Title: Developing simple DNA extraction and PCR-RFLP for *MALE STERILITY 4* (*MS4*) gene in *Cryptomeria japonica* D. Don: Toward an environmentally friendly protocol

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Abstract

This study presents the development of a simple DNA extraction method and a novel PCR-RFLP technique for genotyping the *MALE STERILITY 4* (*MS4*) gene in *Cryptomeria japonica*. Traditional CTAB-based DNA extraction methods, while effective, involve hazardous chemicals and require high-speed centrifugation, which are impractical in many field settings. Our approach utilizes a household dish detergent-based buffer, sodium chloride, and polyvinylpyrrolidone K-30 to extract DNA from *C. japonica* needle leaf tissues. The simplicity of this method makes it more accessible and environmentally friendly. The extracted DNA was successfully used in PCR-RFLP analysis, targeting a single nucleotide polymorphism in the *MS4* gene, demonstrating its efficacy for genotyping. The PCR-RFLP markers reliably discriminated between individual genotypes, confirming the practical application of our simple extraction method, even for conifers containing inhibitory substances. This technique is particularly advantageous for use in arboretums and field stations, where the use of hazardous chemicals and specialized equipment is limited. Our study contributes to genetic resource management by providing an easy, reliable, and safer method for DNA extraction and genetic analysis.

Key words: *Cryptomeria japonica*, sugi, DNA extraction, dish detergent, PCR-RFLP, male sterility

Statements and Declarations

Competing interests: The authors declare no competing interests.

Introduction

Nucleic acid extraction is a starting process for genetic analysis and has been a topic of research for many years (Yadav et al. 2018; Dairawan and Shetty 2020; Schenk et al. 2023). The DNA extraction from plant tissue is difficult compared to that from animal tissue, because it contains poly-saccharide, polyphenolics and other secondary metabolites that prevent nucleic acid extraction. This challenge is particularly pronounced in trees, especially conifers like *Cryptomeria japonica*, which are known for their high levels of polyphenolics and polysaccharides. These compounds interfere with DNA extraction and PCR amplification, making the process more complex than with other plant species. Developing a method that can effectively extract DNA from such challenging plant material is crucial for advancing genetic studies in these species. In 1980, Murray and Thompson (Murray and Thompson 1980) published a DNA extraction method using CTAB (cetyl-trimethylammonium bromide) which efficiently removes polysaccharides from plant tissue. Currently the CTAB-based method is used to extract DNA/RNA from many plant species with wide downstream uses for restriction digestion and PCR experiments without further purification (Doyle and Doyle 1987; Le Provost et al. 2007). One drawback of the method is the use of hazardous chemicals such as chloroform and 2-metcapthoethanol, which are recognized internationally as hazardous substances. Specific handling must comply with the regulations and guidelines of each country. In addition to the use of hazardous chemicals, the CTAB protocol uses centrifugal forces of up to 20,000 g. Arboretums and field stations are rarely equipped with such centrifuges, where genetic markers may be useful for efficient management of genetic resources.

One of the simplest methods to extract DNA is the use of chelating resin, InstaGene (Bio-Rad Laboratories, Inc.), which only requires heating tissue with the resin matrix is needed for DNA extraction. Tsuruta et al. (2021) used InstaGene Matrix to extract DNA from somatic embryogenic cells of *C. japonica* for marker assisted selection of *MS1* (*MALE STERILITY 1*) gene (Tsuruta et al. 2021). The method further applied for leaf, pollen, and inner bark tissue of *C. japonica* from which DNA was extracted for PCR amplification of *MS1* genotyping (Ueno et al. 2022). Although the InstaGene

extraction is simple, the amount of tissue (5–10 mg leaf) used with InstaGene Matrix is a key factor for the success of DNA extraction (Ueno et al. 2022). Too much tissue with InstaGene Matrix results in a viscous solution, leading to failure in PCR. Moreover, the process of trial and error with InstaGene can be a time-consuming task, especially for those inexperienced in DNA experiments.

In this study, we aim to develop a simple DNA extraction method and PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) technique for the recently identified *MS4* (*MALE STERILITY 4*) gene in *C. japonica* (Kakui et al. 2023). The mutation in *MS4* involves a single nucleotide polymorphism (SNP) that results in an amino acid substitution. Although a KASP (Kompetitive Allele Specific PCR) (Dipta et al. 2024) marker for the *MS4* gene was recently developed (Watanabe et al. 2024), its application requires specialized equipment such as a real-time PCR machine, which may not be readily available in arboretums or field stations. Another potential approach is developing allele-specific PCR markers, but this method requires extensive optimization of PCR conditions, even though Locked Nucleic Acids (LNA) are included in PCR primers (Latorra et al. 2003), making it more challenging and time-consuming. Therefore, we focused on developing a PCR-RFLP technique that serves as a practical example of applying our simple DNA extraction method. This approach, which avoids the use of "deleterious" and "poisonous" substances, aims to provide an accessible, environmentally friendly tool for genetic analysis, offering a versatile solution for researchers in various settings.

Materials and Methods

Fresh leaf tissue samples from five *C. japonica* individuals (Table 1) were sampled at Niigata Prefectural Forest Research Institute and kept refrigerated at 4 °C until DNA extraction. One needle leaf (approximately 5 mg) was disrupted in 1 mL of dish detergent buffer (10 % dish detergent ‘Yashinomi Hi-power’ (Saraya Co., Ltd.), 10 % NaCl, and 5% polyvinylpyrrolidone K-30 (PVP) (NACALAI TESQUE) at room temperature using mortar and pestle (Fig. 1). The dish detergent contained 32 % surfactant (sodium alkyl

ether sulfates, alkyl betaines, and fatty acid alkanolamides), auxiliary cleaning agents, and stabilizers. The resulting 'green juice' (approximately 500 µL) was transferred to a 1.5 mL tube, centrifuged for three minutes with a CHIBITAN-R microcentrifuge (Hitachi Koki, Co. Ltd.) at the maximum speed of 6,200 rpm, and 300 µL of supernatant was then collected in a new 1.5 mL tube. Twice the volume (600 µL) of cold 100 % ethanol was added to the tube and mixed with inverted shaking. The solution was centrifuged for 10 min at room temperature using the CHIBITAN-R, and the supernatant was discarded by decantation. 600 µL of 70 % ethanol was then added to the tube to wash the precipitate, spun briefly by CHIBITAN-R, and discarded by decantation. The precipitate was air-dried overnight at room temperature, and dissolved in 100 µL of TE buffer (NACALAI TESQUE).

In order to demonstrate the practical application for the extracted DNA, PCR-RFLP was developed to genotype the male-sterile causative mutation of *MALE STERILITY 4* (*MS4*) in *C. japonica* (Fig. 2) as a model system. PCR primer pairs to amplify the target region were designed by Primer3Plus (Untergasser et al. 2012). The SEQUENCE_TEMPLATE contained the CJt034360 sequence (DDBJ accession number: ICQT01013259) with the *PRIMER_MIN_SIZE* 23, *PRIMER_OPT_SIZE* 23, *PRIMER_MAX_POLY_X* 2, *PRIMER_PAIR_MAX_DIFF_TM* 2, and SEQUENCE_TARGET 301,1 (target causative SNP coordinate (301) along the *SEQUENCE_TEMPLATE*, and the length of 1). The enzymatic function of *MS4* is TKPR1 (tetraketide alpha-pyrone reductase 1), dysfunction of which causes male sterility in not only *C. japonica*, but also *Arabidopsis* (Grienenberger et al. 2010) and rice (Xu et al. 2019). Restriction enzymes to discriminate the mutant and wild type alleles in *MS4* were searched using the REHUNT program (Cheng et al. 2018). Four samples (Table 1), whose *MS4* genotypes were established elsewhere by artificial crossing (Miyajima et al. 2010; Hasegawa et al. 2018; Hirayama et al. 2021) and whose DNA was extracted using a modified CTAB method (Hasegawa et al. 2020), were used to develop PCR-RFLP. The PCRs were carried out in a 10 µL reaction containing 1 µL (about 10 ng) of template DNA, 1 µL of each of primer (2 µM stock solution; forward: AGCCATGAATGGAACAACTGTGT, and reverse: GGAGGAAGGCTCGGTCCTATTAT), resulting in a final concentration of 0.2 µM for each primer, and 5 µL of QIAGEN Multiplex PCR Master Mix (Qiagen). The

thermal profiles used were as follows: the initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 30 sec, 60 °C for 90 sec, and 72 °C for 60 sec with the final extension of 72 °C for 10 min. The resulting products (5 µL) were digested with the restriction enzyme *Hae*III (NIPPON GENE) in a 10 µL of reaction containing 5 µL of PCR products, 1 µL of 10× Buffer M (resulting in a final concentration of 1×) , and 0.1 µL of *Hae*III (high concentration: 30 – 100 units/µL) and incubated for at least three hours or overnight at 37 °C. The PCR products and restriction-digested products were run on 2 % agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

Results and Discussion

The PCR-RFLP developed for *MS4* was successfully discriminated the individual genotypes (Figure 3). When DNAs extracted by the dish detergent buffer was used for this marker system, expected results were also obtained, demonstrating the successful application of the simple DNA extraction method in this study. Because DNA extraction is a basic starting experiment of molecular biology, biology classes in secondary education conduct DNA extraction experiments using commercially available detergent with simple procedures (Ogata et al. 2010; de Andrade et al. 2024). Although these classes commonly use soft tissue plant materials including broccoli flower buds and strawberries, no reports were found for DNA extraction from coniferous plants using dishwashing detergent. In the current study, we developed a simple DNA extraction method with commercially available dishwashing detergent for use in marker assisted management of arboretum or field stations, where the use of hazardous chemicals is not preferable and powerful centrifuges are not equipped. When DNA extracted with this simple detergent buffer was run on agarose gels, DNA bands were rarely observed. This is probably due to the small amount of DNA extracted and fragmented. However, PCR successfully amplified the 1.5 kb fragment, though the amplification of primer dimers or non-specific amplification occurred (Fig. 3). While polymerases, such as MightyAmp DNA polymerase (Lu et al. 2012), may be suitable for use with crude DNA that contains PCR inhibitors, this study used HotStartTaq DNA

polymerase in Multiplex Kit (Qiagen) which is widely used for genetic analysis with microsatellite markers for clonal identification and parentage analysis. Additionally, since the DNA extracted using this method could also be utilized with restriction enzymes, which are generally more sensitive to inhibitors than polymerases in PCR, it suggests that the quality of the extracted DNA is high enough for various molecular biology applications.

The DNA extraction buffer used in this study (Fig. 1) comprised commercially available dishwashing detergent, NaCl, and PVP, each contributing to the effectiveness of the extraction process. The dishwashing detergent acts as a surfactant, breaking down cell membranes and denaturing proteins, thus facilitating the release of DNA into the solution. There are several brands of dishwashing detergent in the market. Although each product has different ingredients, comparative studies have indicated that any dishwashing detergent could extract DNA (Ogata et al. 2010). Because the concentration of detergent is different for each brand, preliminary experiments to determine the most effective concentration of detergent is necessary. In the current study, we initially tried to use 1 µL of 'green juice' extracted by an extraction buffer containing only 2 % dish washing detergent, directly as a template DNA for PCR. Although 1.5 kbp PCR products were successfully obtained, we failed to digest the PCR products by *Hae*III restriction enzyme, probably due to inhibiting contaminants in the 'green juice.' We therefore included salting-out/salting-in for proteins, and ethanol precipitation for DNA in the protocol (Fig. 1). NaCl is indispensable to precipitate DNA by ethanol, while it is expected to work as salt to prevent proteins from dissolving in or precipitating from the buffer. Instead of using dishwashing detergent, laundry detergent may be used (Nasiri et al. 2005). However, measuring a specific amount of detergent powder and dissolving it into a buffer may be more tedious than using liquid type detergent, though any commercially available products commonly found in the laboratory could be a key factor in terms of cost and convenience. The PVP, the other ingredient in the extraction buffer (Fig. 1), was included, because it efficiently removes phenolic compounds, which interact with DNA and prevent DNA extraction (Heikrujam et al. 2020). Extraction buffer without PVP led to failure in *Hae*III restriction digestion in the

current protocol for *C. japonica*, though PCR was successful (data not shown). If samples contain less phenolic compounds or only PCR application is needed, PVP could be removed from the extraction buffer.

In conclusion, we successfully developed a simple and effective DNA extraction method that is applicable to conifers, a plant group traditionally difficult to process due to inhibitory substances. This method's successful application in genotyping the MS4 gene in *C. japonica* suggests its potential utility in a wide range of plant species, making it a valuable tool for genetic analysis in settings where access to specialized equipment is limited. This approach broadens the scope of molecular biology applications and offers a practical solution for DNA extraction in diverse and challenging environments.

Acknowledgments

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Figure legends

Fig. 1 Workflow of the simple DNA extraction method

Fig. 2 Schematic representation of *MS4* (*TKPR1*) and PCR-RFLP in *Cryptomeria japonica*

Yellow rectangles indicate CDS (coding sequences), which are divided by intron sequences shown by black lines. *MS4* consists of five exons and introns with a total of 3,323 bp from the start to the stop codon. The CDS is translated into 333 amino acid residues, the 82nd of which is cysteine (C) and arginine (R) for the wild type and the mutant, respectively. This is the causative mutation of male sterility by *MS4*. The restriction enzyme, *Hae*III, can recognize the GGCC, the mutant nucleic acid sequence, while the

wild type sequence cannot be cut by the enzyme. The PCR-RFLP primer pairs are shown by red triangles; the PCR products from *ms4* allele contain two *Hae*III recognition sequences shown by black triangles, the C82R site, and that in the third intron common with the products from the wild type allele. The resulting electrophoretic profile, with gel wells in gray, shows thee, four, and two bans for *ms4*/*ms4*, *Ms4*/*ms4*, and *Ms4*/*Ms4* genotype, respectively

Fig. 3 Photographic images for agarose gel electrophoresis

(a) Development of PCR-RFLP for *MS4* gene: PCR products (lanes 1–4) and *HaeIII*-digested products (lanes 5–8) for `Shindai 8,` `S8HK5,` `Kamikiri Niigata-58,` `Higashikanbara-5` with genotypes of *ms4*/*ms4*, *Ms4*/*ms4*, *Ms4*/*ms4*, and *Ms4*/*Ms4*, respectively. (b) DNA extracted by the simple extraction using the dish detergent buffer was used for PCR-RFLP genotyping. *Hae*III-digested products for `Shindai 8,' `Kamikiri Niigata-58,` `S8 × Gosenshi-1,` `Setsugai Niigata-1,` and `Kitakanbara-1` are in lanes 9–13. The smallest bands (less than 100 bp) may result from primer dimers or unspecific amplification products. In both (a) and (b), 100 bp ladder was run on the lane M

Tables and Figures

Table 1 Japanese cedar samples used in the current study

No[#]	ID	Male flower phenotype	*MS4* genotype[$]
1	Shindai 8	Sterile	*ms4/ms4*
2	S8HK5	Fertile	*Ms4/ms4*
3	Kamikiri Niigata-58	Fertile	*Ms4/ms4*
4	Higashikanbara-5	Fertile	*Ms4/Ms4*
5	S8 × Gosenshi-1	Fertile	*Ms4/ms4*
6	Setsugai Niigata-1	Fertile	*Ms4/Ms4*
7	Kitakanbara-1	Fertile	*Ms4/Ms4*

[#] For development of the PCR-RFLP maker, trees with No 1–4 were used, while for development of the simple DNA extraction method, trees with No 1 and 4–7 were used.

[$] Genotypes were known previously for trees with No 1–5 (Miyajima et al. 2010; Hasegawa et al. 2018; Hirayama et al. 2021), while they were unknown for trees with No 6–7 before the present study.

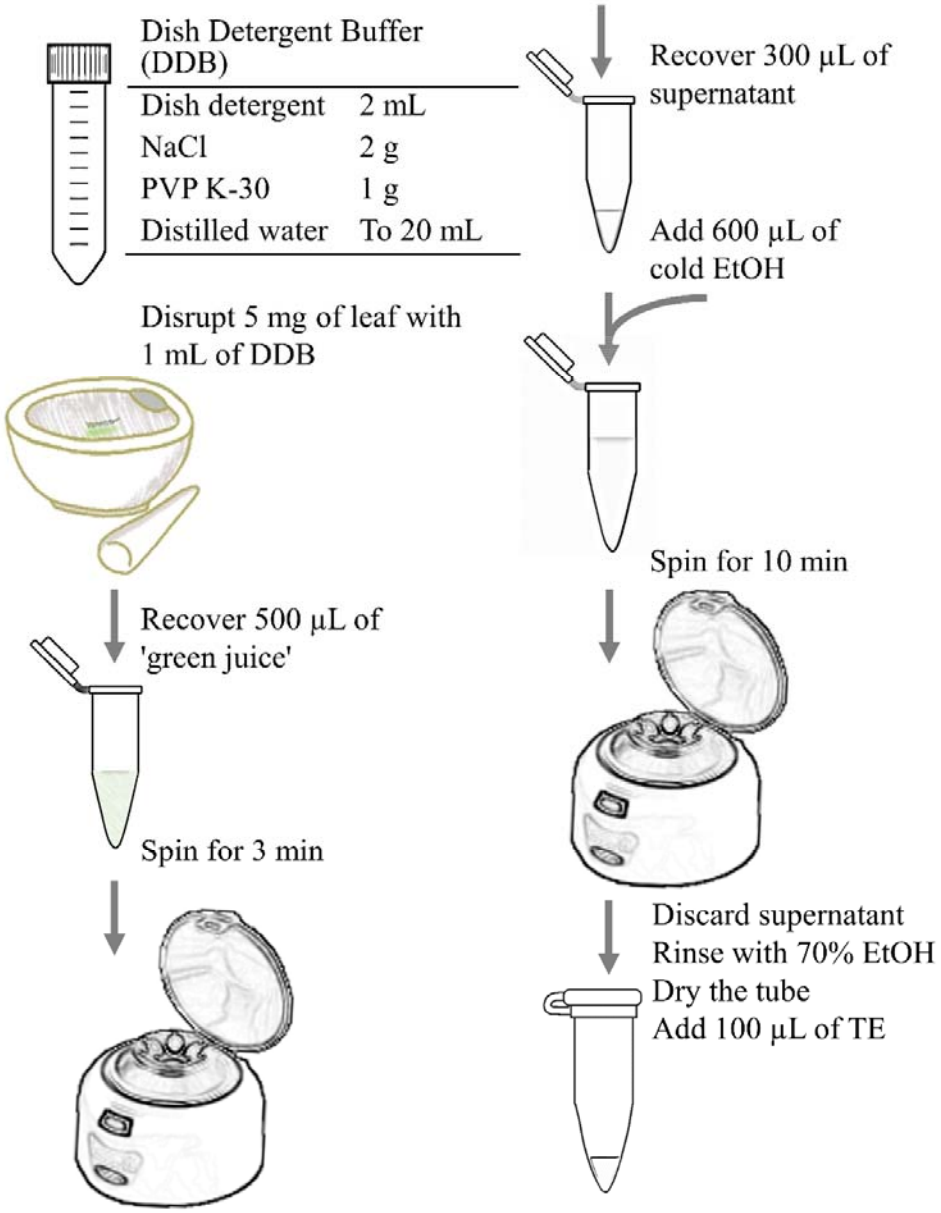
Dish Detergent Buffer (DDB)
Dish detergent 2 mL
NaCl 2 g
PVP K-30 1 g
Distilled water To 20 mL
Disrupt 5 mg of leaf with 1 mL of DDB
Recover 500 µL of 'green juice'
Spin for 3 min
Recover 300 µL of supernatant
Add 600 µL of cold EtOH
Spin for 10 min
Discard supernatant
Rinse with 70% EtOH
Dry the tube
Add 100 µL of TE

Fig. 1

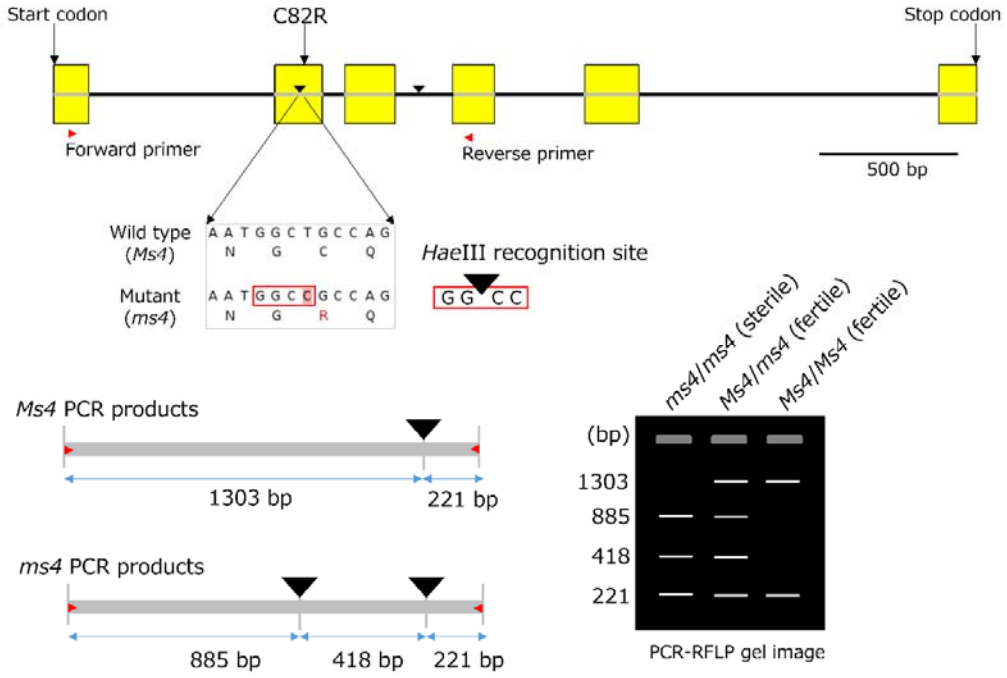
Start codon
C82R
Stop codon
Forward primer
Reverse primer
500 bp
Wild type
(Ms4)
A A T G G C T G C C A G
N G C Q
Mutant
(ms4)
A A T G G C C G C C A G
N G R Q
HaeIII recognition site
G G C C
Ms4 PCR products
1303 bp
221 bp
ms4 PCR products
885 bp
418 bp
221 bp
ms4/ms4 (sterile)
Ms4/ms4 (fertile)
Ms4/Ms4 (fertile)
(bp)
1303
885
418
221
PCR-RFLP gel image

Fig. 2

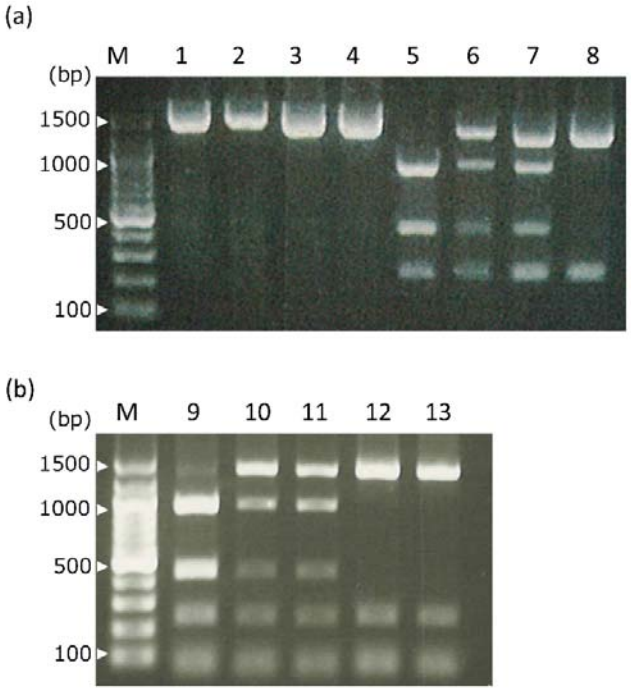
(a)
M 1 2 3 4 5 6 7 8
(bp)
1500
1000
500
100
(b)
M 9 10 11 12 13
(bp)
1500
1000
500
100

Fig. 3