Short Communication *Lignin* **1**, 42-50 (2020)

The Lignin Society *Lignin*

Detection Protocol for a Mutant Allele on the *CINNAMYL ALCOHOL DEHYDROGENASE 1* Locus of the *Morus* Species and Search Trial for the Allele in the Natural Mulberry Population of Okushiri Island

Hana Hachisuka,[a] Sakiko Fukuda,[a] Mizuki Iwase,[a] Shinta Inagaki,[b] Hirokazu Tomiyama,[c] Hisato Okuizumi,[d] Akio Koyama,[e] and Shinya Kajita [a,*]

[a]*Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei 184-8588, Japan;*
[b]*Okushiri Board of Education, Okushiri 043-1401, Japan;*
[c]*Tsukuba Technical Support Center, National Agriculture and Food Research Organization, Tsukuba 305-8634, Japan;*
[d]*Genetic Resources Center and* [e]*Division of Biotechnology, National Agriculture and Food Research Organization, Tsukuba 305-0856, Japan;*
**Corresponding author: kajita@cc.tuat.ac.jp*

Received: 17 August 2020; revised in form: 2 December 2020; accepted: 4 December 2020

Abstract
The mulberry cultivar with unusual red-colored wood, "Sekizaisou," was discovered in the bushland of Okushiri Island, Hokkaido, Japan, around a century ago. The leaves of this cultivar were used as feed for sericulture on the island for a short duration from 1916. Although propagules of Sekizaisou have been preserved by sequential vegetative propagation in several public research institutes in Japan, Sekizaisou is believed to have already become extinct on the island. Recently, a point mutation in the first exon of the *CINNAMYL ALCOHOL DEHYDROGENASE 1* locus in Sekizaisou was identified to be responsible for the change in the color of the wood and the structural alteration of lignin. In this study, we performed genotyping of the allele in nearly 600 mulberry individuals grown naturally on Okushiri Island for the rediscovery of Sekizaisou and its wild relatives. A simple protocol for the detection of the mutant allele using polymerase chain reaction followed by direct Sanger sequencing was applied. Although no individuals with the mutant allele were identified in the present study, our results will provide an insight into the flow of the mutant gene in the natural mulberry population.
***Keywords**: DNA sequencing; Genotyping; Polymerase chain reaction*

INTRODUCTION

Mulberry trees are widely cultivated for silk production, fruit, and wood, and as fodder for livestock [1-4]. Particularly, mulberry leaves are excellent feed for silkworms (*Bombyx mori*) because they contain all essential chemical components, such as proteins, carbohydrates, and vitamins, for their growth and development. Sericulture, which started in China over 5,000 years ago, includes mulberry tree cultivation and silkworm rearing on mulberry leaves to produce silk cocoons. After the emergence of synthetic fibers in industrialized countries, sericulture has become less important; however, it is still a major agricultural industry in some countries, such as China and India. Mulberry wood also shows great potential as a feedstock for biofuel and chemical pulp production in rural areas, especially places where sericulture is well-developed [4-6].

Accession numbers of thousands of mulberry germplasm, including those of various morphological mutants with abnormal stems and leaves, have been stored worldwide [7,8]. These bioresources are key components in biological and agronomical research and for breeding programs.

Despite recent advances in the genomic information on mulberry species [9-11], only a few genes responsible for various morphological mutants have been identified [12-15]

According to Yoshimura and Saito [16], an old man discovered a mulberry tree with drooping branches and unusual red-colored wood in the bushland of Okushiri Island, Hokkaido, northern Japan, around the year 1912. Sekizaisou was named after the color of its wood by Yoshimura and Saito [16]. The leaves of this tree were used as feed for sericulture since 1916 owing to their remarkable properties for silk cocoon production compared to those of other local mulberries planted on the island [16].

Although Sekizaisou is thought to have become extinct on the island due to flooding of the Aonae River within the mulberry cultivation area, grafted plantlets established in Tokyo in 1922 have been preserved by sequential vegetative propagation ever since, and the propagules remain in several institutions in Japan to date [16-18]. Despite its favorable resistance against typical mulberry diseases, comparable damage caused by insect attacks compared to other mulberry cultivars [7], and excellent feed characteristics, as reported by Yoshimura and Saito [16], Sekizaisou has not been given much attention as a resource for conventional mulberry breeding owing to its small leaf size and drooping-branch phenotype (Fig. 1).

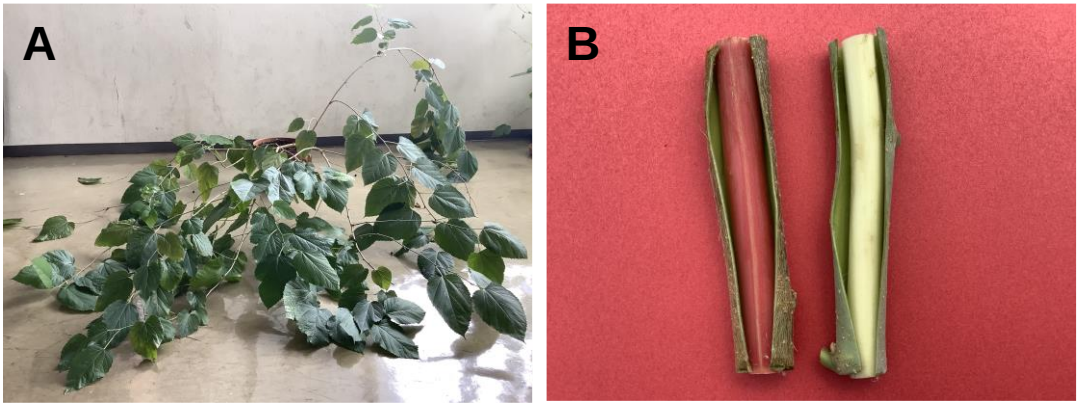

Fig. 1. The typical phenotype of Sekizaisou. (A) A two-year-old Sekizaisou was cultivated in a pot. Unlike the other mulberry cultivars, Sekizaisou possesses branches that droop without a supporting pole. (B) Branches obtained from Sekizaisou (left) and Nezumigaeshi (right) were debarked. The color of the xylem of Sekizaisou was bright red, whereas that of Nezumigaeshi was whitish yellow.

Red, orange, and brown coloration of wood without any chemical staining is a typical characteristic of plants with altered lignin structure [19]. Recently, a point mutation in the first exon of the *CINNAMYL ALCOHOL DEHYDROGENASE 1* (*CAD1*) locus was identified to be responsible for the change in the color of the wood using draft genome sequencing of Sekizaisou [15]. CAD1 converts hydroxycinnamaldehydes to corresponding alcohols in the last step of lignin monomer (monolignol) biosynthesis. Chemical characterization of Sekizaisou wood revealed that substantial amounts of hydroxycinnamaldehydes, especially sinapaldehyde, were integrally incorporated into its lignin. Metabolic profiling also revealed the accumulation of large amounts of sinapaldehyde and its derivatives [15]. To the best of our knowledge, Sekizaisou is the first naturally occurring homozygous dicotyledonous tree deficient in *CAD1*.

A *CAD*-deficient mutant (*cad-n1*) of loblolly pine has also been reported [20]. A not very straight stem and slower growth have been reported in *cad-n1/cad-n1* homozygotes than in wildtype (*CAD/CAD*) trees after 10 months and 12 years of cultivation, respectively [20,21]. In contrast, *CAD/cad-n1* heterozygous trees, compared with wildtype *CAD*/*CAD* trees, showed promoted growth, xylem volume, and wood density [22-24]. Although there is substantial phylogenetic distance between mulberries (angiosperms) and loblolly pines (gymnosperms), it is interesting to note whether the progeny of Sekizaisou, which is heterozygous for the mutant *cad1* allele, expresses beneficial growth traits similar to those of pines. If the *CAD1/cad1* heterozygous genotype in mulberry showed a positive effect on

growth, as observed in the pine mutants, it might have contributed, in part, to the survival of the progeny of Sekizaisou on Okushiri Island under natural conditions.

During the small-scale search for the rediscovery of Sekizaisou on the island by Koyama *et al.* (2004) [25], no mulberry individual with the red-wood phenotype was found. It is difficult for homozygous *cad1/cad1* progenies derived from crossing Sekizaisou and/or its relatives to survive under natural conditions owing to their severe branch-drooping phenotype, which is undoubtedly a disadvantage in survival competition (Fig. 1). In contrast, heterozygous *CAD1/cad1* progenies may survive to some extent because their stems are expected to grow upward, as is usual [15]. In this study, we attempted to confirm whether the extinction of the *cad1* allele in the genome of the natural mulberry population, including heterozygotes, grown on Okushiri Island occurred. We investigated the genotypes of *CAD1* alleles in mulberry individuals grown in the location where Sekizaisou used to be planted. This study gives an insight into effect of the mutation on survival of mulberries in the natural environment.

EXPERIMENTAL

Materials and Methods

Nucleotide sequence analysis

Sequence information of the *Morus CAD1* was obtained from GenBank and DNA Data Bank of Japan. The accession numbers of these sequences are as follows: Sekizaisou (LC476972), Nezumigaeshi (LC476973 and LC476974), Heyebai (CP050230.1, chromosome 7), and *Morus notabilis* (XM_010097985 and XM_010097987). Since the two *CAD1* sequences of *M. notabilis* are identical, only one of them was used for further analysis. Multiple sequence alignment was performed using the ClustalW algorithm [26].

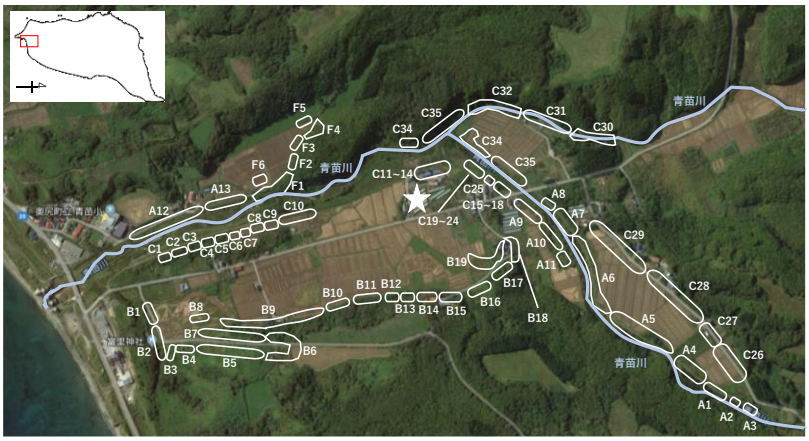

Fig. 2. Aerial photograph of the Tomisato area from where we collected the mulberry leaves. The red square indicates the location of the Tomisato area on the island. Three groups of six colleagues collected the leaves. IDs with different alphabets and numbers indicate the places from where the leaves were collected. One to ten leaves were collected from each place with an ID. The white star-mark indicates the location of the erstwhile house of the discoverer of Sekizaisou (Mr. Yoshimura). The blue line indicates the position of the Aonae River. The original photograph was obtained from Google maps.

Collection of mulberry leaves

Leaves of mulberry individuals grown naturally around the Tomisato area of the island were collected on July 19, 2019. Six colleagues were divided into three parties for the collection of the leaves from different places in the area. Permission to do so was obtained from the representatives of the private fields and the National Forest. Fig. 2 shows an aerial photograph of the Tomisato area, which is comprised of paddy fields, natural bushes, and planted forests. Enclosing tags with white lines indicate

the places from where we collected the leaves. Each tag corresponds to one to ten leaves collected per place. A leaf from each mulberry individual grown naturally in the area was transferred to a small plastic bag, which was labeled and stored temporarily in cooling boxes. Photographs of each mulberry individual and leaf obtained therefrom were taken using a digital camera and the geographical position of the collection was recorded using GPS equipment. The collected leaves were transported to the laboratory on July 20, 2019, and kept in a freezer (-35℃) until total DNA extraction was performed.

Total DNA extraction

Few frozen leaves collected from Okushiri Island was pulverized in a 2-mL plastic tube, which contained the extraction buffer (50 µL; 200 mM Tris-HCl, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate; pH 7.5) and zirconia beads. After pulverization, fresh extraction buffer (150 µL) was further added and mixed using a vortex mixer. After centrifugation, the soluble fraction was transferred to a fresh tube, and isopropanol (200 µL) was added to precipitate the total DNA. The resultant total DNA was dissolved in nuclease-free water (30 µL) after drying. For control experiments, total DNA was extracted from the leaves of Sekizaisou (accession no. JP165851 in Genebank of the National Agriculture and Food Research Organization, NARO), Nezumigaeshi (JP165725), and F1 hybrid progenies [15] derived from crossing Sekizaisou (seed parent) and Kokusou 21 (JP165813, pollen parent). These leaves were collected from the experimental field of NARO (Tsukuba, Japan). All chemicals used in this study were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) unless noted.

Amplification of CAD1 fragments by polymerase chain reaction (PCR) and direct sequencing of the amplified DNA

DNA fragments that contained the 5'-untranslated region (UTR), the first exon, the first intron, and a part of the second exon of the *CAD1* alleles in each individual were amplified using PCR. Total DNA prepared from Sekizaisou, Nezumigaeshi, and F1 progeny of Sekizaisou and Kokusou 21 were also used as the template. Composition of the reaction mixture was as follows: GoTaq DNA polymerase (0.1 µL; 5 units/µL; Promega K.K., Tokyo, Japan), 5x GoTaq buffer (0.4 µL), dNTPs (0.4 µL; 2.5 mM), forward and reverse primers (0.5 µL), total DNA (1 µL) prepared as described above, and nuclease-free water (13.5 µL). Thermal conditions for amplification were as follows: 94℃ for 3 min; 35 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 1 min; and 72℃ for 5 min. The nucleotide sequences of the primers were as follows: MaCAD1-5UTR-Fw2, 5'-CTCATTCAGAAACAGTGATC-3' and Ma-Seq1-Rv, 5'-CTCGGCATGTCCAACTATCC-3'. To reduce the number of samples to be sequenced, three independent reaction mixtures were mixed together after PCR. The PCR primers were removed from the reaction mixture using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific K.K., Tokyo, Japan) and the nucleotide sequence of the amplified DNA was determined using the ABI Prism 3130xl Genetic Analyzer with the BigDye Terminators v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific K.K.).

RESULTS AND DISCUSSION

Multiple Alignment of the CAD1 Partial Sequence in the *Morus* Species

Among the many *CAD1* nucleotide sequences deposited in public databases, only six sequence accessions of the *Morus* species, including Sekizaisou, have been recorded. Four of them originated in *Morus alba* (Sekizaisou [15], two sequences of Nezumigaeshi [15], and Heyebai [27]), and two, which have the completely same sequence, originated in *M. notabilis* [9]. Fig. 3 shows the multiple alignments of partial nucleotide sequences corresponding to the 5'-UTR (blue letters), the first exon (red), the first intron (purple), and a part of the second exon (orange). Although 10 nucleotide differences and four nucleotide insertions were observed in the first intron of the two *M. alba* cultivars (Nezumigaeshi and Heyebai) compared with that of *M. notabilis*, only a single nucleotide difference at the seventh nucleotide of the first exon was detected between them. These results suggested that the sequences of the first and second exons in *CAD1* were highly conserved in the *Morus* species.

In contrast, an additional difference in the first exon could be observed only between Sekizaisou and the others (arrow in Fig. 3). A guanine insertion in the first exon of the Sekizaisou *CAD1* was observed [15]. This resulted in a frameshift mutation and a premature stop codon, causing a loss of function of the Sekizaisou CAD1 protein. If the progeny of Sekizaisou has survived somewhere on the island, it can be expected to possess this point mutation in its genome.

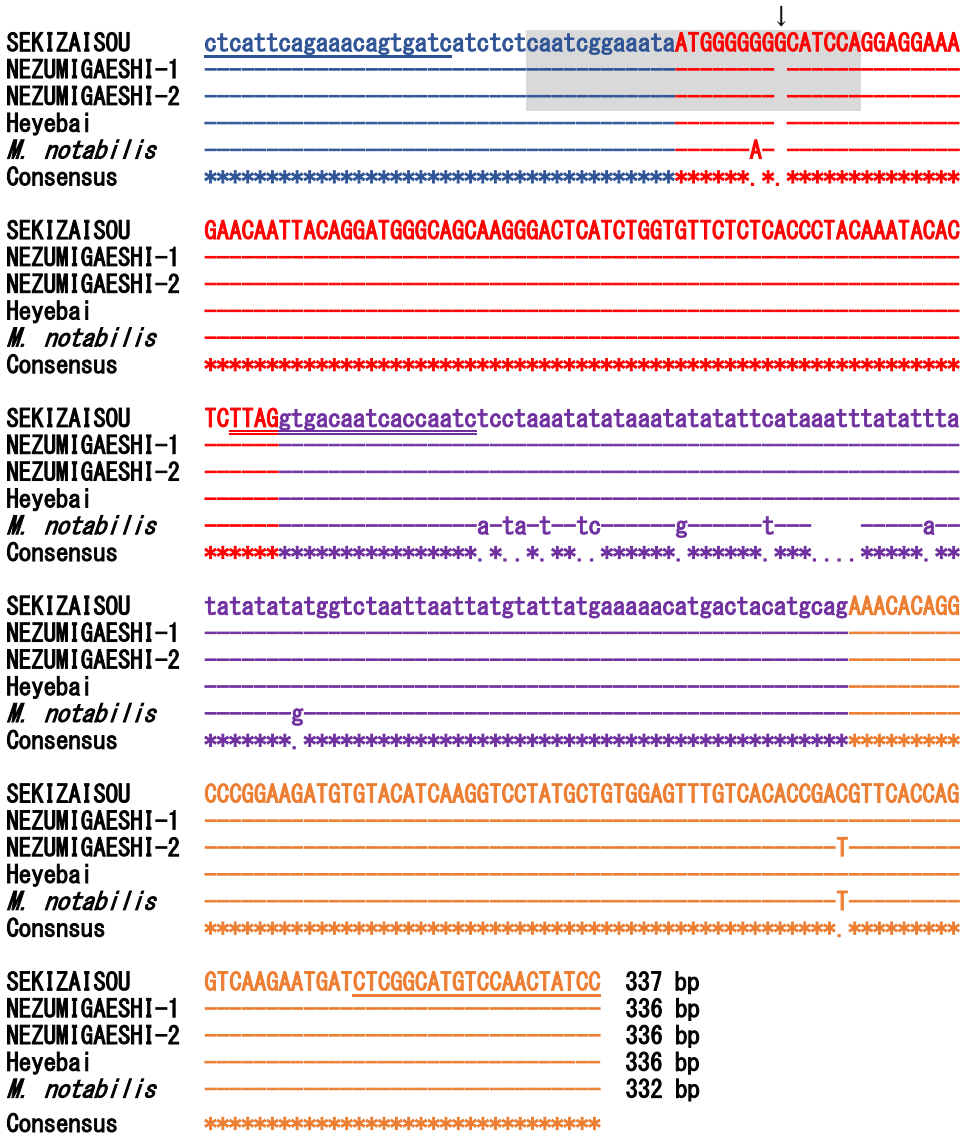

Fig. 3. Multiple partial sequence alignment of *CAD1* from different mulberries. Blue, red, purple, and orange letters correspond to the 5'-untranslated region, first exon, first intron, and second exon, respectively. Asterisks indicate the conserved nucleotides among the different *CAD1* sequences. Dots indicate replacement with other nucleotides or gaps in the sequences. Sequences corresponding to the PCR primers are underlined. Sequences corresponding to the primers used for direct sequencing are double-underlined. Complementary sequences of the shadowed sequences corresponding to the sequencing chromatograms are shown in Fig. 4. Arrow indicates a gap caused by a nucleotide insertion observed in the *cad1* allele of Sekizaisou.

Detection of the Mutant Allele by PCR Amplification and Subsequent Direct Sequencing of the Partial CAD1 Fragment

To complete the protocol for the detection of the mutant *CAD1* allele in mulberry individuals, we analyzed the partial *CAD1* sequence in Sekizaisou and F1 hybrids derived from Sekizaisou and Kokusou 21 (*Morus latifolia*), in addition to Nezumigaeshi (Fig. 4). All of them were diploid, and thus, showed two alleles at the *CAD1* locus. Sekizaisou showed a mutation in both alleles, whereas Nezumigaeshi showed two wildtype alleles without mutations at the *CAD1* locus. In contrast, the F1 hybrids showed two *CAD1* alleles, one with the mutation derived from Sekizaisou and the other derived from Kokusou 21. Based on the sequence similarity of *CAD1* among the various *Morus* species (Fig. 3), the *CAD1* sequence, at least the first exon and a part of the 5'-UTR, in Kokusou 21 was expected to be identical to that in Nezumigaeshi and *M. notabilis*; however, no sequence information of *CAD1* in Kokusou 21 is available at present.

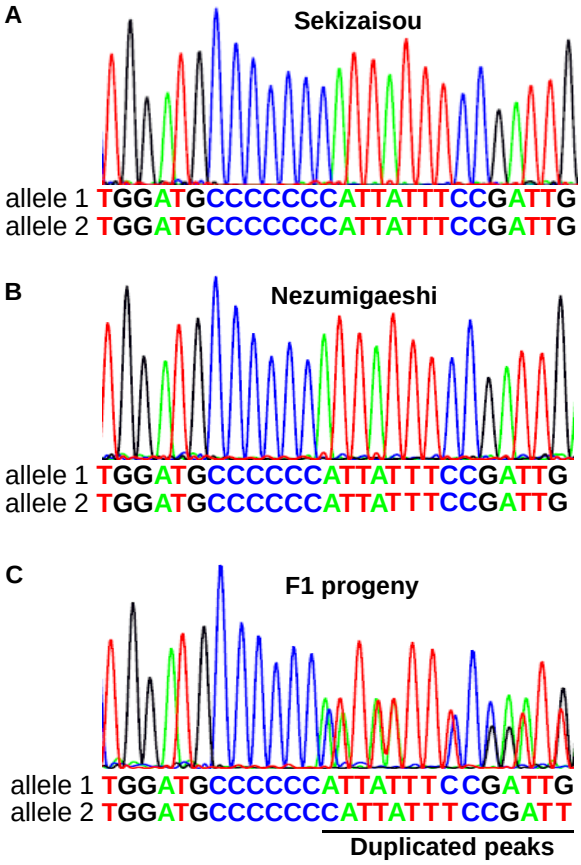

Fig. 4. Partial sequencing chromatograms of the translation start site of the amplified *CAD1* (*cad1*) alleles from Sekizaisou (A), Nezumigaeshi (B), and F1 progeny (C). The chromatograms correspond to the non-coding strand of each allele (the complementary strands of the shadowed sequences in Fig. 3). Sekizaisou showed two *cad1* alleles with the point mutation, whereas Nezumigaeshi showed two *CAD1* alleles without the mutation. F1 showed both *CAD1* and *cad1* alleles. No duplicated peaks can be observed for Sekizaisou (A) and Nezumigaeshi (B). In contrast, duplicated peaks can apparently be observed on the right side of the F1 chromatogram (C). The duplications indicate that the F1 progeny has two different alleles at the *CAD1* locus of its genome.

Partial *CAD1* fragment(s) from both alleles in Sekizaisou, F1, and Nezumigaeshi were amplified using PCR using total DNA as the template and primers corresponding to the 5'-UTR and the second exon, whose sequences are highly conserved among the different *Morus* species (Fig. 3). After amplification, the resultant DNA was sequenced directly using Sanger sequencing. Fig. 4 shows the partial sequencing chromatograms corresponding to the complementary strands of the shadowed sequences derived from Sekizaisou, F1, and Nezumigaeshi (indicated in Fig. 3). For Nezumigaeshi and Sekizaisou, clear peaks without any noise can be observed in each chromatogram, as shown in Fig. 4A and 4C, respectively. In contrast, duplicated peaks can be detected in the right part of the chromatogram for the F1 progeny (Fig. 4C). This duplication was due to the existence of the mutant allele with a nucleotide (guanine/cytosine) insertion of either allele at the *CAD1* locus of the F1 plant, as described above. Thus, the duplicated peak can be used as a diagnostic marker of the mutant *cad1* allele in the genome of mulberry individuals.

Determination of the Areas of Okushiri Island for Collection of the Mulberry Leaves

Before collecting the leaves of individual mulberry plants grown naturally on the island, prior literature on Sekizaisou was reviewed to determine the areas from where the leaves could be collected. The earliest report on Sekizaisou was published by Yoshimura and Saito [16], who also named Sekizaisou. According to them, an old man discovered a mulberry tree with drooping branches and unusual red-colored wood in the bushland of Okushiri Island over a century ago. He brought it to his home and planted it in his garden. It is known that his family name was Yoshimura. He was said to be a blacksmith in Hiroshima before he moved to Okushiri Island. Based on the information on the old man's name and job, we searched for his relatives on the island and finally found his great-grandson, Mr. Ichio Yoshimura. He told us that his great-grandfather's erstwhile house used to be located in Okushiri area (present name, Tomisato area), which is close to the Aonae River (star-marked in Fig. 2).

According to Teikichi Hotta [17,18], who was a mulberry taxonomist and used to be a professor at Kyoto Institute of Technology, many Sekizaisou were grown spontaneously along with the Aonae River in the Taisho era. However, afterward, most of them perished due to repeated flooding of the river. Based on the results described above, we decided to collect the leaves of the mulberry individuals grown in the Tomisato area, which has a high mulberry plant density.

Collection of Leaves, PCR Amplification of the Partial CAD1 Fragment, and DNA Sequencing

The leaves of mulberry individuals were collected, as described in the Materials and Methods section. Six hundred and forty-two leaves of independent mulberry individuals from the determined area, as shown in Fig. 2, were collected. However, no individual with red-colored wood was observed during this investigation, indicating that we had failed to rediscover Sekizaisou. After extraction of total DNA from 642 leaves independently, the partial *CAD1* fragment was amplified using PCR, as described above. Fig. 5 shows one of the results of agarose gel electrophoresis of the amplified DNA we analyzed. Although amplification of most of the total DNA could be achieved, no amplification of 30 out of the 642 isolated total DNA samples was detected. Direct Sanger sequencing was performed to analyze the nucleotide sequence of the amplified DNA (612 samples). The results showed that no clear sequencing chromatogram could be obtained for 26 out of the 612 amplified products. Although sequencing of the amplified products derived from 586 independent mulberry individuals was successful, no duplicated peaks, the diagnostic marker of the mutant *CAD1* allele, could be detected for any of the samples we analyzed. These results suggested that the mulberry individuals grown in the area we searched were unlikely to have the *cad1* allele in their genome.

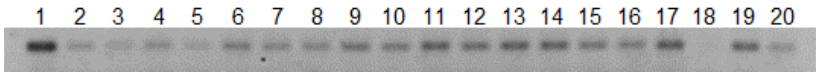

Fig. 5. A typical result of agarose electrophoresis of the amplified DNA. PCR amplification was performed using the total DNA extracted from 20 independent mulberry individuals as the template. Amplified DNA with the expected size, except in lane #18, could be detected.

The possible reasons why we failed to find out the individual with *cad1* allele are as follows: (1) progenies harboring the *cad1* allele were also extinct entirely as Sekizaisou did on the island; (2) the area where we searched was inappropriate for collection of the leaves. A farmer on the island whose name was Eikich Fukuda had been started to use leaves of Sekizaisou as feed for silkworm from 1916 [16]. Thus, it is proposed that he propagated Sekizaisou vegetatively for the feed production, the resultant propagules could cross with other mulberry plants naturally, and then Sekizaisou leaved some offspring on the island. Unfortunately, to the best of our knowledge, his erstwhile address and the place where he cultivated Sekizaisou could not be identified. However, it would be difficult to exclude the possibility that the progeny of Sekizaisou with the *cad1* allele still remains somewhere on the island. To confirm whether the *cad1* allele was extinct or still remains, we should widen the area of our future search for collecting leaf samples. Investigation with next-generation sequencing technology instead of the

traditional Sanger protocol we used in the present study should improve our efficiency for identification of genotype of the allele in many mulberry individuals grown naturally on the island.

CONCLUSIONS

1. A simple protocol using PCR amplification of the alleles at the *CAD1* locus and subsequent direct sequencing was set up to detect the *cad1* mutant allele.
2. Genotyping of 586 mulberry individuals grown naturally in the Tomisato area, where Sekizaisou used to grow spontaneously, was performed. Our study indicated that, neither Sekizaisou nor its progeny with the mutant allele could be identified.
3. To confirm the effect of the *cad1* mutant allele on the survival of mulberry individuals in the natural environment and the extinction of the allele in the natural mulberry population on the island, further investigation of mulberry leaves collected from other areas of the island is required.

ACKNOWLEDGEMENTS

This study was partly supported by KAKENHI (Grant-in-Aid for Scientific Research 18H02250 and 19K22326). We thank the National Agriculture and Food Research Organization Genebank for the distribution of the mulberry cultivars. We would also like to express our gratitude to Hiroaki Yamanouchi, Akemi Shimizu, and the people of Okushiri Island for their cooperation in this study.

REFERENCES

1) Hamamura, Y., Food selection by silkworm larvae, *Nature*, **183**, 1746-1747 (1959).
2) Saddul, D. *et al.*, The potential of mulberry (*Morus alba*) as a fodder crop: The effect of plant maturity on yield, persistence and nutrient composition of plant fractions, *Asian-Australasian J. Anim. Sci.*, **17**, 1657-1662 (2004).
3) Ercisli, S., Orhan, E., Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits, *Food Chem.*, **103**, 1380-1384 (2007).
4) Lu, L. *et al.*, The role of marginal agricultural land-based mulberry planting in biomass energy production, *Renew. Energy*, **34**, 1789-1794 (2009).
5) Guha, A., Reddy, A.R., Leaf functional traits and stem wood characteristics influencing biomass productivity of mulberry (*Morus* spp. L) genotypes grown in short-rotation coppice system, *Bioenergy Res.*, **6**, 547-563 (2013).
6) Rahman, M. M., Jahan, M.S., Evaluation of mulberry plant as a pulping raw material, *Biomass Conv. Bioref.*, **4**, 53-58 (2014).
7) Machii, H. *et al.*, A list of morphological and agronomical traits of mulberry genetic resources, *Misc. Publ. Natl. Inst. Seric. Entomol. Sci.*, **29**, 1-307 (2001).
8) Sohn, K.-W., *Conservation Status of Sericulture Germplasm Resources in the World - I*, The Food and Agriculture Organization of the United Nations, Rome (2003).
9) He, N. *et al.*, Draft genome sequence of the mulberry tree *Morus notabilis*, *Nat. Commun.*, **4**, 2445 (2013).
10) Mathithumilan, B. *et al.*, Development and characterization of microsatellite markers for *Morus* spp. and assessment of their transferability to other closely related species, *BMC Plant Biol.*, **13**, 194 (2013).
11) Dai, F. W. *et al.*, Phenotypic and transcriptomic analyses of autotetraploid and diploid mulberry (*Morus alba* L.), *Int. J. Mol. Sci.*, **16**, 22938-22956 (2015).

12) Jiao, F. *et al*., Developmental characterization of leaf dorsoventral mutant 'Ryoumenguwa' in mulberry (*Morus alba* L.), *J. Insect Biotechnol. Sericol*., **73**, 141-149 (2004).
13) Sopian, T. *et al*., Characterization of mulberry mutant growth response to gibberellin and abscisic acid application and molecular analysis, *J. Insect Biotechnol. Sericology*, **78**, 23-32 (2009).
14) Yamanouchi, H. *et al*., Inheritance of a weeping character and the low frequency of rooting from cuttings of the mulberry variety 'Shidareguwa', *Plant Breed*., **128**, 321-323 (2009).
15) Yamamoto, M. *et al.*, A century-old mystery unveiled: Sekizaisou is a natural lignin mutant, *Plant Physiol*., **182**, 1821-1828 (2020).
16) Yoshimura, M., Saito, K., Kuwa no ichihinsyu Sekizaisou ni tsuite (A mulberry cultivar, Sekizaisou) (in Japanese), *Sakurakai Zasshi* **15**, 107-111 (1924).
17) Hotta, T., Contributions to the knowledge of the systematics of Morus in Japan I, *Trans. Sapporo Nat. Hist. Soc.*, **14**, 193-206 (1936).
18) Hotta, T., Sekizaisou oyobi murasakiguwa ni tsuite (in Japanese), *Sapporo Norin Gakkai Hou*, **137**, 9-17 (1937).
19) Chanoca, A. *et al.*, Lignin engineering in forest trees, *Front. Plant Sci*., **10**, 912 (2019).
20) MacKay, J.J. *et al*., Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase, *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 8255-8260 (1997).
21) Dimmel, D. R. *et al*., Pulping and bleaching of CAD deficient wood, *J. Wood Chem. Technol.*, **21**, 1-17 (2001).
22) Wu, R. L. *et al*., Average effect of a mutation in lignin biosynthesis in loblolly pine, *Theor. Appl. Genet*., **99**, 705-710 (1999).
23) Yu, Q. *et al.*, Differences in wood density and growth of fertilized and nonfertilized loblolly pine associated with a mutant gene, *cad-n1*, *Can. J. For. Res*., **35**, 1723-1730 (2005).
24) Yu, Q. *et al.*, Association of the cad-n1 allele with increased stem growth and wood density in full-sib families of loblolly pine, *Tree Genet. Genomes*, **2**, 98-108 (2006).
25) Koyama, A. *et al.*, Exploration and collection of mulberry (*Morus* spp.) in Okushiri Island (in Japanese), *Annu. Rep. Explor. Introd. Plant Genet. Res.*, **20**, 40-51 (2004).
26) Thompson, J. D. *et al*., CLUSTAL W: improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res*., **22**, 4673-4680 (1994).
27) Jiao, F. *et al*., Genome and population genomic analysis provide insights into the evolution and improvement of domesticated mulberry (*Morus alba*), *Mol. Plant*, **13**, 1001-1012 (2020).