Original Article

Unraveling the genetic diversity of local Thai Cannabis strains using Inter-Simple Sequence Repeat (ISSR) molecular markers

การศึกษาความหลากหลายทางพันธุกรรมของสายพันธุ์กัญชาพื้นเมืองไทยโดยใช้เครื่องหมายโมเลกุลอินเตอร์ซิมเพิลซีเควนซ์รีพีต (ไอเอสเอสอาร์)

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Abstract *Cannabis sativa*, an emerging economic crop in Thailand, has been cultivated in various regions of the country. The objective of this research was to examine the genetic relationships of local Thai Cannabis strains using five inter-simple sequence repeat (ISSR) molecular markers. A total of 50 Cannabis samples were evaluated, encompassing named local strains, unidentified landraces, and a hemp variety. The analysis yielded a total of 52 DNA bands, of which 51 were polymorphic, ranging in size from 300 to 1,500 bp. Based on UPGMA cluster analysis at a genetic distance cutoff of 0.25, the accessions were clearly separated into four distinct groups. Group 1 (bootstrap score: 96.6%) comprised local landraces such as Chiang Rai Dang Kan Kew and Green Thai Stick. Group 2 (bootstrap score: 92.6%) included high-cannabinoid and regional varieties, notably the CBD strain, Kanchanaburi, and Thai Stick. Group 3 (bootstrap score: 95.8%) predominantly clustered the MJUS breeding lines alongside accessions from Sakon Nakhon and Nakhon Phanom. Group 4 (bootstrap score: 77.2%) consisted of the Isara series and Kra Le. This study demonstrated that ISSR markers are highly effective in distinguishing the genetic structure of local Thai Cannabis strains, providing valuable baseline data for germplasm conservation, phytochemical standardization, and future medical breeding programs.

Keywords: Thai cannabis, ISSR marker, Genetic diversity

บทคัดย่อ กัญชา เป็นพืชเศรษฐกิจที่กำลังได้รับความสนใจในประเทศไทย และมีการเพาะปลูกในหลายพื้นที่ทั่วประเทศ การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ทางพันธุกรรมของสายพันธุ์กัญชาพื้นเมืองไทย โดยใช้เครื่องหมายโมเลกุล Inter-Simple Sequence Repeat (ISSR) จำนวน 5 ไพรเมอร์ ทำการวิเคราะห์ตัวอย่างกัญชาทั้งหมด 50 ตัวอย่าง ซึ่งประกอบด้วยสายพันธุ์ท้องถิ่นที่มีชื่อเรียก สายพันธุ์พื้นเมืองที่ไม่ทราบชื่อ และสายพันธุ์กัญชง ผลการวิเคราะห์พบแถบดีเอ็นเอทั้งหมด 52 แถบ โดยเป็นแถบที่แสดงความหลากหลายทางพันธุกรรมจำนวน 51 แถบ มีขนาดตั้งแต่ 300 – 1,500 คู่เบส จากการวิเคราะห์การจัดกลุ่มด้วยวิธี UPGMA ที่ค่าระยะห่างทางพันธุกรรม 0.25 สามารถจำแนกตัวอย่างออกเป็น 4 กลุ่มอย่างชัดเจน ได้แก่ กลุ่มที่ 1 (ค่า bootstrap 96.6%) ประกอบด้วยสายพันธุ์พื้นเมือง เช่น Chiang Rai Dang Kan Kew และ Green Thai Stick กลุ่มที่ 2 (ค่า bootstrap 92.6%) ประกอบด้วยสายพันธุ์ที่มีสารแคนนาบินอยด์สูงและสายพันธุ์จากภูมิภาคต่าง ๆ โดยเฉพาะสายพันธุ์ CBD, Kanchanaburi และ Thai Stick กลุ่มที่ 3 (ค่า bootstrap 95.8%) ส่วนใหญ่ประกอบด้วยสายพันธุ์ปรับปรุง MJUS ร่วมกับตัวอย่างจากจังหวัดสกลนครและนครพนม และกลุ่มที่ 4 (ค่า bootstrap 77.2%) ประกอบด้วยสายพันธุ์ในกลุ่ม Isara และ Kra Le ผลการศึกษานี้แสดงให้เห็นว่าเครื่องหมายโมเลกุล ISSR มีประสิทธิภาพสูงในการจำแนกโครงสร้างทางพันธุกรรมของสายพันธุ์กัญชาพื้นเมืองไทย และสามารถใช้เป็นข้อมูลพื้นฐานที่สำคัญสำหรับการอนุรักษ์ทรัพยากรพันธุกรรม การกำหนดมาตรฐานทางพฤกษเคมี และการพัฒนาสายพันธุ์กัญชาเพื่อประโยชน์ทางการแพทย์ในอนาคต

คำสำคัญ กัญชาไทย, เครื่องหมาย ISSR, ความหลากหลายทางพันธุกรรม

Introduction

Cannabis (*Cannabis sativa*) has historically been cultivated for a dual purpose: as a psychoactive substance and for its fiber. In recent years, it has garnered increasing attention in Thailand, primarily due to its remarkable medicinal properties. While cannabis has been utilized across various cultures for therapeutic applications, modern scientific validation of these uses has emerged only in the past few decades, particularly concerning its efficacy in treating conditions such as chronic pain, epilepsy, and multiple sclerosis (Kumar et al., 2020). The recent legalization of medical cannabis in Thailand has sparked renewed interest in local cannabis strains, propelled by their unique genetic profiles and potential for pharmaceutical applications (Tanchob et al., 2021).

The genetic diversity inherent in Cannabis sativa is instrumental in determining the cannabinoid content of various strains. Strains with high concentrations of tetrahydrocannabinol (THC) are predominantly associated with psychoactive effects, while those dominated by cannabidiol (CBD) are recognized for their therapeutic benefits without the accompanying psychoactive side effects (Hill et al., 2018). This genetic variability within cannabis populations is essential for breeding programs, especially as the demand for specific cannabinoid profiles continues to escalate in both medical and industrial cannabis sectors (Kovalchuk et al., 2019).

Thailand boasts a rich repository of local cannabis strains that have been cultivated and selectively bred over centuries. These strains have adapted to the unique environmental conditions of Thailand, making them invaluable resources for research and development. For instance, strains such as Chiang Rai Dang Kan Kew, Ja Meun Sri, Green Thai Stick, and Kew Ngou are well-documented for their distinctive profiles, particularly their elevated THC content. Additionally, strains like Pai Wiang Haeng, Kanchanaburi Thamuang, and Phetchaburi Tanao Si have emerged as significant contributors to the local cannabis biodiversity (Wong et al., 2020). However, despite the rich historical and agricultural significance of these strains, research into their genetic structure and diversity remains limited, posing challenges for conservation and future breeding endeavors (Sarker et al., 2017).

In this context, the study aims to fill this research gap by investigating the genetic diversity and phylogenetic relationships among these local Thai cannabis strains utilizing inter-simple sequence repeat (ISSR) molecular markers. ISSR markers were chosen for their capacity to detect polymorphisms at multiple loci across the cannabis genome, making them particularly effective for non-model organisms that exhibit complex and highly variable genomes (Hernandez et al., 2019). Moreover, the reproducibility and extensive genomic coverage offered by ISSR markers render them particularly useful for distinguishing between genetically similar populations. These attributes make ISSR an excellent tool for developing DNA fingerprints of cannabis strains, thereby enabling precise categorization and supporting conservation initiatives.

The primary objective of this study is to investigate the genetic diversity and phylogenetic relationships among these local Thai cannabis strains utilizing ISSR molecular markers.

Materials and methods

Sample collection

We collected 37 local cannabis strains and hemp from various regions in Thailand. Among these, 24 strains were sourced from the Buriram team, including

Nakhon Phanom, Sakon Nakhon Phu Phan Thai Stick, Koh Chang, Chiang Rai Dang Kan Kew, Green Thai Stick, Pai Wiang Haeng, Pai Kao Daeng, Phetchaburi Tanao Si, Kanchanaburi Thamuang, Krawea, Thai Stick, Kanchanaburi, Sakon Nakhon Lang Ban 2 , CBD strain, Kew Ngou, Muang Huai Krai, Ja Meun Sri, Kra Le, and Isara codes A-D, along with two unidentified strains. The samples, consisting of both flower and leaf parts from fresh material, were all cultivated by local farmers in Thailand. Additionally, 12 strains (MJUS codes 01-12), originally from the United States, were included. These strains had been bred for over three years at the experimental farm under the Natural Agriculture Research and Development Center, Maejo University. Hemp was used as an outgroup for comparison and was derived from Maejo University as well. The codes and names of all strains are detailed in Table 1.

Table 1. Codes and local names of 50 cannabis samples collected from each site around Thailand

Codes	Collecting part	Strain/group	Codes	Collecting part	Strain/group
1	leave	Hemp (out group)	26	leave	Green Thai Stick
2	leave	Hemp (out group)	27	leave	Pai Wiang Haeng
3	flower	MJUS09	28	leave	Pai Kao Daeng
4	leave	MJUS12	29	leave	Phetchaburi Tanao Sri
5	leave	MJUS11	30	leave	Kanchanaburi Thamuang
6	leave	MJUS10	31	leave	Kanchanaburi Thamuang
7	leave	MJUS05	32	leave	Krawea
8	leave	MJUS06	33	leave	Thai Stick
9	leave	MJUS07	34	leave	Kanchanaburi
10	leave	MJUS08	35	leave	Kanchanaburi
11	leave	MJUS01	36	leave	Kanchanaburi
12	leave	MJUS02	37	leave	Kanchanaburi
13	leave	MJUS04	38	leave	Kanchanaburi
14	leave	MJUS03	39	leave	No name strain
15	leave	Nakhon Phanom	40	flower	Kanchanaburi
16	flower	Sakon Nakhon Phu Phan Thai Stick	41	flower	Kanchanaburi
17	leave	Koh Chang	42	leave	Kanchanaburi
18	leave	Koh Chang	43	leave	No name group
19	leave	Chiang Rai Dang Kan Kew	44	leave	Sakon Nakhon Lang Ban 2
20	leave	Chiang Rai Dang Kan Kew	45	leave	Thai stick
21	leave	Chiang Rai Dang Kan Kew	46	leave	Kra Le
22	leave	CBD strain	47	leave	Isara A
23	leave	Kew Ngou	48	leave	Isara B

24	leave	Muang Huai Krai	49	leave	Isara C
25	leave	Ja Meun Sri	50	leave	Isara D

DNA extraction

DNA extraction from cannabis (*Cannabis sativa*) leaves was performed using the Plant Genomic DNA Extraction Kit by MaroGen. Fresh cannabis leaf tissue (approximately 50–100 mg) was collected and ground into a fine powder using liquid nitrogen to ensure effective cell lysis. The powdered leaf tissue was then processed according to the manufacturer's protocol, which includes steps for cell lysis, DNA precipitation, and purification using silica-based columns to ensure high-quality DNA extraction. After elution of the DNA, the quantity and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer. Absorbance readings at 260 nm and 280 nm were taken to determine the DNA concentration, while the 260/280 ratio was used to evaluate the purity of the DNA, with a ratio between 1.8 and 2.0 indicating DNA free of protein contamination. This method ensures the efficient extraction and quantification of cannabis DNA, suitable for downstream applications such as PCR and sequencing.

ISSR primer screening

The ISSR-PCR method was conducted using five primers (ISSR10, ISSR6, ISSR2, ISSR7, and ISSR4) to evaluate genetic polymorphisms in *Cannabis sativa*. Genomic DNA was extracted from *Cannabis sativa* leaves using a standard CTAB extraction protocol. The quality and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer, measuring absorbance ratios at 260/280 nm and 260/230 nm to ensure the DNA was pure and free of contaminants. DNA samples with 260/280 ratios between 1.8 and 2.0 were considered pure for PCR amplification. The PCR reactions were prepared in a 25 µL volume containing 50–100 ng of template DNA, 0.4 µM of each ISSR primer, 1.5–2.5 mM $MgCl_2$, 200 µM dNTPs, 1X PCR buffer, and 1–2 U Taq polymerase. The thermal cycling conditions included an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 1 minute, an annealing step (optimized for each primer: 55.0°C for ISSR10, 56.0°C for ISSR6 and ISSR7, 55.5°C for ISSR2 and ISSR4), and a 72°C extension for 2 minutes, with a final extension at 72 °C for 10 minutes. The amplified products were resolved on a 1.5–2% agarose gel stained with ethidium bromide and visualized under UV light. Band patterns were analyzed for polymorphism, and repeatability tests were performed to confirm consistent results. $MgCl_2$ concentration and annealing temperatures were adjusted if necessary to enhance clarity and reproducibility of the bands.

Table 2. ISSR Primer Sequences and Annealing Temperatures

primers	annealing temperature	nucleotide sequence
ISSR10	55.0 C	5′ AAC AAC AAC AAC AAC AAC AAC AAC 3′
ISSR6	56.0 C	5′ AGA GAG AGA GAG AGA GGT 3′
ISSR2	55.5 C	5′ AGA GAG AGA GAG AGA GT 3′
ISSR7	56.0 C	5′ GTG TGT GTG TGT GTG TC 3′
ISSR4	55.5 C	5′ GAG AGA GAG AGA CC 3′

PCR and fingerprinting

The ISSR-PCR method was conducted using five primers (ISSR10, ISSR6, ISSR2, ISSR7, and ISSR4) to evaluate genetic polymorphisms in *Cannabis sativa*. Genomic DNA was extracted from *Cannabis sativa* leaves using a standard CTAB extraction protocol. The quality and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer, measuring absorbance ratios at 260/280 nm and 260/230 nm to ensure the DNA was pure and free of contaminants. DNA samples with 260/280 ratios between 1.8 and 2.0 were considered pure for PCR amplification.

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Once the polymorphic bands were obtained, they were used to create a DNA fingerprint for *Cannabis sativa*. The banding patterns generated by the ISSR primers were compared across samples to identify specific genetic markers, which serve as unique identifiers for individual plants or populations. The DNA fingerprints created through ISSR-PCR allow for distinguishing between different *Cannabis sativa* genotypes, providing insights into genetic diversity, population structure, and potential breeding strategies. This technique is particularly valuable for applications such as plant variety protection, forensic identification, and the tracking of genetic variations in breeding programs. The DNA fingerprinting process involves documenting polymorphic band patterns and using statistical methods to evaluate genetic similarity or differences between individuals or populations.

Statistic and Data analysis

For DNA fingerprinting of Cannabis strains using the ISSR (Inter-Simple Sequence Repeat) technique, statistical analysis involves scoring the presence or absence of DNA bands as binary data, where "1" indicates the presence of a band and "0" indicates its absence. This binary matrix is then analyzed to assess genetic diversity and relationships among the cannabis strains. The percentage of polymorphic bands (PMB) is calculated to quantify genetic variation, with higher PMB values indicating greater diversity among the strains. Pairwise genetic similarity between cannabis strains is calculated using similarity coefficients such as Jaccard or Dice coefficients. To further visualize the genetic relationships, cluster analysis is conducted, typically using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), generating a dendrogram or phylogenetic tree. Bootstrap analysis is performed to assess the confidence in clustering, providing support values for each branch of the tree. This approach allows for the grouping of cannabis strains based on their genetic profiles and helps in evaluating the genetic diversity within the population.

Results

The phylogenetic tree in the image separates the samples into four distinct groups based on genetic similarity, which could be described as follows: Group 1: This group contained the largest number of strains, indicated by the highest bootstrap values (e.g., 99.1, 99.7). It included several related samples with a strong genetic connection, indicating high confidence in the grouping. Group 2: Group 2 also contained closely related samples, but with slightly lower bootstrap values compared to Group 1. It contained several subgroups with values like 92.6 and 99.2, indicating strong support for the relationships within this group. Group 3: Group 3 showed a smaller number of strains or samples, with moderate to high bootstrap values such as 95.8 and 91.1. It contained subgroups 3A, 3B, etc., reflecting genetic substructuring. Group 4: Group 4 had two subgroups (4A and 4B) with a strong bootstrap value of 98.9, indicating high confidence in these relationships. This group was genetically distinct from the other groups.

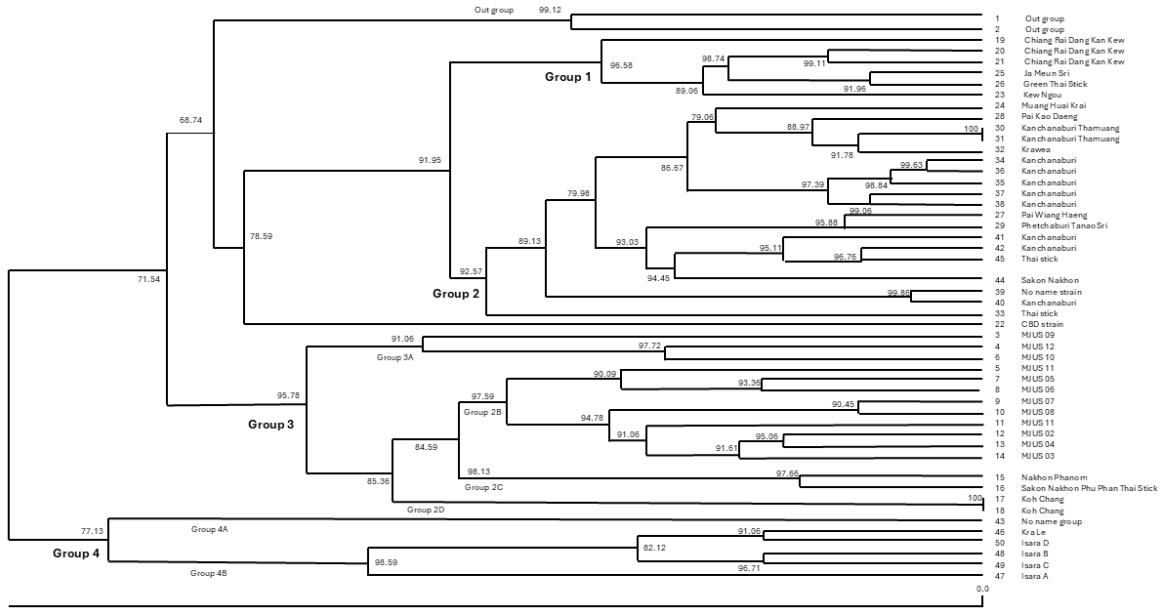


Figure 1. This phylogenetic tree illustrated the evolutionary relationships and genetic distances between various cannabis (*Cannabis sativa*) samples, grouped into four main clusters (Group 1, Group 2, Group 3, and Group 4), with bootstrap values indicating the confidence level for each branch.

The ISSR-PCR analysis using five primers (ISSR10, ISSR6, ISSR2, ISSR7, and ISSR4) demonstrated significant polymorphic amplification across various DNA regions. The annealing temperatures for these primers ranged from 55.0°C to 56.0°C, with the amplified base pair sizes varying from 300 to 1,500 bp. The total number of bands produced ranged from 7 to 14, with the majority being polymorphic. Notably, ISSR10, ISSR2, ISSR7, and ISSR4 yielded 100% polymorphic bands, reflecting their high efficiency in detecting genetic variation, while ISSR6 showed a slightly lower percentage (85.7%) due to the presence of one monomorphic band. Overall, these primers generated a total of 52 bands, 51 of which were polymorphic, leading to an average polymorphism rate of 98.1%. This high percentage highlighted the robustness of ISSR markers in assessing genetic diversity, making them effective tools for molecular

genetic studies. (Table 3).

Table 3. This table presented the base pair sizes, total number of bands, polymorphic bands (PB), monomorphic bands (MB), and the percentage of polymorphic bands (%PB)

Primers	Annealing temperature	Base sizes (bp)	Total bands	PB	MB	%PB
ISSR10	55.0 °C	300-1,500	14	14	0	100
ISSR6	56.0 °C	350-950	7	6	1	85.71
ISSR2	55.5 °C	350-1,500	9	9	0	100
ISSR7	56.0 °C	400-1,250	12	12	0	100
ISSR4	55.5 °C	300-1,500	10	10	0	100
Mean			**52**	**51**	**1**	**98.1%**

Table 4 presented the genetic similarity index among three cannabis strains: Sakon Nakhon Hang Krarok Phu Phan (Group A), Hang Krarok Kiew (Group B), and Hang Krarok (Group C). The genetic similarity between Sakon Nakhon Hang Krarok Phu Phan and Hang Krarok Kiew was 91.1%, while its similarity with Hang Krarok was slightly lower at 88.8%. On the other hand, Hang Krarok Kiew shared a 93.1% genetic similarity with Hang Krarok, making these two the most genetically similar strains. These percentages suggested that although all three strains are closely related, Hang Krarok Kiew and Hang Krarok are more genetically similar to each other than to Sakon Nakhon Hang Krarok Phu Phan.

Table 4. showed the genetic similarity index (similarity percentage) among three cannabis strains: Sakon Nakhon Hang Krarok Phu Phan, Hang Krarok Kiew, and Hang Krarok.

Similarity index (%)	Sakon Nakhon Hang Krarok Phu Phan (Group A)	Hang Krarok Kiew (Group B)	Hang Krarok (Group C)
Sakon Nakhon Hang Krarok Phu Phan (Group A)	100.0	91.1	88.8
Hang Krarok Kiew (Group B)	91.1	100.0	93.1
Hang Krarok (Group C)	88.8	93.1	100.0

Table 5 showed the genetic similarity index among Sakon Nakhon Hang Krarok Phu Phan (Group A), Chao Muen Si (Group B), and Phetchaburi Tanao Si (Group C). Here, Chao Muen Si and Phetchaburi Tanao Si are the most closely related, with a similarity index of 96.9%, whereas Sakon Nakhon Hang Krarok Phu Phan shows a similarity of 91.1% with Phetchaburi Tanao Si and 87.4% with Chao Muen Si, indicating greater genetic diversity between Sakon Nakhon Hang Krarok Phu Phan and the other two strains.

Table 5. presented the genetic similarity index (similarity percentage) among three cannabis strains: Sakon Nakhon Hang Krarok Phu Phan, Chao Muen Si, and Phetchaburi Tanao Si.

Similarity index (%)	Sakon Nakhon Hang Krarok Phu Phan (Group A)	Chao Muen Si (Group B)	Phetchaburi Tanao Si (Group C)
Sakon Nakhon Hang Krarok Phu Phan (Group A)	100.0	87.4	91.1
Chao Muen Si (Group B)	87.4	100.0	96.9
Phetchaburi Tanao Si (Group C)	91.1	96.9	100.0

Discussion

The genetic diversity uncovered in this study through ISSR marker analysis highlighted the rich evolutionary background and practical applications of cannabis (Cannabis sativa) strains native to Thailand. The strains analyzed showed clear genetic clustering into distinct groups, demonstrating their unique evolutionary trajectories and practical applications, particularly for medicinaand recreational uses.

Historically, cannabis has played an important role in Southeast Asia for centuries. The diverse genetic backgrounds of these strains are the result of a combination of natural selection, geographic isolation, and selective breeding by humans. Clarke and Merlin (2016) traced the historical spread of cannabis from Central Asia to Southeast Asia, where local Thai strains adapted to the environmental conditions of the region. These strains, known for their potency and high THC content, became especially popular in the global cannabis market during the 1970s, particularly with the export of "Thai Stick" cannabis.

The strain Chiang Rai Dang Kan Kew, appearing in the genetic analysis, is known for its resilience and adaptability to northern climates, making it valuable for cultivation in similar regions. The strain is also prized for its uplifting effects due to its high THC content, which makes it popular for both recreational and medicinal use, particularly in treating fatigue and depression. Other similar studies, such as those by Merlin (2003), confirmed the adaptability of cannabis to various climates and emphasized how selective breeding has shaped strains like Chiang Rai Dang Kan Kew to thrive in local conditions.

Similarly, Sakon Nakhon Hang Krarok Phu Phan and Hang Krarok Kiew, which showed a high genetic similarity index of 91.07%, were indicative of local breeding efforts in northeastern Thailand, where farmers selected for specific traits, such as robust growth and high resin production. These strains are particularly well-suited for producing cannabis extracts used in medicinal applications, especially in treating chronic pain, nausea, and insomnia. Research by Sawler et al. (2015) supported these findings, indicating that cannabis strains within the same region often share genetic similarities due to selective breeding for specific therapeutic compounds like cannabinoids and terpenes.

The strains appearing in this study are not only valuable for their genetic diversity but also for their potential benefits in modern medicine. For instance,

Hang Krarok, a strain with traditional roots in Thai agriculture, has shown potential in medical research for its strong analgesic and anti-inflammatory properties. It is currently being investigated for its effectiveness in treating a range of conditions, including arthritis and neurological disorders. The high polymorphism seen in this study aligned with global trends in cannabis research, where genetically diverse strains are sought for the development of new therapeutics

In addition to medicinal uses, the high diversity and distinct groups identified through this study could inform future breeding programs aimed at optimizing cannabis strains for various industrial purposes, such as the production of hemp fiber or high-CBD strains for non-psychoactive medical treatments. Clarke and Merlin (2016) have noted that maintaining genetic diversity within cannabis populations is crucial for preserving its adaptability and usefulness across different applications.

In summary, the study underscored the significant genetic diversity and practical utility of Thai cannabis strains, which have both historical significance and contemporary relevance. As cannabis becomes increasingly recognized for its medicinal benefits worldwide, maintaining and further studying these distinct strains will be crucial for future advancements in cannabis research and industry. The findings of this study aligned with international research on cannabis evolution and its applications, showcasing the importance of preserving local strains for their unique properties and potential benefits.

Conclusion

This study revealed significant genetic diversity among local Thai cannabis strains using ISSR molecular markers. The clustering into four major groups highlights the unique evolutionary adaptations and potential applications of these strains. The high polymorphism rate (98.1%) confirmed ISSR as an effective tool for genetic fingerprinting and conservation. These findings provided a strong foundation for breeding programs aimed at medical, industrial, and agricultural applications. Preserving and studying these strains will ensure their continued value in future research, sustainable agriculture, and pharmaceutical development.

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