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Restriction profiles of *trn*L (UAA) intron as a tool in *Cannabis sativa* L. identification

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Abstract. The author reports a method to identify unknown samples of plant material as *Cannabis sativa* L. This method involves PCR amplification of the *trn*L intron of the chloroplast DNA (cpDNA) in five different accessions of *C. sativa* from various geographic areas, as well as in one accession of *Humulus lupulus* L., which belongs to the only other genus of family Cannabaceae. The usage of *trn*L intron, amplified and successively digested with appropriate restriction endonucleases, has allowed the construction of a *Cannabis* profile which can be used in forensic investigations for the identification of samples suspected to be *Cannabis*.

Riassunto. Viene riportato un metodo per identificare campioni incogniti di *Cannabis sativa* L. mediante reazione di polimerizzazione a catena (PCR) dell'introne del *trn*L del DNA plastidiale (cpDNA) e successiva digestione con appropriate endonucleasi di restrizione. Sono stati utilizzati cinque ceppi di *C. sativa* provenienti da diverse aree geografiche ed un ceppo di *Humulus lupulus* L. quale rappresentante dell'altro genere della famiglia delle Cannabaceae. Il metodo riportato permette la costruzione di un modello caratteristico per *Cannabis*, da utilizzare nelle indagini forensi per identificare, campioni sconosciuti sottoposti a perizia.

Key words: *Cannabis sativa,* Chloroplast DNA, Hemp, *Humulus,* restriction mapping, *trn*L intron.

INTRODUCTION

In many countries the cultivation of *Cannabis sativa* L. (hemp) for fibre is allowed, but cultivation for drug use is forbidden. The procedures commonly in use in the forensic laboratory for

Cannabis identification, are based on botanical characteristics of the material and on the presence of chemical compounds.

Botanical identification of material for forensic purposes is normally carried out by microscope examination and it concentrates on leafy material. The type and nature of cystolith hairs on the leaves, as well as the cellular structure of the seeds are observed.

Chemical identification of the cannabinoids, on contrary, is carried out by using a variety of different methods (thin-layer chromatography, gas chromatography/mass spectrometry, gas chromatography or HPLC).

The methods reported above, although working fairly well in various cases, show severe limitations. For example, as far as botanical investigation is involved, NAKAMURA (1969) described more than 80 different plant species containing cystolith hairs similar to those found in *Cannabis*. Thus, it is conceivable that suspicious plant material could be erroneously identified as *Cannabis*.

Another problem, related to chemical tests, is that the cannabinoids and especially the tetrahydrocannabinol (THC), are unstable in many solvents (PARKER et al., 1974) and are readily oxidized (GOUGH, 1991). Moreover, the absence of detectable THC in an unknown sample does not prove that it is not marihuana. In fact SMALL & BECKSTEAD (1973) report that 117 of 350 plants of *C. sativa* examined contained no THC.

The task of the expert in charge of forensic investigations can be particularly arduous in those cases in which the plant material under study has been previously treated (e.g., minced, desiccated or macerated), poorly stored by the criminals or by the police forces after seizure, or has been seized in very small amounts.

Contact with *Cannabis* problems and the experience acquired by my research group in this field, based on the co-operation with the Police Investigation Department, suggests that in extreme cases, the botanical identification as well as the chemical tests for presence of cannabinoids can be almost impossible.

For these reasons, a method of plant identification in a way almost completely independent from the quantity of starting material and from its state of preservation would be extremely

useful to the forensic expert. Such methods are forensically relevant, as in different countries of various continents, cultivation of *C. sativa* is forbidden *per se*, regardless of the cannabinoid content.

In recent years, the study of DNA has become increasingly interesting for the general forensic expert, especially in terms of identifying criminals by using traces of their body fluids in cases of violent crimes with no witnesses. These techniques rely on a paramount advancement in the field of molecular biology, i.e., the Polymerase Chain Reaction (PCR).

For example, by using PCR techniques molecular biologists succeeded in extracting DNA from plant fossils (GOLENBERG et al.,1990) and from human mummies (PAABO, 1985; WOODWARD et al.,1994).

In a previous paper (SINISCALCO GIGLIANO & DI FINIZIO, 1994) the authors have tested the feasibility of using *trn*L intron of the chloroplast DNA (cpDNA) in the *Cannabis* identification. This non-coding region, is common to the all plant kingdom (KUHSEL et al., 1990).

The choice of *trn*L intron is that this region is included between two highly conserved tRNA genes [*trn*L (UAA) 5' exon and *trn*L (UAA) 3' exon] therefore the utilized primers amplified exclusively this region (TABERLET et al., 1991) secondly the sequencing of this region can be increased for evolutionary studies and for identifying intraspecific genetic markers (PALMER et al., 1988; CLEGG et al., 1991).

Another reason for the choice of this region is that on the basis of a previous paper (SINISCALCO GIGLIANO & DI FINIZIO, 1994) the authors have shown that the *trn*L intron is homogeneous in length in different *Cannabis* accessions and appear to be an ideal molecule to construct a profile of cpDNA fragment for forensic identifications.

The object of this paper is test the feasibility of using the *trn*L intron in the construction of a *Cannabis* profile from known material and then the comparison of these results with a sample suspected to be *Cannabis*.

MATERIAL AND METHODS

Plant materials

In the present study, five different cultivars of *Cannabis* were used; from France (accession number CJBN 716/85), Afghanistan (CPRO-dlo 883271), Nepal (CPRO-dlo 891191), The Netherlands (SS 241) and Italy (OBN 0148-F). Also one sample of *Humulus lupulus* L. from Italy (OBN 2801-F) was used.

DNA extraction

DNA was extracted from dried or fresh leaves. Extraction was carried out by using a protocol described by CAPUTO et al. (1991) but appropriately scaled and modified.

Samples were ground in liquid nitrogen using a small mortar and pestle and a sample not exceeding a volume of approx. 300 µl was carefully transferred into a 1.5 ml disposable microcentrifuge tube. Immediately after the nitrogen had evaporated, 800 µl of extraction buffer [50 mM Tris-HCl pH 8.0; 20 mM EDTA pH 8.0; 0.2% bovine serum albumin (BSA); 1% polyvinylpyrrolidone (PVP) and 0.1% ß-mercaptoethanol] were added to the tissue powder. Cells were lysed by adding sodium dodecyl sulfate (SDS) and sodium N-lauroylsarcosinate to a final concentration of 2% each and incubated for 15 min in a water bath at 67 °C. Samples were briefly cooled in an ice bath and proteins were precipitated by adding 0.3 vol 5 M potassium acetate, followed by 20 min incubation on ice and 20 min centrifuging in an Eppendorf microfuge at maximum speed (approx. 14,000 x g) at 4 °C.

The supernatant was extracted twice or three times with chloroform-isoamyl alcohol (24:1) and DNA precipitated by adding 2 vol ethanol and 0.1 vol 3 M sodium acetate. Samples were briefly frozen in an ultrafreezer and then centrifuged for 15 min at the same conditions as above. The pellet was then resuspended in approx. 500 µl redistilled water.

DNA was precipitated again with 1/9 5M NaCl and 20% polyethylene glycol (PEG-8000) (equal volume). Vials were then frozen in liquid nitrogen and stored at –80 °C for 30 min. Finally, the DNA precipitate was collected by centrifuging for 15 min as

above, washed again in 70% ethanol and resuspended in a suitable volume of redistilled water.

PCR reaction

The *trn*L intron was amplified by polymerase chain reaction (PCR) using two universal primers as used in the cpDNA amplifications of various plant species (TABERLET et al., 1991).

The first of the two primers annealed with the *trn*L 5' exon (5'-CGAAATCGGTAGACGCTACG-3') and the second with the *trn*L 3' exon (5'-GGGGATAGAGGGACTTGAAC-3') of the cpDNA.

PCR was carried out in a Perkin Elmer Cetus 9600 thermal cycler, for 30 cycles. The final volume for PCR mixture was 100 μl and consisted of 2-10 ng DNA sample; 10 μl buffer [500 mM KCl, 100 mM Tris-HCl pH 9.0, 1% Triton X-100 and 25 mM $MgCl_2$]; 1μl primer (0.25 μM); 0.2 mM each of the four dNTPs; 2.5 units *Taq* polymerase.

Initial conditions were as follows: 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 45 sec extension at 72 °C. Samples were denatured for 5 min at 94 °C before the beginning of the first cycle. Extension time was increased by 3 sec per cycle and further prolonged for 7 min at the end of the last cycle.

PCR products were then column-purified using Microcon 100 microconcentrators (Amicon, cat. 42413) and loaded onto a 2% agarose gel and electrophoresed under the conditions reported below.

Restriction endonuclease digestion

A number of restriction endonucleases were evaluated. The final selection was made so as to choose endonucleases which produced characteristic fragments in *Cannabis* which could be clearly visualized in a gel system.

The restriction endonucleases tested, which produced characteristic fragments, are reported in Tab. 1.

PCR purified fragments were then digested with the selected restriction endonucleases according to the manufacturer's specification.

Tab. 1 - Restriction endonucleases tested which produced characteristic fragments in *trn*L intron of *Cannabis* and *Humulus*. For each enzyme the clavage sequence is reported.

Enzyme	Isoschizomer	Specificity*
Bbv II	*Bpi* I	GAAGAC(N_2)↓
Bet I		W↓CCGGW
Bsa AI	*Msp* YI	YAC↓GTR
Bsp MII	*Kpn* 2I	T↓CCGGA
Csp 6I		G↓TAC
Gsu I		CTGGAG(N_{16})↓
Mae II		A↓CGT
Mbo II		GAAGA(N_8)↓
Rsa I		GT↓AC
Sna BI	*Eco* 105I	TAC↓GTA
Ssp I		AAT↓ATT

*N = A, G, C or T; R = A or G; W = A or T; Y = C or T.

Electrophoresis and agarose gel

Digested samples were loaded onto a 2% agarose gel prepared in 1 x TBE buffer (90 mM Tris-borate, 2 mM EDTA pH 8.0) containing 50 ng/ml ethidium bromide and electrophoresed at 9 V/cm. The length of the fragments was estimated by using a 100 bp DNA ladder as a marker.

The DNA bands were visualized using an ultraviolet light transilluminator (254 nm) and photographed using 667 Polaroid film.

RESULTS AND DISCUSSION

Cannabis and *Humulus trn*L intron DNA fragments obtained by PCR are shown in Fig. 1.

The list of restriction endonucleases tested that produced characteristic fragments is reported in Tab. 2. For each enzyme the number of cuts and length of fragments produced are reported.

The fragments obtained after digestion with the some of the most representative restriction endonucleases tested are shown in Fig. 2.

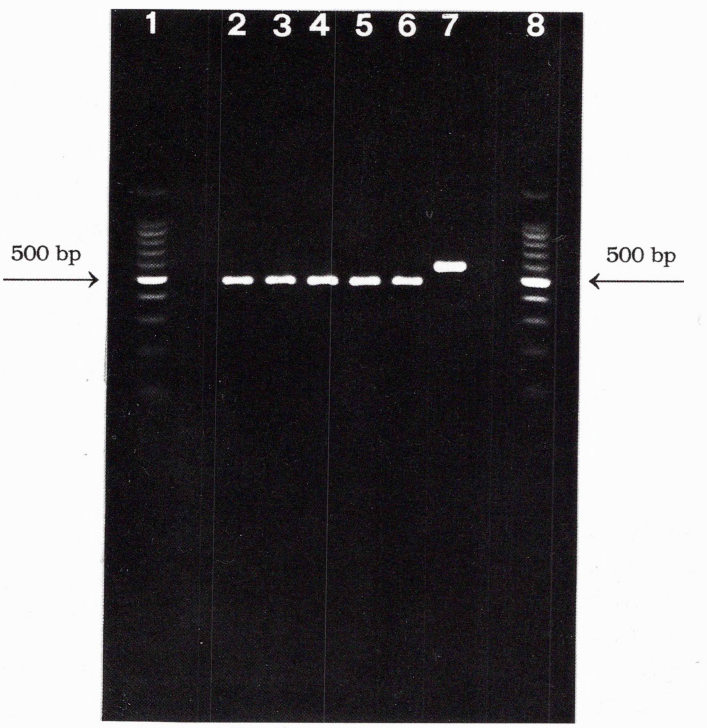

Fig. 1 - *Cannabis* and *Humulus trn*L intron fragments obtained by PCR. Lanes 2-6, *Cannabis*; lane 7, *Humulus*; lanes 1 and 8, 100 bp DNA ladder. The bright fragment is 500 bp long.

Tab. 2 - Restriction endonucleases tested. For each enzyme, the number of cuts and the length of the fragments produced are reported.

Enzyme	Number of cuts in *Cannabis*	Number of cuts in *Humulus*	Fragments produced in *Cannabis* (bp)	Fragments produced in *Humulus* (bp)
Bbv II	1	1	360 - 130	460 - 130
Bet I	2	1	130 - 110 - 250	130 - 460
Bsa AI	0	1	490	290 - 300
Bsp MII	1	0	230 - 260	590
Csp 6I	0	1	490	290 – 300
Gsu I	0	1	490	240 - 350
Mae II	0	2	490	270 - 20 - 300
Mbo II	1	2	370 - 120	280 - 180 - 130
Rsa I	0	1	490	290 - 300
Sna BI	0	1	490	290 - 300
Ssp I	1	1	310 - 180	400 - 190

The raw PCR fragments obtained (Fig. 1) show that the *trn*L intron is homogeneous among the five different accessions of *Cannabis* (approx 490 bp), but different in *Humulus* (approx 590 bp).

As we used external primers, each amplified fragment includes the *trn*L intron region plus an upstream fragment relative to the *trn*L (UAA) 5' exon and an downstream fragment relative to the *trn*L (UAA) 3' exon.

However, by comparing the angiosperm sequences of the *trn*L intron region available in the literature and consulted by accessing the GENBANK database, we excluded from the length of the amplified regions the fragment upstream (25 bp) and downstream (49 bp) of the *trn*L intron. On this basis, the *trn*L intron of the taxa in study are approximately 413 bp in *Cannabis* and 508 bp in *Humulus*.

The restriction endonuclease *Bsp* MII is the only enzyme that produced fragments in the *trn*L intron of *Cannabis* but not in *Humulus*; while *Bsa* AI, *Csp* 6I, *Gsu* I, *Mae* II, *Rsa* I and *Sna* BI produced fragments in the *trn*L intron of *Humulus* but not in that of *Cannabis*.

The restriction endonucleases *Bbv* II, *Bet* I, *Mbo* II, and *Ssp* I, on the contrary, produced fragments in both *Cannabis* and *Humulus*.

All five accessions of *Cannabis* produced the same fragments after digestion with the restriction endonucleases tested. Fig. 2 shows the fragments obtained, for one of the *Cannabis* accession tested, with the most representative enzymes used.

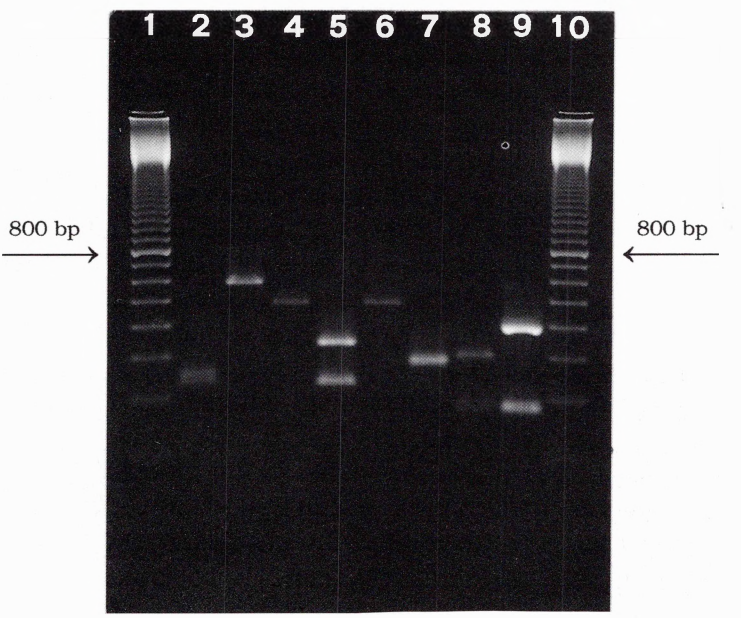

Fig. 2 - *Cannabis* and *Humulus trn*L intron fragments obtained by PCR and digested with the some of the most representative restriction endonucleases tested. *Cannabis* (lane 2) and *Humulus* (lane 3), digested with *Bsp* MII; *Cannabis* (lane 4) and *Humulus* (lane 5), digested with *Gsu* I; *Cannabis* (lane 6) and *Humulus* (lane 7), digested with *Rsa* I; *Cannabis* (lane 8) and *Humulus* (lane 9), digested with *Ssp* I. Lanes 1 and 10, 100 bp DNA ladder. The bright fragment is 800 bp long.

The two fragments produced in *Cannabis* by using *Bsp* MII are 230 and 260 bp; while that produced by using *Ssp* I are 180 and 310 bp.

The fragments produced in *Humulus* from *Gsu* I, *Rsa* I and *Ssp* I are 240 and 250 bp; 290 and 300 bp; 190 and 400 bp respectively.

Our results show that the *trn*L intron is an ideal molecule to construct a restriction map of *Cannabis* DNA, which can be used in forensic investigations for the identification of a sample suspected to be *Cannabis*. However, in order to attribute the unknown sample to *Cannabis*, all fragments produced by the set of the endonucleases tested must be present.

As an alternative, this method may be used to exclude samples that are suspected to be *Cannabis*, as a first approach, in order to reduce the number of samples which are to be sequenced.

The approach indicated here, has the merit that very small amounts of material can be processed (as low as 50 mg dried material). Also it is not expensive and does not require access to a sequencing facility. The only relatively sophisticated piece of equipment required is a PCR thermocycler. This apparatus is becoming increasingly cheaper, and accordingly increasingly frequent in any forensic laboratory.

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