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Preliminary Data on the Usefulness of Internal Transcribed Spacer I (ITS1) Sequence in *Cannabis sativa L.* Identification

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ABSTRACT: A method is described to identify an unknown sample of plant material of forensic interest as *Cannabis sativa L.* The method consists in comparing the sequence of the nuclear ribosomal DNA Internal Transcribed Spacer I (ITS1) of the unknown sample with a *Cannabis* sequence. Our preliminary results show that the ITS1 is an ideal molecule for the identification of a sample suspected to be marijuana.

KEYWORDS: forensic science, substance abuse detection, *Cannabis sativa,* Hemp, Internal Transcribed Spacer I, Ribosomal DNA

Cannabis sativa L. (hemp) is a dioecious annual plant from which various products of economic interest can be obtained: oil from seeds, fiber for rope and fabric from stems, and a psychoactive drug from flowers and leaves. This last characteristic has fostered a great interest in *Cannabis* among forensic scientists. A common forensic problem, in fact, is the identification of fresh or dried plants or leaves of *C. sativa.*

Botanical analysis of *Cannabis* for forensic purposes is normally carried out by microscopic examination and concentrates, especially, on the type and nature of cystolith hairs on the leaves, as well as on the cellular structure of the seeds.

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

The techniques reported above, although working fairly well in general terms, show severe limitations in cases in which they are not appropriately selected and in connection to the experimental material. For example, when a microscopic investigation is the unique test involved, Nakamura (1) described more than 80 different plant species containing cystolith hairs similar to those found in *Cannabis.* Thus, it is conceivable that some suspicious plant material could be erroneously identified as *Cannabis.* In these cases, however, by using both microscopical and chemical techniques, *Cannabis* may be distinguished from all botanical material discussed by this author (1).

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Another problem, related to chemical test, is that the cannabinoids, and especially the tetrahydrocannabinol (THC) are unstable in many solvents (2) and are readily oxidized (3). Moreover, the absence of detectable THC in an unknown sample does not prove that it is not marijuana. In fact, Small and Beckstead (4) reported that 117 of the 350 plants of *Cannabis* examined by them 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 in study has been previously treated (e.g., minced, desiccated, or macerated), has been poorly stored, or has been seized in very small amounts. In these extreme cases, unfortunately, the botanical identification as well as the chemical tests for presence of cannabinoids can be almost impossible.

We are often requested to identify material which is poorly preserved or of inadequate amount, and so cannot be analyzed and often not even identified. For these reasons, a method granting 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.

Recently, genetic methods, and in particular RAPD analyses (5) have been used in addition to chromatographic assays to identify various cultivars of *Cannabis.* However, these method, although invaluable for discriminating between cultivars, may not always provide the botanical identification of a seized sample.

Previously, the author has developed another method to identify unknown plant material as *C. sativa* (6). The method involves PCR amplification and sequencing of the Internal Transcribed Spacer II (ITS2) of the nuclear ribosomal DNA (n-rDNA).

The n-rDNA is made of repeated units present in a few hundreds to more than 10 thousands copies in each nucleus (7). Each unit consists of three subunits (18S, 5.8S and 25S), which code for the ribosome itself. These regions are separated by two quite variable Internal Transcribed Spacer (ITS1 and ITS2). Units are in turn separated by highly variable Inter Genic Spacer (IGS).

In the above-said paper (6), the sequence of ITS2 was examined for five different strains of *C. sativa,* showing that ITS2 was invariant among *Cannabis* strains and different from that of other plant species.

The object of this paper is testing the feasibility of using PCR amplification and ITS1 sequencing as another test which can be used in forensic investigations for the identification of plant material suspected to be *Cannabis.* The choice of this region (ITS1) is also founded on a previous paper (8) in which the authors have shown that the ITS1 is homogeneous in length in different *Cannabis* accessions.

Material and Methods

Plant Materials

For the present study five different cultivars of *Cannabis*, originating from France (accession number CJBN 716/85), Afghanistan (CPRO-dlo 883271), Nepal (CPRO-dlo 891191), The Netherlands (S S 241) and Italy (OBN 0148-F), were used. One sample of *Humulus lupulus* L. coming from Italy (OBN 2801-F) was also used. *H. lupulus* belongs to the only other genus of family Cannabaceae. For each *Cannabis* and *Humulus* cultivars, six individuals were tested.

DNA Extraction

DNA was extracted from dried (0.05–1 g) or fresh (0.1–1 g) leaves. Extraction was carried out by using a protocol described by Caputo et al. (9) opportunely scaled and modified.

Samples were ground in liquid nitrogen using a small pestle and mortar, and a sample not exceeding a volume of approximately 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% β-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 300 μL 5 M potassium acetate, followed by 20 min incubation on ice and 20 min centrifuging in an Eppendorf microfuge at maximum speed (approximately 14,000 × g) at 4°C. The supernatant was extracted twice or three times with chloroform-isoamyl alcohol (24:1) and DNA precipitated by adding 2.4 mL ethanol and 0.12 mL 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 approximately 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.

The DNA was quantified by visual inspection on an agarose gel, by comparison with an appropriate set of DNA markers of known concentration.

DNA Amplification

The ITS1 region was amplified by polymerase chain reaction (PCR) using two primers; one which anneals in the 3′ region of the 18S (5′-GGAGAAGTCGTAACAAGGTTTCCG-3′) and the other in the 5′ region of the 5.8S (5′-ATCCTGCAATTCACACCAAGTATCG-3′) rDNA genes, respectively.

PCR was carried out, for 30 cycles, in a thermal cycler Cetus 9600 (Perkin Elmer, Norwalk, CT).

The final volume for PCR mixtures was 100 μL and consisted of 2–10 ng DNA sample, 10 μL buffer (500 mM KCl; 100 mM Tris-HCl pH 9; 1% Triton X-100; 25 mM $MgCl_2$), 1 μL primer (0.25 μM), 0.2 mM each of the four dNTP and 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 of 3 sec per cycle; extension was further prolonged for 7 min at the end of the last cycle.

Sequence Reaction

PCR fragments were then column-purified using Microcon 100 microconcentrators (Amicon) and double-strand sequenced in both directions by using a modification of the Sanger dideoxy method (10), as implemented in a double strand DNA cycle sequencing system with fluorescent dyes.

Sequence reactions were then loaded into a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Electrophoresis was carried out on a 5.75% acrylamide gel with a constant 32 W power; this usually produced voltages ranging from 1200 to 1500 V and currents ranging between 20 and 25 A.

Sample and gel preparation, as well as electrophoresis conditions were those suggested by the manufacturer in the 373A DNA Sequencing System user manual (Part n. 903204, revision A, June 1994).

Sequences were aligned by using the Clustal V software (11). ITS1 length was calculated by aligning the sequences obtained with the 3′ terminus of 18S and with the 5′ terminus of 5.8S of various sequences available in the literature.

Results and Discussion

The raw PCR fragment obtained for all *Cannabis* samples and *Humulus* had a length of approximately 360 base pairs (bp) (Fig. 1).

The DNA from all five hemp samples had an ITS1 225 bp long with a GC content of 58.2%. The hops ITS1 was 226 bp long with a GC content of 56.6%. The five *Cannabis* sequences obtained are identical.

However, the hops sequence was 90% identical to the sequence of the hemp samples (Fig. 2).

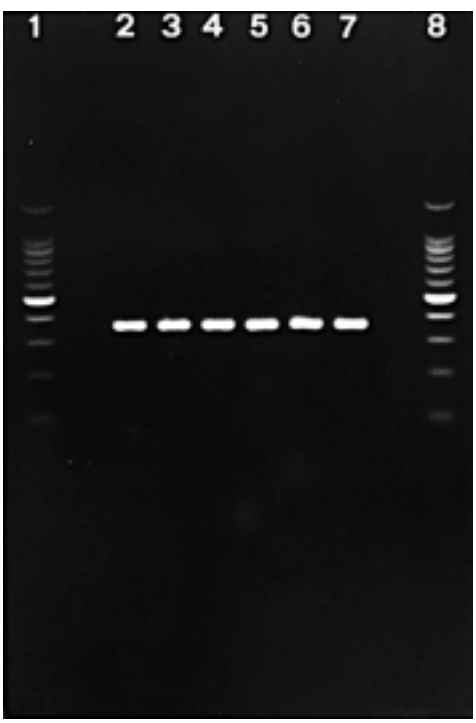

FIG. 1—Cannabis *and* Humulus *DNA fragments obtained by PCR. Lanes 2-6*, Cannabis; *lane 7*, Humulus; *lanes 1 and 8, 100-bp DNA ladder (the bright fragment is 500 bp long).*

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Cannabis    TCGAAACCTGCAACAGCAGAACGACCCGTGAACACGTTTTAAACAGC-TT    49
Humulus     TCGATACCTGCAACAGCAGAACGACCCGTGAACACGTTTTAAACAACCTT    50
            **** ************************************** * **

Cannabis    GGGCGGGCGAGAGGAGCTTGCTCCTTGGACCCGCCCGCACCTGCTGGGAG    99
Humulus     GGGTGGGCGAGAGGAGCTTGCTCCTTGGACCCGCCCTCACCTGCTAGGAG   100
            *** ******************************** ******** ****

Cannabis    AAATCTCGGCGGGCTAACGAACCCCGGCGCAATCTGCGCCAAGGAACAAT   149
Humulus     AAATCCTGGCGGGCTAACGAACCCCGGCGCAATCTGCGCCAAGGAACAAT   150
            *****  ****************************************

Cannabis    AAAAGATTATCGCGTGGCTCGTGCGGTGGCCCGGAGACGGTGTCCGCCAA   199
Humulus     AAAAGATTAGCGCGTTTCTCGTGCGGAGACCCGGAGACGGTGCTCGCCGC   200
            ********* *****  ********* * ************  ****

Cannabis    TCGAGATGCGTGTTTATCGAAATGTC   225
Humulus     TCGAGTTGCGTGTTCTTCAATATGTC   226
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FIG. 2—*ITS1 sequences of* Cannabis sativa *and* Humulus lupulus *(an asterisk indicates identity).*

The *Cannabis* ITS1 sequence was also compared with more than 330,000 sequences present in GENBANK (4223 of which being ITS1 sequences), so as to be sure that hemp ITS1 sequence is indeed diagnostic.

Our protocol represents a distinct improvement, in that it requires very small amounts of tissue, either fresh or dried, and even not properly stored, does not require purification of high molecular weight DNA in low melting point agarose, and furthermore gives useful information from samples that have been stored in poor conditions.

In fact, to reproduce the conditions of forensic work, the DNA was extracted from samples of one cultivar (OBN 0148-F) which had previously been collected, roughly cut in pieces, sealed in plastic bags and kept at room temperature for 15, 30, and 45 days. For all these samples, although the decomposition process was ongoing, especially for those examined after 45 days of treatment, DNA extraction and the successive PCR amplification were possible.

The ITS1 sequences obtained from fresh or dried samples, as well as those obtained from samples OBN 0148-F, before and after the treatments, were identical. The method described in this paper is laborious and requires access to an automated sequencing apparatus, so it can only be carried out in a suitably equipped laboratory; however it only requires very small amounts of material.

An alternative to automated sequencing that we successfully employed, is the method reported in a previous paper (12) which utilizes the amplification of ITS1 and the successive digestion with appropriate restriction endonucleases in the construction of *Cannabis* fingerprints. This method, slightly less accurate than sequencing, has been devised for specific purposes: it can be accomplished in a short time, does not require access either to a sequencing facility or to any other very sophisticated apparatuses. The only relatively sophisticated piece of equipment required is a PCR thermocycler; this apparatus is becoming increasingly cheaper, and accordingly more frequent in any forensic laboratory.

The approach indicated in the present paper, directed toward a molecular biological identification of *Cannabis*, represents a promising direction for future work.

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