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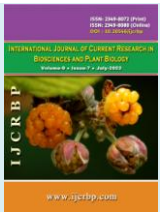

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Thai *Hemp* (*Cannabis sativa* L.) Group: The preliminary genetic diversity of native Thai Cannabis *Isara*01 strain categorized by RAPD marker

R. Kraisittipanit[1, 2], S. Nontati[2] and A. Tancho[2*]

[1]*Program of Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand*
[2]*Natural Farming Research and Development Center, Maejo University, Chiang Mai, Thailand*
[3]*Soil Science Program, Faculty of Agricultural Production, Maejo University, Thailand*

*Corresponding author; e-mail: arnat009@yahoo.com

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Abstract

Background: This study is the first research report on Thai cannabis which is named "*Isara01*". This plant was also known as *KanCha-Isara01* strain. After pushing for cannabis research under the control of the Thai government, our research organization (the Natural Farming Research and Development Center, MaeJo University, Thailand) was therefore instructed to arrange a sample group and study the genetic diversity of 16,000 *Isara01* specimens to be promoted and used in medicine. In this study, RAPD techniques were used to categorize all cannabis for a fundamental data to further work in the future, especially new strain development. Method: We developed the RAPD genetic marker technique for categorization the 133 Thai cannabis strains (*Isara 01*) which are supported by Thai government using five RAPD primers creating fingerprinting and analyzed the genetic relationship by comparison with the amount of phytochemicals such as cannabinoids. Results: 133 Thai cannabis "*Isara01*" were categorized into 4 large groups and 9 sub-groups**.** The RAPD fingerprinting showed a total banding of fingerprint in 35 bands and its devision into 19 polymorphic bands **(**54**.**29**%).** The average of banding was calculated in 7 bands per primer**.** The UPGMA yielded the similarity index as 0**.**9553-0.9923. The comparison ratio between THC/CBD suggested that the highest THC content in cannabis inflorescence in Group D is (37:1).Conclusions: We are completely categorized the Thai cannabis **"***Isara01***"** and it showed intra-genetic diversity and high THC especially group D. Hence, it is suitable for study as a primary model for the development of a new species in Thailand.

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Introduction

Cannabis ***(****Cannabis* spp.***)*** is one of the financial and commercial plant in Thailand, it has been classified in the family of Cannabaceae with various common namese.g. Hemp or Marijuana. More than 170 strains were found ***(***Anna and Mitchell, 2019***)*** any reports suggested that they were originated from natural sources and distributed across distinctive climates including South America, Middle East and Asia **.**It has been used

for various benefits and applications for foods or relaxation a long time ago (Hakki et al., 2007). In Thailand, this plant was also known as *KanCha* which has been rarely studied many findings have shown that it contains more than 483 types of bio-chemical compounds. Cannababinoid, is one of the most phytochemical importance for medical use, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Hilig and Mahlberg, 2004; Pacifico et al., 2006; Allen et al., ***2019***; Ahmed and Hijri, 2021; Tahir et al., 2021).In medicine, it was confirmed that more than 65 types of the plant are used for medical purpose.

For medical work. In addition, THC, has been used in various status; for instance, it has been adapted for the psychoactive drug for decreasing hyperactivities, Anorexia, Insomnia and Myoclonic. Meanwhile, CBD has been used for relaxation and elimination of the mental disorder, Anxiety, Migraine, Myositis, Glaucoma, Epilepsy, Parkinson's disease and Alzheimer (Forapani et al., 2001). However, this plant is under control of Thai government due to the illegal activity and misuse of related product; therefore, only the work of research purpose is allowed. Further, the Natural Farming Research and Development Center, MaeJo University, Thailand, is the first organization inthe north of Thailand that is supported by Thai government for the research purpose of Thai Cannabis plantation and local strains.

A wide variety of techniques have been used to identify and classify the categories of cannabis species and strains such as HPLC of GC. Nevertheless, this technique problems on both thermal and phytochemical volatiles degradation, For GC technique, although this technique is more stable when compared to HPLC with the application of thermal method, the cannabinoid extract from matrix may be contaminated by many solvents and rapidly oxidized by oxygen. Moreover, the samples used for identification must be fresh. Hence, the molecular genetic marker, is an advanced tool that is used for stable and rapid examination. Such molecular genetic marker that has been used for determination of many plants such as the use of Random Amplification Polymorphic DNA) RAPD technique (William et al., 1990) which is particularly powerful technique for analyzing the genetic variation and grouping crops in a short manner (Mina et al., 2018).

However, the research on Thai cannabis is still new to the research field in Thailand. Consequently, the Natural Farming Research and Development Center, Maejo University, which is the main cannabis research center in Northern Thailand that has been supported by the Thai government. All cannabis strains from all over the country have been studied, the most known is "*Isara01*" (Fig. 1) whereas more than 16,000 have been planted in this research area. Therefore, to confirm the genetic variation and grouping, we are going to investigate Thai cannabis "*Isara01* "by using RAPD marker and confirm some of their characteristics for the future work.

Materials and methods

DNA extraction

Isara01 seeds from Thai government were planted in an indoor and an outdoor greenhouse. In addition, 133 fresh cannabis leaves were randomly cut from different sites from the Natural Farming Research and Development Center, Maejo University, Chiang Mai. Samples were cleaned the process of genomic DNA extraction by using Pure-linked genomic DNA kit.

Subsequently, genomic DNA (gDNA) was electrophoresed under agarose gel1.0% (w/v) at 220 voltages for 30 mins for quality test. The quality and quantity of gDNA were confirmed again by spectronanometer (260/280 nm) ratios.gDNA was kept at -20 ^{o}C and it was diluted to 10 ng/µl for downstream experiments.

RAPD fingerprinting

One hundred randomized RAPD primers were used for our preliminary screening (data not shown). Only 5 selected primers were considered as suitable for DNA fingerprinting (OPA03, OPA04, OPA13, OPC02 andOPD02) (Table 1). PCR master mix was applied by using 50 µl per reaction, 2 µl of gDNA (20 ng), 1 µlfor primer (10 pmol⁄ul), 25 µl of one PCR master mix (Biohelix), and 22 µl of molecular grade water.

The PCR protocol was set up into 2 main steps first, 5 cycles 94 ^{o}C denaturation 5 min, 50^{o}C annealing 1 min , 72 ^{o}C extension 1 min, followed by 35 cycles 94 ^{o}C denaturation 5 min, 50^{o}C annealing 1 min ,72 ^{o}C extension 10 min. PCR product (5 µl) was mixed with 1 µl of color detector (Ulrapower, Japan) and was studied using electrophoresis with agarose gel 1.8%w⁄vat 220 voltages for 70 mins. Finally, the bands of PCR were

detected by LED light and photographed by a digital camera before analyzing.

Thai cannabis Isara01 Genetic Relationship

Total cannabis band were parallely analysed based on an occurrence of the band on gel electrophoresis**.** Similar or different DNA banding position was scored as 1 or 0, respectively**.** Similarity coefficiency was then determined using Phylip software ***(***model 3**.**69***)***. The samples were clustered by an Unweighted Pair-Group Method, Arithmetic average ***(*** UPGMA***).*** To obtain a phylogenetic tree, all dataset was calculated using 1,000 bootstrap replicates by Seqboot**.** Then, the tree was constructed and visualised using TreeView software ***(***model X***).***

Delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) measurement

Leave and flower cannabis were kelp and washed by sterile water before powder. 1g 10ml of 95**%** ethanol added into cannabis powder before sonicating using Ultrasonic Cleaner ***(***TUC-70/TUC-100, Jeken***).*** Then, solutions were filtrated and analyzed by High Performance Liquid Chromatograph ***(***HPLC***) (***Shimadzu SCL-10A vp***).***

Results and Discussion

Five primers from one hundred RAPD primers were selected from preliminary. The result showed the highest band in primer OPA 13 ***(***8 bands***)*** and another showed in 6, 7, 7, 7 DNA bands in OPA03, OPA04, OPC02 and OPD02, respectively**.** The polymorphic percentage was calculated as 54**.**29**%** while DNA fragment size between 350-1,500 bp. The smallest scorable bands were around 0.3 kb ***(***Table 2***)***. Fig. 2 shows the OPA 03 fingerprint between 133 *Isara 01* hemp cultivation from outdoor.

When all DNA banding was analyzed from fingerprint, it showed similarity coefficient between 0**.**9553-0.9923, was generated into 4 main groups; as for example, 4 main groups; as for example, group A-D with 9 subgroups ***(***A, B1, B2, B3, B4, C1, C2, D1, D2***)(***Fig. 3***)***. Additionally, the main group related to THC and CBD ratio showed the highest THC: CBD ratio in Group D ***(***sub group D1 and D2***)*** at 37:1 ***(***Table 3***).*** This is the first study on Thai hemp ***(****Cannabis sativa****)*** that emphasizes on genetic variation, which deals with the genetic diversity throughout the process of RAPD marker. Only 20 ng DNA concentration ***(***260/280nm***)*** between 1.68-1.94could reproduce all genetic materials at 50 celsius degree annealing temperature in five primers ***(***OPA 03, OPA 04, OPA 13, OPC 02 and OPD 02***)***.

In this study, it revealed that the RAPD genetic marker is a powerful tool for cannabis grouping. In addition, we distinguished 133 Thai *Isara01* cannabis from five RAPD primers fingerprinting. The dendrogram analysis that used UPGMA suggested that the *Isara01* cannabis can be categorized into four main groups, which are also reflected in the values of Nei's genetic distance from Phylip program ranked from 0**.**955 - 0.992 and bootstrap between 86.5 – 100%. However, this data from either morphology or DNA fingerprint not noticeably indicated a non–significant matter between four main clusters exclude some leaves and THC: CBD ratio.

It also showed the clear difference of THC: CBD ratios between four main groups ***(***Group A = 5:1, Group B including B1-B4 = 10: 1, Group C including sub groups C1-C4 = 9: 1, and Group D including sub groups D1-D4 = 37: 1***)***, respectively. However, there was only a slight difference in group C. In addition, the petiole color in this group appeared differently within group, it exhibited red petiole in 1.1 cwith absoluteness, and showed high bootstrap score as 100%. Furthermore, the UPGMA showed the similarity index as 91.6 in sub group C 1.1 and sub group C 1.2. However, it was subsequently to investigate the cannabinoid estimation which showed THC and CBD ratios between four groups with distinctive characteristics except for group B and group C. It was discovered that a slight difference in blade morphology was expressed in group B as for the large blade and five leaf loops, whereas group C has two majoring sub groups that are red petiole sub group and green petiole. Start this sentence with this: The red petiole in group C consisted of seven leaf loops, whereas the green petiole contained five leaf loops, both of which were reflected in the bootstrap values of 100% values from the computation of Nei's standard genetic distance in Phylip program.

Table 1. Selected RAPD primers sequence used for cannabis fingerprinting.

Selected primers	Sequence primer
OPA 03	5′-AGT CAG CCA C-3′
OPA 04	5′-AAT CGG GCT G-3′
OPA 13	5′-CAG CAC CCA C-3′
OPC 02	5′-GTG AGG CGT C-3′
OPD 02	5′-GGA CCC AAC C-3′

Table 2. The average bands of monomorphic bands and polymorphic bands from five primers.

Selected primers	Number of monomorphic bands	Number of polymorphic bands	Total number of DNA bands	DNA fragment (bp)
OPA 03	3 bands	3 bands	6 bands	380-1,200
OPA 04	3 bands	4 bands	7 bands	600-1,500
OPA 13	4 bands	4 bands	8 bands	400-1,500
OPC 02	4 bands	3 bands	7 bands	300-1,250
OPD 02	2 bands	5 bands	7 bands	350-1,500
	total16 bands	total19 bands	35 bands	
average	3.2 band/primer	3.8 band/primer	7.0band/primer	
polymorphic percentage			54.29 %	

Table 3. HPLC Phytochemical content of THC, CBD and THC: CBD ratio from fresh inflorescence cannabis.

Phytochemical test	group A	group B	group C	group D
THC content (% w/v)	1.036	0.921	0.789	0.901
CBD content (% w/v)	0.219	0.091	0.084	0.026
THC:CBD	5:1	10:1	9:1	37:1

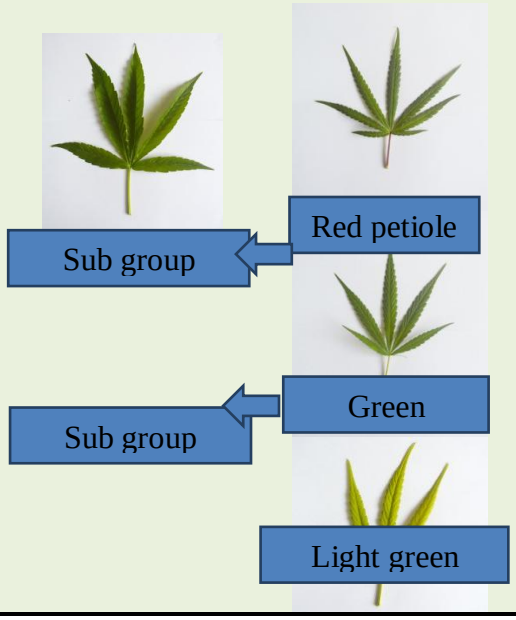

Fig. 1: *Isara01* cannabis (A-F), more than 16,000 cannabis plants manufacturing facility at the Natural Farming Research and Development Center, Maejo University (G-H).

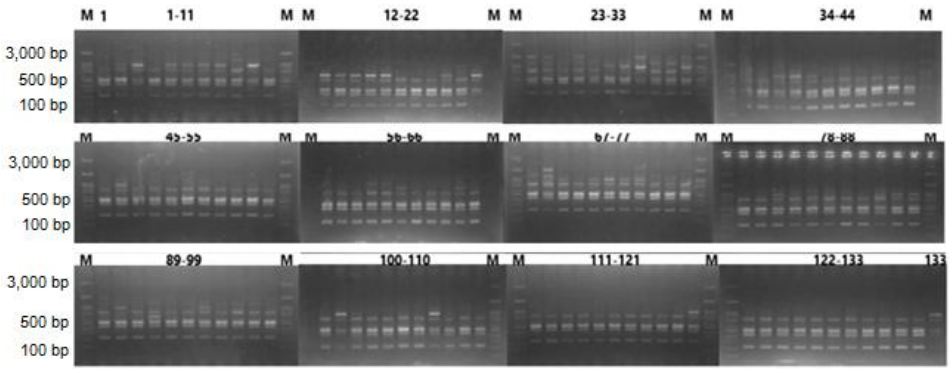

Fig. 2: Amplification products generated by OPA 03 RAPD primer of 133 outdoor *Isara01* cultivars from Natural Farming Research and Development Center, MaeJo University. (M = 100 bp).

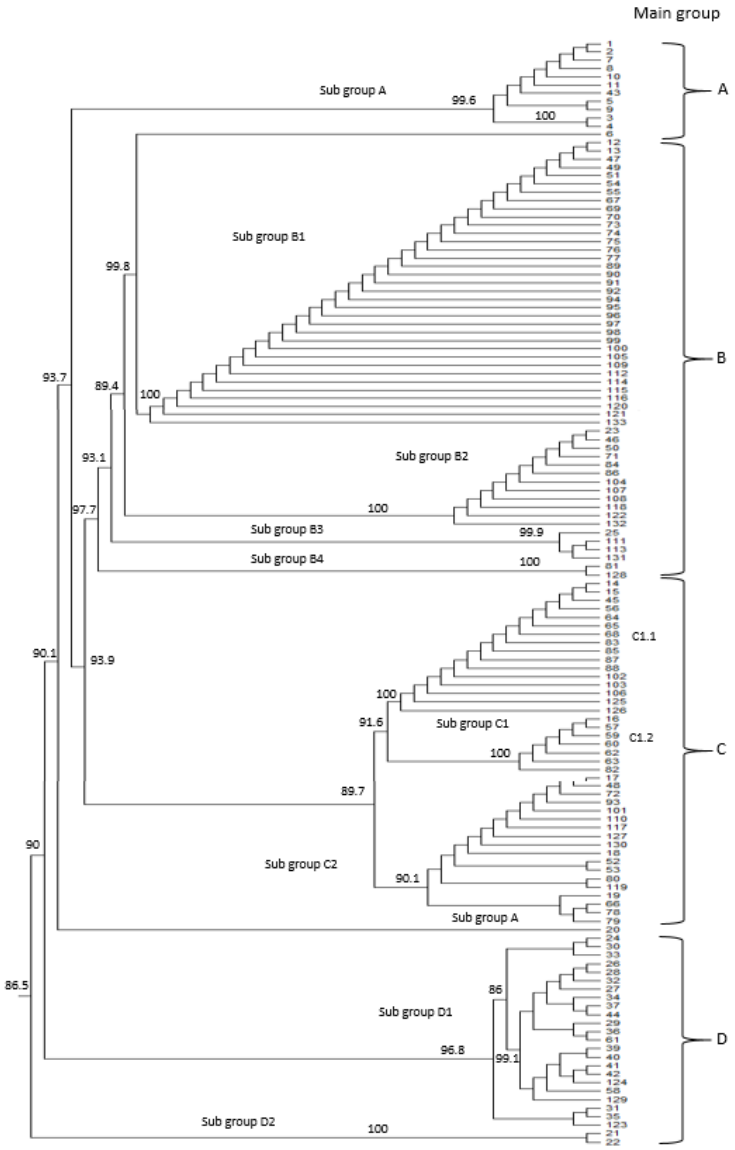

Fig. 3: Dendrogram of 133 Thai cannabis (*Isara01*) by UPGMA was analyzed throughout RAPD genetic primers (i.e., OPA 03, OPC 02, and OPA 04). The pattern showed all four main groups (A-D) and nine subgroups (A, B1, B2, B3, B4, C1, C2, D1, D2). The similarity index was between 0.95 - 0.99.

This hypothesis determined sub group C1.1 and C1.2 which were categorized by gene expression on petiole color, and clearly identified grouping by using five RAPD primers. However, sub group C2 indicated clear leaf color morphology when compared to other sub groups within group C, it was light green color over the blade. However, our study aims to explore the diversity diversity of the group of Thai *Isara01* for further research. Some previous research had supported our study, for example, the study from Italy by Faeti et al., 1996 who studied *C. Sativa* by using RAPD marker to generate 13 cultivars *C. sativa*, and concluded that RAPD was a powerful tool. As a result, the *C. sativa* was generated from three different gene pools ***(***Italy, Hungary, Europe***)***, and RAPD could be identified and categorized into 2 main groups of Italy *C. sativa* strain *(*Carmagnola and Fibranova*)*. Moreover, the study from *(*Forapani et al., *2001)* reported that RAPD marker has high efficiency to distinguish 6 hemp strains by using only 5 primers. This is the fact and it confirms that RAPD has enough efficiency to detect and classify *C. sativa* in strain level. Moreover, having more reports supported our study such as Meijer et al., *(1992)*; Meijer and van Soest *(1992)* and Jagadish et al., *(1996)* who reported that hemp group could be generated from cannabinoid content, fingerprinting analysis using only RAPD marker and geographic difference. This is similar to our study which showed clear classification of Thai *C. sativa Isara* 01 by using RAPD primer and THC: CBD content ratio when matching data together from majoring group A and D. Interestingly, In United Kingdom, the study of the application of RAPD molecular marker was done by grouping *C. sativa* by five primers, sixteen hemps cannabinoid was clearly detected by HPLC and categorized into three main groups. It is found that three primers out of all could be used to differentiate between *C. sativa* with sizes 270 and 280 bp. Hence, it was noted that the data on HPLC content was insufficient for cannabis grouping ***(***Gillan et al., 1995***)***.

Nevertheless, this study is the most recent Thai cannabis research from laboratory. It can be used to support research technique and data collection in the future. Further, some studies demonstrated that some molecular genetic markers were used to perform and generated *C. Sativa* and they could identify species in various methods such as ISSR ***(***Inter-Simple Sequence Repeat ***(***Mareshige et al., 2001***)***, and multiplex PCR ***(***Somkhid et al., 2011***)***. They could be successfully utilized to detect and categorize drug-type and fiber-type *C. Sativa* by the process of *rcbL* and THCA synthase gene marker and the strains development.

Consequently, we have achieved our objectives to group Thai Cannabis *Isara* 01 by using RAPD molecular marker, finally, we can conclude that this marker is accurate enough for Thai hemp strain classification. However, our research organization has been studying the application of modern genetic marker and new PCR or gene targeting barcode identification for new hemp strains, and for high selected cannabis screen and high accuracy. The result showed that Thai cannabis (*Isara01*) has genetic variation both inter and intra strains. The RAPD genetic marker could also be categorized into 4 main groups by the exhibition of some characters of morphology and THC/CBD ratio. The polymorphic percentage in specify the type of the solution was estimated as 54.29%. The similarity coefficient was estimated between 0.9553-0.9923. Remarkably, group D was estimated to have the highest THC: CBD ratio in the leaf. Therefore, our research study confirms that Thai cannabis *Isara01* strain expresses genetic diversity and some characteristics which are suitable for the improvement of the new strains within the cultivation of our research program at the manufacturing facility.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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