The origin and genetic variability of vegetatively propagated clones identified from old planted trees and plantations of *Thujopsis dolabrata* var. *hondae* in Ishikawa Prefecture, Japan

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Abstract

Clonal plantations of *Thujopsis dolabrata* var. *hondae* have been established in Ishikawa Prefecture, Japan, since at least the 1800s. Historical planting of the species has led to the development of vegetatively propagated local cultivars, which originated from 'donor' trees that have often been conserved in sacred groves or avenues at shrines and temples. These donor trees must have been selected from natural populations. In this study we estimated the origin and genetic variability of clones identified among old planted trees and clonal plantations of *T. dolabrata* var. *hondae*, using 19 microsatellite markers. We discovered 12 clones among old planted trees, including five identical to members of a set of 14 we previously identified in plantations (giving 21 clones in total). Based on analyses combining assignment and exclusion tests, we inferred origins of eight of those 21 clones: six may have originated from a natural population distributed in Ishikawa, one from Hokkaido & Aomori, and the other from Iwate & Yamagata, suggesting the clones constituting cultivars have multiple origins. The clones identified in plantations have significantly lower genetic variability, and higher relatedness, indicating that clones of cultivars have a much narrower genetic base than those of natural populations. We suggest new clones selected from natural populations elsewhere, as well as Ishikawa, are needed for future breeding of *T. dolabrata* var. *hondae* to develop clonal forestry for this species.

Keywords: assignment test; clonal forestry; genetic diversity; local cultivar; microsatellite; natural population

Introduction

Clonal forestry refers to extensive deployment of relatively few clones with proven phenotypic superiority (White et al. 2007). Clonal forestry plantations have been established with various tree species, such as *Cryptomeria japonica* (Miyajima 1989; Ohba 1993) and *Chamaecyparis obtusa* (Sato and Miyajima 1956; Miyajima 1989) in Japan, *Picea abies* in Europe (Bentzer 1993), *Pinus radiata* in New Zealand (Arnold and Gleed 1985) and species and interspecific hybrids of *Eucalyptus* (e.g., Van Wyk 1985), *Populus* and *Salix* (Zsuffa et al. 1993) in many countries. In clonal forestry of *C. japonica* in Japan, individuals used for plantations have been spontaneously or instinctively selected during the domestication process over a long period. This has led to the development of many local cutting cultivars, e.g., Measa, Hon-sugi, Aya-sugi and Yabukuguri in the Kyushu region, Okinoyama-sugi in Tottori Prefecture, Boka-sugi in Toyama Prefecture and Sanbu-sugi in Chiba Prefecture (Miyajima 1989; Ohba 1993).

Thujopsis dolabrata var. *hondae* is a coniferous species, distributed from southern Hokkaido to northern Honshu in Japan (Hayashi 1960). Its wood has excellent strength, durability and moisture resistance (Saito 1972), so it has become one of the most important forestry species in Japan. It is mainly harvested in three prefectures: Aomori, Niigata and Ishikawa. In Aomori and Niigata, its wood is mostly produced from natural populations. However, in Ishikawa the wood comes from clonal plantations. These were established in the past from saplings created by layering or unrooted cuttings planted directly in the forests and, in recent years, from rooted cuttings or saplings created by air layering. In Ishikawa Prefecture, the species is known as ‘ate’ and has been planted (mainly on the Noto Peninsula) since at least the 1800s (Saito 1972). Such historical planting has led to the development of more than 20 vegetatively propagated local cultivars, most importantly Ma-ate, Kusa-ate, Eso-ate (Suzu-ate) and Kana-ate. Ma-ate

consists of multiple clones, but a single clone predominates in plantations of each of the other three cultivars (Nakano 1990; Ikeda et al. 2018). A twisted trunk is one of several important traits for identifying the Ma-ate cultivar, although Kana-ate trees may also have a twisted trunk (Nakano 1990; Forest Experiment Station, Ishikawa Agriculture and Forest Research Center 2016). Ikeda et al. (2018) identified clones in plantations using microsatellite markers and found that present plantations had been established from at least 14 clones. The original (donor) trees that gave rise to the clones have often been conserved in sacred groves or avenues at shrines and temples (Saito 1972). People seem to have taken cuttings from these holy trees and distributed them in their local areas. These old planted trees must have been selected from natural populations. The clones used for clonal forestry may have hypothetically originated from natural populations distributed in Ishikawa. An alternative hypothesis, based on oral traditions, is that planting material was brought from natural populations in the Tohoku region (which encompasses Aomori, Iwate and Yamagata Prefectures) about 300, 400 or 800 years ago (Agriculture, Forestry and Fisheries Department, Ishikawa Prefectural Government 1997; Forest Experiment Station, Ishikawa Agriculture and Forest Research Center, 2016). It is still not clear which hypothesis is correct.

Microsatellite marker-based genetic analyses of vegetatively propagated cultivars of *C. japonica*, *C. obtusa* and *T. dolabrata* var. *hondae* used for clonal forestry in Japan have provided valuable information (Matsui et al. 2013; Goto et al. 2008; Ikeda et al. 2018). High genetic variability has been found among 19 major cutting cultivars of *C. japonica* in the Kyushu region, but generally close genetic relationships between clones within the cultivars (Matsui et al. 2013). Clones of a local cutting cultivar of *C. obtusa*, Nango-hi appear to be more closely related to each other, and have lower genetic variability, than individuals in natural populations (Goto, et al. 2008). Clones of the Ma-

ate cultivar of *T. dolabrata* var. *hondae* also tend to have close relationships (Ikeda et al. 2018). All these studies indicate that clones within cultivars tend to be genetically closely related. This may be problematic when crossing clones of conifer cultivars to create new cultivars, because of risks of severe inbreeding depression (Williams and Savolainen 1996). Thus, there are clear needs to raise and maintain higher genetic variability of breeding populations. The natural populations from which clones probably originated are very important genetic resources, and recurrent selection from them would be an efficient way of increasing breeding populations' genetic variability. Identifying such ancestral populations is far from straightforward, because clones originated from various natural populations throughout a long history of domestication (Testolin et al. 2000; Honjo et al. 2008). However, assignment tests based on microsatellite genotypes have proven utility for inferring source populations of individuals of several species (Honjo et al. 2008; Goto et al. 2008; Caldera et al. 2008).

Thus, in this study we used microsatellite markers to evaluate the genetic variability of vegetatively propagated clones identified among old planted trees and plantations of *T. dolabrata* var. *hondae* in Ishikawa Prefecture. We compared the variability of the clones and natural populations of the species. We also attempted to identify the clones' origins, in potential natural source populations, using assignment tests based on microsatellite genotypes. Finally, we discuss implications of the findings for future breeding of *T. dolabrata* var. *hondae*.

Materials and methods

Plant materials

We collected leaves from 24 old planted *T. dolabrata* var. *hondae* trees at 12 shrines, four temples and one private garden (of the Izumi family) in Ishikawa Prefecture (Table 1, Fig.

S1). We also examined the trunk twist of each tree. The two trees in the private garden (old tree nos. 16 and 17), which were believed to be the origin of ate, were the oldest (more than 450 years old) of all the old planted trees. The two old planted trees at Hinomiya Shrine (nos. 13 and 14) were reportedly origins of the Kusa-ate cultivar (Saito 1972). Ikeda et al. (2018) identified 14 clones (designated C-1, -6, -7 and -14, and C-44 to -53) in *T. dolabrata* var. *hondae* plantations in Ishikawa, based on 12 microsatellite genotypes, which we used as genotype data in assignment tests for this study. As potential source populations in assignment tests for clones among old planted trees and plantations, we selected 17 natural populations from the entire distribution range of *T. dolabrata* var. *hondae* and collected leaves from a total of 410 individuals belonging to the natural populations (Fig. 1 and Table S1).

DNA extraction and genotyping

We extracted genomic DNA from the leaves collected from old planted trees and individuals in natural populations and determined genotypes at 19 microsatellite loci (Tdest 1, Tdest 3, Tdest 11, Tdest 14, Tdest 17, Tdest 21, Tdest 24, Tdest 29, Tdest 35, Tdest 37, Tdest 38, Tdest 39, Tdest 42, Tdest 43, Tdest 45, Tdest 49, Tdest 53, Tdest 56 and Tdest 58) developed for *T. dolabrata* var. *hondae* (Sato et al. 2015). We also determined genotypes of the 14 clones from plantations examined by Ikeda et al. (2018) at seven additional microsatellite loci. The methods used for DNA extraction and microsatellite genotyping were as in the previous study (Ikeda et al. 2018).

Data analysis

Clone identification and analysis of clones' genetic relationships

148 We assigned the sampled old planted trees to clones, based on the genotypes at 19
149 microsatellite loci with reference to those of the 14 clones from plantations (Ikeda et al.
150 2018). When old planted trees shared identical multilocus genotypes with known clones
151 from plantations, with no mismatched loci, the old planted trees were considered to be
152 known clones. If the multilocus genotypes of old planted trees did not match those of any
153 known clones from plantations, the old planted trees were considered to be new clones.
154 To estimate genetic relationships between clones, we calculated the coefficient of
155 relatedness (*r*, the fraction of alleles identical by descent shared by individuals; Blouin,
156 2003), using ML-Relate software (Kalinowski et al. 2006). For this, we applied allele
157 frequencies based on data for all sampled individuals in the 17 natural populations
158 included in the study (Fig. 1, Table S1). This coefficient is 1.000 for individuals of the
159 same clone, 0.5 for parents and offspring or full siblings, 0.250 for half siblings, and 0.125
160 for first cousins. We also calculated values of *r* among individuals in each natural
161 population to compare with those among clones in the old planted trees and plantations.
162
163 **Analysis of genetic diversity and structure in natural populations**
164 To estimate genetic diversity within each natural population, we calculated the number
165 of alleles per locus (A), allelic richness based on 12 diploid individuals (A_R), observed
166 heterozygosity (H_O), gene diversity (H_E) and inbreeding coefficient ($F_{IS} = 1-H_O / H_E$)
167 over all loci in each population. The significance of departures from Hardy-Weinberg
168 equilibrium (HWE) over all loci in each population was evaluated by randomization tests.
169 To estimate genetic diversity across all natural populations, we calculated the total
170 number of alleles detected (TN), H_O, average gene diversity within populations and gene
171 diversity in the total population (H_S and H_T, respectively; Nei 1987), and inbreeding
172 coefficient and genetic differentiation measure (F_{IS} and F_{ST}, respectively; Weir and

Cockerham 1984) across all populations at each locus and over all loci. The standardized genetic differentiation measure (G'_{ST}; Hedrick 2005) was also manually calculated. The significance of departures from HWE and genetic differentiation among populations at each locus and over all loci was evaluated by randomization tests. The above calculations, apart from G'_{ST} calculation, were performed using FSTAT version 2.9.4 (Goudet 1995).

To assess the individual-based genetic structure among natural populations of *T. dolabrata* var. *hondae*, the Bayesian clustering method was applied, using STRUCTURE version 2.3.4 software (Pritchard et al. 2000). Simulations were run 10 times for each value of K (number of genetic clusters) from 1 to 10 for 10,000 iterations after a burn-in period of 10,000 iterations with an admixture model, an allele frequency correlated model (Falush et al. 2003) and a LOCPRIOR model (Hubisz et al. 2009). The optimal value of K was determined based on the log probability of data and the ΔK for each K (Evanno et al. 2005) using STRUCTURE HARVESTER software (Earl and VonHoldt, 2012). To facilitate interpretation of the results from STRUCTURE analysis, we used CLUMPP version 1.1.2 (Jakobsson and Rosenberg, 2007). We also used DISTRUCT version 1.1 (Rosenberg, 2004) to visualize the results. Analysis of molecular variance (AMOVA) was performed with GenAlEx version 6.502 (Peakall and Smouse, 2012), based on the genetic structure among natural populations detected by STRUCTURE, which separated the 17 natural populations into four regional groups. These regions were: Hokkaido & Aomori, Iwate & Yamagata, Niigata, and Ishikawa (for details, see 'Genetic diversity and structure in natural populations' in the Results section). Genetic variation of the natural populations was hierarchically divided into three layers (the regions inferred by STRUCTURE analysis, populations and individuals) and variance components for each layer and related Φ-statistics were calculated. The significance of each Φ-statistic was evaluated by a permutation test implemented in GenAlEx.

198

199 **Comparison of genetic variability among clones and among individuals in natural**
200 **populations**

201 To estimate genetic variability among clones, we calculated A, A_R, H_O and H_E for clones
202 identified among old planted trees and in plantations, using FSTAT. Similarly, to estimate
203 genetic variability among individuals within natural populations in the regions inferred
204 by STRUCTURE analysis as well as that across all the populations, we averaged the
205 values of A_R and H_E over populations in each region and over all populations. The A_R and
206 H_E values for the clones detected among old planted trees and in plantation were
207 compared with the average values for all the natural populations, using a Wilcoxon's
208 signed-rank test.

209

210 **Inferring origins of clones**

211 To infer the origin of each clone, we applied a Bayesian-based assignment test (Rannala
212 and Mountain 1997) with the 'leave one out' procedure implemented in GeneClass 2
213 software (Piry et al. 2004), using microsatellite genotype data. This test estimates the
214 likelihood that an individual belongs to a population and assigns the population with the
215 highest likelihood as the origin of the individual. We set the four regions inferred by
216 STRUCTURE analysis as units of origins of clones. To confirm that there was sufficient
217 information for estimating the origin of each clone, we applied a self-assignment test for
218 individuals from natural populations in the four regions. Next we applied an assignment
219 test for each clone, regarding the natural populations in the four regions as potential
220 source populations. To confirm that individuals belong to potential source populations,
221 in addition to an assignment test an exclusion test can be applied (Cornuet et al. 1999).
222 Combinations of assignment and exclusion tests have been used effectively to determine

original populations of individuals in several species (Craig et al. 2000, Hare et al. 2006, Frantz et al. 2006). Thus, we also applied an exclusion test using the Monte Carlo resampling method (Paetkau et al. 2004) with 10,000 replicates implemented in GeneClass2. When the probability that populations in a region were sources of a clone was less than a given threshold ($\alpha = 0.01$) in the exclusion test, we concluded that populations in that region were not sources for the tested clone.

Results

Clone identification and genetic relationships between clones

We identified 12 clones (C-1, -6, -7, -14, -46 and C-54 to -60; Table 1) among old planted trees, five of which (C-1, -6, -7, -14 and -46) were the same as clones found in plantations (Ikeda et al. 2018), while the other seven (C-54 to -60) were newly discovered. Four of the first five clones (C-1, -6, -7 and -14) are commonly used and represent major clones in plantations (Ikeda et al. 2018). Half of the 24 old planted trees were identical to one of the five clones identified in plantations.

Values of the coefficient of relatedness (r) among the 21 clones detected in plantations and old planted trees ranged from 0.000 to 0.799 (Table 2), and were particularly high (>0.5) among C-44, C-53 and C-54, as well as between C-46 & C-49, C-46 & C-59, and C-49 & C-50. Average values of r among clones in old planted trees and plantations were 0.102 and 0.183, respectively; higher than those among individuals within each natural population (0.015 to 0.060; on average, 0.031; Table S1).

Genetic diversity and structure in natural populations

The allelic richness (A_R) and gene diversity (H_E) within each natural population ranged

247 from 5.03 to 6.55 and from 0.567 to 0.693, with averages of 5.88 and 0.652, respectively
248 (Table S1). Inbreeding coefficient (F_{IS}) ranged from -0.112 to 0.093. Two of the 17
249 populations showed significant deviation from Hardy-Weinberg equilibrium (HWE).
250 Over the 19 loci across the 17 natural populations, the average values of the total number
251 of alleles detected (TA), average gene diversity within populations (H_S) and gene
252 diversity in the total population (H_T) were 7.2, 0.652 and 0.677, respectively (Table S2).
253 Values for F_{IS} and two genetic differentiation measures (F_{ST} and G'_{ST}) over the 19 loci
254 were 0.018, 0.039 and 0.114, respectively. Significant deviation from HWE was detected
255 at two of the 19 loci and over all loci. Genetic differentiation among populations were
256 significant at 17 of the 19 loci and over all loci.
257 According to STRUCTURE analysis, log probabilities of the data increased with
258 increasing K up to a plateau at $K = 4$ (Fig. S2), while ΔK values were highest at $K = 2$
259 (Fig. S3). We therefore examined genetic structure among natural populations at $K = 2$,
260 3 and 4. At $K = 2$, the proportions of cluster 2-I in populations were highest in Hokkaido
261 & Aomori, lowest in Niigata & Ishikawa and intermediate in Iwate & Yamagata (Fig. 2).
262 At $K = 3$, clusters 3-I, 3-III and 3-II dominated in Hokkaido & Aomori, Iwate & Yamagata,
263 and Niigata & Ishikawa, respectively, although populations 10 in Aomori and 15 in
264 Niigata also had relatively high proportions of cluster 3-III. At $K = 4$, similar patterns
265 appeared as at $K=3$: clusters 4-I, 4-III and 4-II dominated in Hokkaido & Aomori, Iwate
266 & Yamagata, and Niigata & Ishikawa, respectively, although most populations contained
267 mixtures of clusters. Based on the genetic structure among populations detected by
268 STRUCTURE analysis, we separated the 17 natural populations into four regional
269 groups: Hokkaido & Aomori, Iwate & Yamagata, Niigata, and Ishikawa. At $K = 4$, the
270 values of F_{ST} were about twice as high for clusters 4-II and 4-IV (0.0817 and 0.0675,
271 respectively) as for clusters 4-I and 4-III (0.0360 and 0.0412, respectively) (Fig. 2).

AMOVA results indicated that 8.7, 5.9 and 85.4% of detected genetic variation resided among regions, among populations within regions, and among individuals, respectively (Table S3). All Φ-statistics were highly significant ($P < 0.001$).

Comparison of genetic variability among clones and among individuals in natural populations

Across natural populations in the regions inferred by STRUCTURE analysis, the average values of A_R decreased from north to south, from 5.97 for populations in Hokkaido & Aomori to 5.39 for populations in Ishikawa (Table 3). Average values of H_E decreased from 0.668 to 0.586 in a similar fashion. Average values of A_R and H_E over all the natural populations were 5.88 and 0.652, respectively. The A_R and H_E values for the 12 clones detected among old planted trees were 5.58 and 0.647, respectively; not significantly lower than the averages for all the natural populations. However, values for the 14 clones detected in plantations were significantly lower: 4.90 and 0.616 ($P < 0.01$ and $P < 0.05$, respectively; Wilcoxon's signed-ranks test).

Inferring origins of clones

To confirm that the dataset for reference populations included sufficient information to allow estimation of the origins of clones, we applied a self-assignment test for individuals from natural populations in four regions. This resulted in correct assignment of 362 individuals (88.3% of the total) to their respective sampling regions (Table S4). To infer the clones' origins, we applied a combination of assignment and exclusion tests. Probabilities obtained for the first, second and third most likely source regions for the 21 clones are shown in Table 4. Criteria for assignment to a single region were probability higher than 0.01 for the first region and lower than 0.01 for the second and third regions.

297 Eight clones were assigned only to single regions (C-1, -6, -45, -48, -55 and -59 to
298 Ishikawa; C-7 to Hokkaido & Aomori; C-52 to Iwate & Yamagata), but four (C-14, -46,
299 -49 and -60) were not assigned to any of the four regions and the other 9 were assigned
300 to more than one region.
301
302 **Discussion**

303 **Original trees that gave rise to vegetatively propagated local cultivars**

304 Historical planting of *T. dolabrata* var. *hondae* has led to the development of more than
305 20 vegetatively propagated local cultivars. The four major cultivars are Ma-ate, Kusa-ate,
306 Eso-ate (Suzu-ate) and Kana-ate (Forest Experiment Station, Ishikawa Agriculture and
307 Forest Research Center, 2016). The original (donor) trees of cultivars have often been
308 conserved as sacred trees or in groves in the grounds of shrines and temples (Saito 1972).
309 Ikeda et al. (2018) identified clones of three cultivars (Ma-ate, Kusa-ate and Eso-ate)
310 using microsatellite markers and demonstrated that the Ma-ate cultivar consists of
311 multiple clones (including the three most frequently used in plantations: C-1, -6 and -7).
312 We found that the oldest planted trees (older than 450 years), which were believed to be
313 the origin of ate, were identical to clone C-7 of the Ma-ate cultivar. The other two clones,
314 C-1 and -6, also apparently originated from old planted trees. All the old planted trees of
315 clones C-1, -6 and -7 had a dextrally twisted trunk (Table 1) and bark resembling that of
316 *C. obtusa*, which are characteristics of the Ma-ate cultivar.
317 Ikeda et al. (2018) also demonstrated that plantations of the Kusa-ate cultivar are
318 dominated by a single clone (C-14), which we confirmed originated from old planted
319 trees previously regarded as the cultivar's origin. In addition, Ikeda et al. (2018) argued
320 that two clones detected in plantations (C-44 and -53) were probably trees of the Eso-ate
321 cultivar. However, genotypes of these two clones were not found among the old planted

trees and they are closely related to the Ma-ate clone (C-1), according to coefficients of relatedness (r) estimated in this study. Clone C-54, identified among old planted trees, had high values of r with C-44 and C-53 (0.799 and 0.569, respectively). These values indicate that C-54 has a closer than parent-offspring or full sibling relationship with C-44, and relationship as close as parent-offspring or full sibling with C-53. Clone C-54 may therefore be the original clone of the Eso-ate cultivar, and its derivative clones (C-44 and -53) may have originated from seedlings of C-54 and been used for establishing plantations as the Eso-ate cultivar. Accordingly, this study demonstrates that the original trees of the Ma-ate, Kusa-ate and Eso-ate clones (C-1, -6, -7, -14 and -54) have been conserved as old planted trees at shrines or temples. A similar domestication history has been proposed for vegetatively propagated local cultivars of *C. japonica* in the Kyushu region, Japan. For example, original trees of one of the cultivars, Measa, may have been selected from natural populations, preserved as sacred trees at shrines, and spread to plantations in the Kyushu region (Miyajima 1989; Ohba 1993).

The origins of clones

The STRUCTURE analysis provided indications of four regional groups (Hokkaido & Aomori, Iwate & Yamagata, Niigata, or Ishikawa) of natural populations within the species, *T. dolabrata* var. *hondae*. Therefore, we conducted assignment tests with the four regions inferred by STRUCTURE analysis as units of origins of clones. In the self-assignment test, 88.3% of individuals from natural populations were correctly assigned to their respective sampling regions. According to AMOVA ($\Phi_{RT} = 0.065$), only 5.9% of genetic variation resided among regions, indicating that genetic differentiation among regions is low. Because of this low genetic differentiation among regions, the proportion of individuals assigned to their regions of origin was not particularly high. Use of an

exclusion test in addition to an assignment tests increased the accuracy of assigned origins for some of the clones. Nevertheless, origins of 14 of the 21 clones could not be inferred from results of combined assignment and exclusion tests, either because of the low genetic differentiation among regions or because source populations were not included in the analysis. Goto et al. (2008) also found that origins of eight of 21 clones of a local cutting cultivar of *C. obtusa*, Nango-hi, could not be inferred using a combination of assignment and exclusion tests, because of low genetic differentiation among candidate natural populations (G_{ST} = 0.039; Tsumura et al. 2007). Several hypervariable markers would be needed to assign more clones of *T. dolabrata* var. *hondae* cultivars to their source populations. However, we were able to infer origins of seven of the 21 clones: five were from Ishikawa, one from Hokkaido & Aomori, and the other from Iwate & Yamagata, suggesting that clones of *T. dolabrata* var. *hondae* cultivars are derived from multiple origins. Thus, results of this study support both hypotheses about the origin of clones of this species: some appear to have originated from within Ishikawa Prefecture and others from the Tohoku region.

The three most frequently used clones of the Ma-ate cultivar (C-1, -6 and -7) were inferred to have originated from either inside or outside Ishikawa Prefecture. C-7's inferred origins were natural populations in the Hokkaido & Aomori region. This is consistent with oral traditions that old planted trees were brought from the Tohoku region, which includes Aomori, Iwate and Yamagata Prefectures, in the 12th or 16th century (Nihei and Tsuzi 1917). Inferred origins of the other two Ma-ate clones, C-1 and -6, were natural populations in Ishikawa Prefecture. This suggests that trees of the two clones may be classified as Ma-ate because of phenotypic similarities (in trunk twisting and bark) to the oldest planted C-7 trees, although they have different origins from C-7 and the Ma-ate clone complex has multiple origins. Origins of the Kusa-ate clone (C-14) and Eso-ate

clones (C-44, -53 and -54) could not be inferred in this study. However, Nagahama et al. (1996) suggested that the Kusa-ate cultivar originated from Aomori Prefecture, because the composition of its leaf oil is the same as in individuals from Ohata in Aomori Prefecture.

Genetic variability and relationships among clones

The clones identified in plantations and old trees tended to have lower genetic variability than natural populations. There may be two reasons for this. The first is phenotypic selection during local cultivars' establishment. As a result of selection, the clones of cultivars may have tended to have similar desirable characteristics, such as height, diameter, volume, stem form, crown shape and bark traits. Hence, the genetic variability of the clones selected for cultivars may have declined. Accordingly, selected individuals generally have lower genetic variability than those in natural populations, as reported in other coniferous species, such as *C. obtusa* (Goto et al. 2008), *Pinus radiata* (Moran and Bell 1987) and *Picea glauca* (Rajora 1999). Another reason is that most of the clones may have originated from natural populations in Ishikawa Prefecture, which have lower genetic variability than natural populations in other regions. Six of the eight clones (C-1, -6, -45, -48, -55 and -59) that were only assigned to single regions were inferred to have originated from Ishikawa. The current natural populations in Ishikawa are small, located at the southern limit of *T. dolabrata* var. *hondae*'s range on the Japan Sea side, and geographically isolated from other natural populations. Low population size may lead to loss of genetic variability through genetic drift and inbreeding, while isolation from other populations may limit gene flow and accompanying increases in genetic variability (Frankham 2005). A high F_{ST} value was obtained for the dominant cluster in natural populations in Ishikawa detected by STRUCTURE analysis, suggesting they experienced

considerable genetic drift after divergence from the ancestral population (Falush, Stephens, and Pritchard 2003). Therefore, the natural populations in Ishikawa may have experienced substantial genetic drift without effective gene flow, resulting in substantial reductions in genetic diversity within the populations.

The clones detected in plantations and old planted trees had closer genetic relationships than individuals in natural populations. Similar patterns have been observed in other species. For example, Goto et al. (2008) found that clones of a local cutting cultivar of *C. obtusa*, Nango-hi, had closer relationships than conspecific individuals in natural populations. Genotypes of cultivars have remained constant for hundreds of years through vegetative propagation, and new cultivars tend to be produced by crossings between cultivars (Sefc et al. 2000; Goto et al. 2008). Some of the clones of *T. dolabrata* var. *hondae* cultivars may therefore be families, each consisting of a parent and its offspring, as discussed by Ikeda et al. (2018), or generated by crossing between clones either of the same or different cultivars. Such production of clones in cultivars has putatively occurred in the history of the *C. obtusa* cultivar Nango-hi (Goto et al. 2008).

Conclusions

Our results suggest that clones of the Ma-ate, Kusa-ate and Eso-ate cultivars used in *T. dolabrata* var. *hondae* clonal forestry originated from old planted trees, many of which have been conserved. It also suggests that clones of cultivars used for clonal forestry originated from natural populations in Ishikawa Prefecture and others in the Tohoku region. The clones identified in current plantations have lower genetic variation, and closer genetic relationships, than the natural populations. Thus, the clones of cultivars used in clonal forestry have a much narrower genetic base. The close genetic relationships between clones may be problematic when crossing clones of cultivars to create new

cultivars because such crossings cause severe inbreeding depression (Williams and Savolainen 1996). Recurrent selection from the natural populations from which these clones originated would be an efficient strategy for maintaining and increasing the genetic variability of breeding populations (Zobel and Talbert 1984). Therefore, new clones from natural populations elsewhere, as well as Ishikawa Prefecture, should be included in future *T. dolabrata* var. *hondae* breeding programs to enhance the sustainability of clonal forestry of the species in the Prefecture. It is also essential to conserve natural populations of *T. dolabrata* var. *hondae* as genetic resources for future breeding of the species.

Data Archiving Statement

The microsatellite genotype data used in this study have been deposited in the Dryad Digital Repository: http: (If the manuscript is accepted, we will submit them to the repository).

Disclosure statement

The authors declare that they have no conflicts of interest.

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571 **Titles and legends to figures**

572

573 **Fig. 1** Locations of 17 natural *Thujopsis dolabrata* var. *hondae* populations used in an
574 assignment test as potential source populations for the clones identified from old planted
575 trees and plantations. Numbers on the map correspond to the population numbers in Table
576 S1

577

578 **Fig. 2** Distribution of genetic clusters in each individual, at numbers of clusters (K) = 2,
579 3 and 4, for the 17 natural populations of *Thujopsis dolabrata* var. *hondae*, obtained by
580 STRUCTURE analysis. F_{ST} values for the clusters indicate their genetic divergence
581 from the ancestral population. Numbers below the chart correspond to the population
582 numbers in Table S1.

583 **Table 1** Designations, locations, diameter at breast height (DBH) and trunk twist
584 characteristics of 24 old planted *Thujopsis dolabrata* var. *hondae* trees sampled from 12
585 shrines, four temples and one private garden in Ishikawa Prefecture, Japan

Old tree no.	Place planted	DBH	Trunk twist	Clone name
1	Yahata Shrine, Sasanami, Suzu	76	No	C-54
2		85	No	C-54
3	Komashihiko Shrine, Kyounen, Wakayama, Suzu	93	No	C-54
4		118	Sinistral	C-55
5	Ohhirume Shrine, Mawatari, Horyu, Suzu	102	No	C-56
6	Kumano Shrine, Omou, Noto, Hosu	83	No	C-54
7		88	No	C-54
8	Hakusan Shrine, Yanagida, Noto, Hosu	84	No	C-57
9	Koumyou Temple, Kitakawachi, Noto, Hosu	98	Dextral	C-7 [a]
10		98	No	C-57
11	Kasuga Shrine, Soyama, Anamizu, Hosu	92	Dextral	C-7 [a]
12		95	Dextral	C-7 [a]
13	Hinomiya Shrine, Hajikashi, Anamizu, Hosu	45	No	C-14[a]
14		55	No	C-14 [a]
15	Sugawara Shrine, Tenjindani, Anamazu, Hosu	102	No	C-58
16	Private garden of Izumi family, Urakami, Monzen, Wajima	>150	Dextral	C-7 [a]
17		>150	Dextral	C-7 [a]
18	Isurugi Shrine, Hirose, Monzen, Wajima	120	Dextral	C-1 [a]
19	Ryugo Temple, Sakami, Shika, Hakui	58	Dextral	C-6 [a]
20	Jonen Temple, Notobe, Nakanoto, Kashima	56	Dextral	C-59
21	Tenpyou Temple, Mt. Sekido, Nakanoto, Kashima	73	Dextral	C-6 [a]
22	Tehayahime Shrine, Azuma, Hodatsushimizu, Hakui	94	Dextral	C-46 [a]
23	Hakusan Shrine, Kamiohta, Tsubata, Kahoku	82	Dextral	C-6 [a]
24	Ohkuninushi Shrine, Torigoe, Tsubata, Kahoku	102	No	C-60

586

587 [a]These clones are the same as clones previously detected in plantations (Ikeda, Tokuda,
588 and Tomaru 2018)

589 **Table 2** Values of the coefficient of relatedness (*r*) among 21 *Thujopsis dolabrata* var. *hondae* clones identified in old planted trees and plantations

	C-1	C-6	C-7	C-14	C-44	C-45	C-46	C-47	C-48	C-49	C-50	C-51	C-52	C-53	C-54	C-55	C-56	C-57	C-58	C-59	C-60
C-1																					
C-6	0.269																				
C-7	0.089	0.163																			
C-14	0.176	0.084	0.044																		
C-44	0.265	0.172	0.051	0.077																	
C-45	0.206	0.226	0.054	0.055	0.390																
C-46	0.248	0.191	0.035	0.167	0.116	0.071															
C-47	0.083	0.088	0.108	0.055	0.306	0.177	0.054														
C-48	0.328	0.312	0.256	0.137	0.309	0.453	0.134	0.115													
C-49	0.235	0.451	0.042	0.124	0.077	0.091	**0.543**	0.146	0.200												
C-50	0.105	0.371	0.111	0.183	0.275	0.072	0.209	0.310	0.101	**0.531**											
C-51	0.230	0.199	0.046	0.061	0.268	0.110	0.071	0.208	0.212	0.148	0.298										
C-52	0.307	0.231	0.180	0.340	0.061		0.112		0.240	0.153	0.149	0.109									
C-53	0.360	0.181		0.089	**0.629**	0.275	0.203	0.162	0.250	0.109	0.101	0.185	0.062								
C-54	0.161	0.116	0.095		**0.799**	0.262		0.178	0.260		0.193	0.206	0.076	**0.569**							
C-55	0.184	0.239	0.091		0.294	0.019	0.048		0.181	0.051			0.201	0.222	0.317						
C-56	0.050	0.162	0.072	0.038	0.034	0.195		0.167	0.066				0.058		0.016	0.053					
C-57	0.019	0.059	0.044	0.139	0.044	0.061	0.049	0.026	0.043	0.045	0.163		0.068		0.140	0.053	0.099				
C-58	0.081		0.068	0.121	0.157	0.060		0.115	0.077		0.091	0.008	0.035		0.136	0.071	0.001	0.049			
C-59	0.203	0.349	0.039		0.089		**0.515**		0.080	0.351	0.115		0.116	0.194	0.087	0.118		0.059			
C-60	0.053	0.084	0.121	0.082		0.003	0.138	0.059	0.135	0.138			0.122	0.044	0.033	0.155		0.214	0.069	0.057	

590

591 Blanks indicate that the values are 0.000. Values in bold are $r \geq 0.50$

Table 3 Comparison of genetic variability among clones identified in plantations and old planted trees and among individuals in natural populations of *Thujopsis dolabrata* var. *hondae*

	Region	Population[a]	*N*	A_R	H_E
Old planted trees			12	5.58	0.647
Plantations			14	4.90	0.616
Natural populations	Hokkaido & Aomori[b]	1–10	23.5	5.97	0.668
	Iwate & Yamagata[b]	11–14	24.0	5.94	0.649
	Niigata	15	31	5.67	0.633
	Ishikawa[b]	16, 17	24.0	5.39	0.586
	All[b]	1–17	24.1	5.88	0.652

N, number of samples; A_R, allelic richness based on 12 diploid individuals; H_E, gene diversity

[a] Numbers correspond to the population numbers in Table S1

[b] Average values over populations are shown

Table 4 Probabilities of the first, second and third most likely regions from which *Thujopsis dolabrata* var. *hondae* clones originated, based on a combination of assignment and exclusion tests

Clone	First		Second		Third	
	Population	Probability	Population	Probability	Population	Probability
C-1	**Ishikawa**	0.013	Iwate and Yamagata	0.000 *	Hokkaido and Aomori	0.000 *
C-6	**Ishikawa**	0.060	Niigata	0.005 *	Iwate and Yamagata	0.000 *
C-7	**Hokkaido and Aomori**	0.026	Ishikawa	0.005 *	Iwate and Yamagata	0.004 *
C-14	Iwate and Yamagata	0.001 *	Hokkaido and Aomori	0.001 *	Niigata	0.001 *
C-44	Ishikawa	0.111	Iwate and Yamagata	0.034	Hokkaido and Aomori	0.017
C-45	**Ishikawa**	0.164	Hokkaido and Aomori	0.008 *	Iwate and Yamagata	0.004 *
C-46	Ishikawa	0.002 *	Hokkaido and Aomori	0.000 *	Iwate and Yamagata	0.000 *
C-47	Hokkaido and Aomori	0.027	Ishikawa	0.012	Iwate and Yamagata	0.008 *
C-48	**Ishikawa**	0.078	Iwate and Yamagata	0.002 *	Hokkaido and Aomori	0.002 *
C-49	Ishikawa	0.008 *	Iwate and Yamagata	0.001 *	Niigata	0.000 *
C-50	Niigata	0.034	Ishikawa	0.015	Iwate and Yamagata	0.012
C-51	Ishikawa	0.177	Niigata	0.133	Iwate and Yamagata	0.130
C-52	**Iwate and Yamagata**	0.011	Ishikawa	0.004 *	Hokkaido and Aomori	0.003 *
C-53	Iwate and Yamagata	0.038	Ishikawa	0.010	Hokkaido and Aomori	0.009 *
C-54	Ishikawa	0.522	Iwate and Yamagata	0.478	Hokkaido and Aomori	0.411
C-55	**Ishikawa**	0.019	Hokkaido and Aomori	0.003 *	Iwate and Yamagata	0.001 *
C-56	Ishikawa	0.682	Niigata	0.300	Iwate and Yamagata	0.244
C-57	Ishikawa	0.215	Niigata	0.046	Hokkaido and Aomori	0.009 *
C-58	Niigata	0.134	Ishikawa	0.090	Iwate and Yamagata	0.068
C-59	**Ishikawa**	0.077	Niigata	0.002 *	Hokkaido and Aomori	0.000 *
C-60	Ishikawa	0.000 *	Hokkaido and Aomori	0.000 *	Iwate and Yamagata	0.000 *

602 Clones in boldface indicate those assigned to a single region, with criteria of probabilities greater than 0.01 for the first region and lower than 0.01

603 for the second and third regions

604 *, probability < 0.01

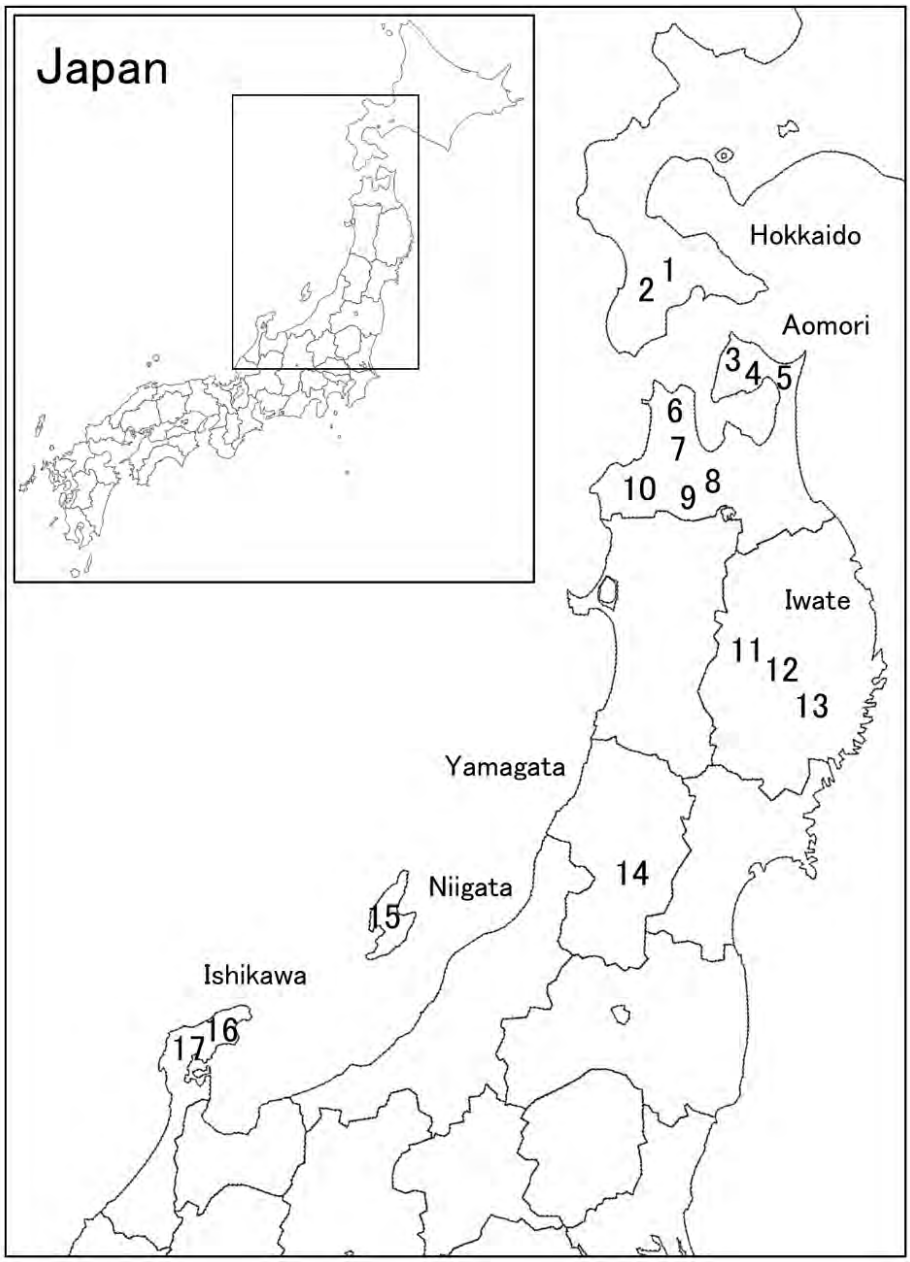

Fig. 1 Locations of 17 natural *Thujopsis dolabrata* var. *hondae* populations used in an assignment test as potential source populations for the clones identified from old planted trees and plantations. Numbers on the map correspond to the population numbers in Table S1

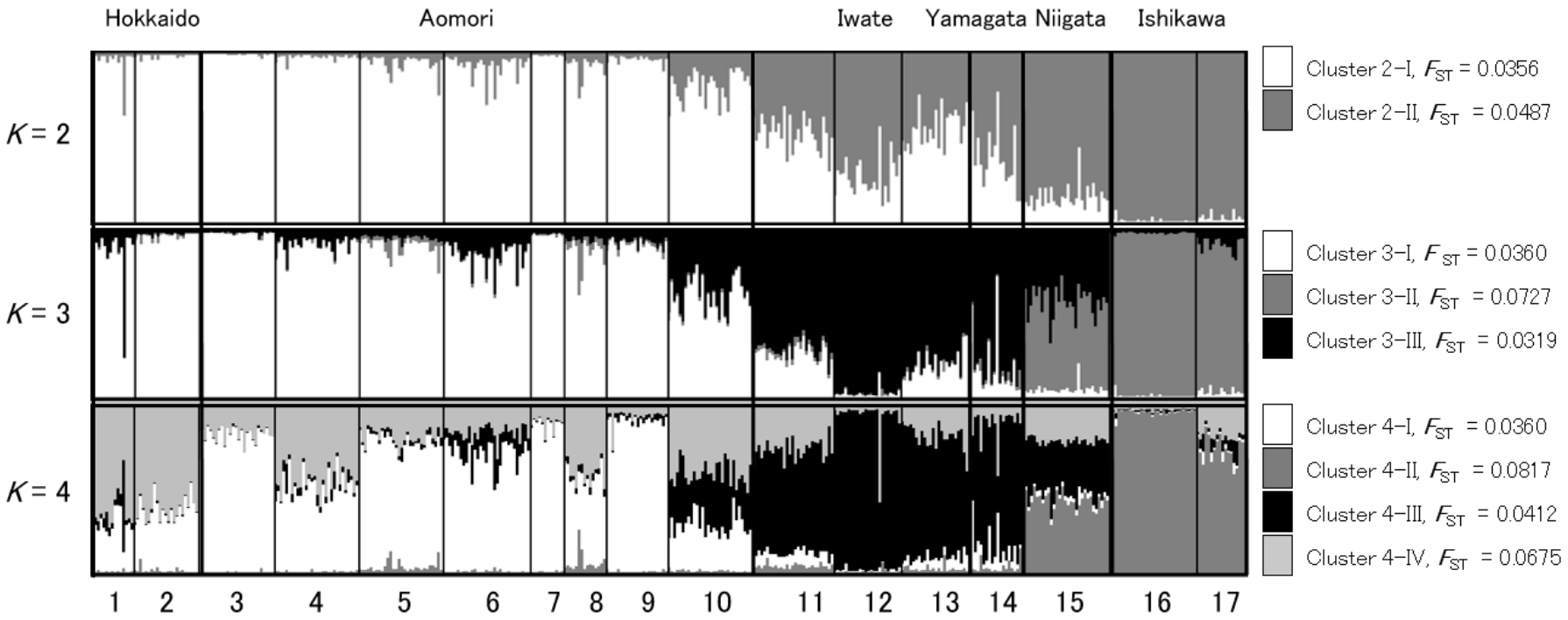

Fig. 2 Distribution of genetic clusters in each individual, at numbers of clusters (K) = 2, 3 and 4, for the 17 natural populations of *Thujopsis dolabrata* var. *hondae*, obtained by STRUCTURE analysis. F_{ST} values for the clusters indicate their genetic divergence from the ancestral population. Numbers below the chart correspond to the population numbers in Table S1.

Table S1 Locality, number of samples and level of genetic variability in old planted trees, plantations and natural populations of *Thujopsis dolabrata* var. *hondae*

Population no.	Locality	*N*	*A*	A_R	H_O	H_E	F_{IS}[a]		*r*
Old planted trees	Ishikawa	12	5.58	5.58	0.588	0.647			0.102
Plantations	Ishikawa	14	5.11	4.90	0.568	0.616			0.183
Natural populations									
1	Minamidate, Assabu, Hokkaido	15	6.32	5.86	0.646	0.662	0.025		0.015
2	Todogawa, Esashi, Hokkaido	23	6.68	5.54	0.593	0.638	0.071		0.034
3	Okoppe, Aomori	27	7.53	5.97	0.673	0.674	0.002		0.028
4	Ohata, Aomori	30	8.00	6.02	0.628	0.676	0.071	*	0.026
5	Higashidori, Aomori	30	7.84	5.93	0.646	0.652	0.010		0.029
6	Nakazato, Nakadomari, Aomori	31	8.68	6.55	0.689	0.693	0.005		0.027
7	Ajigasawa, Aomori	12	5.84	5.84	0.746	0.671	-0.112		0.049
8	Mt. Cyobosan, Aomori	15	6.53	6.12	0.662	0.683	0.032		0.016
9	Owani, Aomori	22	6.84	5.76	0.624	0.652	0.042		0.029
10	Fukaura, Aomori	30	7.79	6.08	0.681	0.675	-0.009		0.028
11	Shizukuishi, Iwate	29	7.89	6.13	0.679	0.675	-0.006		0.028
12	Mt. Hayachine, Iwate	24	6.79	5.56	0.618	0.616	-0.004		0.027
13	Mt. Goyosan, Iwate	24	7.63	6.19	0.672	0.664	-0.012		0.025
14	Kaminoyama, Yamagata	19	6.68	5.87	0.637	0.641	0.006		0.033
15	Sado, Niigata	31	7.26	5.67	0.637	0.633	-0.005		0.033
16	Mt. Horyu, Ishikawa	31	6.42	5.03	0.514	0.567	0.093	*	0.050
17	Suzu, Ishikawa	17	6.47	5.74	0.573	0.605	0.054		0.060
Average		24.1	7.13	5.88	0.642	0.652			0.031

N, number of samples; *A*, number of alleles per locus; A_R, allelic richness based on 12 diploid individuals; H_O, observed heterozygosity; H_E, gene diversity; F_{IS}, inbreeding coefficient; *r*, coefficient of relatedness.

[a]The significance of departures from Hardy-Weinberg equilibrium was tested by randomization tests. Bonferroni correction was used to adjust *P*-values in multiple tests.

* $P < 0.05$.

Table S2 Genetic diversity at 19 microsatellite makers across 17 natural *Thujopsis dolabrata* var. *hondae* populations

Locus	TA	H_O	H_S	H_T	F_{IS}[a]	F_{ST}[b]	G'_{ST}
Tdest1	18	0.920	0.881	0.903	-0.040	0.025 **	0.213
Tdest3	10	0.428	0.452	0.456	0.070	0.011	0.013
Tdest11	14	0.809	0.831	0.849	0.029	0.016 **	0.131
Tdest14	18	0.864	0.884	0.896	0.006	0.013 **	0.118
Tdest17	11	0.654	0.678	0.780	0.040	0.136 **	0.424
Tdest21	28	0.890	0.903	0.926	0.020	0.025 **	0.272
Tdest24	16	0.695	0.725	0.754	0.037	0.037 **	0.144
Tdest29	9	0.461	0.473	0.498	0.043	0.048 **	0.096
Tdest35	33	0.863	0.910	0.939	0.048 *	0.031 **	0.364
Tdest37	4	0.417	0.419	0.418	-0.009	-0.002	-0.004
Tdest38	9	0.666	0.661	0.699	0.006	0.059 **	0.169
Tdest39	7	0.588	0.555	0.576	-0.045	0.033 **	0.086
Tdest42	11	0.557	0.563	0.585	0.004	0.036 **	0.090
Tdest43	14	0.765	0.752	0.783	-0.012	0.045 **	0.165
Tdest45	8	0.520	0.601	0.635	0.143 **	0.061 **	0.140
Tdest49	5	0.150	0.158	0.169	0.031	0.058 **	0.073
Tdest53	20	0.897	0.867	0.880	-0.031	0.017 **	0.119
Tdest56	6	0.661	0.650	0.686	-0.010	0.057 **	0.155
Tdest58	12	0.396	0.418	0.436	0.081	0.040 **	0.072
Average / overall	7.2	0.642	0.652	0.677	0.018 **	0.039 ***	0.114

TA, total number of alleles detected; H_O, observed heterozygosity; H_S, average gene diversity within populations; H_T, gene diversity in the total population; F_{IS}, inbreeding coefficient; F_{ST}, genetic differentiation measure; G'_{ST}, standardized genetic differentiation measure (Hedrick 2005).

[a]The significance of departures from Hardy-Weinberg equilibrium was tested by randomization tests.

[b]The significance of genetic differentiation among populations was tested by randomization tests.

Bonferroni correction was used to adjust *P*-values in multiple tests. * $P < 0.05$, ** $P < 0.01$, *** $P<0.001$.

Table S3 Results of analysis of molecular variance (AMOVA) of 17 natural populations of *Thujopsis dolabrata* var. *hondae* in four regions

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation (%)	Φ-Statistics	Probability
Among regions	3	456.4	1.38	8.7	Φ_{RT} = 0.087	< 0.001
Among populations within regions	13	464.2	0.95	5.9	Φ_{PR} = 0.065	< 0.001
Within populations	393	5356.5	13.63	85.4	Φ_{PT} = 0.146	< 0.001

d.f., degree of freedom

Probabilities were based on permutation tests

Table S4 Results from a self-assignment test for 410 individuals from 17 natural populations of *Thujopsis dolabrata* var. *hondae* in four regions

			Assigned region			
Source region	*N*	%	Hokkaido and Aomori	Iwate and Yamagata	Niigata	Ishikawa
Hokkaido and Aomori	235	92.3	216	16	2	0
Iwate and Yamagata	96	85.3	9	**81**	4	1
Niigata	31	80.6	1	4	**25**	1
Ishikawa	50	80.0	0	1	9	**40**
Total	410	88.3	226	102	40	42

N, total number of samples in each region; %, percentage of samples that were assigned to the regions from which they were sampled

The numbers in boldface indicate the numbers of samples assigned to the originating regions

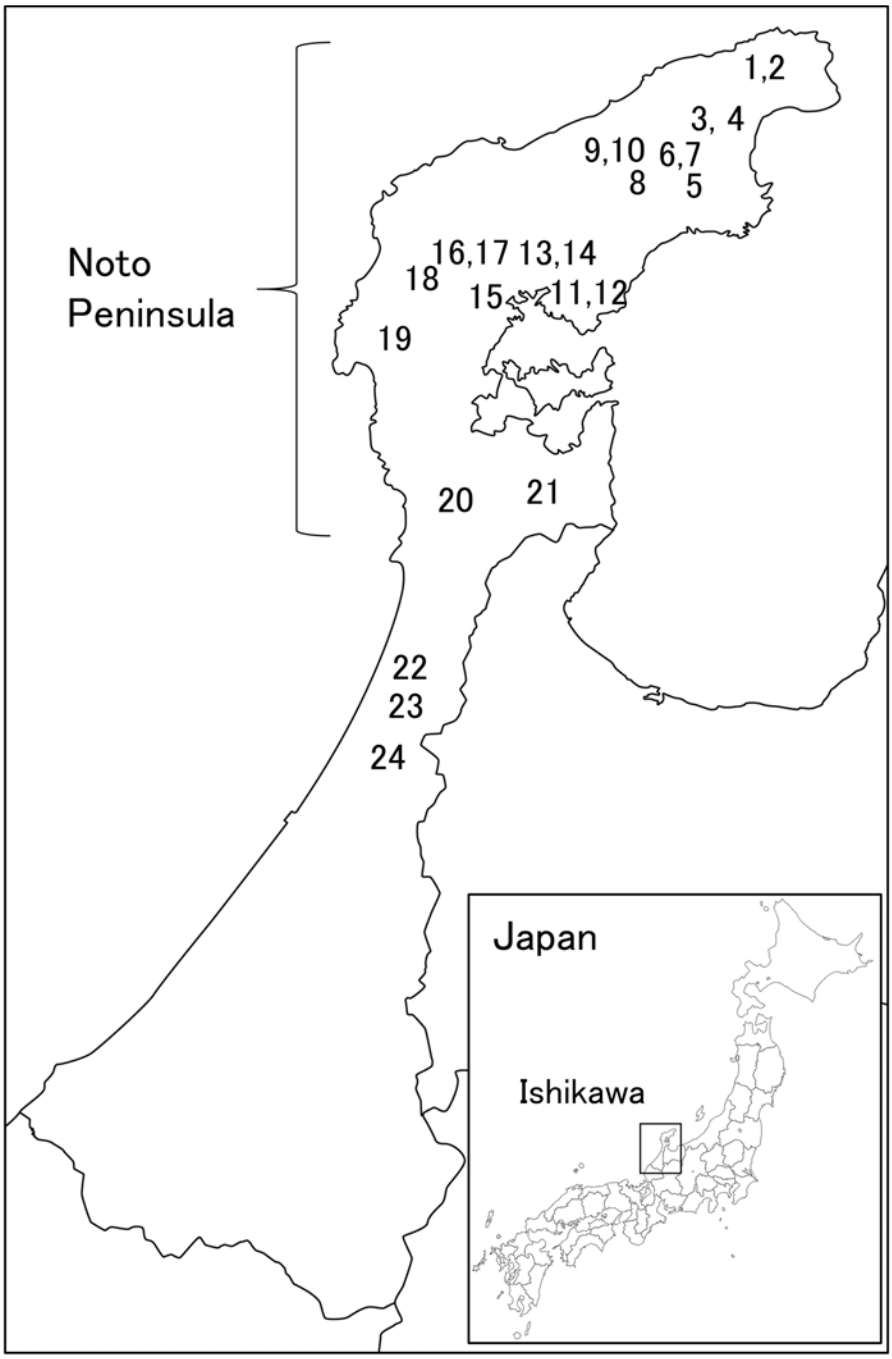

Fig. S1 Locations of the 24 old planted *Thujopsis dolabrata* var. *hondae* trees sampled at 12 shrines, four temples and one private garden in Ishikawa Prefecture, Japan. Numbers on the map correspond to the old tree numbers in Table 1

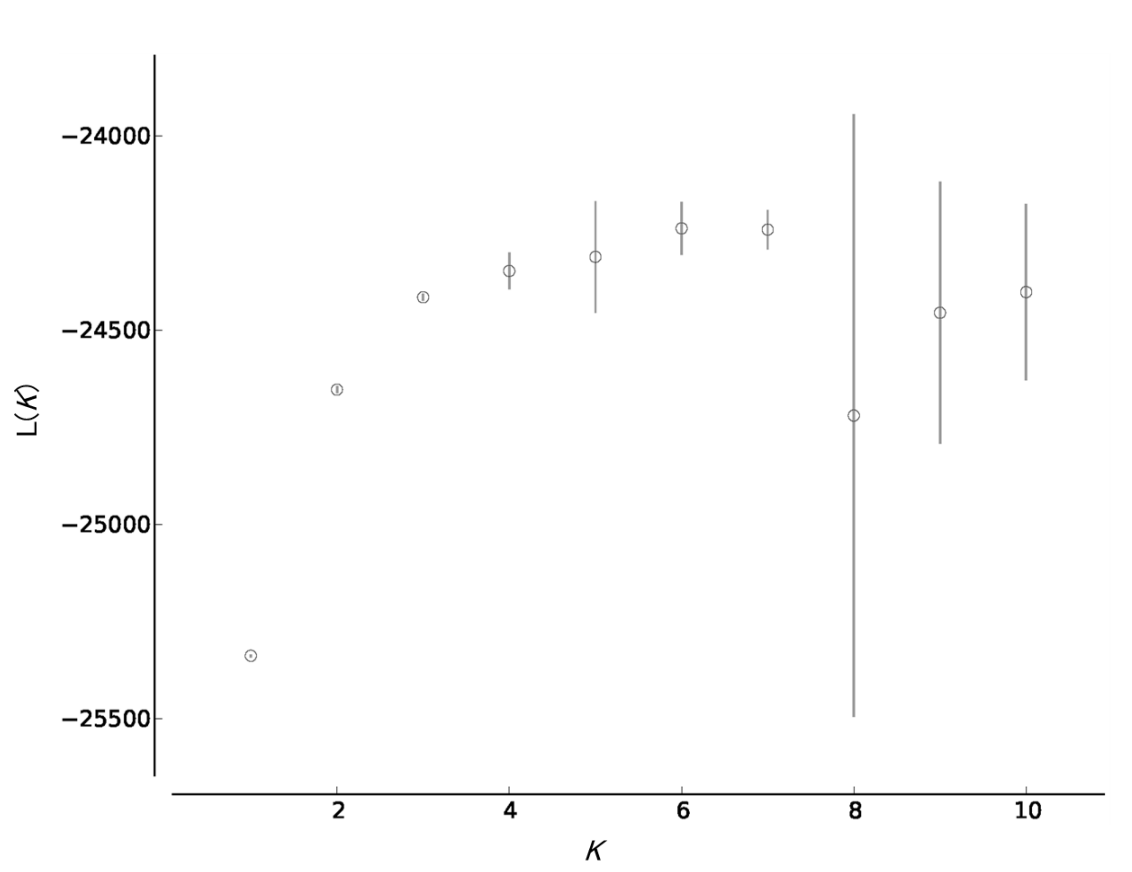

Fig. S2 Change in the mean ± standard deviation of log probability of the data, L (*K*), with increasing number of clusters (*K*), obtained using STRUCTURE analysis of the 17 natural populations of *Thujopsis dolabrata* var. *hondae*

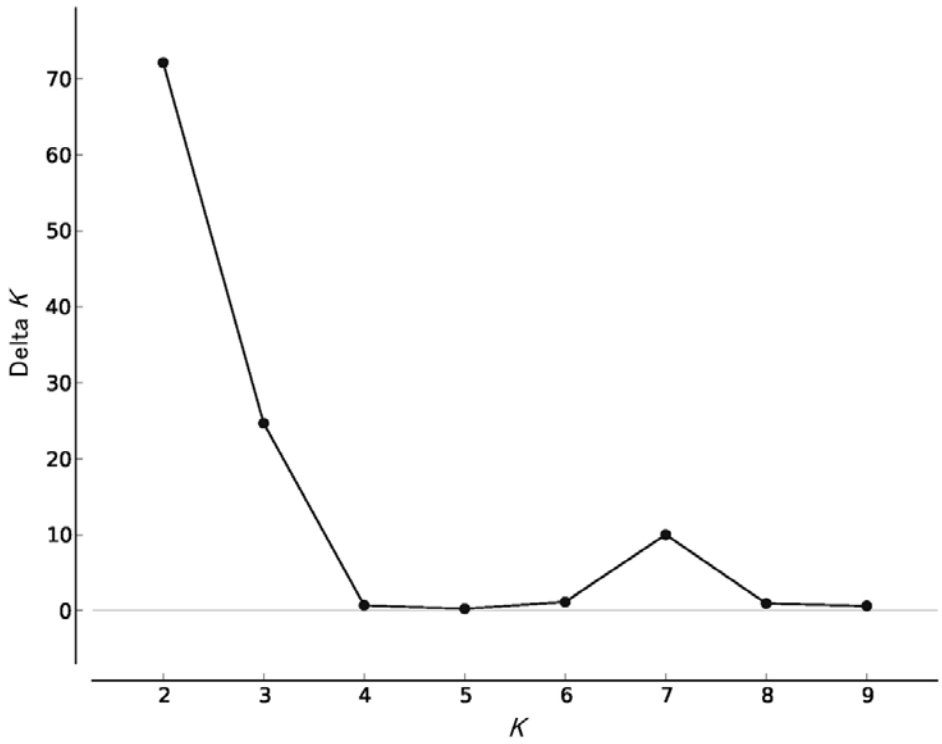

Fig. S3 Change in delta *K* with increasing number of clusters (*K*), obtained using STRUCTURE analysis of the 17 natural populations of *Thujopsis dolabrata* var. *hondae*