IDENTIFICATION OF *Cannabis* STRAINS USING INTER-SIMPLE SEQUENCE REPEATS (ISSRs) MOLECULAR MARKER

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การระบุสายพันธุ์ *Cannabis* โดยใช้เครื่องหมายโมเลกุลแบบ Inter-simple sequence repeats (ISSRs)

นางสาวสุภาพร แก้วมณี

วิทยานิพนธ์นี้สำหรับการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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คำสำคัญ : กัญชา/กัญชง/เครื่องหมายระดับโมเลกุล/ISSR/การระบุสายพันธุ์

กัญชาเป็นพืชในวงศ์ Cannabaceae ซึ่งโดยทั่วไปแบ่งออกเป็นสองประเภทหลัก ได้แก่ กัญชา (Marijuana) และกัญชง (Hemp) โดยกัญชามีปริมาณสารเตตราไฮโดรแคนนาบินอล (THC) มากกว่า 0.3% ต่อมวลแห้งของพืช ในขณะที่กัญชงมีปริมาณ THC น้อยกว่า 0.3% ต่อมวลแห้งของพืช ซึ่งอยู่ในปริมาณที่กฎหมายกำหนดให้อนุญาตได้ ดังนั้น กัญชงจึงเหมาะสมสำหรับการใช้งานทั้งในด้านสุขภาพ ด้านการแพทย์และอุตสาหกรรม อย่างไรก็ตาม การจำแนกความแตกต่างระหว่างกัญชาและกัญชง โดยเฉพาะในระยะการเจริญเติบโตของพืช เป็นเรื่องที่ท้าทายเนื่องจากความคล้ายคลึงกันทางพันธุกรรมและลักษณะทางสัณฐานวิทยา ดังนั้น การประยุกต์ใช้เทคนิคเครื่องหมายโมเลกุล ISSR (Inter-Simple Sequence Repeats) จึงสามารถระบุความหลากหลายทางพันธุกรรมได้อย่างแม่นยำและรวดเร็ว รวมถึงช่วยแยกความแตกต่างระหว่างกัญชาและกัญชงได้อย่างมีประสิทธิภาพ การศึกษาครั้งนี้ได้วิเคราะห์ความหลากหลายทางฟีโนไทป์และพันธุกรรมของสายพันธุ์กัญชาจำนวน 12 สายพันธุ์ที่ปลูกใน "ฟาร์มกัญชา มทส." เพื่อให้ได้การจัดจำแนกสายพันธุ์ที่แม่นยำ ได้ทำการวิเคราะห์ลักษณะทางฟีโนไทป์ร่วมกับโปรไฟล์ DNA ด้วยเครื่องหมาย ISSR และการเพิ่มจำนวนชุดยีนเฉพาะเพื่อควบคุมการสร้างสารแคนนาบินอยด์ด้วยเทคนิค PCR ในเบื้องต้นพบว่าผลการประเมินฟีโนไทป์แสดงให้เห็นความทับซ้อนของลักษณะต่างๆ อย่างมีนัยสำคัญ เช่น ความสูงของต้น (114.67–151 ซม.) และความยาวของใบ (9.3–12.67 ซม.) ทำให้ลักษณะทางสัณฐานวิทยาเพียงอย่างเดียวไม่เพียงพอสำหรับการระบุสายพันธุ์อย่างถูกต้อง การวิเคราะห์เครื่องหมาย ISSR โดยใช้ไพรเมอร์ที่มีความหลากหลายสูงจำนวน 11 ชุด สร้างแถบ DNA ได้จำนวน 782 แถบ และอัตราความหลากหลายทางพันธุกรรมสูงถึง 93.47% ช่วยให้สามารถประเมินความหลากหลายทางพันธุกรรมและจัดกลุ่มสายพันธุ์ได้ เมื่อทำการวิเคราะห์เดนโดรแกรมพบว่าตัวอย่างสามาถแบ่งออกเป็นสองกลุ่มหลัก คือ กลุ่มที่มีสายพันธุ์ที่เกี่ยวข้องกันทางพันธุกรรม 10 สายพันธุ์ และกลุ่มที่ประกอบด้วยสายพันธุ์ที่มีความแตกต่างกันอย่างชัดเจน 2 สายพันธุ์ (RPF1 และ RPF2) นอกจากนี้ เมื่อวิเคราะห์ด้วยยีนที่ควบคุมการสร้างเฉพาะสารแคนนาบินอยด์ด้วย PCR พบว่าเพิ่มความละเอียดในการจำแนก โดยสามารถแยกสายพันธุ์ที่มี THC เด่นชัด (เช่น พันธุ์ฝอยทองและหางกระรอก) ออกจากสายพันธุ์ที่มี CBD เด่นชัด (เช่น Charlotte's Web, RPF1) โดยอาศัยแถบ DNA ขนาด 589-bp และ 169-bp ตามลำดับ วิธีการแบบผสมผสานนี้แสดงให้เห็นถึงข้อจำกัดของลักษณะทางฟีโนไทป์เพียงอย่างเดียว และเน้นย้ำถึง

ความสำคัญของเครื่องมือทางพันธุศาสตร์ในการระบุสายพันธุ์กัญชาได้อย่างถูกต้อง ซึ่งมีความสำคัญต่อโครงการปรับปรุงพันธุ์ การปฏิบัติตามกฎหมาย และการพัฒนาลักษณะพันธุกรรมที่ตอบสนองความต้องการของตลาด

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SOUPHAPHONE KEOMANY : IDENTIFICATION OF CANNABIS STRAINS USING INTER-SIMPLE SEQUENCE REPEATS (ISSRs) MOLECULAR MARKER. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Ph.D., 68 PP.

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Cannabis, plants in the Cannabaceae family, are generally divided into two categories: marijuana and hemp. Marijuana contains more than 0.3% tetrahydrocannabinol (THC) by dry weight per plant, whereas hemp contains less than 0.3% THC by dry weight, which is a quantity within legal limits. Therefore, hemp is suitable for both medical and industrial applications in the health sector. However, distinguishing between marijuana and hemp, particularly during the plant's growth stages, is challenging due to their genetic and morphological similarities. Therefore, the application of Inter-Simple Sequence Repeats (ISSR) molecular marker techniques can accurately and rapidly identify the genetic diversity and distinguish between marijuana and hemp plants. This study investigated the phenotypic and genetic diversities of 12 cannabis strains cultivated at the "SUT Cannabis Farm". To achieve precise strain classification, an integrated approach involving phenotypic trait analysis, ISSR marker-based DNA profiling, and chemotype-specific PCR amplification was employed. Phenotypic evaluations revealed significant overlaps among traits such as plant height (114.67–151 cm) and leaf length (9.3–12.67 cm), limiting the utility of morphological features for reliable strain identification. ISSR marker analysis, utilizing 11 highly polymorphic primers, produced 782 DNA bands with a polymorphism rate of 93.01%, enabling genetic diversity assessments and clustering of strains. A dendrogram revealed two major clusters: one containing 10 genetically related strains and another with two distinct strains (RPF1 and RPF2). Chemotype-specific PCR further refined classification by distinguishing THC-dominant strains (e.g., Foithong, Hangkarok) from CBD-dominant strains (e.g., Charlotte's Web, RPF1), based on the presence of 589-bp and 169-bp amplicons, respectively. This integrated methodology highlights the limitations of phenotypic traits alone and underscores the importance of genetic tools for

accurate cannabis strain identification, with significant implications for breeding programs, regulatory compliance, and market-driven trait development.

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Co-advisor's Signature_____

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µL	=	microliter
A	=	Adenine
bp	=	base pair
C	=	Cytosine
CBD	=	Cannabinoid
CBDAS	=	Cannabidiolic acid synthase
CH_3COOH	=	Acetic Acid
cm	=	centimetre
CTAB	=	Cetyltrimethylammonium bromide
DI water	=	Deionized Water
DNA	=	Deoxyribonucleic Acid
dNTP	=	Deoxyribonucleotide triphosphate
EDTA	=	Ethylene diamine tetra-acetic acid
EtOH	=	Ethanol
FDA	=	Food and Drug Administration
G	=	Guanine
HNO_3	=	Nitric acid
ISSRs	=	Inter-simple sequence repeats
K	=	Potassium
L	=	Liter
M	=	Metre
M	=	Molar
mg/µL	=	milligram per microliter
mL	=	milliliters
mm	=	millimeters
mM	=	millimolar
N	=	Nitrogen

LIST OF ABBREVIATIONS (Continued)

NaCl	=	Sodium chloride
ng/µL	=	nanograms per microliter
NGS	=	Next-generation sequencing
P	=	Phosphorus
pH	=	Potential of Hydrogen
RDPA	=	Randomly amplified polymorphic DNA
rpm	=	revolutions per minute
SNP	=	Single Nucleotide Polymorphisms
SSRs	=	Simple sequence repeats
SUT	=	Suranaree University of Technology
T	=	Thymine
TBE buffer	=	Tris-borate-EDTA buffer
TE buffer	=	Tris-EDTA buffer
THC	=	Tetrahydrocannabinol
THCAS	=	Tetrahydrocannabinolic acid synthase - Cannabis ...
Tris-HCl	=	Tris Hydrochloride
UPGMA	=	Unweighted pair group method with arithmetic average
v/v	=	volume per volume
w/v	=	weight per volume

CHAPTER I
INTRODUCTION

1.1 Introduction

The cannabis plants are classified in the Cannabaceae family, with significant medicinal and industrial uses. The crop is commonly classified into two main categories: (1) medicinal that contains cannabidiol (CBD) levels lower than 0.5% and Δ9-tetrahydrocannabinol (THC) levels greater than 0.5%, and (2) fiber or hemp with THC levels below 0.3% (Hesami et al., 2020). Marijuana has been used for medical purposes, including the production of painkillers and sleeping pills, among others, while hemp has been used for a variety of commercial products, including textiles, paper, pharmaceuticals, food, animal feed, and more. (Rehman et al., 2021).

Currently, cannabis strains that obtain high level of THC (Marijuana) and high level of CBD (Hemp) are readily accessible when extracting the chemical compound found in flower buds (Zhang et al., 2018). Distinguishing between the different strains can still be challenging, but it can be achieved through various means, such as physiological characteristics, chemical compounds (cannabinoids), and DNA molecular markers. Each method has its advantages and limitations, such as (1) isolation by morphology, which allows for easy visual differentiation; (2) chemical compounds (cannabinoids), useful when the plant cannot see and (3) DNA molecular markers, which can identify the desired cannabis in the seedling stage.

Genetic variations at the DNA level can be detected using molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and inter-simple sequence repeat (ISSR) (Liu et al., 2008). Inter-simple sequence repeats (ISSRs) are regions in the genome surrounded by microsatellite sequences. PCR amplification of these regions with a single primer results in multiple amplification products, which can serve as a dominant multi-locus marker system for studying genetic variation in various organisms (Ng & Tan, 2015).

This research focuses on using DNA markers to identify the dominance of THC and CBD in cannabis strains. The use of DNA markers will allow for early identification of the cannabis strains during the seedling stage, thus reducing the time needed for selection during the breeding process. DNA markers offer a more precise and accurate method of strain identification, as both the public and private sectors in Thailand currently rely on morphological characteristics. Several method DNA markers are available, but this research chooses inter-simple sequence repeats (ISSRs) to characterize cannabis strains. This type of marker is suitable for non-plants model such as cannabis, and it requires no prior DNA sequence data is low-cost, easy to use, and can be applied to any type of plant (Barth et al., 2002). Considering these advantages, the ISSR marker will be selected to differentiate between Thai and foreign cannabis strains, with the aim of facilitating future agricultural applications.

1.2 Research objectives

1.2.1. To select a suitable ISSR marker for cannabis chemotype and origin grouping

- Identifies the best primer to use with ISSR (Inter Simple Sequence Repeat) markers.

1.2.2 To identify cannabis strains using the ISSRs marker

- Differentiate and identify different cannabis strains based on their genetic profiles using the ISSR marker technique.

CHAPTER II

A LITERATURE REVIEWS

2.1 Cannabis Botanical Overview

2.1.1 Taxonomic

Kingdom: Plantae

Order: Rosales

Family: Cannabaceae

Genus: *Cannabis*

Species: *Cannabis sativa* L.

Cannabis indica Lam.

Cannabis ruderalis Janisch

Determining when cannabis evolved is challenging as the genus lacks a well-documented fossil record (impressions of leaves or fruits in rocks). In 1883, Friedrich found fossil leaves in Germany, which he named *Cannabis oligogenic* (Figure 1A, B). The species epithet refers to the Oligocene Epoch, although the exact date is not specified. In 1982, Palamarev identified a fossil seed (achene) as "*Cannabis* sp." in Bulgaria (Figure 1C) and dated, it to the Pontian age, 7.3-5.3 million years ago, which is the end of the Miocene Epoch (23.03–5.33 million years ago) (McPartland, 2018).

Cannabis is an annual herb, not typically perennial, and it rarely has single leaves. Instead, its leaves are usually palmately compound, with multiple leaflets. The plant grows up to 1–6 meters tall, with green, hollow, cylindrical stems that are longitudinally ridged. The petioles are variable in length and often longer than 0.5–1.5 cm, depending on the strain. Male and female flowers are produced on separate plants (dioecious) and are typically covered with trichomes, which produce compounds like THC and CBD. Cannabis plants generally bloom during the summer months, from July to August in the Northern Hemisphere, although this can vary based on the strain and environmental conditions.

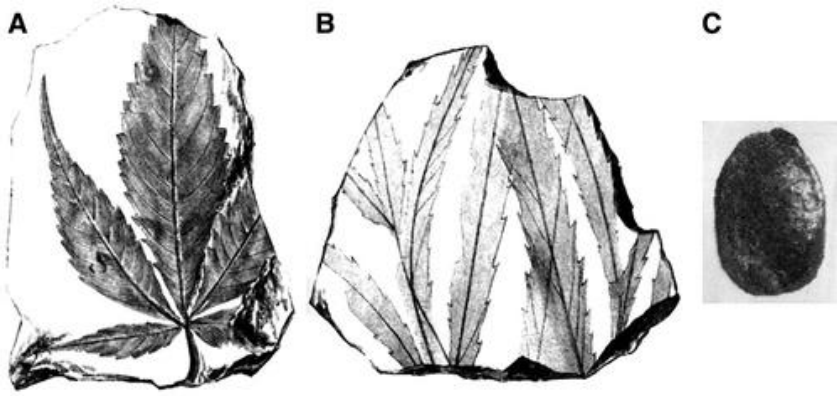


Figure 2.1 Macrofossils identified as cannabis (not to scale). (A, B) Reproduced from a publication whose copyright has expired; (C) Reproduced courtesy of Vladimir Bozukov, Bulgarian Academy of Sciences (Friedrich, 1883); (Palamarev, 1982).

Cannabis strains can be broadly categorized into two types based on their flowering characteristics: auto-flowering and photoperiod.

Auto-flowering Cannabis: This type transitions from the vegetative to the flowering stage based on its age rather than light exposure. Derived from *C.ruderalis*, auto-flowering plants begin to flower automatically after 2–4 weeks of growth and have a shorter lifecycle, typically 8–12 weeks from seed to harvest. They are compact, easy to grow, and ideal for beginners, but they often produce smaller yields compared to photo period plants.

Photoperiod Cannabis: This type requires a specific light cycle to initiate flowering, usually 12 hours of light and 12 hours of darkness. Common in *C.sativa* and *C.indica*, photoperiod plants are often grown for their higher yields and potency. They offer more control over the growth stages but require precise management of light exposure, especially in indoor setups.

These characteristics make auto-flowering strains popular for quick and straightforward cultivation, while photoperiod strains are favored by experienced growers aiming for optimized yields and specific traits.

Cannabis is a perennial herb that is most commonly biennial, and rarely single-leaved. It grows up to 1–6 m tall, with green, hollow, cylindrical, stems that are longitudinally ridged. the petioles are 0.5–1.5 cm long and cylindrical, with a central

groove at the top for to the flower-covered with trichomes. Male and female flowers are produced on separate plants and typically bloom from July to August. The male plant is usually taller, while the female plant is typically stronger. The morphology of cannabis plants is influenced by the strain of seed and environmental factors such as soil type, light, water, nutrients, and space (Raman et al., 2017).

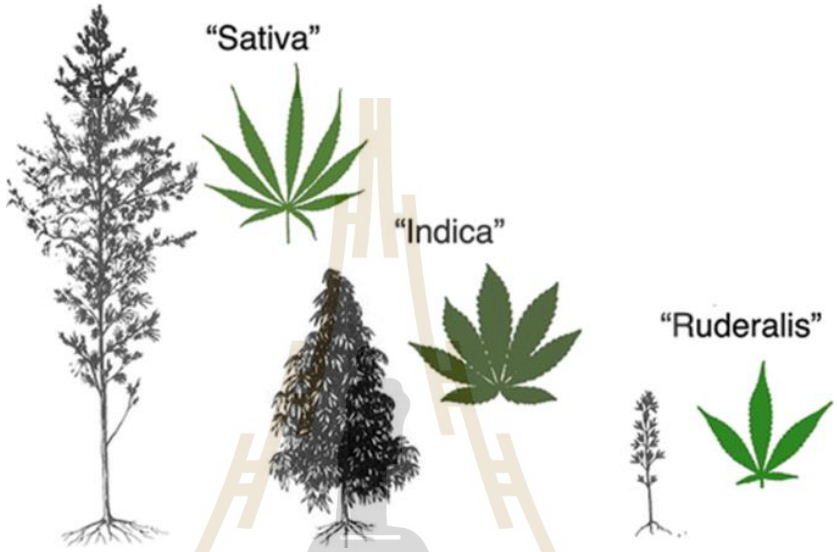


Figure 2.2 Cannabis vernacular taxonomy, courtesy of the Harvard University Herbaria and Botany Libraries (McPartland, 2018).

2.1.2 Cannabis growth phase

The vegetative phase of cannabis plants can vary significantly in duration, especially between indoor and outdoor cultivations. For outdoor plants, the vegetative stage typically lasts between 3 to 16 weeks, depending on environmental conditions and the specific strain (ROYAL QUEEN SEEDS). In indoor cultivation, growers have more control over the light cycle, which influences the transition from the vegetative to the flowering stage. Photoperiod cannabis plants require a change in the light cycle to initiate flowering, typically from 18 hours of light to 12 hours of light per day. This transition is usually induced after 4 to 6 weeks of vegetative growth, but the exact timing can vary based on the grower's objectives and the specific strain (DUTCHPASSION). Ref

It is important to note that while some growers may initiate flowering as early as the 4th week after germination, others may choose to extend the vegetative phase to achieve larger plant size and potentially higher yields. The decision on when to induce flowering indoors depends on various factors, including space constraints, desired plant size, and specific cultivation goals. Optimal growth for cannabis plants occurs at a temperature of 22–26°C, relative humidity of 40-60%, and with the proper ratio of nutrients N, P, and K. It is important to maintain a pH level between 6 and 7 for optimal growth (*The Stages of Cannabis Growth | Clean Leaf Blog*, n.d.). It is crucial to identify gender of your cannabis plants because only female plants should be allowed to grow of harvesting buds. Male and hermaphrodite plants should be separated to avoid pollination, which can lead to fewer buds an abundance of seeds (Alicia, 2020).

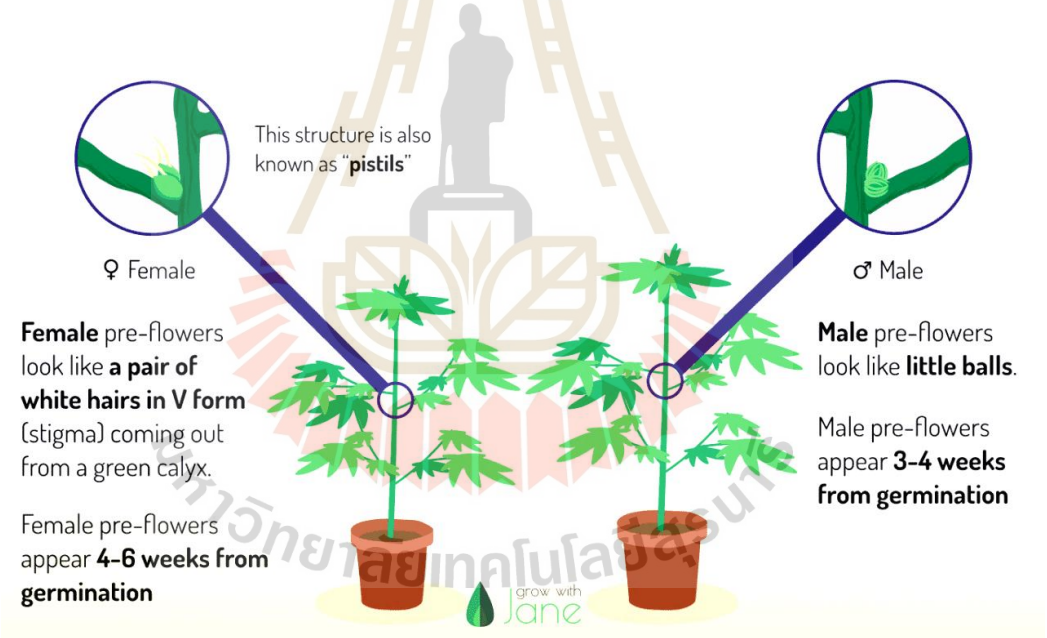


Figure 2.3 Male and female cannabis flowers (Alicia, 2020).

Currently, there is extensive research on cannabis some countries have legalized the use and cultivation of strains containing THC and CBD. New cannabis strains have also been developed to meet the needs and climates of certain countries. Therefore, cannabis has become a plant with very significant potential and

economic value. In addition to its medicinal use, hemp is also grown in many countries for the production of fibers and seeds (Ben Amar, 2006)

2.1.3 Cannabis active compounds

The cannabis plant produces unique compounds called cannabinoids, which are a type of terpene-phenol. The cannabis plant contains at least 85 different cannabinoids, with the two most commonly produced being cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC), but only THC is psychoactive (El-Alfy et al., 2010).

Since the early 1970s, cannabis plants have been classified according to their chemistry, or "chemotype," based on the total amount of THC they produce and the ratio of THC to CBD, which can produce cannabinoids influenced by the environment, the THC/CBD ratio is genetically determined and remains constant throughout the life of the plant. Non-medicinal plants produce relatively low levels of THC and high levels of CBD, while medicinal plants produce high levels of THC and low levels of CBD. It has moderate chemistry and produces moderate amounts of CBD and THC. Female plants of this chemotype may produce enough THC to be used for drug production (Hillig & Mahlberg, 2004). According to the cannabinoids contained in cannabis, cannabis varieties are classified into two types: the narcotic type (*C. indica* or marijuana) with a THC ratio ≥10 and the fiber type (*C. sativa* or hemp) with a THC ratio <0.2. Each type also has different uses, mainly used in food and industrial applications. This includes the production of food products, hemp oil, seeds, and fiber, while the medicinal plants "marijuana" is used only for medical and recreational purposes (Hussain et al., 2021).

Active compounds of cannabis are known to contain many active compounds representing different chemical categories. Some of them are key metabolites such as amino acids, fatty acids, and steroids. While cannabinoids, stilbenoids, flavonoids, lignans, terpenoids, and alkaloids are classified as secondary metabolites, the metabolism of this plant is very rich. Over 480 compounds have been identified in the plants with 180 of them belonging to the family cannabinoids (Hazekamp et al., 2010). Cannabinoids are usually a group of compounds made up of carbon 21 (C21) and are in the form of carboxylic acids. Cannabinoids are the most known secondary metabolites of cannabis. Traditionally, cannabinoids are usually

concentrated in resins. Resins is produced in glandular structures known as trichomes (Figure 4) (Ben Amar, 2006).

Figure 2.4 Phenology of cannabis plant and its inflorescence (Ben Amar, 2006).

The chemical compound found in cannabis is cannabinoid divided into two categories: the psychoactive cannabinoid group is tetrahydrocannabinol (**Δ**9-THC) (Figure 2.5b), and the non-psychoactive cannabinoid is the cannabidiol (CBD) group (Figure 2.5a) (Glodowska, 2016). All genotypes of cannabis contain **Δ**9-THC, but medicinal marijuana (*C. indica*) varieties contain up to 30% of THC, while hemp (*C. sativa*) varieties have the low THC, usually, no more than 0.2% (w/v) (Nissen et al., 2010).

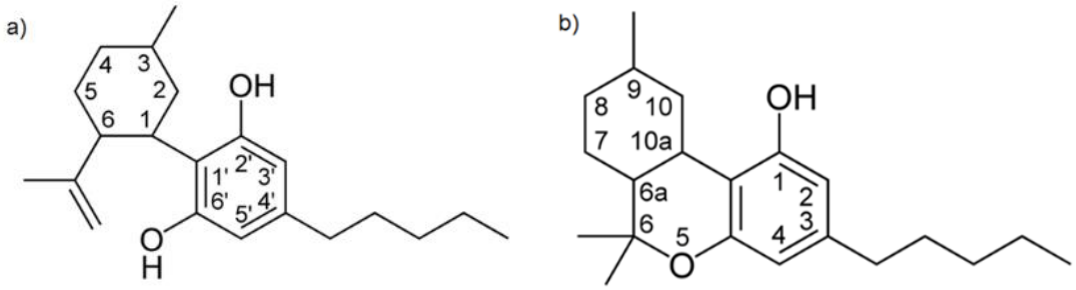


Figure 2.5 Two most important compounds from the cannabinoid family.

a) cannabidiol (CBD) and

b) tetrahydrocannabinol (**Δ**9 -THC) (Glodowska, 2016).

2.2 Comprehensive Uses of Cannabis

Cannabis, specifically its industrial hemp varieties, has long been recognized for its versatility and utility across a wide array of applications, from industrial manufacturing to medical treatments, and recreational and nutritional uses. This literature review examines the diverse uses of cannabis, focusing on its industrial, medicinal, and recreational/nutritional applications, and emphasizes the need for accurate strain identification due to the variability in cannabinoid content and other chemical compounds across different varieties.

2.2.1 Industrial Uses

The industrial uses of cannabis, particularly hemp, have gained significant attention in recent years due to its sustainability and multifaceted applications. Cannabis fibers are well-known for their strength and durability, making them ideal for a variety of industrial applications. These fibers are utilized in the production of textiles, paper, bioplastics, and eco-friendly construction materials, offering an environmentally sustainable alternative to conventional materials (Van Der Werf et al., 2007). The environmental benefits of hemp cultivation, such as its ability to sequester carbon and its minimal water and pesticide requirements, make it a valuable resource in the pursuit of sustainable manufacturing practices (Lachenmeier et al., 2021).

Hemp seeds are another significant by-product, valued for their rich nutritional content. These seeds are a high source of protein, omega-3 fatty acids, and essential amino acids, making them an important component of the food industry (Cocci et al., 2020). Additionally, hemp seeds are utilized in cosmetic formulations due to their moisturizing and anti-inflammatory properties (Zhao et al., 2019). This diverse range of industrial applications highlights the wide-reaching potential of cannabis beyond its use as a recreational drug.

2.2.2 Medicinal Uses

The medicinal properties of cannabis have been extensively studied, particularly the therapeutic effects of cannabinoids like THC and CBD. Both cannabinoids have been found to exhibit pain-relieving and anti-inflammatory properties, leading to their use in treating a range of conditions, including chronic pain, nausea, and muscle spasms associated with conditions like multiple sclerosis (Vermuelen et al., 2020). Moreover, CBD has demonstrated promise in treating epilepsy,

particularly in drug-resistant forms of the condition, with medications such as Epidiolex already approved by regulatory authorities like the FDA (Devinsky et al., 2014).

Cannabis's therapeutic potential, however, is heavily influenced by the specific cannabinoid profile of individual strains. The varying concentrations of THC, CBD, and other minor cannabinoids and terpenes across cannabis strains necessitate precise strain identification, particularly for medicinal applications (Russo, 2016). For clinicians and researchers, accurate strain characterization is essential to ensure that patients receive the appropriate therapeutic benefits, highlighting the importance of reliable testing methods to evaluate the chemical composition of cannabis.

2.2.3 Recreational and Nutritional Uses

Recreational use of cannabis has become increasingly prevalent, particularly with the rise of legalization in various parts of the world. Selective breeding of cannabis strains has led to varieties with higher THC concentrations and specific terpene profiles that cater to recreational users seeking effects (Buchwald et al., 2019). Strains with high THC content are particularly sought after for their psychoactive effects, which range from euphoria to relaxation, while specific terpenes are responsible for the distinct aroma and taste that differentiate cannabis strains (Cox et al., 2021).

In addition to its recreational use, cannabis also offers significant nutritional benefits. Cannabis seeds, rich in protein and essential fatty acids, have garnered attention as a superfood. These seeds contain all nine essential amino acids and are considered a complete protein source, making them an excellent dietary supplement for vegetarians and individuals looking to increase their intake of plant-based proteins (Liu et al., 2019). Moreover, hemp seeds' high levels of omega-3 and omega-6 fatty acids contribute to heart health, and their antioxidant properties further enhance them.

2.3 Molecular Markers in Plant Genetics

Molecular markers are crucial tools for analyzing genetic diversity, understanding phylogenetic relationships, and mapping traits in plants. In cannabis research, these markers provide invaluable insights into the complex genetic structure of cannabis strains, facilitating advances in breeding programs, strain authentication, and the understanding of traits related to cannabinoid production. The ability to precisely identify and map genetic variations plays a critical role in

improving cannabis cultivation, enhancing the efficacy of medical treatments, and maintaining the integrity of cannabis strains.

Molecular markers are specific DNA sequences used to identify genetic variation at specific loci within the genome. They enable precise genetic analysis without the need for phenotypic expression. These markers are generally categorized into two broad types: dominant markers and co-dominant markers.

- Dominant Markers: These markers only indicate the presence or absence of a particular allele, and they do not distinguish between homozygous and heterozygous genotypes. Common examples include RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat).

1) Simplicity: Dominant markers provide straightforward information regarding the presence or absence of specific alleles without needing to determine whether the genotype is homozygous or heterozygous. This simplifies data analysis and interpretation (Khan & Majeed, 2015). Where?

2) No Need for Reference Genomes: Dominant markers can be used without prior knowledge of the organism's complete genome. This is particularly useful for organisms like cannabis, where genomic resources may be limited or incomplete (Wang & Chen, 2010). Where?

3) Cost-Effective: The methods associated with dominant markers, such as RAPD and ISSR, are generally low-cost and require less specialized equipment compared to other techniques like sequencing (Holliday, 1997).

4) High Throughput: Techniques involving dominant markers can be scaled up easily, allowing for the analysis of many samples simultaneously, which is beneficial in breeding programs (Zietkiewicz et al., 1994).

5) Wide Applicability: Dominant markers can be applied to various plant species, making them versatile tools for plant genetics research (Wang & Chen, 2010).

6) Useful for Initial Screening: These markers are effective for initial screenings in breeding programs to quickly identify plants with desirable traits before more detailed analyses are conducted (Khan & Majeed, 2015).

- Co-Dominant Markers: These markers can distinguish between both homozygous and heterozygous genotypes, providing more detailed genetic

information. Examples include SSR (Simple Sequence Repeats) and SNP (Single Nucleotide Polymorphism) markers.

These markers are indispensable in cannabis research, where the genetic diversity of strains, the identification of specific traits (such as cannabinoid content), and the development of new, high-yielding strains are of great interest.

2.3.1 Random Amplified Polymorphic DNA

RAPD markers were one of the first molecular marker systems used in plant genetics due to their simplicity and low cost. RAPD markers utilize short, random primers to amplify DNA segments, producing polymorphic patterns that can be used to assess genetic diversity (Williams et al., 1990). Despite their initial popularity in cannabis research for preliminary genetic studies, RAPD markers have several limitations. They often suffer from poor reproducibility, especially across different laboratories or under varying experimental conditions (Tessier et al., 1999). This lack of consistency limits their utility in large-scale or high-precision studies, such as those required for cannabis strain authentication or trait mapping, where reproducibility is essential.

2.3.2 Simple Sequence Repeats

SSR markers, also known as microsatellites, are highly specific markers that detect variations in short, repetitive DNA sequences. These markers are considered co-dominant, meaning they can differentiate between homozygous and heterozygous alleles. SSR markers are particularly valuable in mapping complex traits, such as cannabinoid biosynthesis genes, because of their high polymorphism and reproducibility (Varshney et al., 2005). In cannabis research, SSR markers have been used to analyze genetic diversity and construct genetic maps for trait selection (Kumar et al., 2017). However, their application requires prior genomic knowledge of the species, which may limit their initial accessibility in research programs focused on newly discovered cannabis strains. The identification of SSR loci in cannabis genomes is also resource-intensive, often requiring sequencing or extensive genomic work prior to marker development.

SSRs can be classified into three groups depending on the number of repeat units: class I, class II, and class III, ranging from >20, 11 to 20 bp, and <11 bp, respectively. Classification of SSR by type of mating unit they were classified into four

groups: (1) perfect microsatellites; (2) incomplete microsatellites; (3) interrupted microsatellites; and (4) composite or hybrid microsatellites. In a perfect microsatellite the repeat order is constant, they are not interrupted by any base (e.g., TATATATATATATA), whereas incomplete microsatellite repetition is interrupted by mismatched bases (e.g., TATATATAGTATATA) in interrupted microsatellite there will be small sequences in the mismatched repeat sequence (e.g. TATATAGCACTATATATATA), whereas in composite microsatellites or in composite or mixed microsatellites there will be small sequences two different repeating sequences are next to each other (e.g. TATATATATACTCTCTCTCTT), although mixed microsatellites are rarely reported in eukaryotes, hybrid microsatellites range from 4% to 25% of total microsatellites. In *Escherichia coli*, it is almost 1.75–2.85% microsatellites. The mixture was reported to be the mixed distribution of microsatellites in prokaryotes differs from eukaryotes due to the increased complexity of the genome (Srivastava et al., 2019).

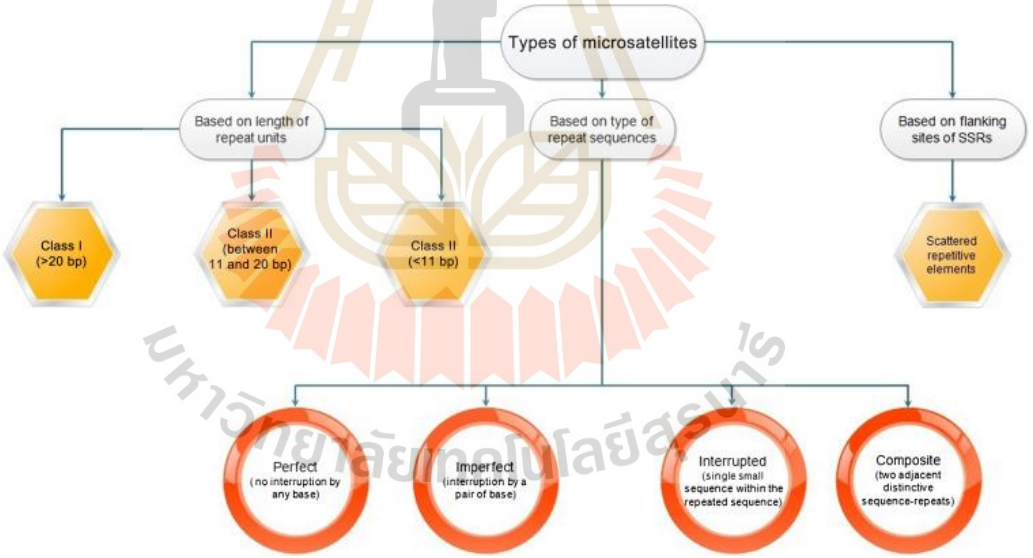


Figure 2.6 Classification of microsatellites (Srivastava et al., 2019).

2.3.3 Inter-Simple Sequence Repeat

ISSR markers amplify DNA regions located between simple sequence repeats (microsatellites) and are particularly useful when genomic information is limited. Unlike SSR markers, ISSR does not require prior knowledge of the cannabis genome, making them an attractive tool for large-scale genetic diversity studies and

strain differentiation. ISSR markers offer several advantages over RAPD, including higher reproducibility, greater resolution, and better robustness in different environmental conditions (Zietkiewicz et al., 1994). These markers have been employed in cannabis to explore genetic variation across different strains, populations, and geographic regions (Chandra et al., 2019). Furthermore, ISSR markers are cost-effective, making them suitable for large-scale studies aimed at characterizing the genetic diversity within cannabis cultivars, an essential aspect for both breeders and forensic purposes.

Microsatellite, also known as simple sequence repeat (SSR) or short tandem repeat (STR) are region in the genome that contains a short DNA pattern (typically 2-5 nucleotides long) repeated multiple times. The inter simple sequence repeat (ISSR) is a segment of DNA flanked at both ends by such microsatellite sequences. Using arbitrarily designed primers containing repeated sequences complementary to microsatellite regions in the genome (ISSR primer). Random DNA segments in the genome could be PCR-amplified (provided that the segments were within the amplified size range) and used as a marker for heredity study of variation, hence the term 'ISSR marker'. Figure 7, shows the basic idea behind ISSR PCR amplification (=ISSR-PCR) (Ng & Tan, 2015).

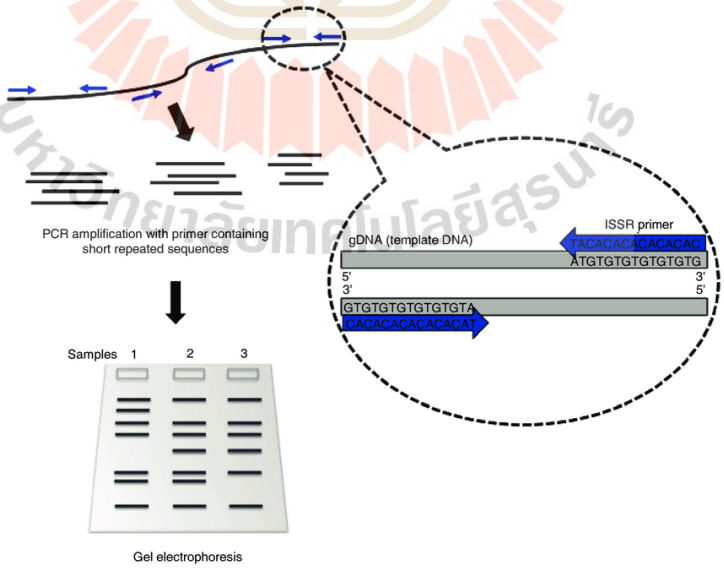


Figure 2.7 Polymerase chain reaction (PCR) amplification using an ISSR primer. (Ng & Tan, 2015).

ISSR markers are effective multilocal markers that can be used diversity analysis, fingerprinting, and genome mapping without prior sequence knowledge. There is faster than the SSR marker and is more reliable than the RAPD marker, but not as polymorphic as AFLP analysis for plants with large and complex genomes. The primer used in ISSR analysis can be based on SSR patterns and provide a wide range of possible amplification products. However, on the other hand, are widely used in plant breeding and evolutionary studies due to their high diversity and distribution in the entire genome (Godwin et al., 1997).

Primers used in ISSR analysis can be based on the SSR patterns (di-, tri-, tetra- or penta-nucleotides) found in microsatellite locations. Which provides a wide range of possible amplification products and can attach to the genome sequence rank either side of the targeted repeat simple sequence. For the ISSR analysis to be successful, pairs of repeating simple sequences must occur within short distances (in base pairs) used by PCR reactions, which produce soluble bands on standard polyacrylamide or agarose gels (Zietkiewicz et al., 1994). Due to the structural differences of the polyacrylamide gel matrix, the effective pore size determined by the electrophoresis method; therefore, depends on the size of the analyzed substance, such as protein or DNA (Stellwagen, 2009).

The ISSR-PCR amplified sequencing technique could be used for DNA fingerprinting and analysis to distinguish botanical traits but could not distinguish between individuals. Microsatellite sequencing and ISSR sequencing also help each other because one side generates a primer for the other (Pradeep Reddy et al., 2002). Simple sequence repeats (SSRs) were widely used as a versatile tool in plant breeding projects, as well as in evolutionary studies because of their high ability to show diversity between species. The SSRs are 1–6 nucleotide and 1–4 nucleotide long DNA sequences, the advantage is that they are widely distributed and abundant in the entire genome, with a high degree of diversity compared to other molecular markers. Disadvantage SSR analysis is an expensive and time-consuming process. Especially, when libraries need to be created for growing many crops creation of high-resolution linkage maps using just the SSR marker is costly, but it is usually more reasonable to combine SSR and AFLP analysis and other advantages of SSR include

co-dominant heritability and ease of analysis and ability to transfer (Charters et al., 1996).

2.4 SSR-based research on cannabis

The advent of molecular markers has revolutionized cannabis research, enabling detailed studies on genetic diversity, strain authentication, and the tracing of genetic lineages. Among the various molecular markers used, ISSR markers have become an essential tool in understanding the complex genetic structure of cannabis. They are particularly valuable in cannabis research for identifying genetic diversity, tracing lineage, and authenticating strains, which are crucial for both breeding programs and ensuring the safety and consistency of medicinal cannabis products. This literature review explores the applications of ISSR markers in cannabis research, focusing on genetic diversity studies, strain authentication, domestication, and hybridization.

2.4.1 Genetic Diversity Studies Using ISSR Markers

ISSR markers are highly valuable for assessing genetic variation within and among cannabis populations. These markers, which amplify regions between microsatellites, have been used to examine genetic diversity in both wild and cultivated cannabis strains. One of the most notable applications of ISSR markers has been in the study of genetic diversity between landrace strains and hybrid varieties. Landraces, which represent traditional cannabis populations adapted to specific geographic regions, are of significant interest due to their potential to serve as a reservoir of genetic traits beneficial for breeding programs.

For example, a 2018 study utilized ISSR markers to evaluate genetic diversity in Iranian cannabis landraces. This study demonstrated significant genetic variation within the landraces, with some strains showing greater diversity than others. The authors highlighted the importance of preserving these landraces as genetic resources for breeding, particularly in enhancing disease resistance, yield, and cannabinoid production (Chandra et al., 2018). By comparing landrace and hybrid populations, researchers have noted that domestication has led to a narrowing of genetic diversity in cultivated cannabis strains. This narrowing is primarily due to selective breeding practices aimed at enhancing specific traits, such as higher THC

content or disease resistance, often at the expense of other genetic traits. The ability of ISSR markers to detect such variations is crucial for understanding the genetic history of cannabis and for maintaining genetic diversity in modern breeding programs.

2.4.2 Strain Authentication and Quality Control

The authentication of cannabis strains is another area where ISSR markers are indispensable. In the medicinal cannabis industry, the identification of specific strains with consistent chemical profiles is paramount for ensuring patient safety. Many cannabis strains exhibit similar morphological characteristics but differ in their chemical compositions, particularly in terms of cannabinoid content and terpene profiles. ISSR markers help differentiate these strains at the genetic level, making them a powerful tool for strain authentication.

A study by Kaushik et al. (2015) highlighted the use of ISSR markers to authenticate cannabis strains based on their genetic fingerprinting. The researchers emphasized that accurate strain identification is critical for the medicinal cannabis market, where precise chemical composition is required for effective treatment outcomes. Inconsistent or mislabeled strains could lead to adverse effects in patients, particularly in medical contexts where the therapeutic properties of specific cannabinoids like CBD and THC are essential. By using ISSR markers, researchers can ensure that strains are consistently identified and that the correct genetic material is used in production, thereby maintaining the quality and safety of cannabis-based medicines.

2.4.3 Domestication Studies of Cannabis

The domestication of cannabis has been a key area of research in understanding how modern cannabis strains have evolved from wild progenitors. ISSR markers have been instrumental in tracing the genetic changes that occurred during the domestication process. By comparing the genetic structure of wild cannabis populations with that of cultivated varieties, researchers can identify the bottlenecks and selective pressures that shaped the genetic makeup of cannabis.

In one study, ISSR markers were used to compare wild cannabis populations in Central Asia with cultivated varieties from Europe and North America. The results suggested that domestication has led to a loss of genetic variation, particularly in populations that have been selectively bred for high THC content (Zhao

et al., 2017). This genetic bottleneck has implications for the long-term sustainability of cannabis breeding programs, as a limited genetic pool can lead to inbreeding depression and a decrease in overall vigor and resistance to pests and diseases. Understanding the genetic consequences of domestication is crucial for breeders aiming to diversify the genetic base of cannabis while maintaining desirable traits.

2.4.4 Hybridization Studies and Trait Inheritance

Hybridization is a common practice in cannabis breeding programs, particularly in the development of new strains with enhanced characteristics, such as increased cannabinoid content, pest resistance, and improved yield. ISSR markers have been widely used to track hybridization events and the inheritance of specific traits in cannabis.

For instance, a study by Saini et al. (2019) examined hybridization events between high-THC and high-CBD cannabis strains using ISSR markers. The researchers were able to track the inheritance of these traits across generations, identifying markers associated with high cannabinoid content. The study also revealed the genetic complexities of hybridization in cannabis, where traits like cannabinoid content do not follow simple Mendelian inheritance patterns but rather involve multiple genes and environmental interactions. ISSR markers proved to be an effective tool for mapping these traits and tracking their inheritance in hybrid cannabis populations, aiding in the development of strains with optimal cannabinoid profiles.

Hybridization studies using ISSR markers are also useful for tracking traits related to pest resistance and environmental adaptability. For example, breeders may use ISSR markers to identify genetic regions associated with resistance to common pests like aphids or mildew. These markers allow for the rapid selection of individuals with desirable traits, accelerating the breeding process and improving the overall resilience of cannabis strains.

2.5 Integration of ISSR with Genomic Tools

The integration of ISSR markers with advanced genomic tools, such as next-generation sequencing (NGS) and single nucleotide polymorphism (SNP) genotyping, has significantly enhanced the ability to study and manipulate the cannabis genome. These technological advances provide deeper insights into the genetic basis of

important traits in cannabis, such as cannabinoid content, disease resistance, and environmental adaptability. This review explores the integration of ISSR markers with genomic technologies and discusses its implications for cannabis research, including its applications in breeding programs and the identification of critical genetic traits.

2.5.1 ISSR and SNP Genotyping

SNP genotyping, a powerful genomic tool, has become a central technique for analyzing genetic variation at a highly detailed level. Combining ISSR markers with SNP genotyping offers a robust approach for mapping genetic traits, enabling researchers to pinpoint quantitative trait loci (QTL) that are associated with specific phenotypic traits. In cannabis research, this approach is particularly useful for identifying genetic loci linked to the production of cannabinoids, which are critical for both recreational and medicinal purposes.

SNP genotyping provides a high-resolution view of genetic variation at individual nucleotide positions across the genome, while ISSR markers, which detect variations in microsatellite regions, offer information on genetic diversity within and between populations. When used in conjunction, ISSR and SNP markers can provide complementary insights into both the broader genetic structure of cannabis and the specific alleles responsible for traits of interest. A study by Singh et al. (2020) demonstrated how combining ISSR markers with SNP analysis can be used to identify QTLs linked to cannabinoid production in cannabis. The integration of these two marker systems allows for a more precise understanding of the genetic factors controlling the biosynthesis of cannabinoids like THC and CBD, offering new opportunities for the development of strains with optimized cannabinoid profiles. Additionally, the use of SNP genotyping alongside ISSR markers enables fine-scale mapping of these QTLs, which is crucial for breeding programs focused on improving the chemical composition of cannabis strains.

2.5.2 NGS Technologies and ISSR Integration

Next-generation sequencing (NGS) technologies have revolutionized the field of genomics by providing the ability to sequence entire genomes quickly and affordably. The combination of NGS with ISSR markers opens new possibilities for understanding the genetic underpinnings of complex traits in cannabis. One of the most exciting applications of NGS in cannabis research is genome-wide association

studies (GWAS), which aim to identify genes linked to specific traits, such as disease resistance or environmental adaptability.

When paired with ISSR markers, NGS-based GWAS can enhance the precision of identifying genetic loci associated with important agronomic traits in cannabis. ISSR markers, which provide broad genetic coverage by amplifying regions between microsatellite repeats, can complement the high-resolution data generated by NGS. By integrating these two approaches, researchers can conduct more comprehensive studies of the cannabis genome, identifying both common and rare alleles that contribute to traits such as pest resistance, drought tolerance, and overall plant vigor. For instance, a study by Zhang et al. (2019) applied NGS technologies to identify genomic regions associated with disease resistance in cannabis. By integrating ISSR markers, the researchers were able to capture genetic diversity across a wide range of cannabis strains, providing more complete information on the genetic basis of resistance to pathogens. This integration allowed them to identify specific genomic regions that could be targeted in breeding programs to develop cannabis strains with enhanced resistance to diseases like powdery mildew and fusarium wilt.

2.5.3 Applications in Cannabis Breeding Programs

The integration of ISSR markers with genomic tools such as SNP genotyping and NGS technologies has significant implications for cannabis breeding programs. One of the most promising applications is marker-assisted selection (MAS), a method that accelerates the development of new cannabis strains with optimized traits. MAS relies on molecular markers to select individuals with desirable genetic characteristics, such as high cannabinoid content, pest resistance, or improved yield. By using ISSR markers alongside SNP and NGS-based tools, breeders can more effectively select plants with the desired traits, improving the efficiency and precision of breeding programs.

For example, a breeding program focused on developing high-THC cannabis strains could use MAS to identify and select plants that carry alleles associated with elevated THC production. This process can be greatly accelerated by integrating ISSR markers, which can provide additional information about the genetic structure of the plants and the inheritance of desirable traits. Furthermore, NGS

technologies allow breeders to map the entire genome of cannabis plants, providing a comprehensive view of the genetic factors influencing cannabinoid production. The combination of these tools enables the selection of plants that are genetically predisposed to produce high levels of THC, reducing the time and cost associated with traditional phenotypic selection methods.

The integration of these genomic tools also facilitates the development of cannabis strains that are better adapted to specific environmental conditions. For example, breeders can use MAS to select for traits such as drought tolerance or pest resistance, improving the resilience of cannabis plants in different growing environments. The ability to select plants with both desirable agronomic traits and optimized cannabinoid profiles opens new possibilities for the commercial production of cannabis, with strains tailored to meet the specific needs of consumers and patients.

The ISSR technique has been widely studied and applied in various fields, including the assessment of genetic diversity in crops like rice. The technique can amplify multiple DNA fragments per reaction, representing loci across the genome. This makes it suitable for fingerprinting rice cultivars and a valuable alternative to single-locus or cross-species approaches. The technique has also been utilized in the Gramineae family to analyze all isogenic species, as well as for corn population analysis and millet accession. However, previous studies have shown that the technique may only detect moderate to low differences in rice (Blair et al., 1999). Nowadays, research on the application of the ISSR technique is gaining more attention and is being used to analyze plants other than rice such as mangoes, mung beans, tobacco, tomatoes, wild rose, wild mulberry, cannabis, and many others.

In 2019, Yamanaka et al. conducted a study to determine the similarity of mangoes and developed a series of 16 SSR markers for mango homology using two genome-rich sites. They used CT duplicates from DNA extracted from 'Tommy Atkins' for genotypic analysis of 28 mango cultivars using these 16 SSRs. The results showed that there were three major clusters using both cluster analysis and PCA, indicating the distribution of genotypes at 'Tommy Atkins' similar varieties were grouped according to their geographical origin and pedigree history. The study also

found that the two main types of mangoes (mono-embryonic and polyembryonic) could be clearly distinguished (Yamanaka et al., 2019).

Poolsawat et al. utilized the Inter-simple sequence repeat (ISSR) technique and a newly developed ISSR-anchored resistance gene analog (ISSR-RGA) to detect powdery mildew (PM) resistance genes in mung beans. These beans were obtained from a cross between CN72, a susceptible strain in Thailand, and V4718, a resistant strain from the Asian Vegetable Research and Development Center and were found to inherit the resistance gene as a single parent gene. Using loci analysis, 5 out of 27 ISSR markers and 3 out of 11 ISSR-RGA markers were found to be significantly associated with PM resistance genes, as confirmed by simple linear regression. Quantitative characterization (QTL) of the results indicated that the ISSR and ISSR-RGA markers were highly effective tools for mapping PM resistance genes in mung beans. The markers closely linked to PM resistance genes would be beneficial for marker-assisted selection in the future development of PM-resistant mung bean cultivars (Poolsawat et al., 2017a).

In the field of cannabis research, ISSR markers have been used. Specifically, in 2008, a study was conducted on cannabis samples confiscated from twenty-three locations in Turkey to determine the genetic relationship between the seized samples and to differentiate between marijuana and hemp. The analysis, which used ISSRs markers, yielded results demonstrating that ISSRs were effective in distinguishing between marijuana and hemp accession (Pinarkara et al., 2009). In 2009, the genetic stability of *C.sativa* plants was evaluated using ISSR markers. The study used 15 ISSR primers which resulted in 115 distinct and reproducible bands. The ISSR profiles of the small propagated plants were monomorphic and comparable to that of the parent plant, confirming the genetic stability between the clone and the parent plant (Lata et al., 2009).

Since most cannabis plants are dioecious, cross-pollination usually increases variation. Once proper primers are obtained that can differentiate between cannabis strains, the next step should confirm that the selected primers are strain-specific using different individual plants from the same cannabis strain. If the obtained primer is stable and can differentiate between strains, the results of the

DNA bands from different individual plants within the same strain should show consistent unique bands.

CHAPTER III

RESERCH METHODOLOGY

3.1 Plant material

Twelve cannabis strains were utilized in this study (Table 3.1). The plant samples were sourced from the "SUT Cannabis Farm", located at Suranaree University of Technology, Suranaree Subdistrict, Mueang District, Nakhon Ratchasima Province, 30000. Leaf samples from all 12 strains were collected, with seven replicates per strain, resulting in a total of 252 plants for DNA extraction. Additionally, three plants from each strain were selected for phenotypic characterization, which was conducted over an 8-week growth period.

Table 3.1 Cannabis strains used in this study.

Name	Origin	Information
Blue Venom (BV)	Blueberry and White Widow	Blue Venom weed strain attributes: G13 Labs are a cannabis seed company which began life in the North of England (*Blue Venom Cannabis Seeds For Sale UK - G13 Labs*, n.d.)
Baox	Hindu Kush and Otto II	BaOx (pronounced bay-ox): Colorado, United States (*BaOx Feminized CBD Hemp Seeds - Windy Hill Hemp*, n.d.)
Cannafuel (CNF)	(Sour Diesel x ACDC) x Cannatonic	Original Sensible Seeds (Hanf, 2025)
Charlotte's Angle (CA)	Crossbreed between Dutch Charlotte and Red Angel.	The Highlife Cannabis Cup in the Netherlands (*Buy CBD Charlotte's Angel ® Seeds Online in Thailand*, n.d.)
Charlotte's Web (CW)	Stanley Brothers	The Stanley Brothers of Colorado (*Charlotte's Web Weed Strain Information*, n.d.)

Table 3.1 Cannabis strains used in this study.

Name	Origin	Information
Golden Tiger (GT)	This pure sativa flower is a complex blend of Koh Chang Thai and Hmong Thai crossed with Malawi and 3rd Generation Malawi	Golden Tiger is sativa bred from crossing Thailand and South African genetics. (*Golden Tiger Weed Strain Information*, n.d.)
Orange Valley (OV)	Hybrid weed strain made from a genetic cross between SFV OG and Agent Orange	Orange Valley: The trailblazers at Relentless Genetics, Tacoma, Washington (*Orange Valley OG Weed Strain Information*, n.d.)
West Slope (WS)	Blueberry and White Widow	West slope hybrid of Colorado, United States (*West Slope Kush B Feminized Hemp Seeds - CBD Hemp Seeds | Fortuna*, n.d.)
Foithong (FT)	Thailand	located species in Sakon Nakhon Province (*Foi Thong (The Landrace Team)*, n.d.)
Hangkarok (HR)	Thailand	Phu Phan mountain range located in Sakon Nakhon Province and Kalasin Province (*Thai Stick*, n.d.)
RPF1	Thailand	Developed from the Hemp and Cannabis Plants Research and Development Project as an economic plant and new alternative plant in the highlands, NSTDA.
RPF2	Thailand	Developed from the Hemp and Cannabis Plants Research and Development Project as an economic plant and new alternative plant in the highlands, NSTDA. (*ขึ้นทะเบียนพันธุ์กัญชง (Hemp) สำคัญอย่างไร?: สวพส.*, n.d.)

3.2 CTAB-based DNA extraction method

The fresh leaves of cannabis plants were ground using liquid nitrogen. Fifty milligrams of the powder were aliquoted into 1.5 mL microcentrifuge tubes, then thoroughly mixed with 700 µL of cetyltrimethylammonium bromide (CTAB) lysis buffer (2% w/v CTAB, 100 mM Tris-HCl, 20 mM EDTA, and 1.4 M NaCl, pH 8.0). The mixture was incubated at 65°C for 30 minutes with inversion every 5 minutes, and then centrifuged at 13,000 rpm for 2 minutes at 4°C. After that, the supernatant was transferred into new 1.5 mL microcentrifuge tubes, and 600 µL of phenol: chloroform: isoamyl alcohol (25:24:1, pH 6.7) were added. The mixture was mixed by vortexing for 2 minutes and then centrifuged at 13,000 rpm for 10 minutes at 4°C. Then, 600 µL of the supernatant was transferred into new 1.5 mL microcentrifuge tubes and added to 600 µL of chloroform: isoamyl alcohol (24:1). The mixture was mixed by vortexing for 2 minutes and centrifuged at 11,000 rpm for 10 minutes at 4°C. Afterward, 500 µL of the supernatant was transferred in new 1.5 mL microcentrifuge tubes and mixed with 3 M sodium acetate (ratio 10:1). This was followed by adding 700 µL of ice-cold isopropanol, vortexing for 2 minutes, and centrifuging at 13,000 rpm for 20 minutes. Following the initial isolation of DNA, the supernatant was removed, leaving behind only the pellet. To wash the pellet, 70% ethanol was added, and the sample was centrifuged at 13,000 rpm for 20 minutes. Subsequently, the supernatant was discarded, and the DNA pellet was air-dried for a period of 30-45 minutes. Once dried, the DNA pellet was dissolved in a TE buffer solution, and 8 µL of RNase (0.2 mg/mL) was added. The quality and quantity of the DNA were assessed through nanodrop and agarose gel electrophoresis, respectively. The methodology used in this study was modified from Gawel & Jarret's research (2009).

3.3 ISSR primers selection

Due to the absence of specific ISSR markers in cannabis and the non-species-specific nature of ISSR markers, the primary primer screening used primer sets from a preliminary screening of mung bean DNA (Table 3.2). ISSR primers that provided clear and reproducible bands were selected to amplify all cannabis strains. Initially, 75 ISSR primers were screened, and 11 ISSR primers that produced

clear and reproducible bands for amplifying all cannabis strains were obtained and then, the ISSR-PCR was used to differentiate isolates of the cannabis strains using ISSR primers The amplification program consisted of an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at a temperature dependent on the primer for 50 seconds, and extension at 69°C for 1 minute. A final elongation step was performed at 69°C for 10 minutes. The amplification products were evaluated through 6% (v/v) polyacrylamide gel electrophoresis, and the DNA bands were observed by staining with silver nitrate.

Table 3.2 ISSR primer sequences (Poolsawat et al., 2017).

Primers code	Primer sequences (5' to 3')	Range of amplified products (bp)	Number of scorable DNA bands
ISSR 810	GAGAGAGAGAGAGAGAT	200-1200	19
ISSR 811	GAGAGAGAGAGAGAGAC	200-1200	20
ISSR 825	ACACACACACACACACT	250-1200	18
ISSR 834	GAGAGAGAGAGAGAGAYT	200-1200	24
ISSR 841	GAGAGAGAGAGAGAGAYC	250–1200	19
ISSR 844	CTCTCTCTCTCTCTCTRC	300–1200	14
ISSR 880	GGAGAGGAGAGGAGA	250–1200	13
ISSR 888	BDBTGTGTGTGTGTGTG	300–1200	20
ISSR 891	HVHTGTGTGTGTGTGTG	200–1200	18
ISSR 895	GAGATTGGTAGCTCTTGATC	250-2070	15
ISSR 899	CATGGTGTTGGT CATTGTTCCA	200–1000	14

B = C, G, T; D = A, G, T; H = A, C, T; N = A, G, C, T; R = purines (A, G); V = A, C, G; Y = pyrimidines (C, T).

3.4 Polyacrylamide gel electrophoresis

After obtaining the PCR product, the DNA pattern was observed through polyacrylamide gel electrophoresis using the following steps:

3.4.1 Preparation polyacrylamide gel

The polyacrylamide gel contained the following substances: 10.08 g of urea, 1.8 mL of 40% acrylamide/bis (19:1), 2.4 mL of 10X TBE buffer, 160 µL of 10% ammonium persulfate (APS), 16 µL of TEMED, and DI water was added to adjust the total volume to 25 mL (6 mL per gel). The mixture was then poured between two glass plates separated by 1 mm spacers and run in the vertical position.

3.4.2 Preparation for running buffer

The TBE buffer used for the experiment contained specific substances, including 108 g of Tris-base, 55 g of boric acid, 10 mL of 3 M EDTA, and a stock solution of 10X TBE, with water added to adjust the volume to 1 L. The pH of the buffer was adjusted to 8.3 (Krueger & Schwartz, 1987). The gel was run using 1X TBE buffer and an electrophoresis system with a constant voltage of 200 volts for 65 minutes.

3.4.3 Staining gels

The polyacrylamide gel was eliminated from the glass plates and soaked in 10% EtOH, positioned on a shaker for 10 mins, after which washed once with DI water. After that, the gel was soaked in 0.7% HNO_3 for 6 mins, the HNO_3 solution was discarded, and the gel was washed two times with DI water. Following this, the gel was soaked in 0.2% $AgNO_3$ for half-hour within the dark, the $AgNO_3$ answer was discarded, and the gel was washed two times with DI water. Subsequently, the gel was washed twice with the developer: first, the gel changed into shaken until the solution started out to exchange color and the developer was discarded; then, the developer was added once more, shaken until the band appeared truly, and the developer solution changed into discarded. The gel was washed once with DI water. After that, the gel was soaked with 3% CH_3COOH for five minutes, the CH_3COOH solution was discarded, and the gel was washed with DI water till all acetic acid changed into removed. Finally, the gel turned into soaked in 10% EtOH for 10 mins. The DNA patterns were observed under the "Molecular Imager Gel Doc XR System."

3.5 ISSR band pattern scoring and statistical analysis

The amplified products have been scored as both binary (1) or absent (0) codes, with every step taken into consideration by a unit person regardless of their depth. The populace-stage range was calculated because the ratio of multiple loci

to the whole wide variety of loci scored for the entire population. The pairwise similarity matrix became created by the usage of the Jaccard similarity (Nouvelles Recherche's Sur La Distribution Florale inside the SIMQUAL layout of NTSYS-laptop (Rohlf, 1988). The dendrogram constructed the usage of the unweighted pairwise clustering approach with arithmetic common (UPGMA) in NTSYS-computer's SAHN module, providing a visual illustration of genetic relationships as determined by means of similarity coefficients (Sneath, 2005).

3.6 THCAS and CBDAS detection in cannabis vegetation

To verify the types of cannabinoids expressed within the dendrogram, the protocol from "Rapid identification of drug-kind and fiber-kind cannabis by means of allele-unique duplex PCR" (Yamamuro et al., 2019) was modified. The technique applied allele-specific primers targeting the THC and CBD genes. PCR amplifications have been finished to distinguish the hashish stress isolates using D-THCAs and F-CBDAS-specific primers (Table 3.3). The amplification process consisted of a preliminary denaturation at 94°C for two minutes, followed by using 30 cycles of denaturation at 94°C for 20 seconds, annealing at 65°C for 50 seconds, and extension at 72°C for 1 minute. A final elongation step becomes achieved at seventy-two °C for 7 mins. After amplification, 5 µL of the PCR aggregate changed into subjected to electrophoresis on a 1% agarose gel at 80 V for 35 minutes, and the PCR products have been visualized after electrophoresis.

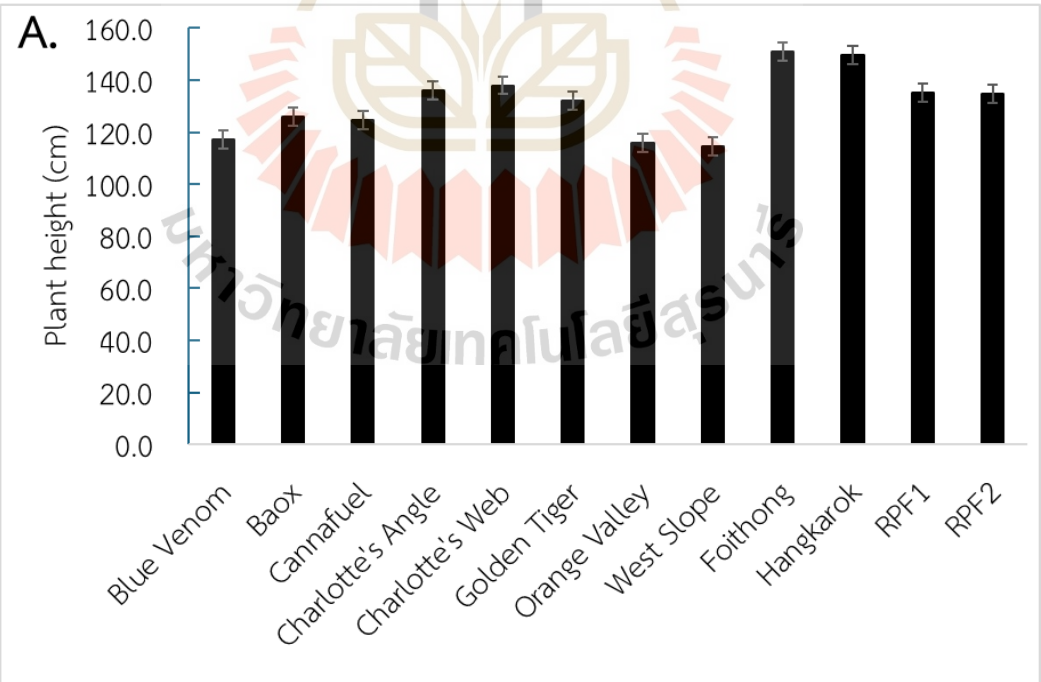
Table 3.3 Specific primers for THC and CBD genes (Yamamuro et al., 2019).

Name of primers	Sequences	Amplification sizes
D-THCAS	Fw: 5′- CCTGAATTCGACAATACAAAATCTTAGATTCAT-3′ Rv: 5′- ACTGAATATAGTAGACTTTGATGGGACAGCAACC-3′.	589 bp
F-CBDAS	Fw: 5′- GTGCTCAACATTCTCCTTTTGGT-3′ Rv: 5′- TGGGTTGTTTTGAGTGTATACGAG-3′.	169 bp

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Plants and Phenotypic Similarities

From a total of 257 cannabis plants representing 12 strains, 3 plants from each strain were randomly selected during an 8-week photoperiod at the Suranaree University of Technology Cannabis Farm for phenotypic analysis. Traits such as plant height, bush shape, and leaf length were evaluated. The results revealed a high degree of phenotypic similarity among the exotic strains, which posed challenges in accurately distinguishing local strains due to overlapping traits. This highlights the necessity for additional intervention or genetic testing to achieve precise classification.



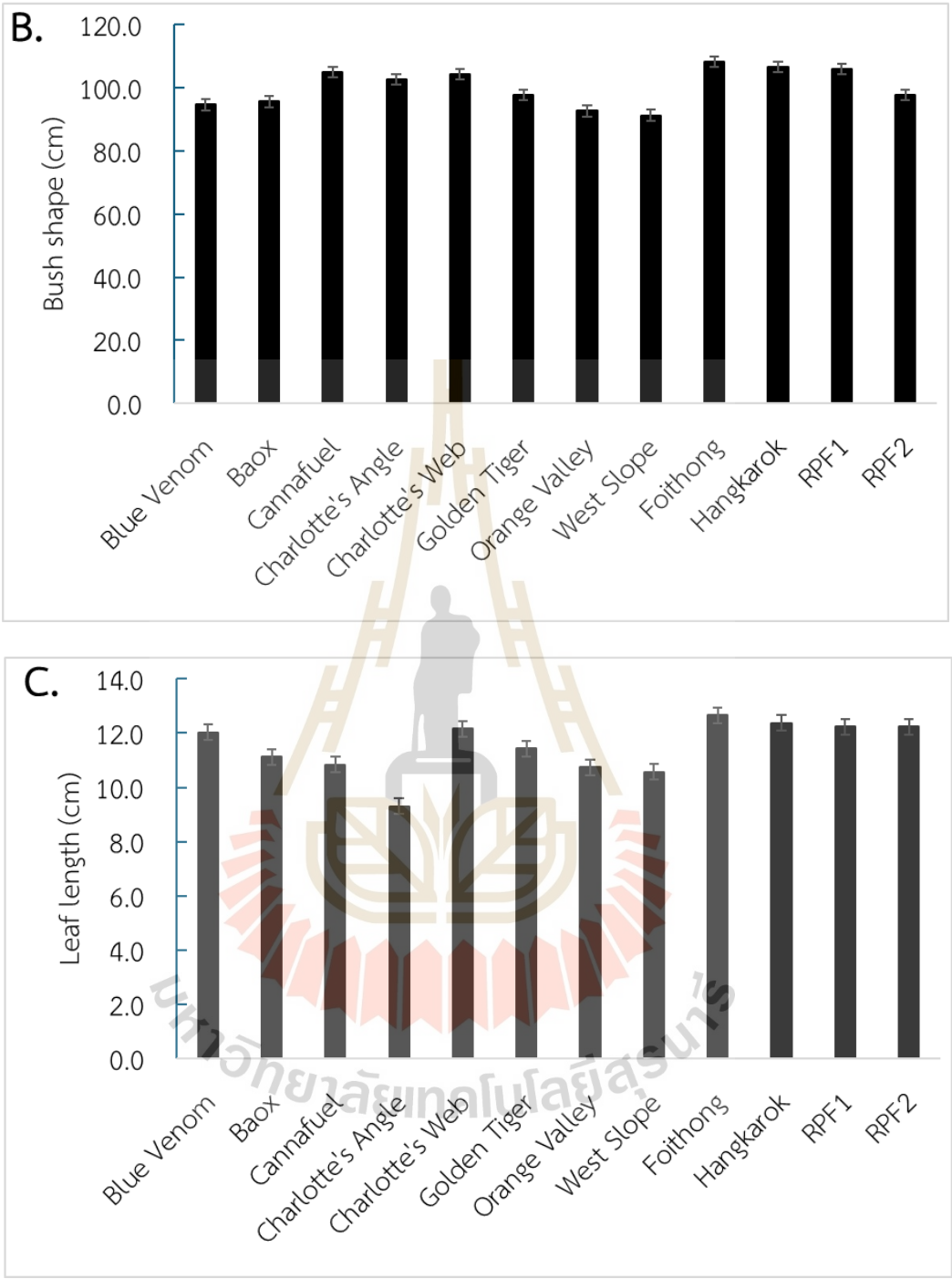


Figure 4.1 Phenotypic Characteristics of Cannabis Strains (n=3).

The phenotypic traits of the 12 cannabis strains, including plant height, bush shape, and leaf length, displayed measurable variations but also significant overlaps, highlighting both the diversity and the uniformity within the group.

Plant height varied from 114.67 cm in 'West Slope' to 151 cm in 'Foithong,' with 'Foithong' and 'Hangkarok' (149.67 cm) showing the tallest growth. These strains stood out significantly compared to the shortest strains, such as 'Orange Valley' (116 cm) and 'West Slope.' Intermediate strains like 'Blue Venom' (117.33 cm) and 'Golden Tiger' (132 cm) represented moderate height ranges, illustrating a spectrum of growth patterns across the strains. For Bush Shape, measured by bush width, ranged from 91.33 cm to 108.33 cm. Similar to plant height, 'Foithong' displayed the most robust growth with the broadest bush shape (108.33 cm), while 'West Slope' (91.33 cm) and 'Orange Valley' (92.67 cm) showed narrower, more compact forms. Most strains, such as 'Charlotte's Web' (104.33 cm) and 'RPF1' (106 cm), exhibited bush shapes within the average range, contributing to a balanced overall appearance. And Leaf Length, showed the least variation, ranging from 9.3 cm in 'Charlotte's Angle' to 12.67 cm in 'Foithong.' Strains with longer leaves included 'Hangkarok' (12.37 cm), 'RPF1' (12.23 cm), and 'Charlotte's Web' (12.17 cm), whereas shorter leaves were observed in 'Cannafuel' (10.83 cm) and 'West Slope' (10.57 cm).

Blue Venom Baox Cannafuel Charlotte's Angle

Charlotte's Web Golden Tiger Orange Valley West Slope

Figure 4.2 Phenotype of cannabis grown in SUT Cannabis Farm.

The results reveal considerable overlap in phenotypic traits, with most strains falling within a narrow range of values for each characteristic. For example, leaf lengths across strains varied by only a few centimeters, with the shortest average (9.3 cm) in 'Charlotte's Angle' and the longest (12.67 cm) in 'Foithong'. Similarly, plant height and bush shape showed overlapping ranges, making visual identification impractical in many cases. While strains like 'Foithong' and 'Hangkarok' displayed larger dimensions across all traits, these differences were not universally distinctive, as other strains exhibited overlapping features. The assessment of morphological traits, such as plant height and bush shape, reveals significant challenges in the visual identification of cannabis strains. Notably, strains like 'Foithong' and 'Hangkarok' demonstrate larger dimensions across various traits; however, these differences are not universally distinctive. Other strains exhibit overlapping features, complicating the task of distinguishing between them based solely on morphology.

Several genetic factors contribute to this phenomenon. Firstly, cannabis is a highly polymorphic species with considerable genetic diversity, leading to a wide range of phenotypic expressions (Hillig, 2004). Many traits, including plant height and bush shape, are polygenic, meaning they are influenced by multiple genes. This polygenic nature can produce a continuous spectrum of phenotypes, resulting in overlapping traits among different strains (Möller et al., 2009). Moreover, environmental influences play a crucial role in shaping plant morphology. Factors such as soil quality, light availability, water,

and temperature significantly affect growth patterns (Chandra et al., 2010 or 2019). Consequently, genetically distinct strains may exhibit similar characteristics when grown under the same environmental conditions, which further complicates visual identification.

Phenotypic plasticity, the ability of an organism to change its phenotype in response to environmental stimuli, also contributes to this overlap. Cannabis plants can adapt their growth forms based on their surroundings, making two genetically different strains appear similar in height and bush shape when cultivated in identical conditions (Sullivan et al., 2016). Additionally, selection pressure during breeding practices can lead to the convergence of certain traits. If multiple strains are subjected to similar selective forces, they may develop overlapping morphological traits, making it difficult to distinguish them based on these characteristics alone (Khan et al., 2015). Finally, the genetic relatedness of strains can further obscure their identification. If strains like 'Foithong' and 'Hangkarok' are genetically like other strains, they may share overlapping traits, reinforcing the challenge of visual identification based on morphology (Cannon et al., 2020). In summary, the difficulty in distinguishing cannabis strains based on plant height and bush shape is attributed to a combination of genetic variation, polygenic inheritance, environmental influences, phenotypic plasticity, selection pressures, and genetic relatedness. These factors underscore the complexity of cannabis morphology and highlight the need for more precise identification methods, such as genetic analysis, to complement traditional visual assessments.

The findings highlight the limitations of relying solely on phenotypic traits for cannabis strain identification. Given the challenges posed by phenotypic overlap, integrating genetic analysis, such as DNA marker studies, could provide a more reliable approach for distinguishing strains. Genetic tools can uncover subtle differences at the molecular level that are not observable phenotypically, enabling accurate classification and better understanding of strain diversity.

4.2 ISSR primers selection and amplification

Out of the 75 ISSR primers selected, 39 successfully generated DNA bands from 12 cannabis strains. Only 11 ISSR primers produced clear bands that can be repeated and have variety. This makes it suitable for additional screening of cannabis strains. These selected primers were highly variable between species. The optimum annealing temperatures were 48°C, 53°C, and 55°C (Table 4.1). The 11 primers amplify a total of 782 scored DNA bands, on average per primer. 71.09- tracks, which included 66 to 75 bands per primer, of which 6.52% (51 bands) were monomorphic and 93.01% (731 bands) were polymorphic.

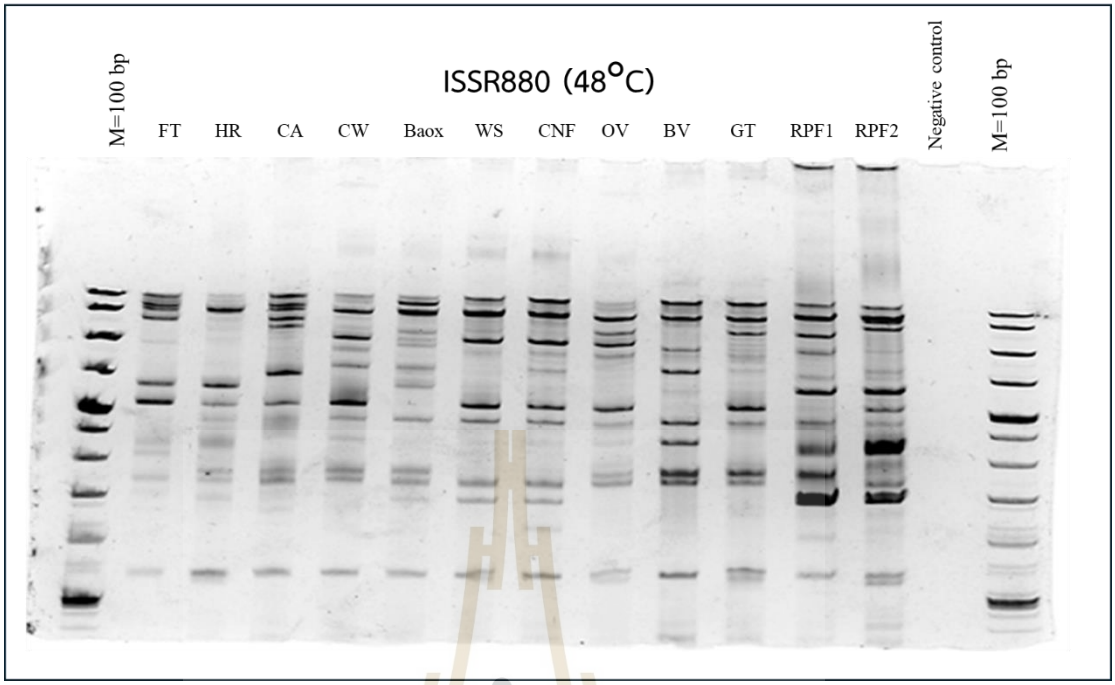
Table 4.1 Information about 11 ISSR primers.

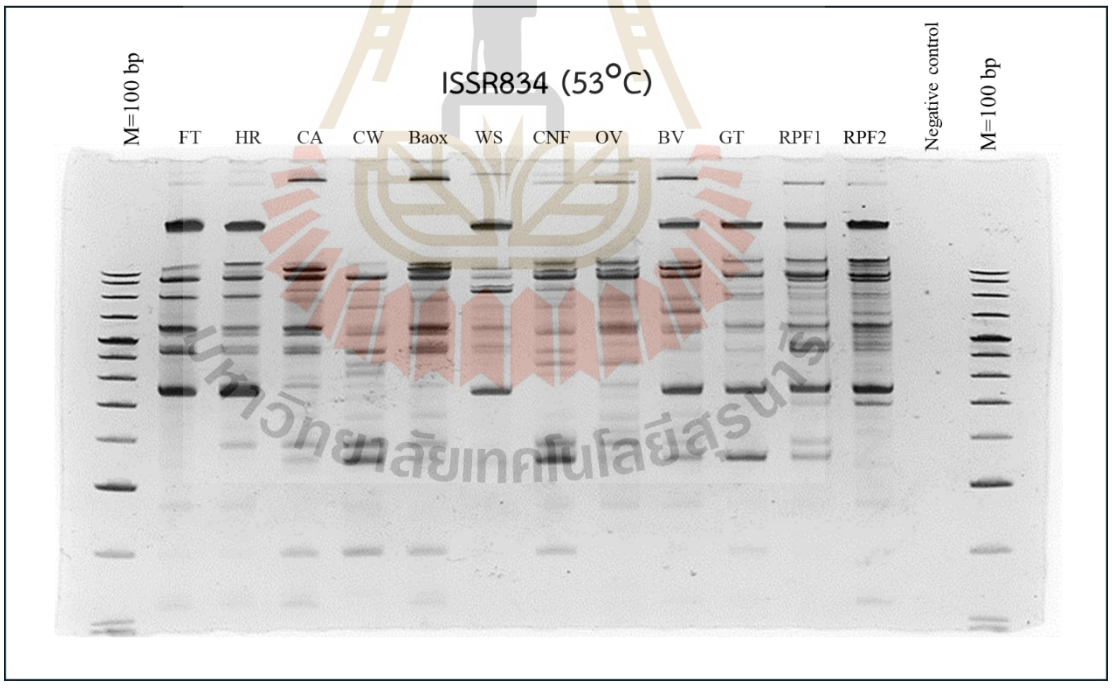
Primers	Sequences (5' to 3')	Annealing temperature (°C)	Number of Polymorphic	Percentage of Polymorphism
ISSR 810	(GA)8T	55	64	93.75
ISSR 811	(GA)8C	55	66	92.42
ISSR 825	(AC)8T	53	69	92.75
ISSR 834	(GA)8YT	53	65	93.85
ISSR 841	(GA)8YC	48	64	90.63
ISSR 844	(CT)8RC	53	68	94.12
ISSR 880	(GGAGA)3	48	69	95.65
ISSR 888	BDB (CA)7	55	66	92.42
ISSR 891	HVH (TG)7	55	68	92.65
ISSR 895	(AG)2TTGGTAG (CT)2TGATC	48	66	90.91
ISSR 899	CATG(GT)2TGGT CATTGTTCCA	48	66	93.94
	Total		731	93.01

B = C, G, T; D = A, G, T; H = A, C, T; N = A, G, C, T; R = purines (A, G); V = A, C, G; Y = pyrimidines (C, T).

Among the 11 primers, a total of 782 comparative total DNA bands were generated, which figure averages to about 71 bands per primer. This means that most of the primer could yield between 66 to 75 bands, which indicates a strong amplification capacity Barrett et al, (2000). It describes that out of the total DNA bands, a large percentage (93.47%) of the bands were polymorphic while only 6.52% were monomorphic making the probability of differentiation within and among sample cannabis strains. However, the low monomorphic band percentage is needed to show the capacity of the ISSR primers in distinguishing different cannabis strains, which is highly relevant for strain identification and breeding programs or genetic resource conservation. Also, for related studies or for evaluation of similar primer efficiency, ISSR based studies on different other plant species may offer insights in as much. Zietkiewicz et al. (1994) highlighted the utility of ISSR markers for identifying polymorphisms in plants. Similarly, Bornet and Branchard (2001) applied this method to other crops.

The significant polymorphism rate detected in this study among other plant species points to the strength of ISSR markers in identifying diversity within a population, which is rare. In this study, 93.01% of DNA generated by the selected ISSR primers were polymorphic, providing an extraordinary level of genetic variability between the cannabis strains. The polymorphism level indicates that these primers are resistant, versatile, and can detect variations within populations which are critical for strain identification as well as for breeding and germplasm conservation. This result is in line with other studies. For instance, Shahabzadeh et al. 2019, showed the high genetic diversity in Tall fescue *(Festuca arundinacea)* populations by tagging the presence of large intraspecific variation with ISSR markers. The results showed that ISSR markers were successful in differentiating between individuals within a single species, suggesting that even closely related populations or individuals may have genetic variation (Shahabzadeh et al., 2020).





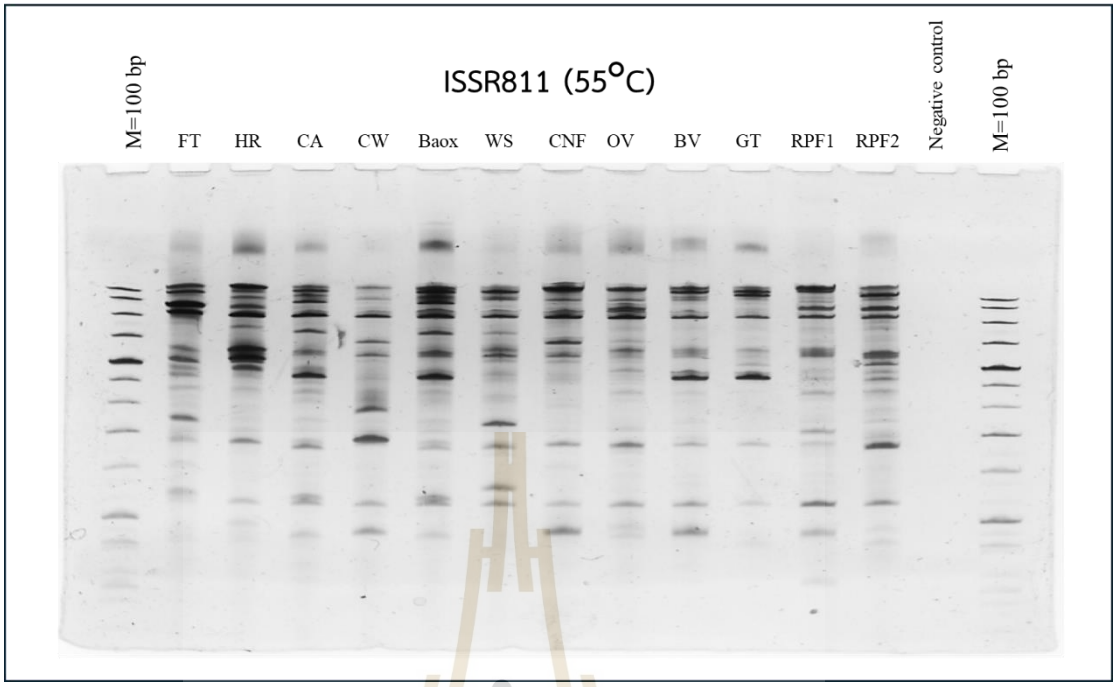


Figure 4.3 DNA pattern of 12 cannabis strains using ISSR markers observe in polyacrylamide gel.

Kojoma et al. (2002) likewise used ISSR markers in *C.sativa* for strain differentiation, which worked successfully. The applicability of such methods for the analysis of cannabis populations, and for breeding purposes was strengthened by improving the ISSR markers used in this study. Such markers provide strain-specific polymorphisms that are required for the authentication and characterization of cannabis strains. According to the data gathered, the rate of polymorphism was very high, and thus the ISSR markers employed herein will be invaluable tools for capture of genetic variation and identification of cannabis strains in improvement programs.

As a conclusion, this and other studies demonstrate the high polymorphism rates such as those seen in the current study confirming the usefulness of ISSR markers for assessing genetic variation within and between individuals and species. It also reaffirms the importance of cannabis research where the discovery and tracing of strains remains dependent on gene markers.

4.3 Genetic diversity

4.3.1 Genetic proximity within cannabis strains

The UPGMA dendrogram (Figure 4.3.1) provides a detailed visualization of the genetic relationships among the 12 cannabis strains analyzed in this study. The clustering patterns reveal distinct groupings, with several strains exhibiting high genetic similarity, as indicated by similarity coefficients exceeding 0.95. These tightly clustered groups highlight strains with closely related genetic backgrounds, likely due to shared breeding histories or environmental adaptations (Sawler et al., 2015).

The analysis also shows intermediate clusters with similarity coefficients ranging from 0.85 to 0.94, suggesting moderately close genetic relationships. Strains in this range might represent divergent lineages within a shared genetic pool, reflecting selective breeding for specific traits such as cannabinoid profiles or growth characteristics (van Bakel et al., 2011).

Interestingly, some clusters exhibit broader genetic distances, with similar coefficients below 0.80, indicating the presence of genetically distinct strains. These outliers may represent unique breeding efforts or adaptations to varying environmental conditions (Nei & Li, 1979). The dendrogram also demonstrates that phenotypic overlap observed in previous analyses may correlate with the genetic proximity of these strains. Strains forming tightly bound clusters with high similarity coefficients are more likely to share overlapping traits, complicating phenotypic classification without genetic confirmation (Fasoula et al., 2004).

This data underscores the importance of combining molecular genetic analysis with traditional phenotypic characterization to achieve accurate strain identification. Furthermore, the presence of both closely related and genetically distinct strains in the dendrogram highlights the influence of selective breeding and the potential for expanding genetic diversity in cannabis cultivation (McPartland et al., 2004). Future research should aim to integrate additional molecular markers and whole-genome sequencing to enhance the resolution of strain classification and better understand the genetic basis of key phenotypic traits.

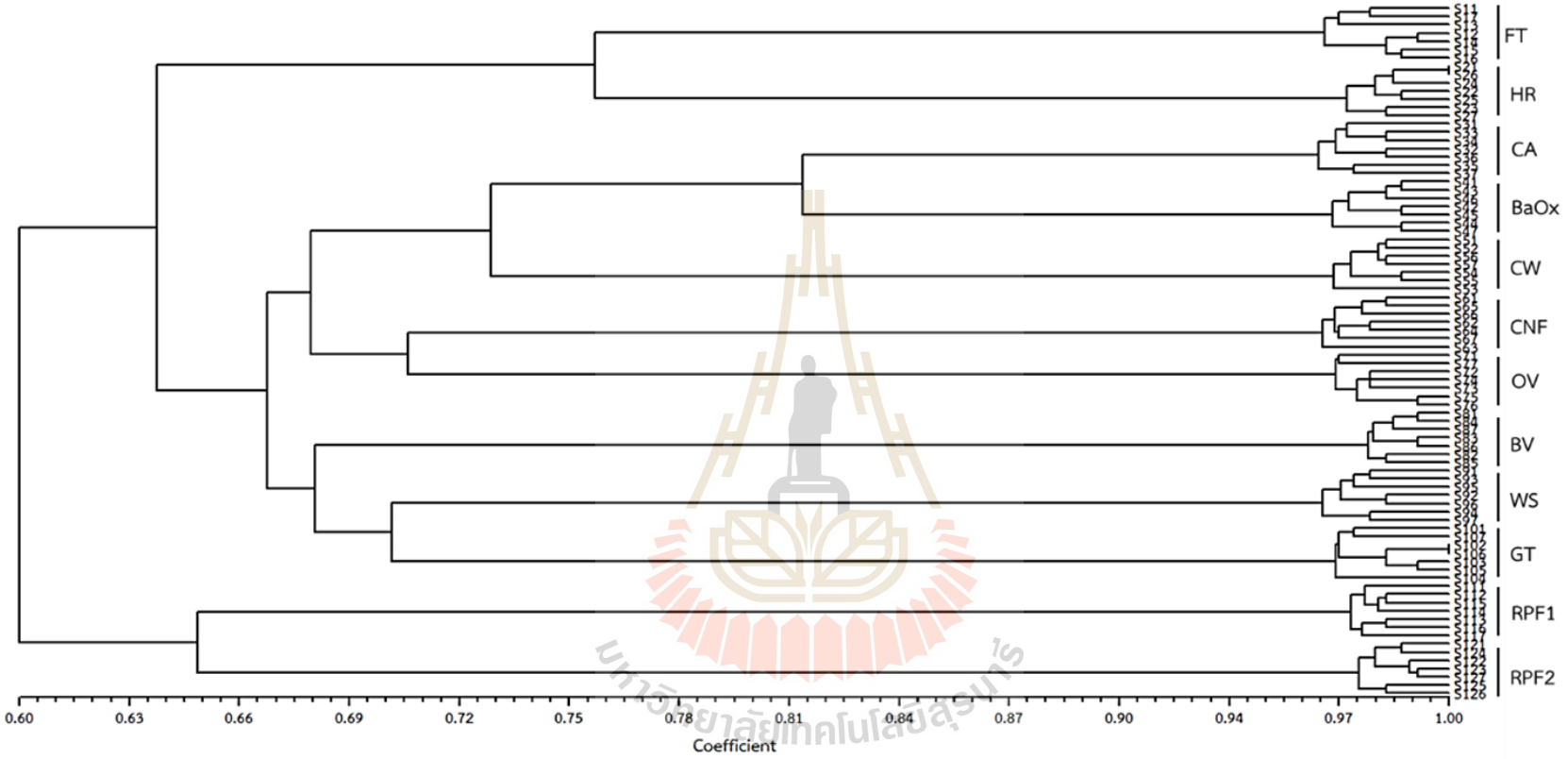


Figure 4.4 Clustering dendrogram within 12 cannabis strains.

The results are consistent with findings from previous studies, where clustering methods based on molecular markers demonstrated the ability to distinguish genetic relationships among plant species. For instance, Salih et al. (2015) utilized ISSR markers to analyze rice populations and identified distinct clusters that corresponded to geographic origins and environmental adaptations. Similarly, Gao et al. (2014) applied ISSR markers to tea plants, revealing genetic clusters that aligned with breeding histories and cultivar origins. In cannabis, Kojoma et al. (2002) successfully employed ISSR markers to differentiate between *C.sativa* strains, highlighting their reliability in elucidating genetic relationships.

In this study, the UPGMA analysis further confirms the utility of ISSR markers in assessing the genetic diversity of cannabis strains. The formation of distinct clusters, particularly those with high similarity coefficients, suggests that specific strains may share genetic lineages resulting from targeted breeding programs or adaptation to similar environmental conditions. These findings provide valuable insights into the genetic structure of cannabis and offer a foundation for future breeding efforts aimed at improving desirable traits.

4.3.2 Genetic Distinction Between Clusters

Polyacrylamide gel electrophoresis was performed on DNA patterns of 12 cannabis strains, with data being used to create a DNA structural relationship dendrogram utilizing UPMGA methods. The data presented were similarity coefficients ranging from 0.61 - 0.84 which show moderate to high genetic distance between the strains, with the lowest similarity coefficient being closer than 0.6, and the proportions being greater than 0.8. The dendrogram produced showed a total of two major clusters demonstrating the two distinct genetic relationships between the strains (Figure 4.5).

Cluster 1 included 10 strains: FoiThong, Hangkarok, Charlotte’s Angel, BaOx, Charlotte’s Web, Cannafuel, Blue Venom, Orange Valley, West Slop, and Golden Tiger. This cluster demonstrated substantial genetic diversity and could be further divided into two sub-clades. The first clade comprised of FoiThong and Hangkarok suggesting that these strains of cannabis are more closely related to each other than to other strains found within cluster one. The second clade included the other 8 strains of cannabis, which were Charlotte’s Angel, BaOx, Charlotte’s Web, Cannafuel, Blue Venom,

Orange Valley, West Slop, and Golden Tiger. This grouping tends to suggest that all these strains of cannabis are genetically similar and may have developed from similar and common genetic traits or even common breeding practices.

Cluster 2, that only accommodates the RPF1 and RPF2 members who are genetically closer to each other than any other members in this study. Their split from cluster 1 also indicates some genetic variations which may be due to separate breeding lines, varying selection conditions, or geographical differences. This separation further emphasizes the genetic uniqueness of RPF1 and RPF2 especially when compared to the greater strain group.

Separation of two major clusters and further sub clade level differentiation of samples within cluster 1 also show the effectiveness of the ISSR markers in demonstrating genetic variation of cannabis strains. The cluster patterns may depend on the breeding processes involved, the geographic areas of evolution or overlapping phenotypic characteristics of the strains, which explains this evolutionary process. This differentiation is important in the development of parent strains of broad genetic resources in the breeding programs so that different gene pools may be combined which in turn may lead to higher expression of the desirable features.

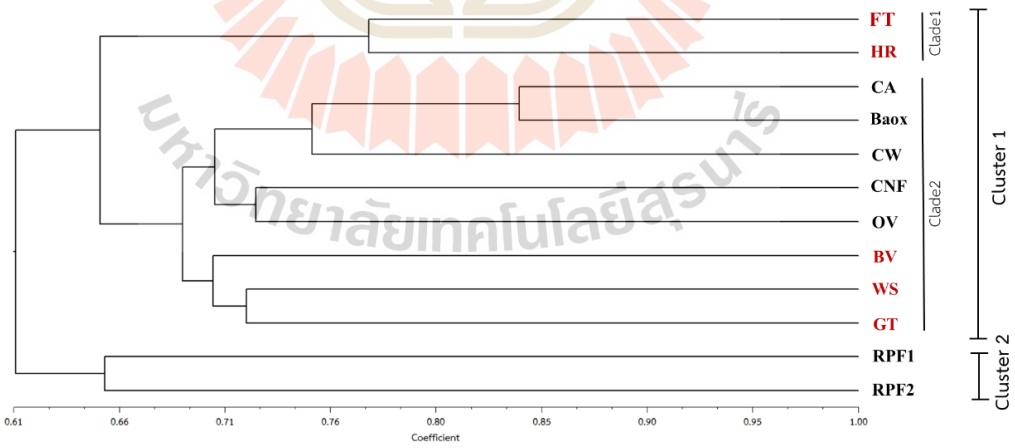


Figure 4.5 Dendrogram showing genetic relationships among 12 cannabis strains, with clear clustering observed between cannabis (indicated in red) and hemp (indicated in black). This separation highlights the genetic distinction between the two groups, providing insights into their diversity and evolutionary relationships.

UPGMA analysis results evaluate the genetic relationships of 12 cannabis strains. A coefficient of similarity ranging from 0.61 to 0.84 indicated a low genetic differentiation, and therefore two main clusters were formed. That genetic variation could indicate differing breeding histories or adaptation to different environmental conditions. When specific markers regarding cannabis are limited, clustering analysis based on ISSR markers makes it easier to elucidate genetic relationships. This might suggest differentiation between two clusters, one larger and one smaller, which could signify the presence of separate genetic lineages or different breeding practices within a species (BuraSilva et al 2021).

Cluster 1 includes a bigger group of genetically heterogeneous isolates further split into two clades. The subdivision reflects a greater genetic homogeneity within each clade, possibly associated with similar phenotypes or joint selection goals (e.g., cannabinoid profile, disease resistance). Cluster 2, where only RPF1 and RPF2 stand apart, also confirms that it is possible that strains belonged to different breeding lineages or acquired some special adaptations for a different setup. Thus, this study identifies the potential contributions of RPF1 and RPF2 as unique genetic reservoirs for the purposes of breeding programs aimed at diversifying the input germplasm.

Such clustering results were in accordance with other plant species that had previously shown genetic separation using ISSR markers. For example, Salih et al. using ISSR markers, it found that rice samples are divided into quite separate clusters corresponding to geographical origin, indicating environmental adaptation and a divergence between breeding (Salih et al.2015). Similarly, Gao et al. ISSR markers have also been used to elucidate the genetic history of many tea plant cultivars; Gao et al. (2014) identified a considerable range of gene diversity in tea plant cultivars and positioned on tree clusters that were concordant with breeding histories and cultivar origins (Gao et al., 2014). In cannabis, Kojoma et al. For example, ISSR markers have provided a reliable means for differentiating Cannabis sativa strains (Kojoma et al., 2002) and may also serve an important role in establishing genetic relationships in cannabis.

Table 4.2 Similarity matrix for leaf samples of 12 cannabis strains based on ISSR markers.

	FT	HR	CA	CW	BaOx	OV	CNF	WS	BV	GT	RPF1	RPF2
FT	1											
HR	0.78	1										
CA	0.62	0.70	1									
CW	0.65	0.64	0.76	1								
BaOx	0.65	0.68	0.84	0.73	1							
OV	0.62	0.62	0.70	0.71	0.67	1						
CNF	0.64	0.66	0.71	0.70	0.74	0.72	1					
WS	0.65	0.62	0.67	0.69	0.70	0.67	0.70	1				
BV	0.67	0.68	0.71	0.67	0.73	0.66	0.68	0.70	1			
GT	0.66	0.64	0.68	0.64	0.69	0.72	0.71	0.70	0.72	1		
RPF1	0.60	0.60	0.59	0.55	0.58	0.62	0.59	0.59	0.63	0.69	1	
RPF2	0.63	0.66	0.62	0.60	0.62	0.58	0.64	0.61	0.61	0.62	0.66	1

The similarity matrix (Table 4.2) represents pairwise genetic similarities among 12 cannabis strains based on ISSR marker analysis. The similarity coefficients ranged from 0.55 to 0.84, indicating moderate to high levels of genetic diversity among the strains. The highest similarity value (0.84) was observed between ‘CA’ and ‘BaOx’, suggesting a close genetic relationship, likely due to shared ancestry or similar breeding history. In contrast, the lowest similarity values were noted for ‘RPF1’ and ‘RPF2’ against most other strains, which may reflect their divergent genetic background. The moderate genetic distances observed (e.g., CA vs. CW, 0.76) suggest opportunities for hybridization to combine desirable traits. For instance, the similarity between ‘CNF’ and ‘OV’ (0.74) may indicate compatibility for breeding programs aimed at improving cannabinoid profiles or environmental resilience. The use of ISSR markers provided reliable differentiation among the strains, as evidenced by the clear clustering patterns. However, the similarity values also highlight limitations in resolving fine-scale genetic differences, suggesting that complementary approaches, such as SNP genotyping, might further elucidate genetic relationships. The results are consistent with previous studies that reported moderate genetic diversity in cultivated cannabis strains due to selective breeding and hybridization. These findings have practical significance for breeders aiming to maintain genetic diversity while optimizing for specific traits.

Identifying genetic clustering is crucial for cannabis breeding programs because it enables breeders to select genetically diverse individuals, which can

enhance heterosis while maintaining trait stability. Understanding these relationships also facilitates the accurate classification and authentication of cannabis strains, which is essential for quality control and regulatory compliance. As emerging markets impose strict regulations on strain identity and cannabinoid profiles, this knowledge becomes even more vital (Ewens, 2020).

4.4 THCAS and CBDAD specifically PCR amplification

The THC and CBD gene-specific primers were used to confirm types of cannabinoids expressed with the dendrogram. The PCR was able to discriminate between these cannabis strains according to their cannabinoid profiles. The result (Figure 4.6.) revealed that the primers were capable of selectively amplifying and visualizing THC-type or CBD-type bands. So, clusters cannabis strains into separate groups based on their cannabinoid content. This enabled the strains to be classified as THC or CBD dominant and offered further opportunities into the study of cannabis varietal genetics by cannabinoid profile.

Figure 4.6 Band patterns using specific THC and CBD primers. The THC gene produced an amplified product of 589 bp (red strains), while the CBD gene yielded a band at 169 bp black strains). Any bands observed beyond the 589 bp marker were not specific to both genes and did not affect the results.

Of the 12 cannabis strains tested, five were classified as THC-dominant types: FoiThong, Hangkarok, Blue Venom, West Slop and Golden Tiger. These strains tested positive for a PCR band associated with the THC gene, identifying them as drug-type cannabis that are characterized by higher levels of this cannabinoid and Psych activity. So, the presence of these in the strains from this THC category just makes sense as per their target used in medical and recreation cannabis market focusing on high THC content. The remaining 7 strains were classified as CBD-dominant types: Charlotte's Angel, BaOx, Charlotte's Web, Cannafuel, Orange Valley, RPF1 and RPF2. These strains were positive for the CBD-specific gene; thus, they are categorized as fiber-type and medicinal cannabis with high levels of CBD and low THC.

In drug-type cannabis, the nonspecific 169 bp DNA fragment is amplified when using the primer pairs F-CBDAS (Fw and Rv), targeting the active THCAS sequence. However, when the primer combination F-CBDAS Fw and D-THCAS Rv is used, this fragment is replaced by a 772 bp product. This is likely due to D-THCAS Rv having a stronger binding affinity for the active THCAS sequence than F-CBDAS Rv. Similarly, in fiber-type cannabis, a nonspecific 589 bp fragment is generated using the primer pairs D-THCAS (Fw and Rv), targeting the active CBDAS sequence. When the primer pair F-CBDAS Fw and D-THCAS Rv is used, this 589 bp fragment is replaced by the same 772 bp product. This change is likely because F-CBDAS Fw binds more strongly to the active CBDAS sequence than D-THCAS Fw. The 772 bp band is common in both drug-type and fiber-type cannabis and can be easily distinguished from the 169 bp and 589 bp bands by size. However, when the DNA template concentration is low, the 772 bp band tends to disappear, leaving only the specific 169 bp or 589 bp fragments. This characteristic makes the 772 bp band a useful marker for distinguishing between drug-type and fiber-type cannabis (Yamamuro et al., 2021).

Allele-specific primers for THC and CBD allow rapid, high-throughput and cost-efficient cannabis typing. Such strategy enables selective breeding programs targeted for high-THC or CBD-enriched strains when required by the market. Also, it is helpful in differentiating genetic strains which is significant for regulatory kings because cannabis must meet the legal THC content to avoid severe fines. In conclusion, our data indicate that unique THC and CBD gene primers provide an accurate and rapid

classification of cannabis according to their cannabinoid profiles which have important ramifications for breeding, standards, and regulations.

A clear clustering of 12 strains according to their cannabinoid profiles, appeared in a dendrogram that was generated after specific primers separating THC and CBD types were applied. Results showed that subjects are clustered into separate groups for THC and CBD. Five THC-dominant strains (FoiThong, Hangkarok, Blue Venom, West Slop and Golden Tiger) clustered separately from the rest confirming their elevated levels of THC. On the other hand, the CBD-dominant strains (Charlotte's Angel, BaOx, Charlotte's Web, Cannafuel, Orange Valley and RPF1 and RPF2) clustered near each other due to their high-CBD low-psychoactive traits.

The dendrogram highlights the clear genetic distinction between THC-dominant and CBD-dominant cannabis strains, strengthening the reliability of our strain classification based on cannabinoid profiles analyzed with specific primers. This genetic insight is highly relevant to cannabis breeding, enabling the development of targeted strains for specific purposes, such as high-THC or high-CBD production.

ISSR markers have been extensively used to reveal genetic diversity and distinguish between plant populations. For example, Gao et al. (2020) demonstrated the effectiveness of ISSR markers in distinguishing genetic variation in Cannabis sativa strains, identifying markers associated with cannabinoid production. Similarly, Patel et al. (2017) employed ISSR markers to assess genetic diversity in Cymbopogon citratus (lemongrass), showcasing their ability to identify genetic variability in closely related populations, which is applicable to cannabis research.

Further supporting this approach, Rana et al. (2019) utilized ISSR markers to study genetic divergence in medicinal plants, underscoring their value in identifying genetic markers linked to specific bioactive compounds. Additionally, Alizadeh et al. (2020) highlighted the utility of molecular techniques such as specific primers in differentiating chemotypes within medicinal plant species, providing evidence for the genetic basis of biochemical variations. These studies collectively validate the methodology employed in this study for separating cannabis strains based on cannabinoid profiles, emphasizing its importance in advancing cannabis breeding and strain development.

CHAPTER V
CONCLUSION

While phenotypic analysis offers a foundational understanding of cannabis plant characteristics, its limitations necessitate the incorporation of genetic tools for accurate strain identification and classification. This integrated approach will support advancements in cannabis breeding, cultivation, and research.

In this study, ISSR markers were effectively employed to characterize 12 cannabis strains based on their genetic properties, along with THC and CBD gene-specific primers. According to the ISSR analysis, the strains showed high genetic diversity characteristics, and two primary clusters reflected their close genetics linkage. These molecular tools were then used to phenotype cannabis strains based on cannabinoid composition, as THC- and CBD-specific primers clearly grouped THC-dominant and CBD-dominant strains.

UPGMA clustering of DNA sequences from ISSR markers further revealed genetic diversity, with cluster 1 containing ten strains that exhibited notable internal variation, indicating a broad genetic basis in this group. Group 2 consisting of RPF1 and RPF2 was shown to be genetically distinct, indicating different reproductive lineages a different or unique modifications. Results with a high polymorphism rate of 93.01% confirm the effectiveness of the ISSR marker in identifying genetic variation in cannabis strains, making it a powerful tool for the identification of marijuana strains, breeding, and enhancement of genetic control breeding programs

In the end, the aggregate of ISSR markers and cannabinoid-unique primers affords a robust technique for hashish stress identification and genetic evaluation. The findings not only effectively enhance the understanding of genetic relationships among hashish traces, but also offer sensible programs in breeding, pressure authentication, and regulatory compliance.

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APPENDIX

Table A1 Primer sequences, range of amplified products, number of scorable DNA bands, number of polymorphic bands between male and female parents, number of loci associated with powdery mildew resistance, and annealing temperature for each of 75 ISSR primers in 'CN72 × V4718' cross.

Primers	Primer sequences	Range of amplified products (bp)	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)	Annealing temperature (°C)
807	(AG)8T	200–1200	16	1	50
808	(AG)8C	200–1200	17	3	50
809	(AG)8G	200–1200	24	2	50
810	(GA)8T	200–1200	19	1	50
811	(GA)8C	250–1200	20	3	50
812	(GA)8A	200–1200	19	1	50
813	(CT)8T	200–2072	19	2	50
814	(CT)8A	200–2072	14	4	50
815	(CT)8G	250–1200	11	0	50
816	(CA)8T	200–1200	20	2	50
817	(CA)8A	300–1200	12	1	50
818	(CA)8G	300–1400	14	3	50
819	(GT)8A	500–1400	11	0	50
820	(GT)8C	200–2072	17	0	50
821	(GT)8T	250–2072	14	1	50
822	(TC)8A	200–2072	25	0	54
823	(TC)8C	250–2072	15	0	50
824	(TC)8G	200–2072	13	0	50
825	(AC)8T	250–1200	18	1	50
826	(AC)8C	200–1200	19	0	50
827	(AC)8G	250–1500	18	4	50
828	(TG)8A	300–1200	12	1	50
829	(TG)8C	300–1200	13	0	50
830	(TG)8G	250–1200	21	3	50
834	(AG)8YT	200–1200	24	1	50

Table A1 (Continue).

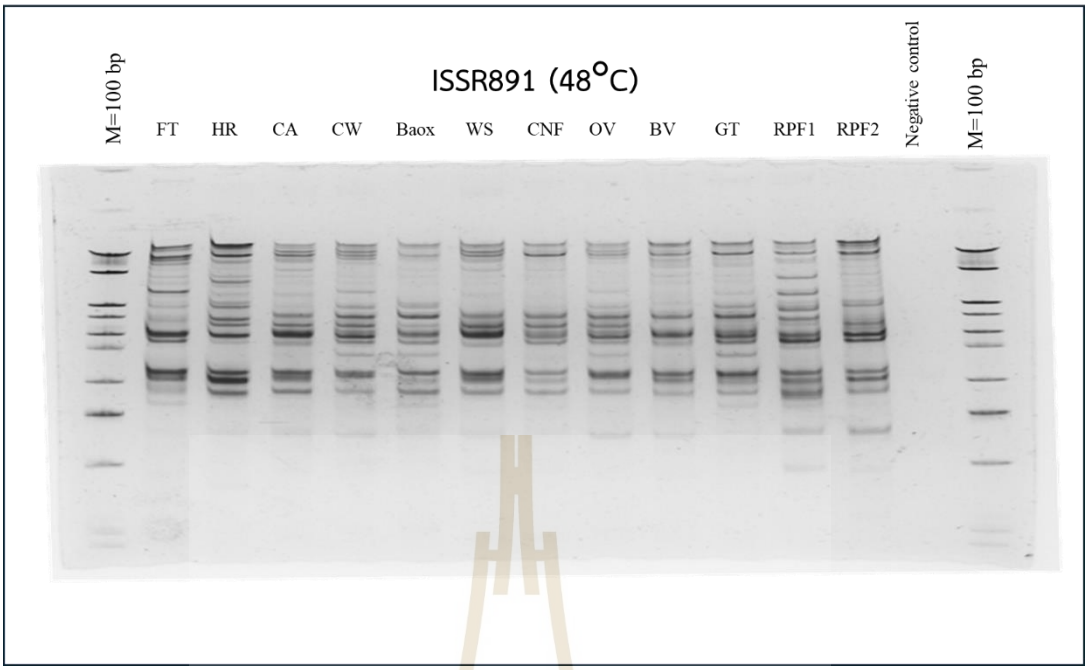
Primers	Primer sequences	Range of amplified products (bp)	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)	Annealing temperature (°C)
835	(AG)8YC	200–1200	26	2	50
836	(AG)8YA	200–1200	24	2	50
840	(GA)8YT	200–1200	19	1	40
841	(GA)8YC	250–1200	19	1	50
841c	(GA)8CC	250–1200	18	2	50
841 t	(GA)8TC	250–1200	18	0	50
842	(GA)8YG	250–1200	23	1	50
843	(CT)8RA	200–2072	11	1	50
844	(CT)8RC	300–1200	14	0	50
845	(CT)8RG	200–2072	16	0	45
846	(CA)8AT	200–1500	14	0	50
847	(CA)8RC	300–1200	15	1	50
848	(CA)8RG	400–1200	14	0	50
849	(GT)8YA	150–2072	14	2	50
850	(GT)8YC	200–1200	19	3	50
851	(GT)8YG	250–1200	12	1	50
853	(TC)8RT	300–2072	13	1	50
854	(TC)8RG	200–2072	12	0	50
855	(AC)8YT	350–1200	15	0	50
856	(AC)8YA	250–1200	18	1	50
857	(AC)8YG	200–1200	28	3	50
858	(TG)8RT	200–1200	23	2	50
859	(TG)8RC	350–1200	17	1	50
860	(TG)8RA	250–1400	11	1	50
861	(ACC)6	100–2072	18	0	50
862	(AGC)6	150–1000	12	0	50
864	(ATG)6	200–1500	27	2	50
865	(CCG)6	200–2072	15	0	50
866	(CTC)6	200–1500	15	1	50
867	(GGC)6	100–1000	12	0	50

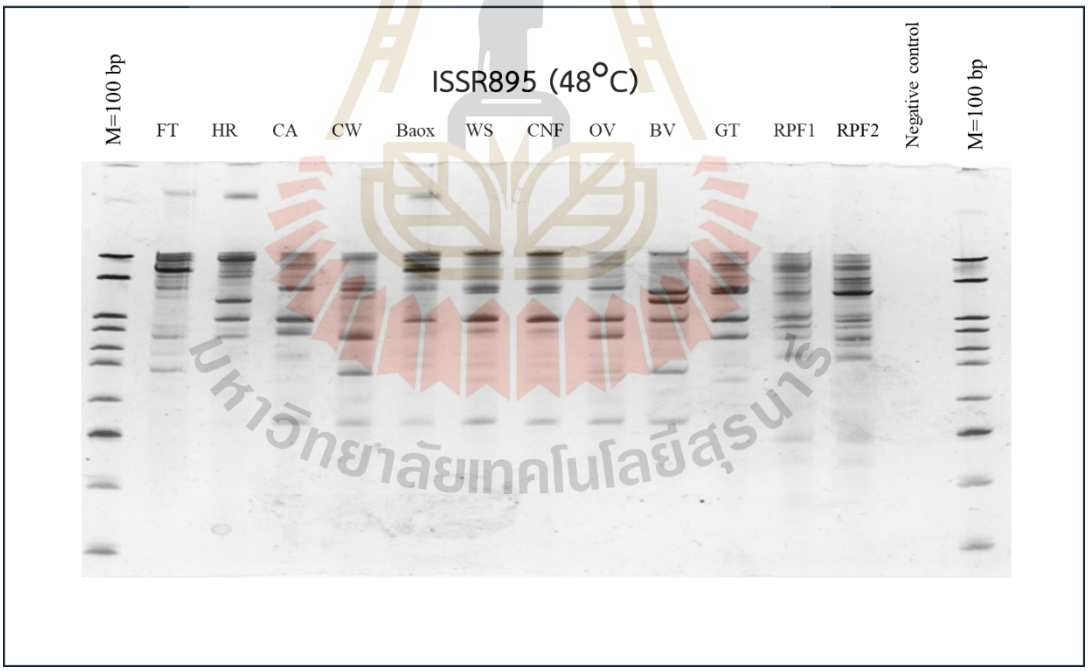
Table A1 (Continue).

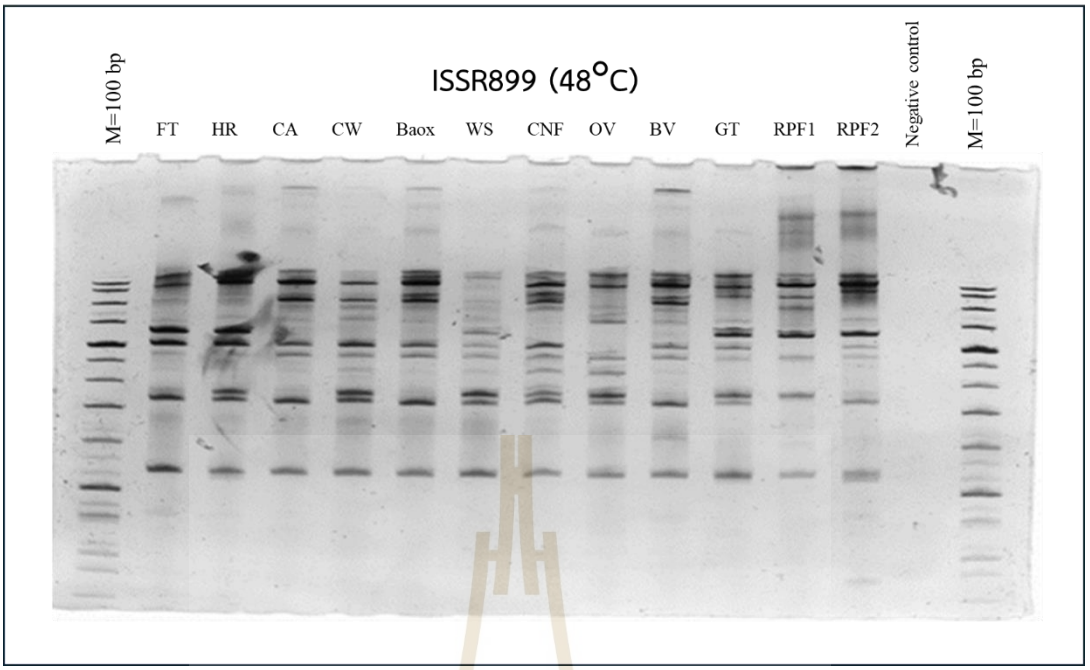
Primers	Primer sequences	Range of amplified products (bp)	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)	Annealing temperature (°C)
867	(GGC)6	100–1000	12	0	50
868	(GAA)6	350–1200	13	1	50
869	(GTT)6	200–2072	17	0	50
872	(GAGA)4	250–2072	11	0	44
873	(GACA)4	350–1200	15	0	50
876	(GATA)2 (GACA)2	400–1200	14	2	50
878	(GGAT)4	300–1400	16	1	50
879	(CTTCA)3	250–2072	12	0	44
880	(GGAGA)3	250–1200	13	3	50
881	(GGGTG)3	200–2072	20	0	50
884	HBH (AG)7	200–1200	25	4	50
885	BHB (GA)7	200–1200	26	2	50
886	VDV (CT)7	250–1500	26	2	50
887	DVD (TC)7	250–1500	26	5	50
888	BDB (CA)7	300–1200	20	3	50
889	DBD (AC)7	200–1200	16	0	50
890	VHV (GT)7	200–1200	18	2	50
891	HVH (TG)7	200–1200	18	1	50
895	(AG)2TTGGTAG (CT)2TGATC	250–2072	15	1	40
899	CATG(GT)2TGGT CATTGTTCCA	200–1000	14	0	40
900	ACTTCCC(CA)2GG TTA(CA)2	200–2072	25	0	50
	Total		1297	90	
	Average		17	1.2	

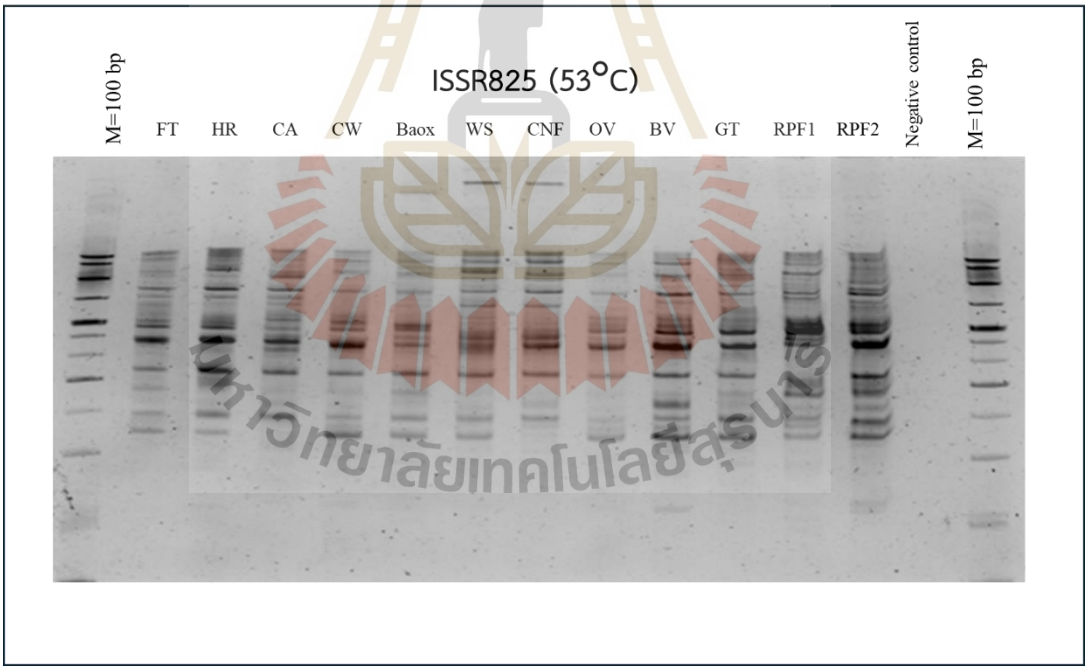
B = C, G, T; D = A, G, T; H = A, C, T; N = A, G, C, T; R = purines (A, G); V = A, C, G.

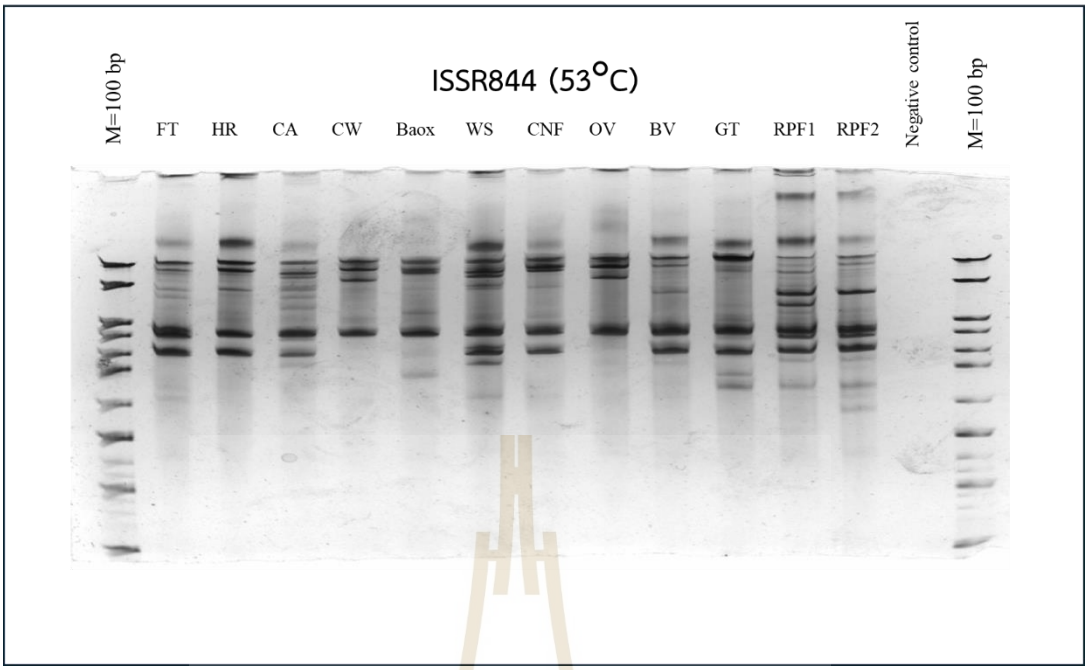
Y = pyrimidines (C, T).

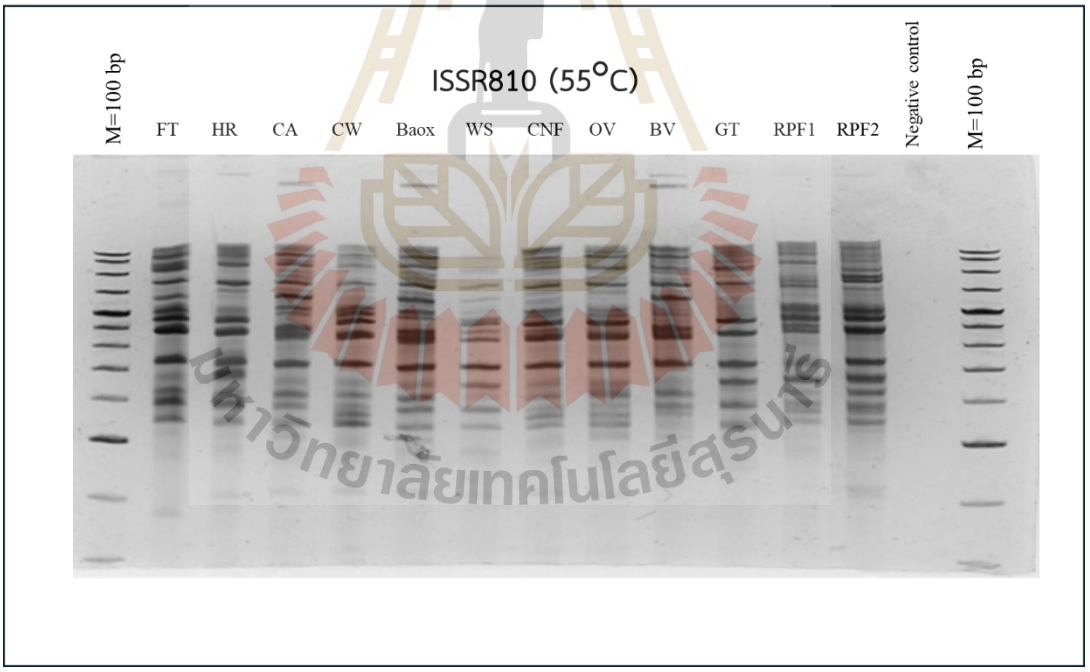












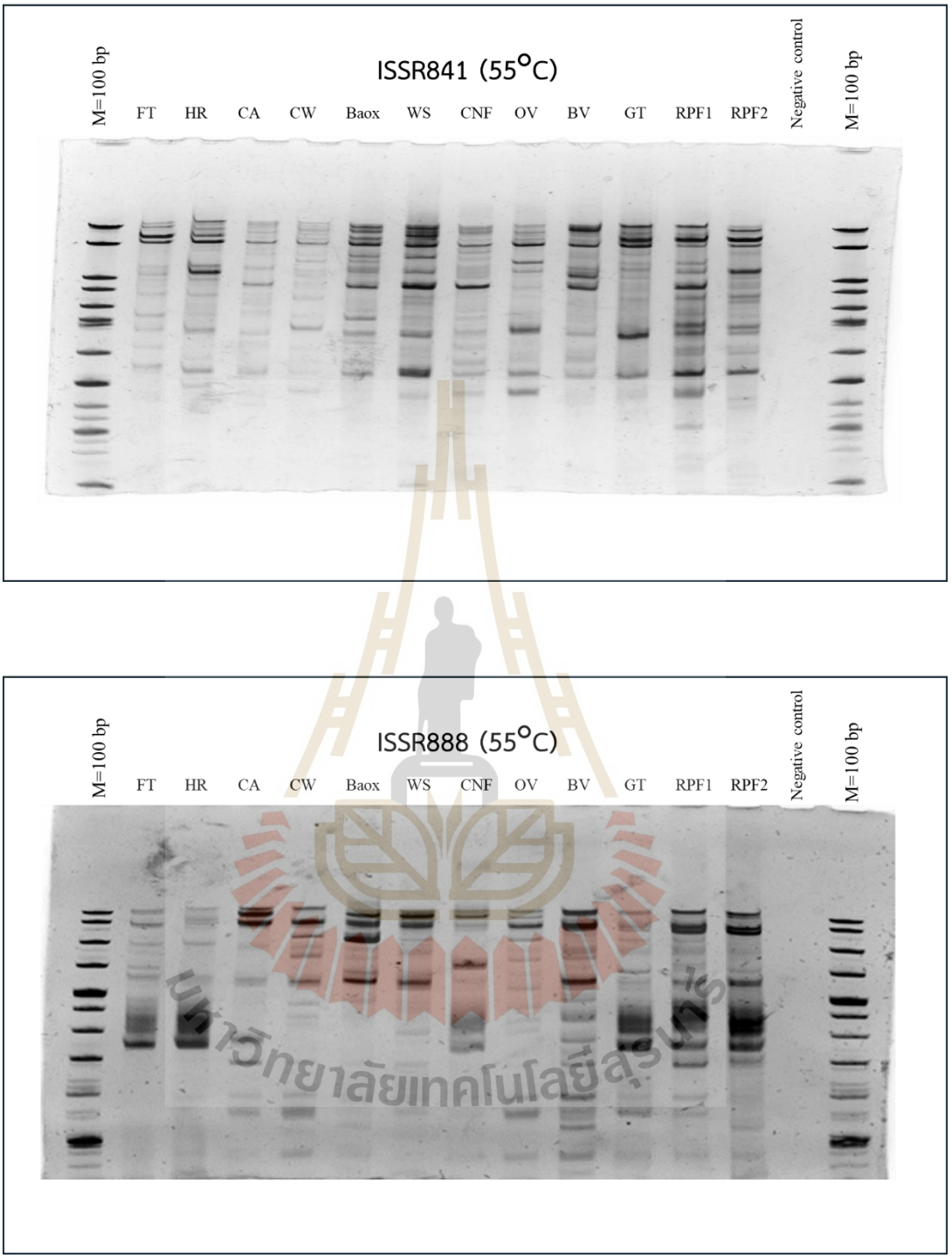


Figure B1 DNA pattern of 12 cannabis strains using ISSR markers observe in polyacrylamide gel.

Figure B2 Cannabis growing at 'SUT cannabis farm'.

Table A2 Data of phenotypic characteristics of cannabis strains (n=3).

Strains	No.	Height	Average	Bush shape	Average	Leaves	Average
Blue Venom	1	115	117.33	94	94.67	12	12.03
	2	117		94		12.3	
	3	120		96		11.8	
Baox	1	122	126.00	97	95.67	11	11.13
	2	123		95		11.3	
	3	133		95		11.1	
Cannafuel	1	132	124.67	105	105.00	10.6	10.83
	2	112		107		10.9	
	3	130		103		11	
Charlotte's Angle	1	133	136.00	102	102.67	9.5	9.30
	2	140		102		9.3	
	3	135		104		9.1	
Charlotte's Web	1	135	138.00	106	104.33	12.1	12.17
	2	137		104		12.2	
	3	142		103		12.2	
Golden Tiger	1	122	132.00	98	97.67	11.5	11.43
	2	141		97		11.4	
	3	133		98		11.4	

Table A2 Data of phenotypic characteristics of cannabis strains (n=3).

Strains	No.	Height	Average	Bush shape	Average	Leaves	Average
Orange Valley	1	114	116.00	94	92.67	10.4	10.73
	2	119		92		10.8	
	3	115		92		11	
West Slope	1	116	114.67	91	91.33	10.5	10.57
	2	111		92		10.7	
	3	117		91		10.5	
Foithong	1	150	151.00	108	108.33	12.6	12.67
	2	155		109		13	
	3	148		108		12.4	
Hangkarok	1	148	149.67	106	106.67	12.5	12.37
	2	147		107		12.4	
	3	154		107		12.2	
RPF1	1	134	135.33	105	106.00	12.1	12.23
	2	137		104		12.4	
	3	135		109		12.2	
RPF2	1	133	134.67	97	97.67	12.2	12.23
	2	137		98		12.2	
	3	134		98		12.3	

BIOGRAPHY

Miss. Souphaphone Keomany was born on december 11, 1997 in Khammouane, Laos. She graduated with a bachelor's degree from Institute of Crop science, from Savannakhet University in 2020. In 2021, she enrolled at the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, and received a scholarship from One Research One Graduate (OROG) of the Thailand Research Fund.