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Random Amplified Polymorphic DNA and Restriction Fragment Length Polymorphism analyses of *Cannabis sativa*

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Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) methods were used for the analysis of three different strains of *Cannabis sativa* that included the Mexican (drug type), Tochigishiro (fiber type) and Nara cultivated strains. *Cannabis sativa* samples were grouped into three chemotypes, drug type, fiber type and intermediate drug type by GC analysis. The RAPD analysis was able to distinguish the three chemotypes clearly, however, in each type, some genetic diversities was shown. The RFLP analysis showed that the genetic distance between the Tochigishiro and Nara cultivated strains was small, whereas that between the Mexican strain and the other two strains was large, and that there was no genetic diversity within each strain. These results indicated that the RAPD and RFLP analyses, using DNA as a marker, applied in combination might be useful for distinguishing between the strains having chemotypes.

Keywords: *Cannabis sativa*; RAPD analysis; RFLP analysis; GC analysis; THC; CBD

Cannabis sativa is a plant widely distributed throughout the world, mostly in the temperate and tropical zones.[1] These are probably derived from the same original plant cultivated some 5,000 – 10,000 years ago in Central Asia. Today, it is considered to be a single "unstabilized species" with over 100 varieties or races produced by the genetic plasticity, environmental influences, and human manipulations.[1] Originally, the plant was cultivated for the oil contained in its seeds, fiber from stems and for obtaining cannabinol-containing drug materials such as marijuana (marihuana) from its flowering tops. Marijuana, a green, brown, or gray mixture of dried and shredded flowers and leaves of *C. sativa*, is the most widely distributed illegal drug in the world. All the drugs from this plant, such as sinsemilla (from buds and flowering tops of female plants), hashish ("hash" for short; sticky resin from female plant flowers) and hash oil (tar-like liquid distilled from hashish), are mind-altering or psychoactive drugs.[1] The plant contain about 420 constituents including over 60 naturally occurring cannabinoids, of which Δ^9-tetrahydrocannabinol (THC)[2] is the main and the most active chemical constituent that affects the brain.[1] Because of its psychotic symptoms, acute anxiety and other serious adverse effects, the hemp plant *Cannabis sativa* is under the strict government control in many countries. And accordingly, it is often needed to identify its origin. Various efforts have been made for characterizing and distinguishing *C. sativa* strains by using morphological, chemical or biochemical methods.

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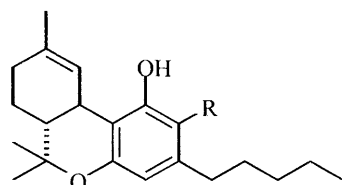
For example, the plant has been grouped into several chemotypes by the use of gas chromatography (GC) and high performance liquid chromatography (HPLC), the "drug type" that mainly producing THC (or Δ^9-tetrahydrocannabinolic acid; THCA),[2] the "fiber type" containing low concentrations of THC and high concentrations of cannabidiol (CBD) (or cannabidiolic acid; CBDA)[2] and "intermediate chemotypes" producing comparable concentrations of these two. However, the chemotyping is not always reliable for drug profiling, because the amounts of cannabinoids are somewhat dependent on the growth conditions and stages, and when chromatographic methods are used, one needs to consider the unstability of the cannabinoids.[1]

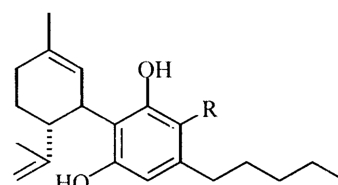
Recently, various DNA profiling techniques have been reported to be very effective in assessing the genetic relationships between species, varieties and even individuals. There are two major techniques; Random Amplified Polymorphic DNA (RAPD),[3, 4] which analyses the variations in the number and size of the fragments amplified by Polymerase Chain Reaction (PCR) using random primers, and Restriction Fragment Length Polymorphism (RFLP),[5] which analyses the variations in the number and size of the fragments produced by digestion of DNA with restriction endonucleases, and hybridized with unique probes. These two methods have significant advantages over morphological, chemical or biochemical methods as it directly assesses the genotype rather than the phenotype and the genotype is not easily affected by growth conditions or stages. The methods are applicable for any type of DNA and DNA prepared from any parts of the sample, and do not need a large amount of sample tissue.

Recently, Gillan *et al.* reported that the RAPD analysis enabled differentiation between *C. sativa* samples that cannot be differentiated by HPLC analysis[6] and Jagadish *et al.* reported that it consistently distinguishes between *C. sativa* samples from distinct sources.[7] However, they did not refer to the cannabinoid contents of each sample.

In the present paper, we describe the preliminary applications of RAPD and RFLP analysis for the DNA extracted from three different chemotype strains of *Cannabis sativa*; Mexican (drug type), Tochigishiro (fiber type), and Nara cultivated strains.



R = H : Δ^9-tetrahydrocannabinol (THC)
R = COOH : Δ^9-tetrahydrocannabinolic acid (THCA)



R = H : cannabidiol (CBD)
R = COOH : cannabidiolic acid (CBDA)

Fig. 1. Structure of Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and their precursor forms, THCA and CBDA.[2]

MATERIALS AND METHODS

Plant Materials The seeds of the Mexican strain were kind gifts from Prof. Syoyama (Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan), those of the Tochigishiro strain, which is used for industrial fiber production, was from the Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences (Tsukuba, Ibaraki, Japan), and those of the Nara cultivated strain, which was cultivated in Japan in the past for fiber production, was from the Kinki Regional Narcotics Control Office. The seeds were cultured on 1/2 Murashige and Skoog (MS) solid medium and the fresh young leaves of one- to two-month old *C. sativa* plant of each type were used for the preparation of samples for genomic DNA, and also for GC analysis.

Preparation of genomic DNA Genomic DNA (*ca* 0.1 – 1 mg) for the RAPD and RFLP analyses was

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prepared as follows. Fresh young leaves (*ca* 100 – 500 mg) of each *C. sativa* were immediately frozen in liquid nitrogen, ground to a fine powder and mixed with 10 ml of Dellaporta's alkaline extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol; pH 8.0).[8] Then 1 ml of 20 % SDS solution was added to the mixture, and the mixture was incubated at 65 °C for 10 min. After addition of 2 ml of 5 M potassium acetate, the mixture was kept on ice for 20 min and centrifuged at 7500 rpm for 15 min at 4 °C. To the supernatant 8 ml of isopropanol was added, and gently mixed. After one hour or more of incubation at –20 °C or –80 °C, the mixture was centrifuged and DNA was collected as a pellet. The co-precipitated RNA was hydrolyzed by treating with 1 μl of RNase A (10 mg/ml). The DNA was further purified by means of phenol/chloroform extraction and precipitation with 0.6 volume of 20 % polyethylene glycol (PEG)/2.5 M NaCl. The DNA was collected by centrifugation, washed with 70 % EtOH and then briefly dried. The genomic DNA thus prepared was dissolved in 100 μl distilled water, and used for the RAPD and RFLP analyses.

DNA fragment amplification by PCR for RAPD analysis Each genomic DNA sample was amplified by using six different PCR primers in a 0.5 ml-microtube containing a total of 25 μl of a PCR mixture consisting of *ca* 10 ng of genome DNA, 120 μM of each dNTP, 12.5 pmol of 10 mer-random primer (Operon Technologies Inc., listed below), 0.5 U of *Taq* DNA polymerase, 1 mM $MgCl_2$, and *Taq* buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1 % Triton X-100, pH 9.0). The control mixture contained all the components of the PCR mixture except the template DNA to check for exogenous DNA contamination. After heating the mixture at 95 °C for 5 min the *Taq* DNA polymerase was added to the mixture, over which one drop of mineral oil was overlaid. The tube was pre-incubated at 72 °C for 30 sec, followed by 40 times of the incubation cycle of 1 min at 92 °C, 1 min at 36 °C, and 2 min at 72 °C, and then post-incubated at 72 °C for 5 min. After the reaction, 5 μl of the solution containing the amplified DNA fragments was subjected to electrophoresis using 1.2 % agarose gel with ethidium bromide (0.5 μg/ml) as the stain, and the gel was photographed under UV light.

Random primers used in this experiment were OPD-03 (GTCGCCGTCA), OPD-05 (TGAGCGGACA), OPD-07 (TTGGCACGGG), OPF-02 (GAGGATCCCT), OPF-04 (GGTGATCAGG), and OPF-07 (CCGATATCCC).

Digestion, hybridization and detection for RFLP analysis Each genomic DNA sample was digested with six different restriction endonucleases in a 1.5 ml-microtube containing 200 μl of a mixture consisting of *ca* 1 μg of genome DNA, 100 U of restriction enzyme (Toyobo, listed below), and the reaction buffer suggested by the manufacturer. After incubation at 37 °C overnight, 10 μl of 5M ammonium acetate and 500 μl of cold EtOH were added to the mixture, and the precipitate formed during the incubation at -20 °C for over 2 hours was separated by centrifugation. The pellet was dissolved in 10 μl of distilled water and then subjected to electrophoresis using 0.8 % agarose gel. The restriction fragment bands on the gel were denatured with 0.25 M HCl, and then transferred to a nylon membrane (Hybond-N^+, Amersham) under alkaline conditions (0.4 N NaOH). A rice ribosomal DNA clone was used as a probe. The plasmid of pRR 217[9] containing the rice ribosomal DNA clone in the *Eco* RI cloning site was digested with *Eco* RI, and the clone was separated by electrophoresis using 1.0 % low melting point agarose gel (Sea Plaqul agarose, FMC BioProduct) and was isolated by cutting out the gel. The isolated rice ribosomal DNA clone was labeled with ^{32}P by using the Random Primer Labeling Kit (Takara Shuzo Co., Ltd.), and then hybridized with the restriction fragments of *C. sativa* DNA on the nylon membrane at 65 °C, overnight. The hybridized probe was detected by using a BAS 2000 station system (Fuji Firm Co., Ltd.).

Restriction endonucleases used in this experiment were *Eco* RI, *Hind* III, *Dra* I, *Bgl* II, *Xba* I and *Eco* RV.

Detection of THC (THCA) and CBD (CBDA) by GC analysis[2] Fresh young leaves (150 mg – 500 mg) of each plant were individually stored at –20 °C until

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use. Each leaf was extracted with 0.5 ml of MeOH with sonication for one hour. One or 2 µl of the MeOH solution was used as the sample for the GC analysis. As the standard samples, 1 mg/ml and 50 µg/ml of THC and CBD, respectively, were used. GC was carried out by using an HP 5890 II apparatus with FID detection and a DB-1-15m column. He was used as the carrier gas at the flow rate of 1 ml/min, the injection temperature was 230 °C, and the detection temperature was 280 °C. Each analysis was made in triplicate.

RESULTS AND DISCUSSION

GC analysis showed that the Tochigishiro strain contained CBD (CBDA), whereas the Mexican strain a large amount of THC (THCA) (TABLE I). This confirmed that the Tochigishiro strain is a fiber type, and the Mexican strain a drug type, and the Nara cultivated strain containing a small amount of THC and no CBD an intermediate drug type.[1]

TABLE I. Cannabinoid Contents in Each Strain of *Cannabis sativa* by GC Analysis[2]

Sample	CBD (CBDA; µg/g fresh levies)	THC (THCA; µg/g fresh levies)
Tochigishiro strain	80.6	< 1.0
Mexican strain	< 1.0	1327.0
Nara cultivated strain	< 1.0	45.4

Each value is an average of several samples, each treated in triplicate.

In the preliminary RAPD analysis with 24 primers (Operon Technologies Inc., OPDs-01 to -12 and OPFs-01 to -12), six primers, OPD-03, OPD-05, OPD-07, OPF-02, OPF-04 and OPF-07, gave reproducible and informative bands with the genomic DNA of *C. sativa*. Therefore, these were used in the present study. A total of 91 distinct bands was obtained from the 36 genomic DNA samples (17 samples from Tochigishiro strain, 12 samples from Mexican strain, and 7 samples from Nara cultivated strain) by using the six primers, and each primer gave 9 to 22 bands. Representative RAPD patterns obtained by using OPD-03 are shown in Fig. 2. Genetic similarity coefficients from the RAPD profiles calculated are listed in TABLE II. All of the strains showed some genetic diversities within each strain, and the diversites were expressed as standard deviations in the table. Although these intra-strain diversities existed, the genetic similarity tree (Fig. 3) showed that these three strains formed individual breeds. These results suggested that the RAPD analysis can distinguish between the three strains, the Tochigishiro, Mexican and Nara cultivated strains of *C. sativa.*

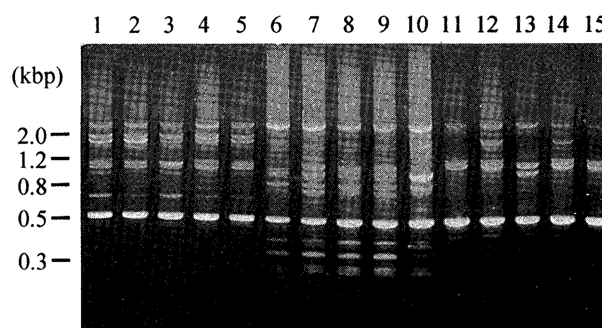

Fig. 2. Representative RAPD patterns obtained by PCR using primer OPD-03 with genomic DNA from *C. sativa*
Lanes 1–5 for samples of Tochigishiro strain; lanes 6–10 for Mexican strain; and lanes 11–15 for Nara cultivated strain.

TABLE II. Genetic Similarity Coefficient from RAPD Profiles for Each Strain of *Cannabis sativa*

	Tochigishiro strain (17 samples)	Mexican strain (12 samples)	Nara cultivated strain (7 samples)
Tochigishiro strain	0.72 ± 0.07		
Mexican strain	0.51 ± 0.04	0.68 ± 0.06	
Nara cultivated strain	0.50 ± 0.05	0.55 ± 0.04	0.74 ± 0.05

Genetic similarity (GS) = $2m_{xy}/(m_x + m_y)$, where m_{xy} is the number of bands existing in both of the two strains x and y, and m_x and m_y are the numbers of total bands in strains x and y.

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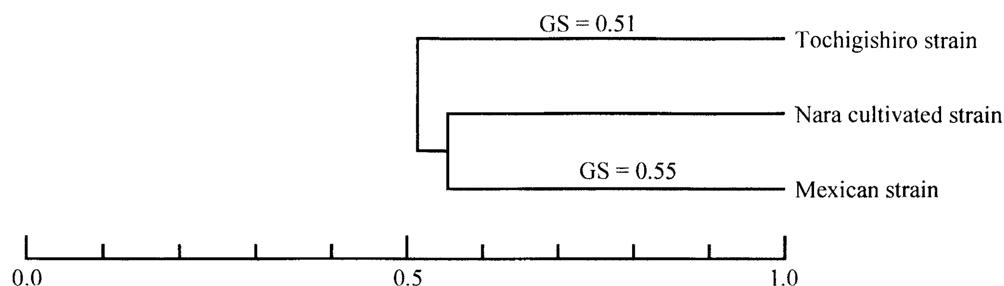

Fig. 3. Genetic similarity tree of *C. sativa* by RAPD profiles
Genetically identical materials should have GS of 1.

In a preliminary test of the RFLP analysis, six restriction endonucleases, *Eco* RI, *Hind* III, *Dra* I, *Bgl* II, *Xba* I and *Eco* RV, were found to digest the *C. sativa* genomic DNA. So these enzymes were used in the present study. A total of 23 distinct bands was obtained from the 14 genomic DNA samples (5 samples from the Tochigishiro strain, 5 samples from the Mexican strain, and 4 samples from the Nara cultivated strain), which were separately digested with six restriction endonucleases and hybridized with rice ribosomal DNA clone. Each restriction endonuclease gave 1 to 6 bands that were hybridized with the clone (TABLE III). There was no genetic diversity observed within one strain according to the RFLP patterns, whereas the RAPD analysis showed some intra-strain diversities. The estimated nucleotide diversities ($\hat{d}$) were calculated from the RFLP profiles, which are listed in TABLE IV. A phylogenetic tree was drawn on the basis of the unweighted pair-group method using arithmetic average (UPGMA)[10] by using the calculated $\hat{d}$ values (Fig. 4).

TABLE III. Length of DNA Fragments in Kilobases after Digestion with Restriction Enzymes for Strains of *Cannabis sativa*

Restriction enzyme	Tochigishiro strain	Mexican strain	Nara cultivated strain
Eco RI	4.4		4.4
		4.0	
	3.3	3.3	3.3
		1.9	
	1.8		1.8
		1.7	
Hind III	10	10	10
Xba I		11	
	9.5		9.5
		8.7	
Eco RV		9.5	
	5.2		5.2
		4.7	
	2.9	2.9	2.9
	1.7	1.7	1.7
	1.0	1.0	1.0
Dra I		7.8	
	5.0	5.0	5.0
	2.1	2.1	2.1
	1.7		
		1.6	1.6
Bgl II	15	15	15
	8.8		8.8
		8.5	

No diversity observed within one strain of Tochigishiro strain (5 samples), Mexican strain (5 samples), and Nara cultivated strain (4 samples).

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TABLE IV. Estimated Nucleotide Diversity by RFLP Profiles for Each Strain of *Cannabis sativa*

	Tochigishiro strain	Mexican strain	Nara cultivated strain
Tochigishiro strain	$\hat{F} = 28/28 = 1.00$ $\hat{G} = 1$ $\hat{d} = 0$		
Mexican strain	$\hat{F} = 18/32 = 0.563$ $\hat{G} = 0.9048$ $\hat{d} = 0.01448$	$\hat{F} = 36/36 = 1.00$ $\hat{G} = 1$ $\hat{d} = 0$	
Nara cultivated strain	$\hat{F} = 26/28 = 0.929$ $\hat{G} = 0.9877$ $\hat{d} = 0.00179$	$\hat{F} = 18/32 = 0.563$ $\hat{G} = 0.9048$ $\hat{d} = 0.01448$	$\hat{F} = 28/28 = 1.00$ $\hat{G} = 1$ $\hat{d} = 0$

Nucleotide diversity was given by the following equations; $\hat{F} = 2m_{xy}/(m_x + m_y)$, $\hat{G} = [\hat{F}(3 - 2G_1)]^{1/4}$, $\hat{d} = -(2/r)\log_e \hat{G}$, where $\hat{F}$ is the ratio of bands shared by two strains, m_{xy} the number of bands existing in both of the two strains x and y, m_x and m_y the numbers of total bands as in strains, x and y, $\hat{G}$ the asymptotic value as $\hat{F}^{1/4}$ equals $\hat{G}_1$, $\hat{d}$ the calculated nucleotide diversity, and r the number of bases recognized by the enzyme.

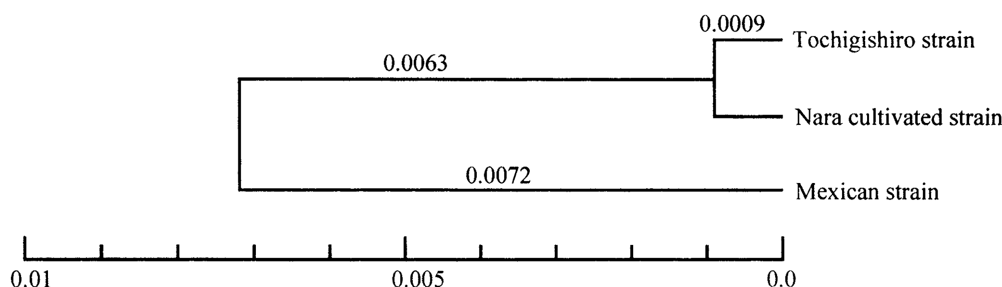

Fig. 4. Phylogenetic tree of *C. sativa* by RFLP profiles
The tree was drawn by using the unweighted pair-group method using arithmetic averages (UPGMA) with calculated $\hat{d}$ values.

This tree indicated that the genetic distance between the Tochigishiro and Nara cultivated strains was near, whereas that between the Mexican strain and the other two strains was far. The results suggested that the RFLP analysis can clearly distinguish between the Mexican strain (drug type) and the other two strains of *C. sativa* with no obscure intra-strain diversities.

The two genetic analysis, the RAPD and RFLP methods, used in combination are useful for distinguishing between the three types of *C. sativa*. The RFLP analysis first clearly distinguishes the Mexican strain (drug type) from the other two strains and then the RAPD analysis distinguishes between the Tochigishiro strain (fiber type) and Nara cultivated strain (intermediate drug type).

The results of our study shows the possibility of the RAPD and RFLP analyses distinguishing between the strains having chemotypes like *C. sativa*.

Further studies are being conducted to collect the RAPD and RFLP analyses data of other strains of *C. sativa*.

ACKNOWLEDGMENTS

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REFERENCES AND NOTES

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2. In fresh leaves, THC and CBD are in precursor forms, THCA and CBDA. Heating under the GC analysis condition, changes THCA and CBDA easily to THC and CBD.

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