-0.016).

EST-SSR (Expressed Sequence Tag - Simple Sequence Repeat) and G-SSR (Genomic Simple Sequence Repeat) are two distinct types of molecular markers used in genetic studies, and they differ not only in their sources but also in their applications and the reasons for variations in their genetic results (Yang et al., 2016). In this study, the mean of genetic parameters, except F_{IS} of G-SSR, were higher than EST-SSR. EST-SSR and G-SSR markers' mean genetic diversity (HE) was 0.373 and 0.775, respectively. These values fall within the lower and moderate levels of genetic diversity, respectively. It's worth highlighting that the genetic parameter results obtained from the analysis of the 21 *P. thunbergii* populations using EST-SSRs and genomic SSRs differed significantly. Moreover, using different molecular markers provides additional insights into how genetic diversity is distributed across biogeographic zones within *P. thunbergii* populations in Japan. The study found that G-SSR markers, which are derived from non-coding regions of the genome and thus subject to fewer functional constraints, exhibited higher genetic diversity than EST-SSR markers. This suggests that non-coding regions accumulate more mutations, leading to greater variability, while coding regions are under stronger selective pressures.

The lower genetic diversity observed with EST-SSRs compared to genomic SSRs aligns with expectations. EST-SSRs are derived from Expressed Sequence Tags, representing the actively transcribed regions or genes within an organism's genome. Since ESTs are primarily associated with functional genes, these markers are situated within coding regions of the genome. Coding regions are under strong purifying selection, as mutations within them can potentially disrupt gene function and impact an organism's fitness (YANG et al., 2016). Consequently, the genetic diversity within coding regions, including EST-SSR loci, tends to be lower. This is because purifying selection acts to remove or constrain genetic variants that might otherwise accumulate in non-coding regions. Thus, EST-SSRs often reveal fewer alleles and lower heterozygosity than G-SSRs, typically derived from non-coding genome regions where selective pressures are less intense (ZHAO et al., 2017).

On the other hand, G-SSRs are typically situated in non-coding regions, which experience fewer functional constraints and where mutations can accumulate more freely. This leads to the higher genetic diversity often observed with G-SSRs, including more alleles and greater heterozygosity. Because these regions are less critical for gene function, they are more permissive to genetic variation (SORKHEH et al., 2016; PARTHIBAN et al., 2018). Different results were shown by F_{IS} of EST-SSR, which were slightly higher than G-SSR. The slightly higher F_{IS} values (inbreeding coefficients) observed for EST-SSRs than G-SSRs can indicate different selective pressures and demographic factors (BLISCHAK et al., 2020).

Higher F_{IS} values for EST-SSRs might suggest that these markers are subject to stronger influences of inbreeding or reduced gene flow. This could be due to their location within genes and the associated selective constraints, which might lead to more structured populations or greater within-population relatedness (LI et al., 2023).

In contrast, G-SSRs located in non-coding regions may reflect different demographic and population dynamics, resulting in lower F_{IS} values (KIM et al., 2015). These findings collectively provide valuable insights into the genetic diversity of *P. thunbergii* populations, emphasizing the significance of specific markers and genetic regions in understanding their genetic variation. The polymorphism percentage measures the degree of genetic variation or polymorphism at specific genetic loci within a population. The results generally showed different percentages of polymorphism loci among populations (Table 3). It means that the percentage reflected the differences in genetic diversity. This diversity can be valuable for understanding the population's adaptability, evolution, and potential responses to environmental changes.

4.2. Analysis of Molecular Variance (AMOVA)

AMOVA provides insights into the distribution of genetic diversity among different levels, such as within populations, among populations, and in groups of populations. The Analysis of Molecular Variance (AMOVA) of EST-SSR in *P. thunbergii* 21 populations (Table 2) indicates that the genetic diversity within the populations was either greater than the diversity observed between populations or between groups. In biogeography, high genetic diversity within populations means that these populations may have adapted to local environmental conditions, leading to a rich reservoir of genetic traits that could be crucial for resilience

and adaptability in changing climates (SAMAD-ZADA et al., 2023).

This suggests that conservation efforts should focus on maintaining the genetic integrity of individual populations, as they harbor significant genetic variation crucial for the species' adaptability and resilience. Furthermore, the findings highlight the importance of habitat preservation and management strategies that promote gene flow among populations to enhance overall genetic health. This biogeographic pattern of genetic diversity through AMOVA within *P. thunbergii* populations aligns with the typical characteristics of outbreeding forest tree species, such as conifers. The AMOVA results in this context support the idea that *P. thunbergii* populations exhibit characteristics of conifer species, with significant genetic diversity within populations, contributing to their resilience and adaptability within their respective ecological niches (TAO et al., 2023).

4.3. Wilcoxon Sign Rank and Polymorphism Loci

Bottleneck events can have important implications for populations' genetic diversity and structure. Thus, it can investigate whether specific genetic loci show deviations from the expected mutation-drift equilibrium, which may suggest a past bottleneck event or other demographic changes in the population's history (SONSTHAGEN et al., 2017).

Within biogeography terms, information related to the bottleneck of the populations is crucial for understanding how environmental factors and historical events have shaped the current distribution of genetic variation. In the analysis of *P. thunbergii* populations using EST-SSR markers, it's notable that a bottleneck event (Table 3, $p<0.05$) was detected in the Tsuda population when employing the Infinite Allele Model (IAM). These suggest that the Tsuda population may have experienced significant stressors that reduced its effective population size, leading to a loss of allelic richness and increased inbreeding. However, it's important to recognize that the most appropriate bottleneck analysis methods for microsatellite markers are the Two-Phase Mutation Model (TPM) and the Stepwise Mutation Model (SMM). These models account for the unique mutation processes associated with microsatellites, offering a more nuanced understanding of genetic drift and population dynamics in response to environmental pressures (SAGAR ET AL., 2023).

The results from both the SMM and TPM analyses, which are generally more appropriate for microsatellite markers, indicate that no bottleneck events occurred in any of the 21 *P. thunbergii* populations. Furthermore, it's significant to consider the TPM model when assessing genetic diversity, as it is considered a better fit for revealing changes in heterozygosity in populations over time (SU et al., 2019).

Therefore, considering the TPM and SMM results for EST-SSR markers, the overall assessment suggests no evidence of recent or historical population bottlenecks that would have led to a significant reduction in the effective population size. This finding underscores the stability of genetic diversity within these populations, which may be attributed to their ecological resilience and adaptive capacity in response to environmental changes. The absence of bottleneck signals in the data is an important finding as it implies that these populations have likely maintained relatively stable sizes and genetic diversity over time in the context of these specific markers and models (BUCKLEY et al., 2022).

The percentage polymorphism of the loci implied that the Kyushu region, Izumo, and Naruse had the highest percentage (85.7%), indicating a rich genetic pool, while the lowest was in Juo (14.7%), which implies that the genetic diversity within the Juo population is comparatively lower. The variations in genetic diversity among geographic zones of *P. thunbergii* populations can be influenced by factors such as historical events, gene flow, and local adaptation, and they provide valuable insights into the genetic structure and history of the studied populations. Environmental pressures and habitat fragmentation may further exacerbate these differences, leading to distinct evolutionary trajectories for each population (DELNEVO et al., 2021). These findings underscore the importance of considering historical and anthropogenic factors in shaping genetic diversity among *P. thunbergii* populations. For instance, the distinct variations observed can be attributed to natural selection pressures and human activities such as reforestation that have historically influenced gene flow and population dynamics throughout Japan (MUKASYAF, 2024). Furthermore, the implications of these biogeographic patterns extend beyond mere interest in genetic diversity. Developing effective conservation strategies to preserve this species' remaining genetic resources is crucial. Given the significant role those local adaptations play in survival, targeted management practices must account for regional differences in genetic diversity to ensure the resilience of *P. thunbergii* against emerging environmental challenges (IWAIZUMI et al., 2018).

5. CONCLUSIONS

The studies provide a comprehensive look at the biogeographic pattern of genetic diversity within 21 *P. thunbergii* populations in Japan using seven EST-SSR markers. These markers revealed slight variations in genetic profiles among populations, such as in the number of alleles, observed and expected heterozygosity, and inbreeding coefficient. Notably, observed heterozygosity (H_O) and expected heterozygosity (H_E) displayed ranges from 0.231 to 0.463 and 0.331 to 0.458, respectively, underscoring the differences in genetic diversity within these populations.

The study's comparison between EST-SSRs and G-SSRs further illuminates the influence of marker choice on genetic diversity assessments. In this study, the mean of genetic parameters, except F_{IS} of G-SSR, was higher than that of EST-SSR. EST-SSRs, situated within coding regions subject to purifying selection, exhibited lower genetic diversity than G-SSRs, which are located in non-coding regions with fewer functional constraints.

Additionally, slightly higher F_{IS} values for EST-SSRs suggest that these markers may experience stronger inbreeding or reduced gene flow, possibly due to their location within genes. In general, the results showed different percentages of polymorphism loci among populations, which means that the percentage reflected the differences in genetic diversity.

This diversity can be valuable for understanding the population's adaptability, evolution, and potential responses to environmental changes. On the other hand, the results of the Analysis of Molecular Variance (AMOVA) of EST-SSR in *P. thunbergii* 21 populations indicate that the genetic diversity within the *P. thunbergii* populations was greater than the diversity observed between populations or between groups. Furthermore, the overall assessment of bottleneck analysis for EST-SSR markers suggests that there is no

evidence of recent or historical population bottlenecks that would have led to the stability of genetic diversity within these populations, which may be attributed to their ecological resilience and adaptive capacity in response to environmental changes.

The absence of bottleneck signals in the data is an important finding as it implies that these populations have likely maintained relatively stable sizes and genetic diversity over time in the context of these specific markers and models. These findings collectively provide valuable insights into the genetic diversity of *P. thunbergii* populations, emphasizing the significance of specific markers and biogeographic regions in understanding their genetic variation.

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