CHAPTER III
RESEARCH METHODOLOGY

3.1 Cannabis

3.1.1 Sample

Thirty cannabis leaf samples of known gender were obtained from the Suranaree University of Technology cannabis Farm (SUT cannabis) in Nakhon Ratchasima (14.871121, 102.025668). The collected samples were stored at -20°C until further analysis.

3.1.2 DNA Extraction

Each cannabis (*Cannabis sativa* L.) leaf sample was first mashed in a mortar with liquid nitrogen until it attained a fine powder consistency, thereafter transferred to a 1.5 mL tube. DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena, Germany) procedure. The concentration and purity of the extracted DNA was evaluated using absorbance measurements at 260 and 280 nm using a spectrophotometer, as well as via gel electrophoresis. The DNA was further used for amplification by the Polymerase Chain Reaction (PCR).

In a simple and rapid DNA extraction technique (adapted 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 with 100 µL of 0.1 mM NaOH using a small pestle for a few seconds. Leaves was also crushed in a mortar using a pestle and after that transferred to the Eppendorf tube. The sample was further neutralized with 100 µL of 0.1 M HCl, and the grinding process continued for an additional 1-2 minutes. Subsequently, 50 µL of the mixture was transferred into a new Eppendorf tube containing 450 µL of 50 mM Tris-HCl, pH 8.0 (diluted 1:10), and vortexed (this technique does not need centrifugation to precipitate big particles). One µL aliquot of the solution was used as DNA template for subsequent analysis.

3.1.3 Specificity Testing of DNA markers for Cannabis sex using PCR

The DNA was amplified with the MADC2 and SCAR119 primers **(Table 3.1)** by PCR reaction in a BIO-RAD thermal cycler (T100™). PCR was performed in a total volume of 25 μL consisting of 0.5 U *Taq* DNA polymerase (homemade), 1X PCR buffer with $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate (dNTP) mixture (New England Biolabs, Beverly, Massachusetts), 0.3 μM 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).

Table 3.1 Primers used for Cannabis 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	

3.2 Date Palm

3.2.1 Sample

Sample of young Date Palm (*Phoenix dactylifera* L.) leaves of both known and unknown sex were collected from 13 cultivars: KL1, Yellow Barhee, Red Barhee, Red India, Um ed Dahan, Khoneizi, Shi Shi, Ghannami, Jozi, Braim, G2, H1, and AP-red1. These samples were obtained from BunMak Date Palm in Nakhon Ratchasima (14.67360, 102.39645) and Phutara Green Park in Buriram (14.69488, 102.50448), Thailand. The collected samples were stored at -20°C until further analysis.

3.2.2 DNA Extraction

Twenty milligrams of date palm leaf samples were mashed in a mortar by protocol modified from Keb-Llanes et al. (2002) with 700 μL of lysis buffer solution containing 100 mM Tris-HCl, 20 mM EDTA, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 4% (w/v) polyvinylpyrrolidone (PVP), 20% (w/v) sodium dodecyl sulfate (SDS), and 10 mM β-mercaptoethanol. The DNA concentration and purity were measured using a Nanodrop spectrophotometer (Thermo Scientific, USA) and visualized by 0.8% agarose gel electrophoresis before being used as a template in PCR (Polymerase Chain Reaction).

3.2.3 Specificity Testing of DNA markers for Date Palm sex using PCR

A total of 40 DNA samples, comprising 20 female and 20 male samples from 13 date palm cultivars, were used as template for LOX 5.1 and GPAT3 gene fragments amplification using PCR for the analysis of a reference gene and male-specific DNA markers, as previously reported (**(Table 3.2)** Jani et al., 2022; Torres et al., 2018). The DNA was amplified using multiplex primer PCR, and the reaction was carried out using a BIO-RAD thermal cycler (T100™). The PCR protocol began with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of 95°C for 20 seconds, annealing at the primer's specified annealing temperature for 20 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 under 2% agarose gel electrophoresis, which was stained with a 1:20,000 dilution of RedSafe™ (iNtRON Biotechnology, WA, USA).

Table 3.2 Primer sequences for Date Palm sex determination and their reference

No	Primer name	Sequences (5'→ 3')	Amplicon size (bp)	Reference
1	GPAT3	**F:** AGAAAACCTGATATGCTCTCTG	450	Torres et al., 2018
		R: TGTGATGCACTTGGTAACTACT		
2	LOX5.1	**F:** CTACACCGCAGAGTTTGTCG	156	Jani et al., 2022
		R: AGATTGGACCCATGAGTTGC		

3.2.4 Development of LAMP technique for determining the sex of date palm LAMP primer design

PCR products were purified using a PCR purification kit to remove any impurities and ensure clean sequencing results. The purified PCR products were then sent to a sequencing facility to obtain the nucleotide sequences of the target regions from each cultivar. After sequencing, the quality of the sequencing data was checked to ensure accuracy and completeness. FastQC was used to assess the quality of the sequence reads. Next, the sequences were imported into sequence alignment software that supports ClustalW (e.g., MEGA, BioEdit). Multiple sequence alignment was performed using ClustalW to identify conserved and variable regions among the 13 date palm cultivars. The alignment results were analyzed to identify regions suitable for LAMP primer design. LAMP primers were designed using NEB® LAMP promer design tool software version 1.4.2 (New England BioLabs Co., Ltd.). Parameters for LAMP primer design, including primer length, GC content, and temperature conditions, were optimized to ensure efficient primer performance.

3.2.5 LAMP Reaction and Detection

3.2.5.1 Commercial set Testing (10X *Bsm* buffer)

LAMP reactions were carried out as described previously by Notomi et al. (2000), with some modifications. The primers used were designed as mentioned in 3.4. In brief, each 25 µL reaction mixture contained 1.6 µM of each FIP and BIP primer, 0.8 µM of each LF and LB primer, and 0.2 µM of each F3 and B3 primer (Integrated DNA Technologies (IDT)®, Seoul, South Korea). The reaction was carried out in 1X ThermoPol® Reaction Buffer, which contains 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton® X-100 (pH 8.8 at 25°C), 1.4 mM deoxynucleoside triphosphate (dNTPs) mixture, *Bst* DNA polymerase (New England Biolabs, Beverly, Massachusetts, USA). The mixture also included 0.8 M betaine (Sigma-Aldrich, St. Louis, Missouri, USA), and 1 µL of 30–50 ng DNA template was used. The reaction mixture was incubated at 62°C for 60 minutes. The results were checked visually by observing the formation of white

precipitate, which indicates the accumulation of magnesium pyrophosphate, a by-product of the LAMP reaction. The presence of turbidity in the reaction tube was considered a positive result, as it reflects successful DNA amplification (Mori et al., 2001; Notomi et al., 2000). To confirm the amplification, the reaction products were also analyzed by electrophoresis on a 2% agarose gel stained with RedSafe™ (1:20,000 dilution; iNtRON Biotechnology, Washington, USA).

3.2.5.2 Phenol red preparation

To prepare a 5 mM phenol red solution, 188.2 mg of phenol red sodium salt (molecular weight ≈ 376.38 g/mol) was measured using an analytical balance (Sigma-Aldrich, 2023). The powder was transferred to a clean beaker and dissolved in approximately 80 mL of distilled water with continuous stirring. Once fully dissolved, the solution was poured into a 100 mL volumetric flask, and distilled water was added to make up the final volume. The pH of the solution was checked using a pH meter and adjusted to 8.0 by 2.5 M NaOH (KemAus™, New South Wales, Australia), as phenol red acts as a pH indicator and changes color based on the pH of the solution (Invitrogen, 2008; Rinkenberger & Horwitz, 1993). For experiments requiring sterility, the solution was filtered using a 0.22 µm syringe filter (Freshney et al., 2015). The prepared phenol red solution was stored at 4°C and protected from light to maintain stability (U.S. Pharmacopeia., 2022).

3.2.5.3 2X LAMP with phenol red variation

LAMP reactions were carried out as described previously by Notomi et al. (2000), with some modifications. The primers used were designed as mentioned in 3.4. In brief, a 25 µL reaction mixture containing 1.6 mM of each FIP and BIP primer, 0.8 mM of each LF and LB primer, 0.2 mM of each F3 and B3 primer, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine (Sigma-Aldrich, St. Louis, Missouri, USA), 0.125 mM phenol red (Loba Chemie, MUMBAI, Maharashtra, India), 1.4 mM dNTPs mixture (New England Biolabs, Beverly, Massachusetts, USA), 3.41 pmol *Bst* DNA

polymerase (homemade), and 1 μL of 30–50 ng DNA template was used. The reaction mixture was incubated at 65°C for 60 minutes. The reaction products were examined visually for a color change in phenol red. A positive result was indicated by a color shift from orange to yellow due to the pH change. Additionally, electrophoresis of the product on a 2% agarose gel stained with a 1:20,000 dilution of RedSafe™ (iNtRON Biotechnology, WA, USA) was performed to confirm amplification.

3.2.6 Lyophilization process

To develop the date palm sex identification kit, the conditions for the LAMP reaction were first optimized, and ready-made solution sets were designed. Prior to the lyophilization process, the buffer solution (containing 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, and 0.125 mM phenol red) was separated from the master mix. This buffer solution was stored at 4°C and later added to reconstitute the reaction with the DNA template. The master mix used for lyophilization consisted of 1.6 mM of each FIP and BIP primer, 0.8 mM of each LF and LB primer, 0.2 mM of each F3 and B3 primer, 1.4 mM dNTPs mixture, and 3.41 pmol *Bst* DNA polymerase (homemade). Sugars were incorporated into the master mix to maintain reaction stability during freeze-drying. Two sugars, 5% D(+)-trehalose dihydrate (ACROS Organics) and sucrose (KemAus™, New South Wales, Australia), were tested for their effectiveness in preserving the reaction components (Jovanović et al., 2006; Kommineni et al., 2022; Zhao et al., 2022). The prepared LAMP master mix was then subjected to a freeze-drying (lyophilization) process. The reaction solution was first prepared and cooled below the freezing point in -80°C freezer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) before being placed in a freeze dryer (CHRIST®, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The condenser temperature was lowered to -85°C, and a vacuum of 0.050 mbar was applied. Once the samples were completely dried, they were removed from the freeze dryer.

3.2.7 Testing of lyophilized product

Each lyophilized reagent tube was resuspended with 24.0 µL of buffer, prepared as described in 3.6. After resuspension, 1 µL of 30–50 ng DNA template was added to each tube to initiate the LAMP reaction. The reaction mixture was then incubated at 65°C for 60 minutes to facilitate DNA amplification. The reaction products were examined visually for a color change of phenol red. A positive result was indicated by a color shift from orange to yellow due to the pH change.

3.2.8 Stabilized Freeze-Dried LAMP

The freeze-dried LAMP master mix was tested for stability at 25°C, 4°C, and -20°C over a period of six months. Evaluations were conducted at 7, 14, 21, and 28 days, as well as at 2 and 6 months to assess its performance. The testing procedure followed the method described in 3.7. Additionally, electrophoresis of the amplification product was performed on a 2% agarose gel stained with a 1:20,000 dilution of RedSafe™ to confirm successful DNA amplification.

3.2.9 Properties of Date Palm sex identification kits

Each lyophilized reagent tube was resuspended with 24.0 µL of LAMP buffer and thoroughly mixed using a portable vortex mixer. Then, 1 µL of DNA template (30–50 ng) was added to initiate the LAMP reaction. The reaction was conducted in a small, thermal block at 65°C for 60 minutes, making it suitable for on-site testing.

To facilitate field deployment, the results were evaluated using simple and rapid detection methods. The LAMP reaction mixture contained phenol red, enabling direct visual detection by the naked eye. A positive reaction was indicated by a color change from orange to yellow, confirming successful DNA amplification.