

DNA Fingerprinting of *Cannabis sativa* Using Inter-Simple Sequence Repeat (ISSR) Amplification

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Abstract

Chemical analysis of cannabinoid, and Inter-Simple Sequence Repeat (ISSR) fingerprinting of DNA were used to identify different samples of *Cannabis sativa* L. for forensic purposes. Three samples were classified into two types, tetrahydrocannabinol (THC) and cannabidiol (CBD) chemo-types, by high performance liquid chromatography (HPLC). The two samples of the CBD type

were not distinguished by their HPLC patterns. ISSR fingerprinting identified polymorphic DNA patterns between these samples. ISSR fingerprinting clearly differentiated between cannabis samples that could not be achieved by HPLC analysis.

Key words

Cannabis sativa · hemp · Cannabaceae · DNA profiling · ISSR · cannabinoid · HPLC

Introduction

Hemp (*Cannabis sativa* L.) is one of the most widely occurring plants. It has been cultivated legitimately around the world for its fiber and seed oil for thousands of years. In a somewhat less reputable manner, the dried flowering tops and leaves have been used as a narcotic, as in marijuana and hashish. *C. sativa* is a markedly variable species in terms of morphologic, geographic and chemical features. Quimby et al. [1] reported morphological variations in Mississippi *C. sativa*. Several investigators have analyzed tetrahydrocannabinol (THC) and cannabidiol (CBD), and identified several different chemical phenotypes of *Cannabis* [2–6].

In many countries, including Japan, possession and cultivation of *C. sativa* is strictly controlled by law. Hence, correct identification of drug material from seized samples is important. Conventional methods for identifying *Cannabis* include chemical coloration, thin-layer chromatography (TLC), gas chromatography-mass spectroscopy (GC-MS), high-performance liquid chromatogra-

phy (HPLC) and histological microscopy. However, a poor quality sample or an inadequate amount of material occasionally renders identification impossible.

Several molecular techniques have been evaluated for their ability to establish genetic relationships among different plants. Some recent studies have classified *C. sativa* by genomic DNA, using random amplified polymorphic DNA (RAPD) or restriction fragment length polymorphisms (RFLP) analyses [7–10]. PCR-RFLP profiles of chloroplast *trnL* intron are also used as a tool in *Cannabis* identification [11]. To distinguish *Cannabis* from other plant species, some nucleotide sequences have been identified. Gigliano et al. [12–15] characterized the sequence of the Internal Transcribed Spacer I and II (ITS1, 2), and Miyahara et al. [16] reported that the sequence of the 5S-rRNA gene spacer region differs from that of other plant species. Recently, we identified a difference in the sequence of the chloroplast inter-genic spacer region among several samples of *C. sativa* [17]. However, sequence analysis is laborious and requires expensive facilities.

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Microsatellites, or simple sequence repeats (SSRs) are short (1 – 5 bp long) tandemly repeated DNA sequences comprised of di-, tri-, tetra-, or penta-nucleotide motifs. SSRs are abundant in eucaryotic genomes. Using a sequence tagged microsatellite or SSR marker is known to be a powerful technique for genetic analysis. However, establishing a microsatellite marker is not simple or quick work. Recently, inter-simple sequence repeat (ISSR) fingerprinting was developed to fingerprint genetic polymorphism [18]. Anchored microsatellite primers were capable of amplifying regions between SSRs. ISSR primers anneal directly to SSRs and therefore no prior sequence knowledge is needed. In the present study, we evaluated the effectiveness of ISSR fingerprinting for identifying different samples of *C. sativa*; samples that HPLC had failed to distinguish between by their cannabinoid content.

Materials and Methods

Plant samples

Three strains of *C. sativa* (#001, #006 and #044) from different sources were used for ISSR fingerprinting and cannabinoid analysis (Table 1). These three samples were prepared from three different samples (#001-a, #001-b and #001-c, respectively). The accession number corresponds to the number used at Izu Experimental Station for Medicinal Plants, National Institute of Health Sciences, Japan. All plant samples were grown in an experimental incubation room to avoid the effect of environmental differences (e.g., temperature, light, and soil) on plant growth and cannabinoid content. Plants were potted with washed and autoclaved sand. Water and fertilizer solution were automatically supplied in fixed amounts. The room was maintained at 25 °C and under constant fluorescent light of approximately 10,000 lux.

DNA extraction

DNA was extracted from fresh 40-day-old plant leaves (150 mg) using the modified cetyltrimethylammonium bromide (CTAB) method of Kohjiyouma et al. [17]. Purified DNA was adjusted to a final concentration of 0.5 ng/μl with sterile water.

SSR primers

For the initial screening, we examined 81 SSR primers of the “set 9” obtained from the Biotechnology Laboratory of the University of British Columbia, Vancouver, Canada. Then we used four pri-

mers, No. 808 (AG)₈C, 811 (GA)₈C, 827 (AC)₈G and 834 (AG)₈YT, (Y: pyrimidine).

PCR amplification

PCR amplification was performed in a total volume of 10 μl. Each reaction was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001 % gelatin (Sigma, Milwaukee, USA), 2.5 mM MgCl₂, 200 μM dATP (PE Biosystems, Norwalk, USA), 200 μM dGTP, 200 μM dCTP, 200 μM dTTP, 0.2 μM primer, 0.25 units AmpliTaq Gold DNA polymerase (PE biosystems), and 1 ng template DNA. Amplification was carried out in a Takara PCR Thermal Cycle MP (TAKARA, Kyoto, Japan) programmed for a first hot start step of 10 minutes at 95 °C, and one cycle each of 1 minute at 94 °C for denaturation, 1 minute at 65 °C for annealing, 2 minutes at 72 °C for extension, followed by 9 cycles with annealing temperature lowered by 1 °C to 56 °C (65 °C→56 °C), then 30 cycles with annealing temperature at 55 °C, and a final extension of 5 minutes at 72 °C.

Electrophoresis and detection of PCR products

The amplified products were electrophoresed on 2 % agarose gels at 50 V for 3 h with Tris-acetate-EDTA (TAE) buffer (pH 8.0). After staining with ethidium bromide, the banding patterns were detected under UV light (254 nm).

Genetic analysis

The band profiles were image analyzed with Kodak Digital Science 1D Image Analysis Software (EASTMAN KODAK, New York, USA), and scored for presence (= 1) or absence (= 0) to calculate simple matching coefficients [19] between each pair of samples for ISSR. Dendrograms based on a dissimilarity index were generated using the unweighted pair-group method with arithmetic averages (UPGMA) [20].

Cannabinoid analysis using HPLC

Quantitative analysis of cannabinoid in the leaves was performed by HPLC. Leaf samples were harvested from the tops of 40-day-old plants and then dried at 50 °C for 12 h with a warm-air blow-dryer. Dried samples were ground to a fine powder. Each powdered sample (50 mg) was extracted with ethanol at room temperature for two hours. The extract was centrifuged at 7,000 g for 5 min. The supernatant was filtered through a membrane filter (0.45 μm, ADVANTEC, Tokyo, Japan). The extracts were then analyzed by an HPLC system composed of a model LC-9A (Shimadzu, Kyoto, Japan), SPD-6 AV spectrophotometric detector, C-R5A chromatopac, CTO-6A column oven and SCL-6B system controller. Waters Symmetry C18 (3.5 μm, φ 4.6 × 100 mm, Waters) was used for a column whose temperature was maintained at 30 °C. Solvent system, MeOH-H₂O (85 : 15, v/v), was used a flow rate of 0.6 ml/min. The effluent was monitored by absorption at 210 nm.

Results and Discussion

Comparison of HPLC analysis of the three samples

THC and CBD contents in the leaves of samples #001, #066 and #044 were analyzed. Chromatograms of HPLC are shown in Fig. 1. The three individual samples of #001 contained mainly THC (1.41 – 1.66%), whereas CBD was present in only very small amounts (0.11 – 0.23%). Both the #066 and #044 samples con-

Table 1 Plant material

Experimental Code	Accession Number ¹	Source
#001-a	0122-98-200	Orechova, Czech Republic
#001-b		
#001-c		
#044-a	0041-98-050	Tochigi, Japan
#044-b		
#044-c		
#066-a	0185-98-200	Marseille, France
#066-b		
#066-c		

¹ Genetic resource numbers from databases of the National Institute of Health Sciences, Japan.

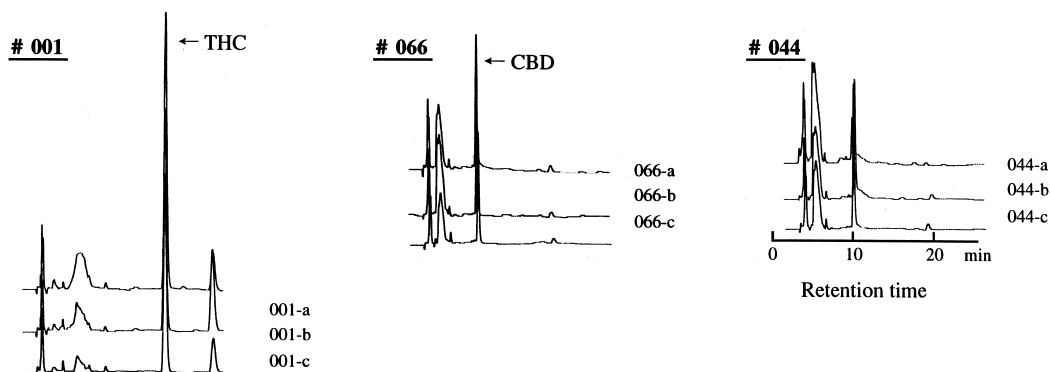


Fig. 1 HPLC profiles of three samples of *Cannabis sativa*.

tained CBD as a major cannabinoid. In samples #066, the average CBD content was 0.92% (0.53–1.49%), while that of THC was 0.10% (0.05–0.18%). Similarly, the average CBD content in samples #044 was 0.67% (0.34–0.94%), with that of THC was 0.12% (0.08–0.19%). From these results, #001 was classified as the THC chemo-type, and #066 and #044 as the CBD chemo-type. However, HPLC chromatograph patterns of #066 and #044 were very similar. We were unable to identify any difference between these two samples based on the HPLC profiles. Thus, we attempted to identify these two samples by ISSR analysis.

ISSR analysis of the three samples

From the initial screening of 81 SSR primers, four SSR primers (No. 808, 811, 827 and 834) produced clear and reproducible bands and were thus used to identify the three different samples. We obtained eight clear bands from primer No. 808, four from primer No. 811, four from No. 827, and nine from No. 834. Fig. 2 shows the ISSR profiles using primer No. 811. Of the 25 fragments produced, 22 were polymorphic. A dendrogram of the three samples (i.e., nine samples; each strain was composed of three individual samples) identified three major groups. The genetic dissimilarity index for #001 and #066 was estimated to be 0.33, and for #044 and a group of #001 and #066 was 0.60. Three individual DNA samples of “Czech Republic”, #001-a, 001-b and 001-c, constituted one group. Likewise, three of “France”, #066a, 066-b and 066-c, and three of “Japan”, #044-a, 044-b and 044-c, constituted one group each. Each strain of *C. sativa* could therefore be classified as a unique genotype based on ISSR fingerprinting, although from different individual DNA samples. Especially in comparing between #044 samples and #066 samples, we could not distinguish them by the HPLC analysis. However, we were able to classify the two samples by the ISSR finger-

printing. Thus, ISSR fingerprinting proved to be a suitable method for estimating the genetic difference among several samples of *C. sativa*.

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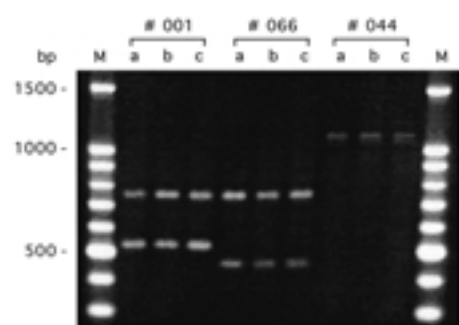


Fig. 2 ISSR fingerprinting of three samples of *Cannabis sativa* using primer #811 (GA)₈ C. M, 100 bp ladder molecular markers (bp).

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