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Cannabis fingerprints by using Random Amplified Polymorphic DNA (RAPD)

GESUALDO SINISCALCO GIGLIANO, ALESSANDRA DI FINIZIO, PAOLO CAPUTO, SALVATORE COZZOLINO[1]

Dipartimento di Biologia Vegetale, Università di Napoli "Federico II", Via Foria 223, 80139 Napoli, Italia. [1]Orto Botanico, Facoltà di Scienze, Universita' di Napoli "Federico II", Napoli, Italia.

Abstract. Four strains of *Cannabis sativa* L. were investigated in order to test the feasibility of strain characterization by using RAPDs for forensic purposes. Six individuals per strain and four random primers were used. UPGMA analysis on the RAPD fragments showed that individuals from the same strains always cluster together, that never a strain is clustered within another and that within-strain distances are always less than between strain distances. Therefore, RAPD analysis can be regarded as an useful tool to characterize and distinguish *Cannabis* strains.

Riassunto. Sono stati saggiati, mediante lo studio dei RAPD, quattro ceppi di *Cannabis sativa* L. alfine di una loro caratterizzazione nelle indagini forensi. Per questo studio sono stati utilizzati sei individui per ciascun ceppo e quattro primer. L'analisi dei frammenti ottenuti (UPGMA) mostra che gli individui dello stesso ceppo formano un gruppo unico e che ciascun gruppo è nettamente distinto dagli altri. I nostri risultati evidenziano, inoltre, che lo studio dei RAPD può essere un valido metodo per caratterizzare ceppi di *Cannabis*.

Key words: *Cannabis sativa*, Forensic plant identification, Plant DNA profiling, RAPD.

INTRODUCTION

Law enforcement agencies must identify seized drug materials. In temperate climates, the principal drug material is *Cannabis sativa* L. (hemp).

The standard chromatographic techniques used to analyse samples of forensic interest require gram amounts of leaf material

for each sample. This material must be fresh, because cannabinoids and especially tetrahydrocannabinol (THC) are readily oxidized (GILLAN et al., 1995). The quantities of THC may vary in relation to the cultivar tested (GIULIANO & SINISCALCO GIGLIANO, 1983), the conditions of cultivation (SINISCALCO GIGLIANO, 1984) and the light intensity (BALDUZZI & SINISCALCO GIGLIANO, 1985). Finally, it is known that the cannabinoids are unstable in many solvents used for the extraction (PARKER et al., 1974).

The authors of the present paper have described 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 (SINISCALCO GIGLIANO, 1995). 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 the forensic investigations for the identification of samples suspected to be *Cannabis*.

In the last year rapid and efficient techniques for DNA analysis have become available, which allow the investigation of infraspecific genotypic differences not previously noted with either kinds of analyses. Detection of genetic variation is essential for a wide range of comparative studies and can aid in assessing relationship among close taxa.

Genetic variation, however, may be investigated in order to find characters which are present in single populations or cultivars for discriminating purposes. In this regard, the study of genetic variation may be of interest for the forensic expert.

Random amplified polymorphic DNA (RAPD) provides a very powerful technique for detecting DNA polymorphisms, as desribed by WILLIAMS et al. (1990). RAPD analysis is based on random amplification of genomic DNA fragments using short primers of arbitrary sequence. DNA amplification is achieved by polymerase chain reaction (PCR). This technique does not depend on hybridization analysis with radioactively labelled probes, requires

small amounts of DNA and no prior knowledge of DNA sequences. The great advantage of RAPD is that it reveals DNA polymorphism without prior sequencing or other characterisation of the genomic DNA concerned. The fingerprint polymorphism revealed by this analysis can be used for detecting genetic variation in *Cannabis* at population or cultivar level (GILLAN et al., 1995) or above (FAETI et al., 1996). In this preliminary study we test the feasibility of using RAPD techniques for the characterisation and fingerprinting of different cultivars of *C. sativa*.

MATERIAL AND METHODS

Plant materials

In the present study four different cultivars of *Cannabis* were used; one from Afghanistan (accession number CPRO-dlo 883271), one from Nepal (CPRO-dlo 891191) and two from The Netherlands (SS-240 and SS-241).

DNA extraction

DNA was extracted from fresh (0.1 - 1 g) leaves using a protocol described by CAPUTO et al. (1991). Samples were ground in liquid nitrogen using a small pestle and mortar 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

was 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 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.

DNA amplification

The PCR was carried out in a thermal cycler Perkin Elmer Cetus 9600. The final volume for PCR mixtures was 25µl and consisted of 2-10 ng DNA sample, 2.5 µl buffer (500 mM KCl; 100 mM Tris-HCl pH 9; 1% Triton X-100; 25 mM $MgCl_2$), 0.25 µl primer (0.25 µM), 0.2 mM each of the four dNTPs and 2.5 units *Taq* polymerase.

Amplification was carried out for 40 cycles and the initial conditions were as follows: 30 sec denaturation at 94 °C, 40 sec annealing at 35 °C, 1 min extension at 72 °C.

The primers employed (Pharmacia Biotech) were:

Primer	Sequence
1	5'-GGTGCGGGAA-3'
2	5'-GTAGACCCGT-3'
3	5'-AAGAGCCCGT-3'
4	5'-CCCGTCAGCA-3'

Electrophoresis and agarose gel

Amplified samples were loaded onto a 1.3% agarose gel (Boehringer) prepared in 1xTBE buffer (0.09 M Tris-borate, 0.002 M 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 (Promega) as marker.

Initially, only a fraction of each amplified sample was loaded; afterwards the samples that presented band variation were selected. The selected samples for each strain were grouped according to the primers employed and loaded onto a single agarose gel.

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

Data analysis

Amplified bands were scored as present (1) or absent (0) and the data were used to calculate the genetic similarity between the examined strains. Faint bands or bands that were not seen in repeated amplifications were not included in the analysis. On the raw presence/absence matrix a calculation of pairwise distances was carried out by employing Euclidean distance, as already done in similar studies (GILLAN et al., 1995). The resulting square similarity matrix was then subjected to UPGMA analysis by using the NEIGHBOR software of the PHYLIP package (FELSENSTEIN, 1993).

RESULTS AND DISCUSSION

The PCR fragments obtained for all samples examined are reported in Fig. 1 and Fig. 2.

A total of 615 bands were amplified by 4 decamer primers. Among those, 567 were polymorphic for presence/absence. The number of bands for each primer varied from 24 to 51, with an average of 35 bands per primer (Tab. 1). The size of the amplified fragments ranged from 300 to 2200 bp. The number of amplified bands differs among the four examined strains, ranging from 20 in strain 15 to 47 in strain 21. This variation could be ascribed to different levels of heterozygosity in the examined strains; this could depend upon different strategies of artificial selection (outcross versus imbreeding) during the origin and isolation of the four strains.

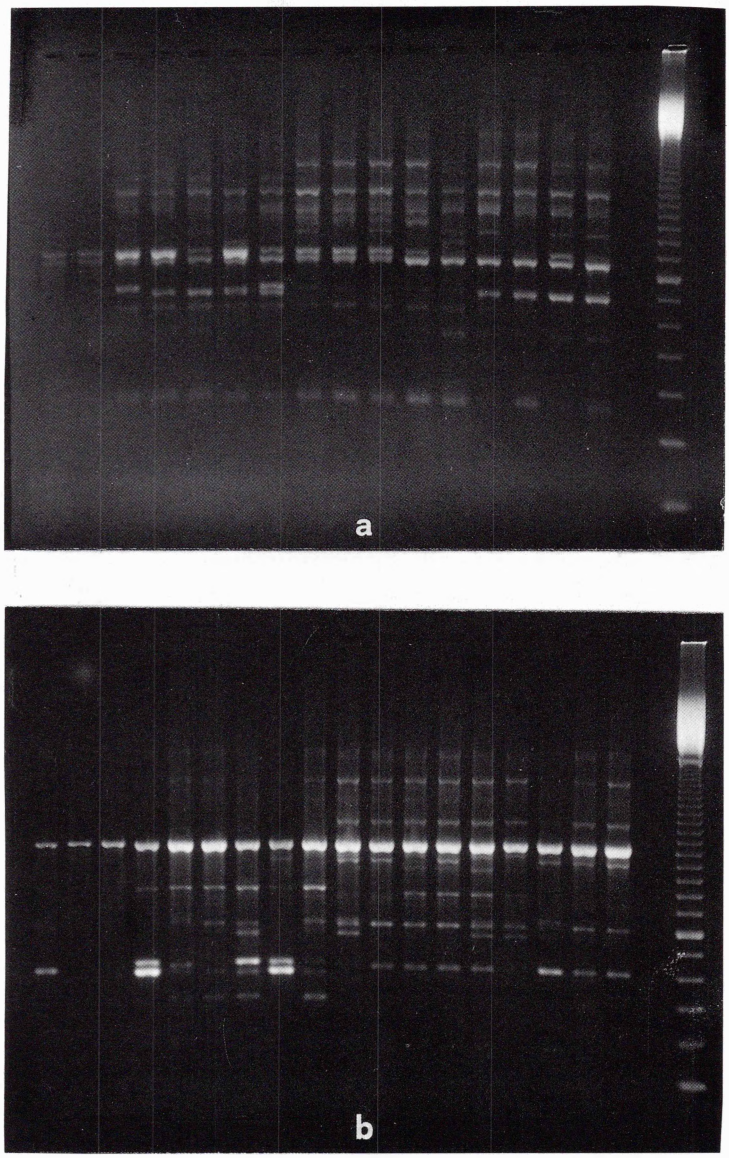

Fig. 1 - Amplification products of *Cannabis sativa* samples.
(a) primer 1; (b) primer 2. 100 bp DNA ladder extreme right;
the bright fragment is 800 bp long.

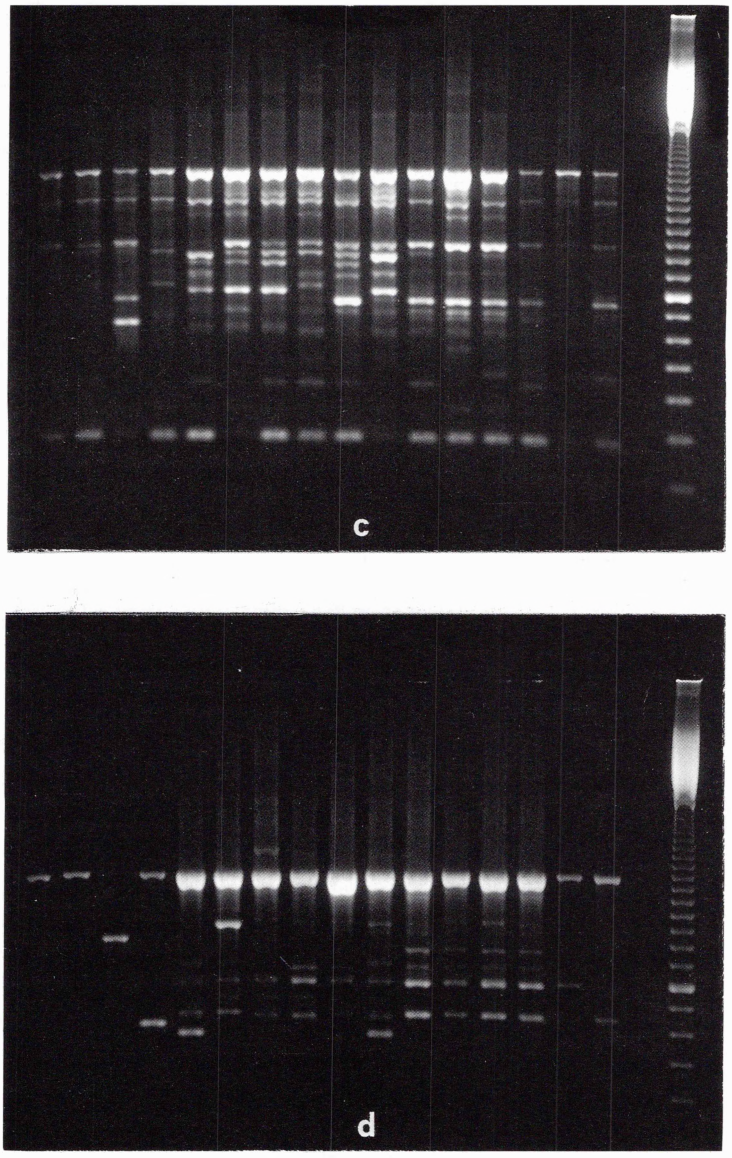

Fig. 2 - Amplification products of *Cannabis sativa* samples.
(c) primer 3; (d) primer 4. 100 bp DNA ladder extreme right;
the bright fragment is 800 bp long.

Tab. 1 - Primers, sequence, number of bands for strain and size of the fragments detected by random PCR amplification of the samples in study.

Primer	Sequence	Number of bands for strains				Size (bp)
		15*	21*	58*	60*	
1	5'-GGTGCGGGAA-3'	2	12	9	10	350 - 1900
2	5'-GTAGACCCGT-3'	3	10	8	10	400 - 2200
3	5'-AAGAGCCCGT-3'	11	16	9	15	300 - 1700
4	5'-CCCGTCAGCA-3'	4	9	3	8	600 - 1500

*The origin of each strain are reported in the material and methods section.

RAPDs, in fact, are presumedly codominantly inherited (WILLIAMS et al., 1990; HADRYS et al., 1992); this implies that unexpected increases in the number of different fragments in one strain may be interpreted as a consequence of outcrossing.

UPGMA analysis of the distance matrix (Tab. 2) yielded a phenogram (Fig. 3), which indicates that PCR amplification using arbitrary primers clearly differentiates the four strains. In fact, members of each strain always cluster together and different strains always belong to different clusters; moreover, the within-strain distances are, for any possible pair of specimens, always shorter than between-strain distances.

A 1000 bp band was present only in strain 60 (primer 4). By using different primers which find unique bands in each strain, these bands may be used as genetic markers for strain recognition or in future breeding programmes.

Tab. 2 - Euclidean distance between all the specimens in study. Individuals are arbitrarily labelled with a letter (A-F).

15A	0.000	1.732	2.236	1.732	2.828	2.236	4.472	4.899	4.899	5.196	4.472	5.292
15B	1.732	0.000	1.414	2.000	3.317	2.000	4.359	5.000	4.796	4.899	4.359	5.196
15C	2.236	1.414	0.000	2.449	3.000	2.449	4.359	5.000	4.796	5.099	4.359	5.196
15D	1.732	2.000	2.449	0.000	3.317	2.449	4.583	5.000	5.000	5.292	4.359	5.385
15E	2.828	3.317	3.000	3.317	0.000	3.606	4.899	5.477	5.477	5.916	5.099	5.831
15F	2.236	2.000	2.449	2.449	3.606	0.000	4.359	5.000	5.000	4.899	4.359	5.196
21A	4.472	4.359	4.359	4.583	4.899	4.359	0.000	3.742	3.742	4.123	3.464	3.742
21B	4.899	5.000	5.000	5.000	5.477	5.000	3.742	0.000	2.828	3.317	3.464	3.162
21C	4.899	4.796	4.796	5.000	5.477	5.000	3.742	2.828	0.000	3.317	3.742	2.828
21D	5.196	4.899	5.099	5.292	5.916	4.899	4.123	3.317	3.317	0.000	3.873	3.000
21E	4.472	4.359	4.359	4.359	5.099	4.359	3.464	3.464	3.742	3.873	0.000	3.742
21F	5.292	5.196	5.196	5.385	5.831	5.196	3.742	3.162	2.828	3.000	3.742	0.000
58A	4.359	4.472	4.472	4.472	4.796	4.690	5.000	5.000	4.796	4.899	4.123	5.196
58B	4.359	4.690	4.690	4.690	4.796	4.690	5.196	5.000	4.796	4.899	4.583	5.196
58C	4.359	4.690	4.690	4.690	4.796	4.690	5.000	4.796	4.583	4.899	4.359	5.000
58D	4.243	4.583	4.583	4.359	4.690	4.583	5.099	4.899	4.690	4.796	4.243	5.099
58E	4.472	4.796	4.796	4.583	4.899	4.796	5.099	4.899	4.690	4.796	4.243	5.099
58F	4.472	4.796	4.796	4.583	4.899	4.796	5.099	4.899	4.690	4.796	4.243	5.099
60A	5.385	5.477	5.477	5.292	5.745	5.099	5.000	4.796	4.796	4.243	4.123	4.359
60B	5.292	5.568	5.385	5.385	5.657	5.196	4.690	4.690	4.899	4.583	4.243	4.243
60C	5.292	5.568	5.385	5.385	5.657	5.196	4.472	4.690	4.690	4.583	4.472	4.243
60D	5.568	5.657	5.477	5.477	5.916	5.292	4.583	4.583	4.583	4.472	4.123	4.123
60E	5.657	5.745	5.745	5.568	6.164	5.385	4.899	4.899	4.899	4.359	4.690	4.472
60F	5.196	5.292	5.292	5.099	5.745	4.899	4.583	4.796	4.583	4.472	4.583	4.359

Tab. 2 - (Continued).

15A	4.359	4.359	4.359	4.243	4.472	4.472	5.385	5.292	5.292	5.568	5.657	5.196
15B	4.472	4.690	4.690	4.583	4.796	4.796	5.477	5.568	5.568	5.657	5.745	5.292
15C	4.472	4.690	4.690	4.583	4.796	4.796	5.477	5.385	5.385	5.477	5.745	5.292
15D	4.472	4.690	4.690	4.359	4.583	4.583	5.292	5.385	5.385	5.477	5.568	5.099
15E	4.796	4.796	4.796	4.690	4.899	4.899	5.745	5.657	5.657	5.916	6.164	5.745
15F	4.690	4.690	4.690	4.583	4.796	4.796	5.099	5.196	5.196	5.292	5.385	4.899
21A	5.000	5.196	5.000	5.099	5.099	5.099	5.000	4.690	4.472	4.583	4.899	4.583
21B	5.000	5.000	4.796	4.899	4.899	4.899	4.796	4.690	4.690	4.583	4.899	4.796
21C	4.796	4.796	4.583	4.690	4.690	4.690	4.796	4.899	4.690	4.583	4.899	4.583
21D	4.899	4.899	4.899	4.796	4.796	4.796	4.243	4.583	4.583	4.472	4.359	4.472
21E	4.123	4.583	4.359	4.243	4.243	4.243	4.123	4.243	4.472	4.123	4.690	4.583
21F	5.196	5.196	5.000	5.099	5.099	5.099	4.359	4.243	4.243	4.123	4.472	4.359
58A	0.000	3.464	3.464	3.317	3.000	3.000	4.899	5.000	5.196	5.292	5.568	5.292
58B	3.464	0.000	1.414	1.732	1.732	1.732	4.243	4.796	5.000	4.899	5.000	4.690
58C	3.464	1.414	0.000	2.236	1.732	1.732	4.243	4.583	4.796	4.690	5.000	4.472
58D	3.317	1.732	2.236	0.000	1.414	1.414	4.123	4.472	4.690	4.583	4.899	4.583
58E	3.000	1.732	1.732	1.414	0.000	0.000	4.123	4.472	4.690	4.583	4.899	4.583
58F	3.000	1.732	1.732	1.414	0.000	0.000	4.123	4.472	4.690	4.583	4.899	4.583
60A	4.899	4.243	4.243	4.123	4.123	4.123	0.000	3.000	3.606	3.162	3.000	3.162
60B	5.000	4.796	4.583	4.472	4.472	4.472	3.000	0.000	2.000	2.236	3.162	3.000
60C	5.196	5.000	4.796	4.690	4.690	4.690	3.606	2.000	0.000	2.646	2.828	2.646
60D	5.292	4.899	4.690	4.583	4.583	4.583	3.162	2.236	2.646	0.000	2.236	2.449
60E	5.568	5.000	5.000	4.899	4.899	4.899	3.000	3.162	2.828	2.236	0.000	2.236
60F	5.292	4.690	4.472	4.583	4.583	4.583	3.162	3.000	2.646	2.449	2.236	0.000

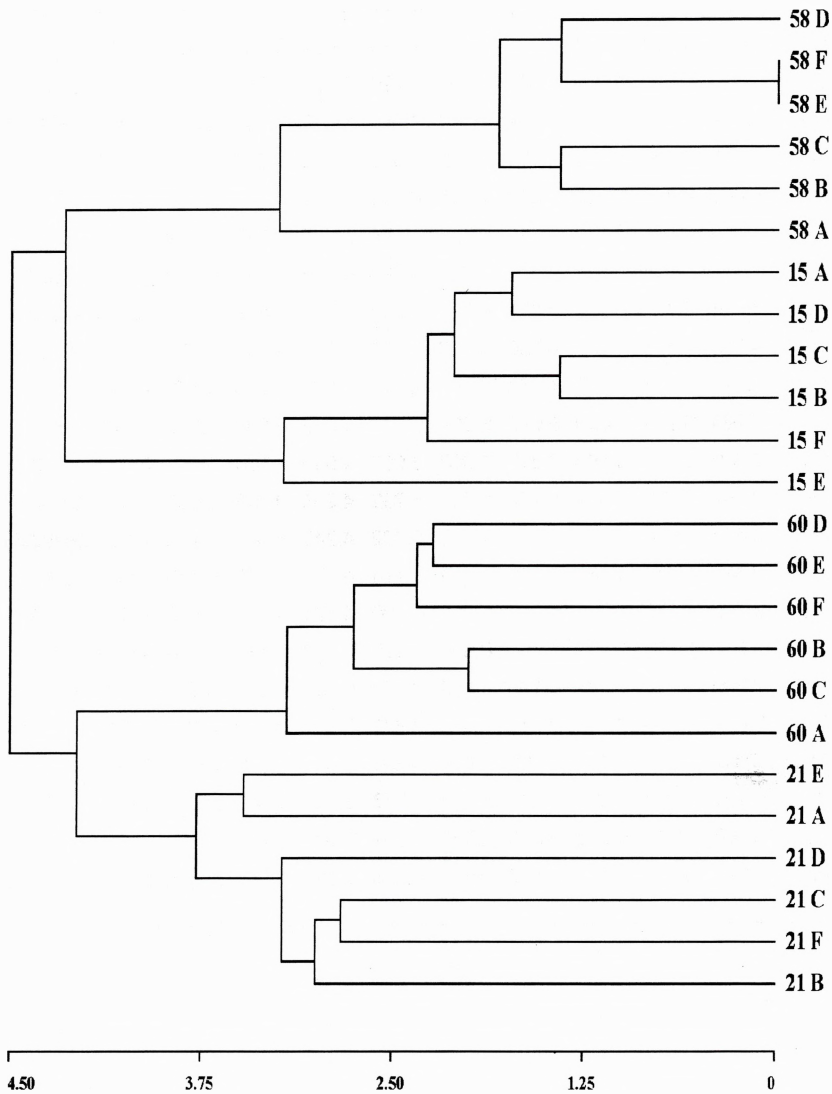

Fig. 3 - Phenogram showing the relationships among specimens.
Specimens are arbitrarily labelled with a letter (A-F).

Our findings represent an expansion of previous work by GILLAN and collaborators (1995). In that paper the authors showed that RAPD technique allowed the characterisation of individuals belonging to a single strain of *Cannabis*. Other researchers (FAETI et al., 1996) demonstrated that genetic diversity in *Cannabis* may be assessed by using RAPDs, finding on average a low number of bands per primer. In this paper, we suggest that RAPD analysis may be usefully employed to characterise different strains; in addition, by extending the study to a large number of different *Cannabis* accessions, the preparation of a database of strain and primer-specific RAPD markers may grant quick identification of a *Cannabis* specimen of unknown origin.

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