

Article

Marker-Assisted Selection of Trees with *MALE STERILITY 1* in *Cryptomeria japonica* D. Don

Yoshinari Moriguchi ^{1,*} , Saneyoshi Ueno ², Yoichi Hasegawa ², Takumi Tadama ¹, Masahiro Watanabe ¹, Ryunosuke Saito ¹, Satoko Hirayama ³, Junji Iwai ⁴ and Yukinori Konno ⁵

¹ Graduate School of Science and Technology, Niigata University, 8050, Igarashi 2-Nocho, Nishi-ku, Niigata 950-2181, Japan; tama52178@gmail.com (T.T.); f20e024j@mail.cc.niigata-u.ac.jp (M.W.); f19e013a@mail.cc.niigata-u.ac.jp (R.S.)

² Department of Forest Molecular Genetics and Biotechnology, Forestry and Forest Products Research Institute, Forest Research and Management Organization, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan; saueno@ffpri.affrc.go.jp (S.U.); yohase@ffpri.affrc.go.jp (Y.H.)

³ Niigata Prefecture Niigata Regional Promotion Bureau, 2009 Akiha-ku Hodojima, Niigata 956-8635, Japan; hirayama.satoko@pref.niigata.lg.jp

⁴ Niigata Prefectural Forest Research Institute, 2249-5 Unotoro, Murakami, Niigata 958-0264, Japan; iwai.junji@pref.niigata.lg.jp

⁵ Miyagi Prefectural Forest Research Institute, 3-8-1 Aoba-ku Honmachi, Sendai 980-8570, Japan; konno-yu943@pref.miyagi.lg.jp

* Correspondence: chimori@agr.niigata-u.ac.jp; Tel.: +81-25-262-6861

Received: 25 May 2020; Accepted: 3 July 2020; Published: 6 July 2020



Abstract: The practical use of marker-assisted selection (MAS) is limited in conifers because of the difficulty with developing markers due to a rapid decrease in linkage disequilibrium, the limited genomic information available, and the diverse genetic backgrounds among the breeding material collections. First, in this study, two families were produced by artificial crossing between two male-sterile trees, ‘Shindai11’ and ‘Shindai12’, and a plus tree, ‘Suzu-2’ (*Ms1/ms1*) (S11-S and S12-S families, respectively). The segregation ratio between the male-sterile and male-fertile trees did not deviate significantly from the expected 1:1 ratio in either family. These results clearly suggested that the male-sterile gene of ‘Shindai11’ and ‘Shindai12’ is *MALE STERILITY 1* (*MS1*). Since it is difficult to understand the relative positions of each marker, due to the lack of a linkage map which all the closely linked markers previously reported are mapped on, we constructed a partial linkage map of the region encompassing *MS1* using the S11-S and S12-S families. For the S11-S and S12-S families, 19 and 18 markers were mapped onto the partial linkage maps of the *MS1* region, respectively. There was collinearity (conserved gene order) between the two partial linkage maps. Two markers (CJt020762_ *ms1-1* and reCj19250_2335) were mapped to the same position as the *MS1* locus on both maps. Of these markers, we used CJt020762 for the MAS in this study. According to the MAS results for 650 trees from six prefectures of Japan (603 trees from breeding materials and 47 trees from the Ishinomaki natural population), five trees in Niigata Prefecture and one tree in Yamagata Prefecture had heterozygous *ms1-1*, and three trees in Miyagi Prefecture had heterozygous *ms1-2*. The results obtained in this study suggested that *ms1-1* and *ms1-2* have different geographical distributions. Since MAS can be used effectively to reduce the labor and time required for selection of trees with a male-sterile gene, the research should help ensure that the quantity of breeding materials will increase to assist future tree-breeding efforts.

Keywords: conifer; linkage map; male sterility; marker-assisted selection

1. Introduction

Molecular marker-assisted selection (MAS), which can reduce the time required for a breeding cycle, is an attractive method for conifers, which have longer generation times than those of most crop species [1]. However, in conifers, the practical use of MAS is limited because it is difficult to develop markers for MAS due to a rapid decrease in linkage disequilibrium, the limited genomic information available, and the diverse genetic backgrounds among the breeding material collections. Nevertheless, the progress with genome analysis technologies has recently accelerated, producing an enormous volume of sequences and the subsequent development of markers linked to a particular target gene.

Sugi (*Cryptomeria japonica* D. Don) is an important forestry species that occupies nearly 4.5 million hectares of planted forest in Japan, which corresponds to approximately 44% of all the planted forest area in the country [2]. The increase in area covered by planted *C. japonica* has triggered pollinosis. *C. japonica* pollinosis is one of the most serious allergies in Japan, affecting 26.5% of the Japanese population [3]. As a countermeasure against *C. japonica* pollinosis, the use of male-sterile individuals is effective. The first male-sterile tree ('Toyama1') in *C. japonica*, which produced no pollen grains, was found in Toyama Prefecture in 1992 [4]. Genetic male sterility was found to be conferred by a major recessive gene [5], *MALE STERILITY 1 (MS1)* [6]. Hasegawa et al. [7] reported that *MS1* was different from a gene reported in *Arabidopsis* (GenBank Accession AJ344210, [8]); however, its protein structure showed functional and structural similarities to wheat male-sterile genes (*ms1* and *ms5*). Since the discovery of this individual, six male-sterile trees homozygous for *MS1* (*ms1/ms1*) have been selected ('Shindai3', 'Fukushima-funen1', 'Fukushima-funen2', 'Tahara-1', 'Sosyun', and 'Mie-funen1') [6,9–14]. The frequency of these male-sterile trees in the forest is considered to be very low, because Igarashi et al. [9] identified only two male-sterile trees in a screening of 8700 trees distributed across a 19-ha planted forest. Male-sterile trees are generally identified by observing pollen release and/or by the direct inspection of the male strobili using a magnifying glass or microscope. In the selected male-sterile trees, the confirmation of the male-sterile gene *MS1* was made based on the results of test crossings. These test crossings led to the discovery of three other male-sterile genes: *MS2*, *MS3*, and *MS4* [5,6,15,16]. In some male-sterile trees such as 'Shindai11' and 'Shindai12', male-sterile genes have not yet been investigated.

Mutations in the *MS1* gene lead to the collapse of microspores after the separation of pollen tetrads [17], whereas that of the *MS2* gene lead to the formation of microspore clumps after normal microsporogenesis [15]. On the other hand, mutations in the *MS3* and *MS4* genes lead to the formation of microspores of various sizes after normal microsporogenesis [15,16]. The four male-sterile genes *MS1*, *MS2*, *MS3*, and *MS4* have been mapped to different linkage groups: the ninth (referred to as LG9 hereafter), fifth, first, and fourth linkage groups, respectively [17–19]. Only one tree with *ms2*, *ms3*, and *ms4* was selected, respectively. Therefore, trees with *ms1* have generally been used for tree improvement and seedling production. Both male-sterile trees and also trees heterozygous for the male-sterile gene are important for tree improvement and seed production as the maternal and paternal parents, respectively. Currently, seven trees heterozygous for *MS1* (*Ms1/ms1*), 'Suzu-2', 'Naka-4', 'Ooi-7', 'Ohara-13', 'Zasshunbo', 'Kamiukena-16', and 'Kurihara-4', have been selected ([4,6,14,20,21], Konno, personal communication). For the precise selection of trees heterozygous for *MS1*, it is generally necessary to produce F_1 trees by artificial crossing and to confirm whether these F_1 trees are male-sterile or -fertile trees. Confirmation is performed by the direct inspection of male strobili using a magnifying glass (or a microscope) or by observing the pollen release.

Due to the large amount of labor required for selection, the number of trees with the male-sterile gene is not sufficient. To reduce the labor of screening, the MAS of trees with the male-sterile gene is necessary. Recently, some markers closely linked to the *MS1* gene or derived from a putative *MS1* gene have been developed [22–25]. Moriguchi et al. [22] and Ueno et al. [25] reported that estSNP04188 and dDcontig_3995-165 were 1.8 cM and 0.6 cM from *MS1* in the T5 family (173 trees), respectively. Hasegawa et al. [23] reported that 15 markers were 0 cM from *MS1* in the F1O7 family (84 trees). Among these, AX-174127446 showed a high rate of predicting trees with *ms1*. Mishima et al. [24] reported two markers from contig "reCj19250" that can be used to select trees with *ms1*. On the

other hand, Hasegawa et al. [7] reported a candidate male-sterile gene CJt020762 at the *MS1* locus, and all the breeding materials with the allele *ms1* had either a 4-bp or 30-bp deletion in the gene (they defined these alleles as *ms1-1* and *ms1-2*, respectively). Both of these were expected to result in faulty gene transcription and function; therefore, they developed two markers [26] from contig “CJt020762”. The lack of a linkage map for these markers constructed from the same family makes it difficult to understand the relative position of each marker.

Therefore, in this study, we (1) checked whether the male-sterile gene of Shindai11 and Shindai12 was *MS1*, based on the results of test crossings, (2) constructed a partial linkage map of the region encompassing *MS1* using 46 markers, and (3) selected the trees with *ms1* by MAS. As there are few studies pertaining to the practicable applications of MAS in conifers, this study should provide a valuable model.

2. Materials and Methods

2.1. Phenotyping of Male Sterility and Single Nucleotide Polymorphism (SNP) Genotyping for Linkage Analysis

We used two families, S11-S and S12-S, in this study. These families were produced by artificial crossing between two male-sterile trees, ‘Shindai11’ and ‘Shindai12’, and a plus tree, ‘Suzu-2’ (*MS1/ms1*), during March of 2016. These trees are considered to be diploid ($2n = 22$), because two or fewer alleles have been obtained from microsatellite analyses and they are able to produce an amount of seeds. Strobili production was promoted by spraying the trees with gibberellin-3 (100 ppm) in July 2018. Approximately five male strobili were sampled from each individual from early November to early December 2018. Each sampled male strobilus was bisected vertically with a razor, and male sterility was determined using a microscope (SZ-ST, Olympus, Tokyo, Japan). Individuals without male strobili and individuals in whom it was difficult to discriminate male sterility were excluded from further analysis. Finally, 130 individuals from S11-S and 138 individuals from S12-S were used to construct a linkage map. Needle tissue was collected from three parent trees (‘Shindai11’, ‘Shindai12’, and ‘Suzu-2’) and all the F_1 trees (268 trees) of two mapping populations. Genomic DNA was extracted from these needles using a modified hexadecyltrimethylammonium bromide (CTAB) method [26,27].

Single nucleotide polymorphism (SNP) markers from contigs “reCj19250” and “CJt020762” [7,24] and SNP markers mapped to LG9 [23,25,28] were used to construct a partial linkage map of the region encompassing the *MS1* locus for each of the two families (because the gene was located in LG9) [18]. For estSNP00204 [22], AX-174127446 [23], and CJt020762 [7], the SNaPshot assay, which extends primers by a single base, was used for genotyping. The primer sequences used to target the three markers in the SNaPshot assay (estSNP00204 [22], AX-174127446 [23], and CJt020762 [7]) are shown in Table S1. Although CJt020762 contained a 4-bp and 30-bp deletion, we used the 4-bp deletion for primer design because there is no polymorphism associated with the 30-bp deletion between the parents of the mapping populations. Multiplex polymerase chain reaction (PCR) was performed using three primer pairs and the Multiplex PCR Kit (QIAGEN, Hilden, Germany). Each reaction contained 2× QIAGEN multiplex PCR master mix, 1 µL primer mix (2.5 µM for each primer), and 40 ng genomic DNA in a total volume of 6 µL. Amplification was performed in the Takara PCR Thermal Cycler (Takara, Tokyo, Japan) using an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1.5 min, and extension at 72 °C for 1 min, with a final extension at 60 °C for 30 min. To remove any primers and dNTPs, 5.0 µL of the PCR products were treated with 2.0 µL ExoSAP-IT reagent (Thermo Fisher Scientific, Waltham, MA, USA), followed by incubation at 37 °C for 30 min and then 80 °C for 15 min to inactivate the enzyme. Single-base extension reactions were carried out in a 5.0 µL final volume containing 0.5 µL SNaPshot Multiplex Ready Mix (Thermo Fisher Scientific), 1 µL primer mix (1.0 µM for each primer), and 2.0 µL of the treated PCR products. The reactions were performed in the Takara PCR Thermal Cycler (Takara) with 25 cycles of denaturation at 96 °C for 10 s and annealing and elongation at 60 °C for 30 s. The final extension products were treated with 1 U shrimp alkaline phosphatase (Thermo Fisher Scientific) and

incubated at 37 °C for 1 h, followed by enzyme inactivation at 80 °C for 15 min. The PCR products (1.0 µL) were mixed with 0.2 µL GeneScan 120 LIZ size standard and 8 µL Hi-Di formamide prior to electrophoresis. Capillary electrophoresis was performed on the 3130xl genetic analyzer using POP-7 (Thermo Fisher Scientific), and the alleles were analyzed using GeneMaker v2.4.0 software (SoftGenetics, State College, PA, USA). For the other 43 SNP markers mapped to LG9, genotyping was performed using the 48.48 Dynamic Array (Fluidigm, South San Francisco, CA, USA). For the 48.48 Dynamic Array, 6.25 ng genomic DNA per sample (at a concentration of 5 ng/µL) were used for specific target amplification. The assays were performed following the protocol provided by the manufacturer. The data obtained were analyzed using Fluidigm SNP Genotyping Analysis software (ver. 4.5.1). The primer information is provided in Table S2.

Chi-square tests were performed for each locus to assess the deviation from the expected Mendelian segregation ratio. Loci showing an extreme segregation distortion ($p < 0.01$) and with many missing data points (more than five individuals) were excluded from further linkage analysis. The linkage analyses were performed using the maximum likelihood mapping algorithm in JoinMap ver. 4.1 software (Kryazma, Wageningen, The Netherlands) with a cross pollination-type population (hk × hk, lm × ll, and nn × np) and two rounds of map calculation [29]. The markers were assigned to the LG9 linkage group using a logarithm of odds ratio threshold of 8.0, which was the same value as in previous reports on *C. japonica* [18,19,22,28]. The maximum likelihood mapping algorithm was used to determine the marker order in the linkage group. The map distance was calculated using the Kosambi mapping function [30]. The default settings were used for the recombination frequency threshold and ripple value.

2.2. MAS of Trees with *ms1*

The needles for MAS selection were collected from breeding materials in Niigata (Tohoku breeding region), Yamagata (Tohoku breeding region), Miyagi (Tohoku breeding region), Shizuoka (Kanto breeding region), Tottori (Kansai breeding region), and Kumamoto (Kyushu breeding region) Prefectures with sample numbers of 238, 163, 30, 34, 72, and 66, respectively. In the samples from Miyagi Prefecture, Kurihara-4, a tree heterozygous for *MS1*, was included. Genomic DNA was extracted from these needles using a modified CTAB method [26,27]. In addition, we also performed MAS selection using previously extracted DNA from 47 *C. japonica* trees in the Ishinomaki natural population of Miyagi Prefecture, where clonal analysis was performed in 2017 [31].

Based on the sequence information of CJt020762, Hasegawa et al. [26] developed two primer pairs that sandwiched the two deletions, respectively. These two markers were used for MAS selection in this study. PCR amplifications were performed in 10 µL reaction volumes containing 5 ng of genomic DNA, 1× PCR Kapa2G buffer with 1.5 mM MgCl₂, 0.2 µL of 25 mM MgCl₂, 0.2 µL of 10 mM each dNTP mix, 0.4 µL of 5 µM forward primers labeled with dye (CJt020762_*ms1*-1_F and CJt020762_*ms1*-2_F), 0.2 µL 5 µM reverse primers (CJt020762_*ms1*-1_R and CJt020762_*ms1*-2_R), 5 ng template DNA, and 0.5 U KAPA2G Fast PCR enzyme (KAPA2G Fast PCR kit; KAPA Biosystems, Wilmington, USA). Amplification was performed on the Takara PCR Thermal Cycler (Takara) under the following conditions: initial denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation for 15 s at 95 °C, annealing for 15 s at 60 °C, extension for 1 s at 72 °C, and a final extension for 1 min at 72 °C. The PCR products and the DNA size marker (LIZ600; Thermo Fisher Scientific) were separated by capillary electrophoresis on the ABI 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The DNA fragments were detected using the GeneMarker software (ver. 2.4.0; SoftGenetics, State College, PA, USA).

3. Results and Discussion

3.1. Linkage Maps of the *MS1* Region

Of the 130 progenies in S11-S family produced by artificial crossing between ‘Shindai11’ and ‘Suzu-2’, 75 were male-fertile and 55 male-sterile. On the other hand, of the 138 progenies in S12-S family produced by artificial crossing between ‘Shindai12’ and ‘Suzu-2’, 65 were male-fertile and 73 male-sterile. The segregation ratio between the male-sterile and male-fertile trees in the S11-S and S12-S progenies did not deviate significantly from the expected ratios of 1:1 ($\chi^2 = 0.31$ ($p = 0.08$) and 0.46 ($p = 0.50$), respectively). These results clearly suggested that the male-sterile gene of ‘Shindai11’ and ‘Shindai12’ was *MS1*. Based on observations using a microscope, Miura et al. [32] reported that the male-sterile phenotype of ‘Shindai11’ and ‘Shindai12’ was similar to those of ‘Fukushima-funen1’, ‘Fukushima-funen2’, and ‘Shindai3’, which are regulated by the *MS1* gene [6,9]. These previous observational results obtained by microscopy were consistent with the results in this study.

The 19 and 18 markers were mapped onto the partial linkage maps of the region encompassing *MS1* for the S11-S and S12-S families, respectively (Figure 1). There was collinearity (conserved gene order) among the two partial linkage maps. Two markers (CJt020762_ *ms1-1* and reCj19250_2335) were mapped to the same position as the *MS1* locus in both maps. Of these markers, reCj19250_2335 could not be selected ‘Ooi-7’ heterozygous for *MS1* (*ms1-2/MS1*), suggesting that reCj19250 was not the causative gene of *MS1*; the marker did not select trees with *ms1* with 100% accuracy [7,23]. As genome sequencing has now been completed in *C. japonica*, the question of whether these markers are located close to each other within the genome will probably be investigated in the near future. The gene CJt020762 was found to code for a lipid transfer protein, and the loss of protein function was predicted in breeding materials with *ms1* due to the 4-bp and the 30-bp deletions in the coding region [7]. CJt020762 also showed functional and structural similarity with wheat male-sterile genes; the mRNA sequence from CJt020762 showed a two-fold higher expression in male-fertile strobili than in male-sterile strobili [7]. Therefore, we used CJt020762 for the MAS in this study.

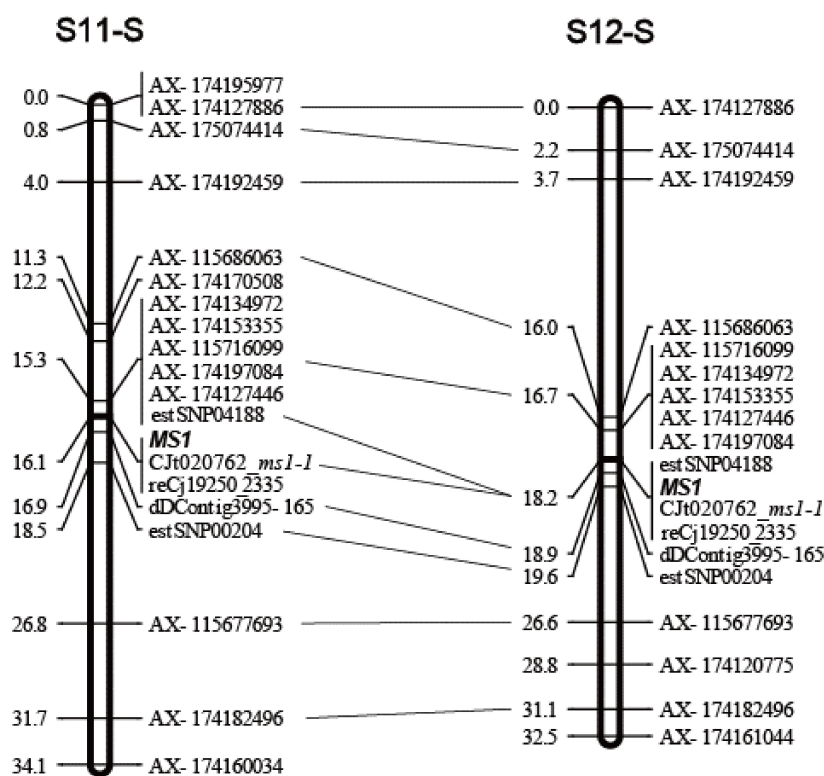


Figure 1. Partial linkage maps of the region encompassing *MS1* in the S11-S and S12-S *C. japonica* families.

3.2. MAS of Trees with *ms1*

In the MAS results of this study, we found that five trees in Niigata Prefecture ('Kashiwazakishi-1', 'Setsugai Niigata-6', 'Setsugai Murakami-2', 'Setsugai Aikawa-8', and 'Kamikiri Niigata-55') and one tree in Yamagata Prefecture ('Taisetsu Yamagata-8') had heterozygous *ms1-1*, and three trees in Miyagi Prefecture ('Kurihara-4' and two trees in the natural population) had heterozygous *ms1-2*. Two male-sterile trees in Niigata Prefecture ('Shindai11' and 'Shinadai-12') used as the mother trees of the mapping families had homozygous *ms1-1*. The two trees with *ms1-2* in the Ishinomaki natural forest (Ishinomaki_J284 and Ishinomaki_J278) were considered to have a parent–child relationship according to their genotypes. Because Hasegawa et al. [7] also found trees with *ms1-2* in this forest, trees with *ms1-2* may be distributed at a high frequency in this forest. Through further selections from this natural forest, it may be possible to obtain more breeding materials for male sterility.

Because half of the offspring in the mapping family 'Fukushima-funen1' (*ms1-1/ms1-1*) × 'Ooi-7' (*Ms1/ms1-2*) [23] showed male sterility, both of the trees with *ms1-1* and *ms1-2* can be used in a breeding program. Therefore, MAS should target both the *ms1-1* and *ms1-2* alleles. In Niigata Prefecture, where three male-sterile trees ('Shindai3' (*ms1-1/ms1-1*), 'Shindai11' (*ms1-1/ms1-1*), 'Shindai12' (*ms1-1/ms1-1*)) have been found thus far [6,7,32], five trees heterozygous for *MS1* (*Ms1/ms1-1*) were newly found among the 238 trees. This higher number of trees with *ms1* may be due to the large number of samples analyzed in this study. However, considering the achievements attained in the past selection, the rate of trees with *ms1* in Niigata Prefecture appears to be higher than the rates of other prefectures. In Miyagi Prefecture, where one tree heterozygous for *MS1* ('Kurihara-4', *Ms1/ms1-2*) was found thus far [Konno, personal communication], two trees with *ms1-2* (they have a parent–child relationship) were newly found among the 77 trees. Thus, the deletion mutations detected in Niigata Prefecture (*ms1-1*) and Miyagi Prefecture (*ms1-2*) were different. These results suggest that *ms1-1* and *ms1-2* may have different geographical distributions. Among the four breeding regions in *C. japonica*, the Tohoku breeding region has a relatively large amount of breeding materials for male sterility (Figure 2). However, the breeding materials for male sterility in the Kanto and Kansai breeding regions are still fewer than those in the Tohoku breeding region, and there are no breeding materials for male sterility in the Kyushu breeding region.

In this study, 650 trees were examined for male-sterile alleles. The precise selection of trees heterozygous for *MS1* using a magnifying glass or a microscope requires considerable labor, time (approximately 5 years: 1 year to promote flowering, 1 year for seed production, and 3 years to confirm male sterility), and space (approximately 56 seedlings per 1 m²). Labor includes gibberellin treatment, artificial crossing (i.e., pollen collection, male strobilus removal, pollination bag setting and pollen application), seed collection, field plowing, seed sowing, watering, fertilizer application, weeding, pesticide application, gibberellin treatment, and the observation of male strobili. For the examination of 650 trees using this method, firstly, we have to prepare 19,500 F₁ seedlings (age, 3 years) by 650 artificial crossings (30 F₁ seedlings per clone); in contrast, the MAS performed in this study (consisting of fragment analysis using a sequencer) requires approximately two weeks to complete, including DNA extraction (approximately 100 samples per day) and genotyping (approximately 200 samples per day). Since MAS is effective for reducing the amount of labor and time required to select trees with the male-sterile allele, the research should help ensure that the quantity of breeding materials will increase to assist future tree-breeding efforts. Hasegawa et al. [26] developed allele specific PCR (ASP) and amplified length polymorphism (ALP) markers to analyze *ms1-1* and *ms1-2*, respectively, without a sequencer. In a laboratory without a sequencer, these markers are effective for performing MAS. If both *ms1-1* and *ms1-2* must be analyzed, the total estimated cost of fragment analysis by sequencer (approximately 236.7 US\$ for 96 samples) is very similar to that of the ASP and ALP marker-based analysis (approximately 241.6 US\$ for 96 samples) (Table S3). These estimates may be further reduced through the development of DNA extraction methods with greater efficiency and lower cost.

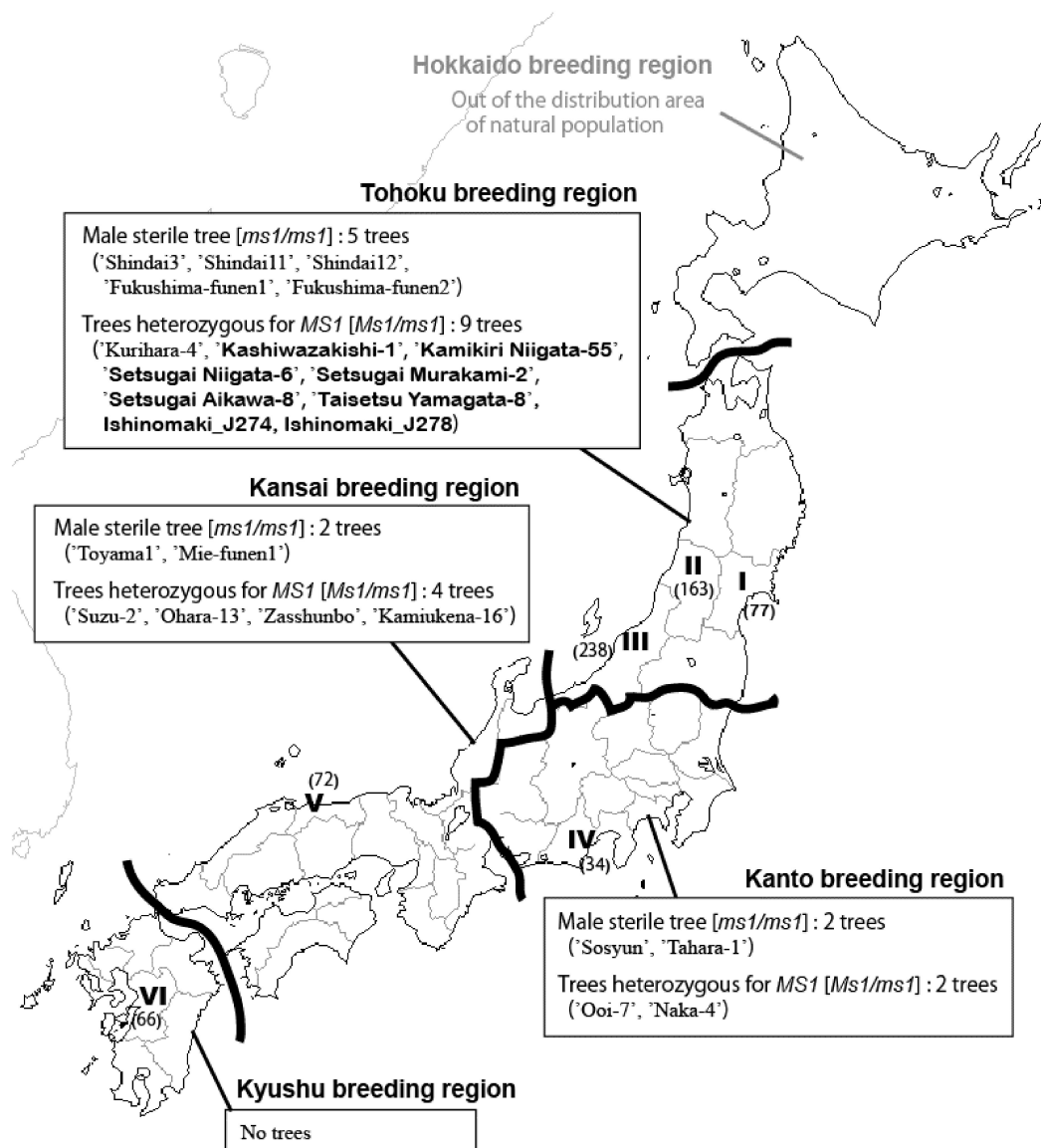


Figure 2. Breeding materials with MALE STERILITY 1 in four *C. japonica* breeding regions. The bold font shows the selected trees in this study. Roman numerals indicate the prefectures where marker-assisted selection (MAS) was performed (I: Miyagi, II: Yamagata, III: Niigata, IV: Shizuoka, V: Tottori, VI: Kumamoto). Numbers in parentheses indicate the sample size.

4. Conclusions

In this study, we performed MAS for 650 trees from six prefectures of Japan using CJt020762_*ms1-1* markers and found that five trees in Niigata Prefecture (‘Kashiwazakishi-1’, ‘Setsugai Niigata-6’, ‘Setsugai Murakami-2’, ‘Setsugai Aikawa-8’, and ‘Kamikiri Niigata-55’) and one tree in Yamagata Prefecture (‘Taisetsu Yamagata-8’) had heterozygous *ms1-1*, and three trees in Miyagi Prefecture (‘Kurihara-4’ and two trees in the natural population) had heterozygous *ms1-2*. The results obtained in this study suggested that a difference in geographical distribution between *ms1-1* and *ms1-2*. In this study, we selected trees with *ms1* among 650 trees, without producing 19,500 F₁ seedlings by 650 artificial crossing (30 F₁ seedlings per clone). This MAS is able to complete within approximately two weeks. Because MAS can effectively reduce the labor and time for the selection of trees with the male-sterile gene, research should help ensure that the quantity of breeding materials will increase to assist future tree-breeding efforts.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/7/734/s1>, Table S1: Primer sequence of the SNaPshot assay, Table S2: Primer sequence for a 48.48 Dynamic Array, Table S3: Cost comparison of ASP and ALP markers by means of agarose gel and the fragment analysis using a sequencer in marker-assisted selection (MAS) of 96 samples.

Author Contributions: Conceptualization, Y.H., S.U., S.H. and Y.M.; material preparation and phenotype data curation, T.T., S.H., J.I., Y.K. and Y.M.; marker development and genotype data collection, S.U., Y.H., T.T., M.W., R.S. and Y.M.; funding acquisition, Y.M.; writing—original draft, Y.M.; writing—review and editing, Y.H., S.U., M.W. and T.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the grants from Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) and NARO Bio-oriented Technology Research Advancement Institution (BRAIN) (the Science and technology research promotion program for agriculture, forestry, fisheries and food industry (No. 28013B)) and the grants from NARO Bio-oriented Technology Research Advancement Institution (BRAIN) (Research program on development of innovative technology (No. 28013BC)).

Acknowledgments: The authors would like to thank Y. Abe, Y. Komatsu for assistance with laboratory works. We also thank Y. Sato for artificial crossing. We also thank Y. Ito, S. Ikemoto, M. Sonoda, K. Yokoo, T. Hakamata and T. Miyashita for providing samples.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Neale, D.B.; Kremer, A. Forest tree genomics: Growing resources and applications. *Nat. Rev.* **2011**, *12*, 111–122. [[CrossRef](#)]
2. Forestry Agency. *Statistical Handbook of Forest and Forestry*; Forestry Agency, Ministry of Agriculture, Forestry and Fisheries: Tokyo, Japan, 2014; pp. 8–9. (In Japanese)
3. Baba, K.; Nakae, K. The national epidemiological survey of nasal allergy 2008 (compared with 1998) in otolaryngologists and their family members. *Prog. Med.* **2008**, *28*, 2001–2012. (In Japanese)
4. Taira, H.; Teranishi, H.; Kenda, Y. A case study of male sterility in sugi (*Cryptomeria japonica*). *J. Jpn. For. Soc.* **1993**, *75*, 377–379. (In Japanese with English summary).
5. Taira, H.; Saito, M.; Furuta, Y. Inheritance of the trait of male sterility in *Cryptomeria japonica*. *J. For. Res.* **1999**, *4*, 271–273. [[CrossRef](#)]
6. Saito, M. Breeding strategy for the pollinosis preventive cultivars of *Cryptomeria japonica* D Don. *J. Jpn. For. Soc.* **2010**, *92*, 316–323. (In Japanese with English summary). [[CrossRef](#)]
7. Hasegawa, Y.; Ueno, S.; Wei, F.J.; Matsumoto, A.; Uchiyama, K.; Ujino-Ihara, T.; Hakamata, T.; Fujino, T.; Kasahara, M.; Bino, T.; et al. Identification and genetic diversity analysis of a male-sterile gene (*MS1*) in Japanese cedar (*Cryptomeria japonica* D. Don). *Sci. Rep.* **2020**. under review. [[CrossRef](#)]
8. Wilson, Z.A.; Morroll, S.M.; Dawson, J.; Swarup, R.; Tighe, P. The *Arabidopsis* MALE STERILITY1 (*MS1*) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors. *Plant J.* **2001**, *28*, 27–39. [[CrossRef](#)]
9. Igarashi, M.; Watanabe, J.; Ozawa, H.; Saito, Y.; Taira, H. The male sterile sugi (*Cryptomeria japonica* D. Don) was found in Fukushima Prefecture (I): Selection of the search ground and identification of the male sterility. *Tohoku J. For. Sci.* **2004**, *9*, 86–89. (In Japanese with English abstract).
10. Takahashi, M.; Iwaizumi, M.G.; Hoshi, H.; Kubota, M.; Fukuda, Y.; Fukatsu, E.; Kondo, T. Survey of male sterility on sugi (*Cryptomeria japonica* D. Don) clones collected from Kanto Breeding Region and characteristics of two male-sterile clones. *Bull. Natl. For. Tree Breed. Cent.* **2007**, *23*, 11–36. (In Japanese with English abstract).
11. Fujisawa, T.; Saito, H.; Fujimiya, T.; Taira, H.; Saito, M. Selection and practical application of male-fertile cedar from elite trees in Kanagawa prefecture. In Proceedings of the 120th Japanese Forest Society Meeting, Kyoto, Japan, 26 March 2009. Abstract Number Pb01-06 (In Japanese).
12. Ueuma, H.; Yoshii, E.; Hosoo, Y.; Taira, H. Cytological study of a male-sterile *Cryptomeria japonica* that does not release microspores from tetrads. *J. For. Res.* **2009**, *14*, 123–126. [[CrossRef](#)]
13. Yamada, H.; Yamaguchi, K. Survey of male sterility of sugi (*Cryptomeria japonica* D. Don) clones collected from Kansai Breeding Region and characteristics of the male-sterile clone. *Appl. For. Sci.* **2009**, *8*, 33–36. (In Japanese)

14. Kawai, K.; Kubota, M.; Endo, K.; Isoda, K. Trial of efficient method for screening *Cryptomeria japonica* trees heterozygous for male-sterile gene by segregation of male-sterile seedlings derived from self-pollinated progeny. *Bull. FFPRI* **2017**, *444*, 265–266. (In Japanese)
15. Yoshii, E.; Taira, H. Cytological and genetical studies on male sterile sugi (*Cryptomeria japonica* D. Don), Shindai 1 and Shindai 5. *J. Jpn. For. Soc.* **2007**, *89*, 26–30. (In Japanese with English summary). [[CrossRef](#)]
16. Miyajima, D.; Yoshii, E.; Hosoo, Y.; Taira, H. Cytological and genetic studies on male sterility in *Cryptomeria japonica* D. Don (Shindai 8). *J. Jpn. For. Soc.* **2010**, *92*, 106–109. (In Japanese with English summary). [[CrossRef](#)]
17. Saito, M.; Taira, H.; Furuta, Y. Cytological and genetical studies on male sterility in *Cryptomeria japonica* D. Don. *J. For. Res.* **1998**, *3*, 167–173. [[CrossRef](#)]
18. Moriguchi, Y.; Ujino-Ihara, T.; Uchiyama, K.; Futamura, N.; Saito, M.; Ueno, S.; Matsumoto, A.; Tani, N.; Taira, H.; Shinohara, K.; et al. The construction of a high-density linkage map for identifying SNP markers that are tightly linked to a nuclear-recessive major gene for male sterility in *Cryptomeria japonica* D. Don. *BMC Genom.* **2012**, *13*, 95. [[CrossRef](#)] [[PubMed](#)]
19. Moriguchi, Y.; Ueno, S.; Higuchi, Y.; Miyajima, D.; Ito, S.; Futamura, N.; Shinohara, K.; Tsumura, Y. Establishment of a microsatellite panel covering the sugi (*Cryptomeria japonica*) genome, and its application for localization of a male sterile gene (*ms-2*). *Mol. Breed.* **2014**, *33*, 315–325. [[CrossRef](#)]
20. Isoda, K.; Kawai, K.; Yamaguchi, K.; Kubota, M.; Yamada, H. Screening of trees heterozygous for male-sterile gene in Kansai breeding region. *Ann. Rep. Tree Breed. Cent.* **2013**, *2013*, 55–59.
21. Saito, M.; Aiura, H.; Kato, A.; Matsuura, T. Characterization of the cutting cultivar of *Cryptomeria japonica* D. Don, Zasshunbo, with a heterozygous male-sterility gene in a heterozygous state, selected from Toyama Prefecture. *Jpn. For. Genet. Tree Breed.* **2015**, *4*, 45–51. (In Japanese with English abstract).
22. Moriguchi, Y.; Uchiyama, K.; Ueno, S.; Ujino-Ihara, T.; Matsumoto, A.; Iwai, J.; Miyajima, D.; Saito, M.; Sato, M.; Tsumura, Y. A high-density linkage map with 2,560 markers and its application for the localization of the male-sterile genes *ms3* and *ms4* in *Cryptomeria japonica* D. Don. *Tree Genet. Genomes* **2016**, *12*, 57. [[CrossRef](#)]
23. Hasegawa, Y.; Ueno, S.; Matsumoto, A.; Ujino-Ihara, T.; Uchiyama, K.; Totsuka, S.; Iwai, J.; Hakamata, T.; Moriguchi, Y. Fine mapping of the male-sterile genes (*MS1*, *MS2*, *MS3*, and *MS4*) and development of SNP markers for marker-assisted selection in Japanese cedar (*Cryptomeria japonica* D. Don). *PLoS ONE* **2018**, *13*, e0206695. [[CrossRef](#)] [[PubMed](#)]
24. Mishima, K.; Hirao, T.; Tsubomura, M.; Tamura, M.; Kurita, M.; Nose, M.; Hanaoka, S.; Takahashi, M.; Watanabe, A. Identification of novel putative causative genes and genetic marker for male sterility in Japanese cedar (*Cryptomeria japonica* D. Don). *BMC Genom.* **2018**, *19*, 277. [[CrossRef](#)] [[PubMed](#)]
25. Ueno, S.; Uchiyama, K.; Moriguchi, Y.; Ujino-Ihara, T.; Matsumoto, A.; Fu-Jin, W.; Saito, M.; Higuchi, Y.; Futamura, N.; Kanamori, H.; et al. Scanning RNA-Seq and RAD-Seq approach to develop SNP markers closely linked to *MALE STERILITY 1 (MS1)* in *Cryptomeria japonica* D. Don. *Breed. Sci.* **2019**, *69*, 19–29. [[CrossRef](#)] [[PubMed](#)]
26. Hasegawa, Y.; Ueno, S.; Fu-Jin, W.; Matsumoto, A.; Ujino-Ihara, T.; Uchiyama, K.; Moriguchi, Y.; Kasahara, M.; Fujino, T.; Shigenobu, S.; et al. Development of diagnostic PCR and LAMP markers for *MALE STERILITY 1 (MS1)* in *Cryptomeria japonica* D. Don. *BMC Res. Note* **2020**. under review. [[CrossRef](#)]
27. Murray, M.; Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325. [[CrossRef](#)]
28. Moriguchi, Y.; Ueno, S.; Saito, M.; Higuchi, Y.; Miyajima, D.; Ito, S.; Tsumura, Y. A simple allele-specific PCR marker for identifying male-sterile trees: Towards DNA marker-assisted selection *Cryptomeria japonica* breeding program. *Tree Genet. Genomes* **2014**, *10*, 1069–1077. [[CrossRef](#)]
29. Van Ooijen, J.W.; Voorrips, R.E. *JoinMap, Version 3.0: Software for the Calculation of Genetic Linkage Maps*; University and Research Center: Wageningen, The Netherlands, 2001.
30. Kosambi, D.D. The estimation of map distances from recombination values. *Ann. Eugen.* **1944**, *12*, 172–175. [[CrossRef](#)]

31. Doğan, G.; Tadama, T.; Kohama, H.; Matsumoto, A.; Moriguchi, Y. Evidence of clonal propagation in *Cryptomeria japonica* D. Don distributed on Pacific Ocean side in Japan. *Silvae Genet.* **2017**, *66*, 43–46. [[CrossRef](#)]
32. Miura, S.; Nameta, M.; Yamamoto, T.; Igarashi, M.; Taira, H. Mechanisms of male sterility in four *Cryptomeria japonica* individuals with obvious visible abnormality at the tetrad stage. *J. Jpn. For. Soc.* **2011**, *93*, 1–7, (In Japanese with English summary). [[CrossRef](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).