

SIMPLE DNA EXTRACTION AND VALIDATION OF MOLECULAR MARKERS FOR EARLY SEX DETERMINATION IN NEW THAI *Cannabis sativa* L. CULTIVARS

Passakorn Phobphimai¹, Sumeth Imsoonthornruksa², Ratasark Summart^{1,3}, Natteewan Udomsil³ and Mariena Ketudat-Cairns^{1,4 *}

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Abstract

Precision Agriculture (PA) utilizes cutting-edge technology and data management to optimize the efficiency and precision of farming techniques. Early and precise sex determination is essential for the production of *Cannabis sativa* L., as female plants have a higher concentration of cannabinoids, making them more desirable for therapeutic purposes. The objective of this study is to create a rapid and dependable method for detecting male cannabis plants right after young leaves immerge utilizing molecular markers. Thirty cannabis leaf samples of some new cultivars of known sex from Suranaree University of Technology (SUT) cannabis farm were used in the study. DNA was extracted with an innuPREP Plant DNA Kit. The DNA was amplified by Polymerase Chain Reaction (PCR) with MADC2 primers, and the products were evaluated by agarose gel electrophoresis. The results of our study showed that male plants displayed a single DNA band of 390 base pairs, whilst female plants showed two bands of approximately 560 and 870 base pairs. These findings are consistent with prior studies, validating the dependability of the MADC2 marker in identifying the gender of new cannabis cultivars. Easy and rapid DNA extraction from cannabis leaves was also developed. This study emphasizes the significance of molecular approaches in improving cannabis farming. Implementing such strategies has substantial potential for enhancing cannabis breeding and production.

Keywords: *Cannabis sativa*, Sex determination, Molecular marker, MADC2, PCR

¹ School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand. E-mail: filmphob@gmail.com; ratasark_s@hotmail.co.th; ketudat@sut.ac.th*

² Wow Innotech Co. Ltd. 459/7 Moo 4 Chai Mongkol District Mueng Nakhon Ratchasima, Nakhon Ratchasima 30000, Thailand. E-mail: tonikuya@hotmail.com

³ Division of Food Technology, Mahidol University Kanchanaburi Campus, Kanchanaburi 71150, Thailand. E-mail: natteewan.udo@mahidol.edu

⁴ Center for Biomolecular Structure, Function and Application, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand.

* Corresponding author

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Introduction

Precision Agriculture (PA) involves the use of technology and data management to optimize the efficiency and accuracy of farming practices. The primary goal of integrating technology in agriculture is to reduce costs by enabling farmers to predict future occurrences more accurately.

Cannabis sativa L., an annual plant in the Cannabaceae family, is dioecious, meaning it has separate male and female plants. Male cannabis plants produce pollen necessary for reproduction, while female plants can produce flowers with pistils. For medicinal applications, female cannabis plants are preferred due to their higher cannabinoid production compared to male plants or monoecious varieties, which produced self-pollinated seeds with lower cannabinoid yield (Abrams, 2017). Generally, the sex of cannabis plants can be identified 4-8 weeks after planting. Cannabis farmers prefer to harvest unfertilized female flowers for extracting cannabinoids. The demand for a high-throughput screening methodology to rapidly identify male plants after seed germination is increasing. Cannabis employs an XY/XX sex determination system (Mandolino *et al.*, 2002). However, it has been proposed that an X-to-autosome balance system, similar to other flowering plants, might also be involved (Ming *et al.*, 2007, 2011; Razumova *et al.*, 2016; Punja and Holmes, 2020).

Previous research focused on developing male-associated DNA markers (Mandolino *et al.*, 1999), leading to the identification of two distinct genotypes in the male-associated DNA from cannabis (MADC2) region of male and female Cannabis. PacBio long-read sequencing of a male plant along with targeted sequencing of 48 unrelated male genotypes was used to identify the MADC2 male-specific genomic signature (Mandolino *et al.*, 1999, 2002). This prompted the investigation of methodologies for identifying male cannabis and

their ability to distinguish male genotypes from non-males.

Implementing molecular techniques to identify DNA markers for sex determination in new cannabis cultivars in Thailand will enhance farmers' ability to manage their crops efficiently and accurately. This technology can help farmers select female seedlings before planting, reducing costs and increasing the yield of unfertilized cannabis flowers.

Materials and Methods

Plant material and DNA Isolation

Thirty cannabis leaf samples of known gender were obtained from the Suranaree University of Technology cannabis Farm (SUT cannabis) (Table 1). Each cannabis leaf sample was initially ground in a mortar with liquid nitrogen until it became fine powder and was then placed in a 1.5 mL tube. DNA was extracted by the innuPREP Plant DNA Kit (Analytik Jena, Germany) protocol. The extracted DNA concentration and purity was evaluated by its absorbance at 260 and 280 nm wavelengths in a spectrophotometer and by gel electrophoresis. The DNA was then used for amplification via the Polymerase Chain Reaction (PCR).

For the simple and rapid DNA extraction method (modified from Wang *et al.*, 1993; Werner *et al.*, 2002; Araujo *et al.*, 2021), 5 mg of young cannabis leaf tissue was ground in an Eppendorf tube containing 100 µL of 0.1 mM NaOH with a small plastic pestle for a few minutes. Alternatively, leaves can be ground in a mortar with a pestle and then transferred to the Eppendorf tube. The sample was then neutralized with 100 µL of 0.1 M HCl, and the grinding process continued for an additional 1-2 minutes. Next, 50 µL of the mixture was pipetted into a new Eppendorf tube containing 450 µL of

Table 1. Leaves samples from SUT Cannabis farm

No	Cultivar	Sex	No	Cultivar	Sex	No	Cultivar	Sex
1	SUT10 #3	Female	11	Charlott angle (CA4)	Female	21	SER 13	Male
2	Hangkrarok	Male	12	Charlott web (CW2)	Female	22	SER 14	Male
3	Golden Tiger 5 (GT5)	Female	13	ACDC 1	Female	23	SER 15	Female
4	Golden Tiger 6 (GT6)	Female	14	ACDC 2	Female	24	SER 16	Female
5	Blue venom 1 (BV1)	Female	15	SER 7	Female	25	SER 17	Male
6	Blue venom 2 (BV2)	Female	16	SER 8	Female	26	SER 18	Female
7	Baox #1	Male	17	SER 9	Male	27	SER 19	Female
8	Baox #5	Male	18	SER 10	Female	28	SER 20	Female
9	Orange valley #1 (CBD14)	Female	19	SER 11	Female	29	SER 21	Female
10	Orange valley #2 (CBD14)	Female	20	SER 12	Male	30	SER 22	Female

Table 2. Primer used for sex determination

Primer	Primer Sequences (5'→3')	Amplicon size (bp)
MADC2	F: GTGACGTAGGTAGAGTTGAA	390 (Male)
	R: GTGACGTAGGCTATGAGAG	560 and 870 (Female)
SCAR119	F: TCAAACAACAACAAACCG	119 (Male)
	R: GAGGCCGATAATTGACTG	

50 mM Tris-HCl, pH 8.0 (diluted 1:10), and vortexed (This method does not require centrifugation to precipitate large particles). One μ L aliquot of this solution was used as a DNA template for subsequent analysis.

PCR Conditions

The DNA was amplified with the MADC2 and SCAR119 primers (Table 2) by PCR reaction in a BIO-RAD thermal cycler (T100™). PCR was performed in a total volume of 25 μ L consisting of 0.5 U Taq DNA polymerase (homemade), 1X aliquot buffer, 0.1 mM of each deoxynucleoside triphosphate (dNTP) mixture (New England Biolabs, Beverly, Massachusetts), 0.3 μ M of each primer, and 20-50 ng of template DNA. The same PCR protocols as Mandolino *et al.* (1999) were used for MADC2 and SCAR119 markers. The PCR started with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at the primer's specified annealing temperature for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. Subsequently, the PCR products were analyzed by 2% agarose gel electrophoresis, stained with a 1:20,000 dilution of RedSafe™ (iNtRON Biotechnology, WA, USA). The presence and size of PCR products were visualized over a UV transilluminator (BIORAD® Gel Doc XDR) and analyzed with Quantity One software (BIORAD®, CA, USA).

Results and Discussion

Comparison of MADC2 and SCAR119 markers

Genomic DNA was extracted from 30 cannabis leaf samples collected from SUT cannabis farm. The total genomic DNA from five selected samples was amplified with the MADC2 and SCAR119 primers. The MADC2 primers amplification product showed a single DNA band of approximately 390 bp in all-male plants, while female and monoecious plants exhibited two bands of approximately 560 bp and 870 bp, respectively (Figure 1(a)). The SCAR119 primers amplification product showed a single DNA band of

approximately 119 bp in all-male plants, with no bands observed in female and monoecious plants.

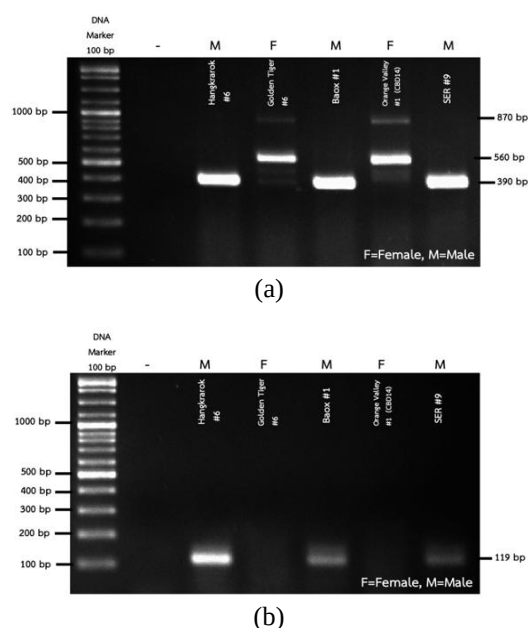


Figure 1. Visualization of PCR products for sex determination of cannabis by MADC2 (a) and SCAR119 (b) primers.

Our sex determination results with the SCAR119 marker generally align with those obtained with the MADC2 marker in males. This finding is consistent with the report by Faux *et al.* (2014), which states that male plants exhibit a single band of 119 bp. However, it is challenging to discern whether the absence of a band in female plants is due to a false negative result or if the plants are indeed female, as no band is observed. Therefore, we opted to use the MADC2 primers to amplify the new cannabis varieties found in Thailand, as it provides a clearer distinction between male and female plants.

MADC2 marker amplification products

The total genomic DNA of all 30 cannabis leaf samples from SUT cannabis was amplified with the MADC2 primers (Figure 2).

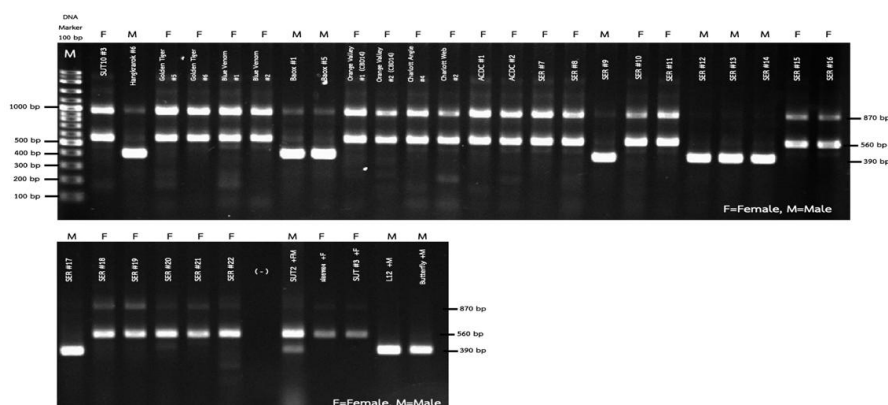


Figure 2. Visualization of PCR products for sex determination of cannabis by MADC2 primer

According to Mandolino *et al.* (1999), MADC2 is a genetic marker that can be amplified by PCR to produce a 390 bp amplicon, which is linked to the manifestation of the male phenotypic morphology. This study involved the testing of 30 cannabis leaf samples obtained from SUT cannabis, Suranaree University of Technology. Some of these cultivars were newly bred and developed at SUT and now are widely grown in Thailand because of their high productivity and favorable characteristics. Prior to analysis, the gender of all samples was already known. The results of our study confirm the idea described earlier. This is evidenced by the PCR results, which revealed genetic profiles that align with the known male and female plant genotypes, corresponding to the observed sexes of the samples. The consistent results of the MADC2 marker in determining the sex of Thai cultivars further support its dependability. In addition, Tehen *et al.* (2010) conducted studies on sex determination in cannabis utilizing multiple MADC markers, which consistently and accurately identified female plants in every case.

Female cultivars are preferred in medicinal applications because they have the capacity to produce substantial quantities of secondary metabolites, including cannabinoids, terpenes, and flavonoids, which have diverse therapeutic and recreational characteristics (Torres *et al.*, 2022). Efficiently identifying and cultivating female plants is essential for maximizing the production of these important chemicals.

Prior research has emphasized the significance of sex determination in the breeding and culture of cannabis. To avoid pollination and maintain the quality and yield of female flowers, it is common practice to eliminate male plants (Hermsmeier *et al.*, 2004). Identifying the sex of plants at an early stage enables better allocation of resources and enhanced management of crops (van Bakel *et al.*, 2011).

Moreover, the progress in next-generation sequencing (NGS) technologies has facilitated the creation of efficient tools for genetic analysis in cannabis. Sawler *et al.* (2015) showed the application of Next-Generation Sequencing (NGS) in identifying sex-linked markers, which establishes a strong foundation for genomic research in cannabis. These technologies provide an unparalleled level of detail and precision, which allows for the development of more advanced breeding methods (Vergara *et al.*, 2019).

The use of MADC2 as a dependable indicator for determining the sex of Thai cannabis cultivars is consistent with prior research and promotes the progress of cannabis breeding initiatives. Utilizing molecular technologies for sex determination improves cultivation efficiency and facilitates the development of high-quality plants with desirable features.

Simple and rapid DNA extraction

A total of 12 cannabis leaf samples, randomly selected from a pool of 30 samples collected from SUT cannabis, were subjected to genomic DNA extraction by a simple and rapid method. The extracted DNA was then analyzed for amplification with the MADC2 primers. The amplification products from the MADC2 primers revealed DNA bands that were consistent with the previously identified sex of the samples (Figure 3). However, the more rapid, easy and simple DNA extraction method provides DNA samples that are not very clean. Therefore, in some of the cannabis male samples, 3 bands of 870, 560 and 390 bp can be observed. The present of the 390 bp band can be used to identify the sex of the plant. This consistency confirms the reliability of the simple and rapid extraction method, and the primers can be used accurately to determine the sex of the cannabis plants.

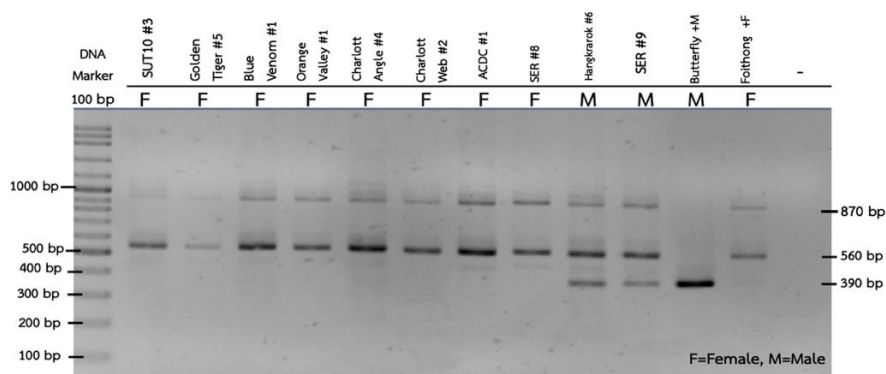


Figure 3. Visualization of PCR Products for Sex Determination of Cannabis Using the MADC2 Primer from simply and Rapidly Extracted DNA

The DNA extraction method utilized in this study proved to be both simple and rapid, requiring approximately 5-10 minutes for DNA template preparation, including sample grinding, and an additional 1.5-2 hours to complete the PCR, followed by 30 minutes agarose gel electrophoresis. Compared to traditional extraction methods, this approach is also significantly more cost-effective, utilizing only three chemicals (NaOH, HCl, and Tris- HCl) as opposed to the 10-15 chemicals required in the method described by Bellstedt *et al.* (2010). This method demonstrated superiority over other quick DNA extraction methods for certain plant species, particularly in terms of success rate, cost efficiency, and speed of the experiment. Furthermore, it showed better storability of the extracted DNA, as noted by Osmundson *et al.* (2013). Notably, this method did not require the use of a centrifuge to precipitate large particles, yet it was still effective in producing amplicons. The efficiency and cost-effectiveness of this method make it an attractive alternative for routine DNA extraction in plant studies. The ability to perform the extraction without specialized equipment, such as a centrifuge, further enhances its applicability in various laboratory settings, particularly those with limited resources. The rapid preparation and processing times also facilitate quicker experimental workflows, making this method highly suitable for high-throughput studies.

Conclusions

This study demonstrated the efficacy of the MADC2 marker in identifying the sex of cannabis plants. DNA from 30 leaf samples, encompassing 13 different cannabis varieties, was analyzed, and the genotype results of the MADC2 marker

corresponded accurately with the phenotypic sex of all samples. These findings confirm the reliability of the MADC2 marker for sex determination in cannabis. Additionally, the successful extraction of DNA from cannabis leaves by the simple and rapid method further underscores the practical applicability of these techniques. Future studies should focus on refining these molecular techniques to improve the applicability of sex determination methods in field use, ultimately enhancing cannabis breeding and cultivation practices

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