A STUDY OF INFRASPECIFIC FLAVONOID VARIATION OF *CANNABIS SATIVA* L. (CANNABACEAE)

by

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ABSTRACT

The genus Cannabis has been treated taxonomically by several authors, but two main hypotheses predominate. Small and Cronquist (1976) concluded that the genus is monotypic and contains only Cannabis sativa L. Subspecies were named according to the economic purpose of the plants, i.e. fibre or drug, and varieties were assigned according to habitat (wild or cultivated). Schultes (1974) believed the genus is polytypic, with Cannabis sativa L. representing the fibre plants, C. indica Lam. the drug plants, and C. ruderalis Janis. the wild and weedy plants.

A study of flavonoid variation was undertaken to determine which of the taxonomic schemes would be supported by this variation. Individual plants were examined by thin-layer chromatography and presence/absence data for nine flavonoids were analyzed by three types of computer programs.

The infraspecific variation of flavonoids in the genus Cannabis supports the monotypic theory of Small and Cronquist (1976). No consistent, distinguishable groups were noticeable in the data. Wild taxa were more closely related to their cultivated progenitors than they were to each other. A division of the genus Cannabis into several species would not be supported by flavonoid variation.

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1. INTRODUCTION

The family Cannabaceae consists of two economically important genera, Humulus (hops) and Cannabis (marijuana). Although these genera have been placed in Moraceae by Schultes (1969) and Hayward (1938), there is now general agreement that they belong in a separate family (Benson, 1957; Cronquist, 1968; Fernald, 1950; Hutchinson, 1969; and Ram and Nath, 1964). As both genera have been cultivated for a very long time, their evolution has been strongly controlled by man. Instead of adapting to natural selection pressures, these plants have been subjected to selection for attributes which man prefers. Humulus spp. are used in the brewing industry while Cannabis is a triple purpose plant, providing hemp fibre, seed oil, and narcotic resin.

The use of Cannabis can be traced back 8500 years when it was reported in Chinese documents (Schultes, 1969). Many references were found in Roman and Greek writings wherein its use in making rope and cloth was described (Schultes, 1973). In India, Cannabis has been, and still is, used for a wide variety of medicinal and religious purposes.

Cannabis sativa was first described by Carl von Linné in 1753, from material which probably represented commercial hemp crops grown in Europe at the time. The plant has been cultivated there for many years, according to palynological and historical evidence (Stearn, 1974). In 1783, Lamarck described a second species Cannabis indica and indicated its origin as "East Indies", a location which Schultes (1974) interpreted as "eastern India". Lamarck distinguished his new species by its shrubby,

more profusely branched stature and its harder, more cylindrical stem. He pointed out that the plant contained little or no fibre and had a strong tobacco-like odour (Schultes, 1974). In 1924, Janischewsky named a third species of Cannabis which he found growing wild in Western Siberia. Cannabis ruderalis Janis. was described as having morphological and biological characteristics different from C. sativa L. The seeds of this weedy plant were covered by remains of the perianth and, due to a caruncle-like growth at the base, fell from the plant on maturation (Schultes, 1974).

With the increased interest in the narcotic properties of marijuana, the taxonomy of Cannabis sativa L. has recently undergone detailed scrutiny. Two taxonomic schemes have emerged. Small and Cronquist (1976) have concluded that the genus is monotypic, consisting solely on Cannabis sativa L. They recognize two subspecies based on chemical characters: ssp. sativa (fibre-type) and ssp. indica (drug-type). They segregated two varieties in each subspecies according to their habitat. Cannabis sativa ssp. sativa var. sativa and C. sativa ssp. indica var. indica represent cultivated plants while C. sativa ssp. sativa var. spontanea and C. sativa ssp. indica var. kafiristanica exemplify the wild or feral habit. Schultes (1974), after examination of representative specimens from Afghanistan, Russia, and the United States, maintained that there were three species in the genus: Cannabis sativa L. representing the fibre plants, C. indica Lam. the drug plants, and C. ruderalis Janis. the wild plants. Anderson (1974) compared the anatomy of a Cannabis stem from Afghanistan with a stem from Kansas and concluded

that the two plants belonged to different species. Emboden (1974) also believes the genus is polytypic.

In their analysis of the genus, Small and Beckstead (1973) examined the cannabinoid content of 350 stocks of Cannabis and found that these compounds reflected man's artificial selection. Members of the genus could be separated into two main groups; those which produced a resin high in tetrahydrocannabinol (THC) and those which produced greater amounts of cannabidiol (CBD) in the resin. Small and Cronquist (1976) concluded that man's selection for drug cultivars had resulted in the high THC strain while his selection for fibre cultivars results in the high CBD strain. Small and Cronquist found a correlation between geographic origin and cannabinoid content; those with high THC concentrations were equatorial in distribution while those plants with high CBD in the resin were from more northerly regions.

The purpose of the present study was to determine the flavonoid composition of Cannabis and to relate this to existing taxonomic schemes. Flavonoids were chosen because they show considerable structural diversity, they are chemically stable, and they are rapidly and easily identified (Harborne, 1975). Furthermore, the value of flavonoids in systematic studies is well known (Harborne, 1967, 1974; Heywood, 1974). Since man has made no practical use of flavonoids of Cannabis, their occurrence and variation may reflect natural selection pressures, rather than artificial selection. However, as man selected for plants capable of producing greater amounts of fibre or drug, he might have been unconsciously selecting for changes in flavonoid composition.

II. METHODS

Seeds for material examined in the present study were obtained from Ernest Small, Biosystematics Research Institute, Agriculture Canada (see Table I).

Table I.

Population #	Number of Individuals	Country of Origin (Small, 1976)
1	8	ruderal, A.W. Haney, Champagne Co., Illinois,USA
2	13	ruderal, H. Scholtz, Berlin, Germany
3	7	D. Mulindwa, Dept. Agriculture, Kampala, Uganda
4	6	Ministry of Agriculture and Natural Resources, Reduit, Mauritius (Bulk Extract)
5	6	Instytut Przemyslu Wlokien Lykowych, Bialobreskie, Poland
6	4	"fibridia", M. Arnoux, Fed. Nat. Prod. de Chanvre, Le Mans, France (originally from Germany)
7	9	University of Mississippi (originally from India)
8	2	T.C. Nel, Pretoria, South Africa
9	3	Phnom-Penh, Cambodia

Seeds were germinated in flats and, after approximately two weeks growth, the seedlings were transplanted into four inch pots and returned to the growth chamber. For growth conditions, see Table 2. After flowering was complete, plants were harvested individually and air-dried in a plant press. Voucher specimens are housed at UBC.

Table II. Growth Conditions

Chamber:	CONVIRON PGW-36	
Lighting:	Bank 1: 6 a.m.- 9 p.m.	Planted (flats): 13 MAY 76
	Bank 2: 9 a.m.-7 p.m.	Transplanted (pots): 16-22 MAY 76
	Bank 3: 10 a.m.-4 p.m.	Humidity: 60%
Temperature:	Day 28 C.	10 hour photoperiod
	Night 10 C.	

For flavonoid identification, six plants of population #4 (33 gm. dry weight) were combined and extracted with n-hexane to remove resinous material. They were then extracted repeatedly with boiling 80% methanol until the extract was colorless. All methanol extracts were pooled and evaporated to dryness on a rotary evaporator. The residue was mixed with Celite in hot water and filtered under vacuum. The aqueous filtrate was saturated with sodium chloride (NaCl) and extracted first with ethyl acetate and then with n-butanol. Both organic solutions were evaporated to yield a bulk flavonoid-containing powder.

The powder was dissolved in 20% methanol for column chromatography. The first column used in this study consisted of Sephadex LH-20 which was eluted, first with water, and then with methanol:water solutions ranging from 10 - 100% methanol, in steps of 10%. Finally, the column was washed with acetone. In this way, flavonoid diglycosides are separated from monoglycosides. Aglycones are removed with the acetone wash. The columns were run in a darkroom where they could be

monitored with an ultraviolet (UV) lamp at 3660 nm. Fractions were evaporated and analyzed by thin-layer chromatography (TLC), usually in organic (#1) and aqueous (#2) solvent systems (see Table 3). Those fractions with similar compounds were combined.

Table III. Chromatographic media and solvent systems.

	Solvent	Medium	Use
#1	Benzene/methanol/2-butanone/ water (55:20:22:3)	Polyamide DC 6.6	Thin layer chromatography
#2	Water/n-butanol/acetone/ dioxane (70:15:10:5)	Polyamide DC 6.6 " SC 6	TLC Column chromatography (CC)
#3	Acetic Acid (10%)	Cellulose	TLC
#4	1,2-dichloroethane/methanol/ 2-butanone/water (50:25:21:5)	Polyamide DC 6.6 " SC 6	TLC CC
#5	Water/2-butanone/acetone/ formic acid (60:30:9:1)	Polyamide DC 6.6 " SC 6	TLC CC

Avicel (microcrystalline cellulose) columns are used to separate flavonoids and their glycosides based on both absorption and partition (Markham & Mabry, 1975). Avicel was suspended with water in a water-saturated solution of ethyl acetate:petroleum ether (60:40) and packed into a column. A sample of flavonoid, adsorbed onto Avicel, was added and the column was eluted with an ethyl acetate:petroleum ether solution beginning at 60:40 and progressing in increments of 10% ethyl acetate. Increments (5-10% each) of 2-butanone (MEK) were added if compounds failed to elute with 100% ethyl acetate. In this manner, compounds of low polarity (few hydroxyl groups) were removed from the stationery phase (water) before

compounds with more hydroxyl groups. Fractions were monitored by TLC using solvents #1 and #2 (see Table III). Fractions containing similar compounds were combined. For preparative work, columns of Avicel were eluted with 10% acetic acid.

Polyamide SC 6 proved to be effective as a column medium and was especially useful for clean-up of fractions prior to instrumental analysis. This medium is also very versatile and can be eluted with several different solvent systems. For example, diglycosides separate well with solvent #2, while monoglycosides of flavonols and C-glycosyl flavones can be resolved with solvent #4 (see Table III). Columns of Polyamide SC 6 can be run quickly, washed and reused in a short period of time.

For thin-layer chromatography, plates of Polyamide DC 6.6 (.3 mm.), spread by hand, provided consistent, reproducible R_f values. Plates of cellulose were used occasionally. Developed chromatograms were air-dried and sprayed with a 0.1% solution of diphenyl boronate (in methanol:water, 1:1), a compound which produces characteristic colors after reaction with flavonoids. After 5 - 10 minutes, chromatograms were viewed under UV light at 3660 nm. Pure fractions were chromatographed in several solvent systems and R_f values were recorded. If impurities were present, the solution was streaked on a TLC plate and developed in a suitable solvent. Once separation was accomplished, the compound was outlined under UV light and scraped off the plate with the medium. The purified compound was then eluted from the medium with methanol.

When pure flavonoid material was obtained, the compound was subjected to anumber of analytical techniques. First, a sample was hydrolyzed with dilute trifluoroacetic acid (5-10 drops in 2 mls. water) at 100 C. for two hours. The hydrolysate was evaporated to remove excess acid, water was added and the solution was extracted with ethyl acetate. The ethyl acetate fraction which contained the aglycones was evaporated; the residue was taken up in a small volume of methanol and chromatographed on Polyamide. The water fraction containing sugars was evaporated to dryness, taken up in one or two drops of methanol:water (1:1), and was chromatographed on Cellulose in solvent #6 (see Table III). The sugar chromatograms were sprayed with p-anisidine phthalate, heated at 100 C. for ten minutes, and viewed under UV light.

Another sample of the purified compound was analyzed by UV spectrophotometry according to the procedures outlined by Mabry et. al., (1970). With various shift reagents, spectra were recorded and patterns of hydroxylation on the flavonoid skeleton were determined. Results were compared with published data (Mabry et. al., 1970). In some cases, enough flavonoid was obtained to enable the proton magnetic resonance spectrum (PMR) of the trimethylsilyl derivative to be determined. These spectra were also compared to published data (Mabry et. al., 1970).

For individual plant analysis, leaves and flowers were stripped from pressed specimens and placed in a Soxhlet extraction thimble. Each sample was extracted with n-hexane for eight hours to remove resinous material. Samples were then extrated with methanol (80%) for eight hours. Each methanol solution was treated as above for the bulk extract, yielding a flavonoid-containing powder. Semi-quantitative analysis was

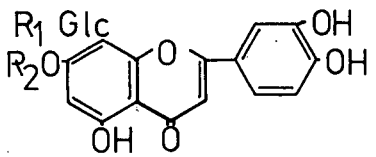
done in the following manner: 500 mg. powder was dissolved in 1.0 ml. methanol and 5 μl. of the solution was spotted on the TLC plate. Plates were developed in solvent #1, air-dried thoroughly and developed in the second direction in solvent #2 (see Table III). Compounds were located on the chromatograms by means of color reaction and assigned a rank of zero or one indicating presence or absence. These data were used for numerical analysis.

After individual analyses were complete, plant extracts containing novel or minor compounds were combined and the minor flavonoids were isolated.

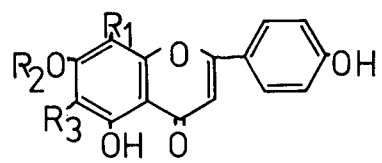
III. RESULTS

The flavonoids of Cannabis were first examined by Bate-Smith (1962). He identified kaempferol in a hydrolyzed leaf extract. In 1973, Paris and Paris studied material which had been grown in France and found luteolin, apigenin, apigenin-7-glucoside, orientin, orientin-7-glucoside, vitexin, and vitexin-O-glucoside. In a study of pollen of Cannabis plants grown at the University of Paris, Paris et. al. (1975) identified apigenin and luteolin. Gellert et. al. (1975) examined plants from six different countries and found that those from Hungary were poor in flavonoids while those from Thailand and South Africa were rich in flavonoids. They identified quercetin and kaempferol after hydrolysis of extracts. In 1976, Paris and Paris examined the flavonoids of Cannabis for a third time, using more material from the same source as in 1973 (see above). In this study, they were able to identify a total of ten compounds; their structures are presented in Figure 1.

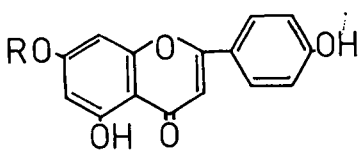
Figure 1. Flavonoids of Cannabis sativa as determined by Paris & Paris (1976).



Orientin (R_1=R_2=H)
Orientin-Glc (R_1=Glc,R_2=H)
Orientin-7-Glc (R_1=H,R_2=Glc)
Orientin-7-GlcRha (R_1=H,R_2=GlcRha)



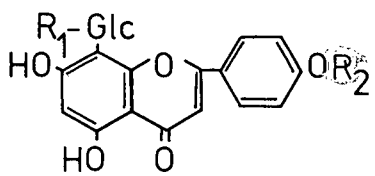
Vitexin-7-Glc (R_1=R_2=Glc,R_3=H)
Vitexin-7-GlcRha (R_1=Glc,R_2=GlcRha,R_3=H)
Isovitexin (R_1=R_2=H,R_3=Glc)
Isovitexin + Glc + Ara (R_3=Glc)
Isovitexin + Glc + Rha (R_3=Glc)



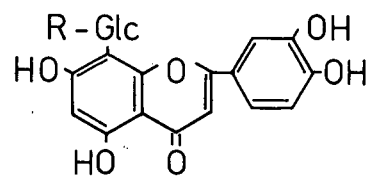
Apigenin-7-O-(p-coumaric acid)-glucoside (R=Glc-coumaric acid)

The present study confirms the presence of flavonol glycosides and C-glycosyl flavones in Cannabis sativa L. Some previously reported flavonoids were not found in the present study while some occurrences are described herein for the first time. This is the first study of variability of flavonoids in the genus. A total of nine flavonoids were identified; their structures are presented in Figure 2.

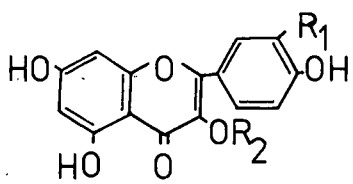
Figure 2. Flavonoids of Cannabis sativa L. as determined in the present study.



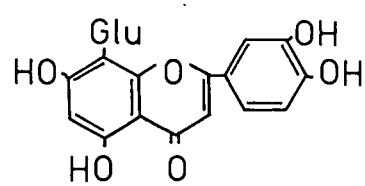
Vitexin (R_1=R_2=H)
Cytisoside (R_1=H,R_2=CH_3)
Vitexin-glucoside (R_1=Glc,R_2=H)
Cytisoside-glucoside (R_1=Glc,R_2=CH_3)



Orientin (R=H)
Orientin-glucoside (R=Glc)



Quercetin-3O-glucoside (R_1=OH,R_2=Glc)*
Quercetin-3-O-diglucoside (R_1=OH,R_2=GlcGlc)
Kaempferol-3-O-diglucoside (R_1=H,R_2=GlcGlc)

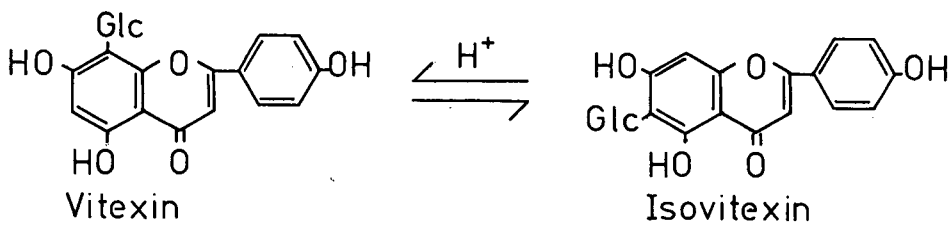


C-Glycuronic luteolin

* = identified as a minor constituent

The first compound identified was orientin (8-C-glucosyl luteolin) which gave, on acid hydrolysis, an equilibrium mixture of orientin and iso-orientin. This Wessely-Moser rearrangement (Chopin and Bouillant, 1975) involves cleavage of the ether linkage, rotation of the A-ring, and reformation of the heterocyclic ring. The result is the interchange of positions C-6 and C-8 (see Figure 3). The compound with the higher R_f in aqueous solvent (see Table III) is the 8-glucosyl isomer.

Figure 3. Wessely-Moser rearrangement of a C-glycosyl flavone.



The B-ring substitution of orientin (ortho-dihydroxyl) was recognizable by its characteristic bright lemon-yellow fluorescence which developed after it was sprayed with the diphenyl boronate reagent. This was supported by UV analysis (see Table V) and comparison of UV spectra with published data (Mabry et. al., 1970). The compound was chromatographed against an orientin standard which had been identified by PMR analysis (Nicholls, unpublished). A glycoside of orientin was isolated which gave the same UV spectrum as orientin and, on acid hydrolysis, yielded glucose and orientin. Since UV analysis indicated no hydroxyl substitution and the aglycone was orientin, it was concluded that the glucose was linked to one of the C-

glucosyl hydroxyl groups. The compound was not obtained in sufficient quantity to permit PMR analysis, so it is represented only as orientin-x"-O-glucoside.

Vitexin (8-C-glucosyl apigenin) was recognizable on TLC because its reaction with diphenyl boronate produced an olive-green fluorescence. Its structure was confirmed by UV analysis (see Table V). Hydrolysis in TFA produced the characteristic Wessely-Moser rearrangement product; isovitexin appeared as a lower spot in aqueous-solvent TLC analysis (see Table III). A glycoside of vitexin was also isolated and, after hydrolytic and UV analysis, was partially identified as a vitexin-x"-O-glucoside.

During plant-to-plant variation studies, one specimen was noted to have both 8-C-glycosyl and 6-C-glycosyl iosmers of luteolin and apigenin. Although the small amount eluded isolation, isovitexin and iso-orientin are included as minor constituents.

The third C-glycosyl flavone from Cannabis had higher R_f values in organic solvents (see Table III) but co-chromatographed with vitexin in aqueous solvents. The UV spectrum of the compound closely resembled acacetin (4'-O-methyl apigenin). Proton magnetic resonance analysis revealed a methoxyl group resonance at δ 3.8 (see Figure 4). Hydrolysis produced a Wessely-Moser rearrangement product, indicating a C-glycoflavone. It was concluded that the structure must be 4'-O-methyl vitexin, which is the known cytisoside (Chopin et. al., 1975). The existence of a cytisoside-x"-O-glucoside was supported by hydrolytic and TLC procedures. This is the first reported occurrence of cytisoside and its glucoside in Cannabis sativa.

Figure 4. Proton magnetic resonance spectrum of cytisoside-glucoside.

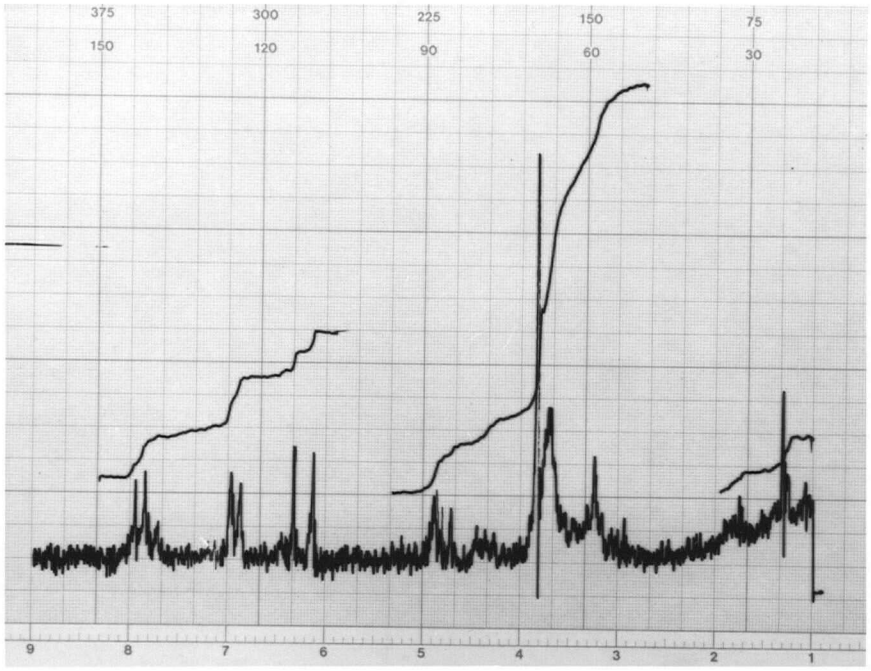


Table IV. TLC Results

	R_f value (for solvent see Table III)			
	#1	#2	#4	#5
Orientin	0.13*	0.30	0.22	0.18
Vitexin	0.33	0.39	0.43	0.22
Cytisoside	0.60	0.44	0.71	0.25
Quercetin-3-O-diglucoside	0.20	0.66	0.35	0.46
Kaempferol-3-O-diglucoside	0.29	0.68	0.48	0.44
Orientin-x"-O-glucoside	0.16	0.70	0.29s	0.50
Vitexin-x"-O-glucoside	0.27	0.69	0.48	0.51
Cytisoside-x"-O-glucoside	0.45	0.71	0.72	0.12
C-glycuronic-luteolin	0.00	0.00	0.00	0.12

(*s indicates streaking on TLC plate.)

Table V. Ultraviolet absorption data.

	UV Maxima (max)					
Compound	Methanol	NaOAc	H_3BO_3	$AlCl_3$	HCl	NaOMe
Orientin (Band II)	258,270	275	264	276	278	274
(Band I)	353	390	375	427	362,389	414
Vitexin	271	279,303s	271	278,306s	279,306s	278,330
	331	363	331	348	346	395
Cytisoside	273	288	284	278,304	278,304	278,300s
	328	352	332	348,382	342,380	370
Quercetin-3-OGlcGlc	262,302s	266	264,302s	278,330	274,300s	282,330
	366	382	383	430	358,400	430
Kaempferol-3-OGlcGlc	267,300s	274,300s	267,300s	277,300s	277,300s	282,320s
	354	370	370	354	346,400	410
Orientin-x"-OGlc	270	276	262	278,302s	278,302s	276,312
	350	382	374	429	429	410
Vitexin-x"-OGlc	274	276	274	278,306s	278,306s	278,330
	332	358	328	350,398	346,396	398
Cytisoside-x"-OGlc	273	288	284	278,304s	378,304s	278,300s
	328	352	332	348,382	342,380	370

The presence of flavonols in Cannabis was suggested by the work of Bate-Smith (1962) and Gellert et. al. (1974). In the present work, quercetin was suspected to be present when orange-yellow fluorescent spots were observed on chromatograms. Once isolated, the compound was hydrolyzed with TFA and produced only quercetin and glucose. Ultraviolet spectra of the compound were compared with published data (Mabry et. al., 1970). Final identification, made by comparison of this compound with a standard in several solvent systems, was quercetin-3-O-diglucoside. A kaempferol derivative was indicated by its lime-green coloration under UV light and by its UV spectra. Comparison with standard material by TLC and hydrolytic procedures confirmed the structure as kaempferol-3-O-diglucoside.

The last major compound proved the most difficult to isolate. on 2-D maps (see Figure 5), it appeared as a lemon-yellow streak which began at the origin and progressed halfway up the chromatogram in aqueous solvent. Because of its low R_f values in most solvents, isolation of a pure compound was not accomplished. The compound did, however, migrate slightly in acidic conditions on TLC (see Table IV). Acid hydrolysis produced a Wessely-Moser rearrangement product, indicating a C-glycoflavone. Base hydrolysis (in 0.1 N NaOH for 5-15 min.) did not change the R_f values, thus eliminating the possibility of an acylated derivative. This compound was tentatively identified as C-glycuronic luteolin.

Flavonoid profiles of individual plants are presented in Table VI.

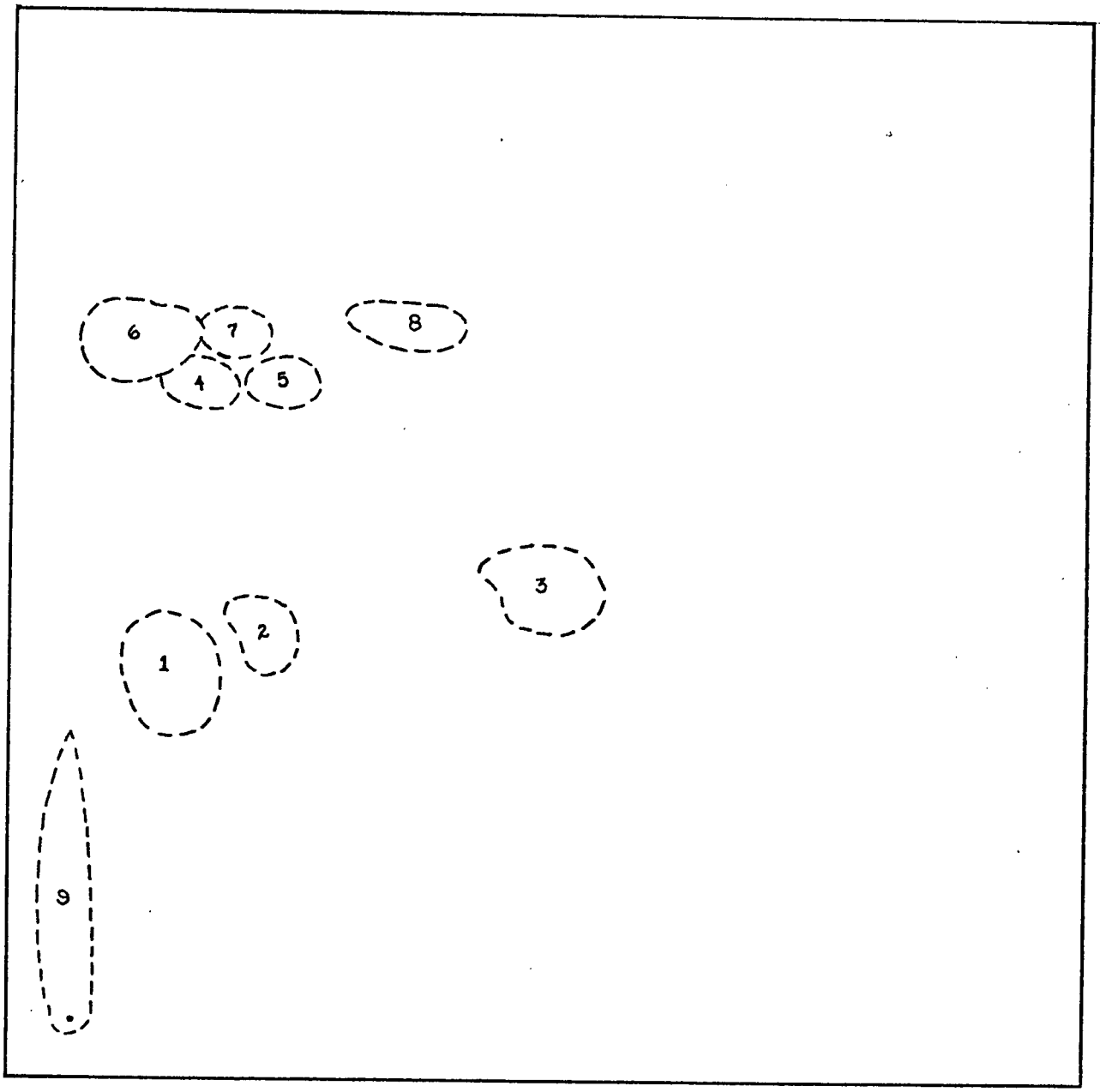


Figure 5. Relative positions of flavonoids of Cannabis sativa L. on two dimensional chromatogram.

1 = Orientin
2 = Vitexin
3 = Cytisoside
4 = Quercetin-diglucoside
5 = Kaempferol-diglucoside
6 = Orientin-glucoside
7 = Vitexin-glucoside
8 = Cytisoside-glucoside
9 = C-glycuronic luteolin

Table VI. Raw data from two-dimensional thin-layer chromatograms.

Plant #	Small & Cronquist's varieties	Compound Occurrence*								
		O	V	C	QGG	KGG	OG	VG	CG	CGL
1	indica	+	+	-	+	+	+	+	-	+
2	"	+	+	+	+	+	+	+	+	-
3	"	+	+	-	+	+	+	-	-	-
4	"	+	+	-	+	+	+	-	-	-
5	"	+	+	-	-	+	+	+	+	-
6	"	+	+	+	-	+	+	+	+	-
7	"	+	+	-	+	+	+	+	+	-
8	"	+	+	+	+	+	+	+	+	-
9	"	+	+	+	+	+	+	-	+	-
10	"	+	+	-	+	+	+	+	-	-
11	"	-	-	-	+	+	+	+	-	-
12	"	+	+	-	+	+	+	-	+	-
13	"	-	-	-	+	+	+	+	-	-
14	"	+	+	-	+	+	+	+	-	-
15	"	+	+	-	+	+	+	+	-	-
16	kafiristanica	+	+	+	+	+	+	+	+	-
17	"	+	+	+	+	+	+	+	+	-
18	"	+	+	+	+	+	+	+	+	-
19	"	+	+	+	+	+	+	+	+	-
20	"	+	+	+	+	-	+	+	+	-
21	"	+	+	+	+	+	+	+	+	-
22	"	+	+	+	+	-	+	+	+	-
23	sativa	+	+	+	+	+	+	-	+	+
24	"	+	+	-	+	+	+	+	-	+
25	"	+	+	-	+	+	+	+	+	+
26	"	+	-	-	+	+	+	+	-	+
27	"	+	+	-	+	+	+	-	+	+
28	"	+	+	-	+	+	+	-	-	+
29	"	-	-	-	+	+	-	-	-	+
30	"	+	+	-	+	+	+	-	+	+
31	"	-	-	-	+	+	-	-	-	+
32	"	+	+	-	+	+	+	+	-	+

Table VI. Cont'd

Plant #	Small & Cronquist's varieties	Compound Occurrence O	V	C	QGG	KGG	OG	VG	CG	CGL
33	spontanea	+	+	-	+	+	+	-	-	+
34	"	-	-	-	+	+	-	-	-	+
35	"	+	+	-	-	-	-	-	-	+
36	"	-	-	-	-	-	+	-	-	+
37	"	+	+	-	+	+	-	-	-	+
38	"	-	-	-	+	+	-	-	-	+
39	"	+	+	-	+	+	+	+	-	+
40	"	+	+	-	+	+	+	-	-	-
41	"	-	-	-	+	+	-	-	-	+
42	"	-	-	-	+	+	-	-	-	+
43	"	+	+	-	+	+	-	-	-	+
44	"	+	+	+	+	+	+	-	+	+
45	"	-	+	-	+	+	+	+	+	+
46	"	+	+	-	-	+	+	+	+	+
47	"	-	-	-	+	+	-	-	-	+
48	"	+	+	-	+	+	-	-	-	+
49	"	-	-	-	+	+	-	-	-	+
50	"	-	-	-	+	+	-	-	-	+
51	"	-	-	-	+	+	-	-	-	+
52	"	-	+	-	+	+	-	-	-	+
53	"	-	-	-	-	+	-	-	-	+
	Mean ($\bar{x}$) =	.71	.74	.26	.89	.93	.70	.47	.40	.59
	Standard Deviation (s) =	.45	.45	.45	.31	.27	.46	.50	.49	.50

* Compounds are coded as follows:

O = Orientin

V = Vitexin

C = Cytisoside

QGG = Quercetin-diglucoside

KGG = Kaempferol-diglucoside

OG = Orientin-glucoside

VG = Vitexin-glucoside

CG = Cytisoside-glucoside

CGL = C-glycuronic luteolin.

IV. BIOSYNTHESIS OF FLAVONOIDS

The C_{15} skeleton of flavonoids is derived from two separate biosynthetic pathways; the B-ring and the central C_3-unit arise from phenylalanine while the A-ring is derived from the polyacetate pathway. Once formed, the C_{15} skeleton is modified in a variety of ways, producing the wide range of flavonoids known to occur in nature. Presumably, each modification requires a unique enzyme or group of enzymes, reflecting a change in the genetic makeup of the plant.

Hydroxylation of the flavonoid skeleton may occur at different stages in the biosynthetic pathway. Hess (1964) concluded that the C_6-C_3 portion (phenylalanine-derived) of the flavonoid was substituted before entering the pathway. Recent studies by Saleh et. al. (1978) support Hess' hypothesis. Others have indicated that B-ring hydroxylation occurs at the flavonol level (Wallace, 1975), or flavone level (Styles and Ceska, 1975). Wallace (1975) also found that B-ring hydroxylation of C-glycosyl flavones occurs at the flavanone (or C-glycosyl flavanone) stage of biosynthesis. Methylation of B-ring hydroxyl groups is probably a one-enzyme reaction, with S-adenosyl methionine as the methyl donor (Halbrock, 1971).

Glycosylation of flavonoids is also under strict genetic control and proceeds in a stepwise manner from mono- to di-glycosides (Miles and Hagen, 1968). Various genetic loci controlling glycosylation have been described including a gene 'Fg' which controls the transfer of glucose to the carbon-bound sugar of isovitexin. This gene segregates independently from one that controls an enzyme responsible for glycosylation of the 7-OH position of isovitexin (van Brederode and van Nigtevecht, 1974).

Extrapolation of biosynthetic data produces a biosynthetic scheme as shown in Figure 6. Although it is interesting to draw parallels from such a scheme, it may be dangerous to do so. Harborne (1967) stated "every plant that has been extensively studied has a unique genetic system for controlling flavonoid synthesis; unique in the sense that the extent of gene interaction present varies from one plant to another".

Levy (1977) suggested that biosynthetic data should be used in taxonomic studies. He calculated "minimum biosynthetic-step distances" (MBSD) and "biosynthetic-step identities" (BSI) and used them to assess relationships between populations. Levy states that construction of putative biosynthetic pathways for flavonoids is relatively simple because:

"i) within species and among related groups, most flavonoid diversity is substitutional,

ii) flavonoid substitution occurs in a discrete, stepwise manner,

iii) different flavonoid nuclei are not interconvertible after substitution has occurred."

While the first and third suppositions are probably correct, the second conclusion is questionable. If two flavonoids differ by only one substitution, (for example, quercetin and kaempferol) then it may be reasonable to infer that they differ by only one biosynthetic step. However from Figure 6, it is obvious that such a conclusion cannot be drawn concerning the similarity between orientin and vitexin, for although they are similar, they represent a difference of several steps. Also, one might conclude that

a flavone (for example, apigenin) and a C-glycoflavone (vitexin) differ by only one step, a sugar substitution, but this too would be a fallacy. In fact, Levy (1977) draws this similarity between luteolin-7-glucoside and orientin.

A biosynthetic pathway, as shown in Figure 6, can be helpful in determining the wide variety of reactions possible in a plant and may be used to assess relationships between plants. Interpretation of such a scheme must be done cautiously to ensure that reticulate portions of the pathway (for example, cytisoside-glucoside production) are fully understood. Also, detailed knowledge of each step in the pathway must be obtained in order to determine if plieotropy is operating. For these reasons, biosynthetic relationships were not used in the study, and each compound was given equal weight in the numerical analysis.

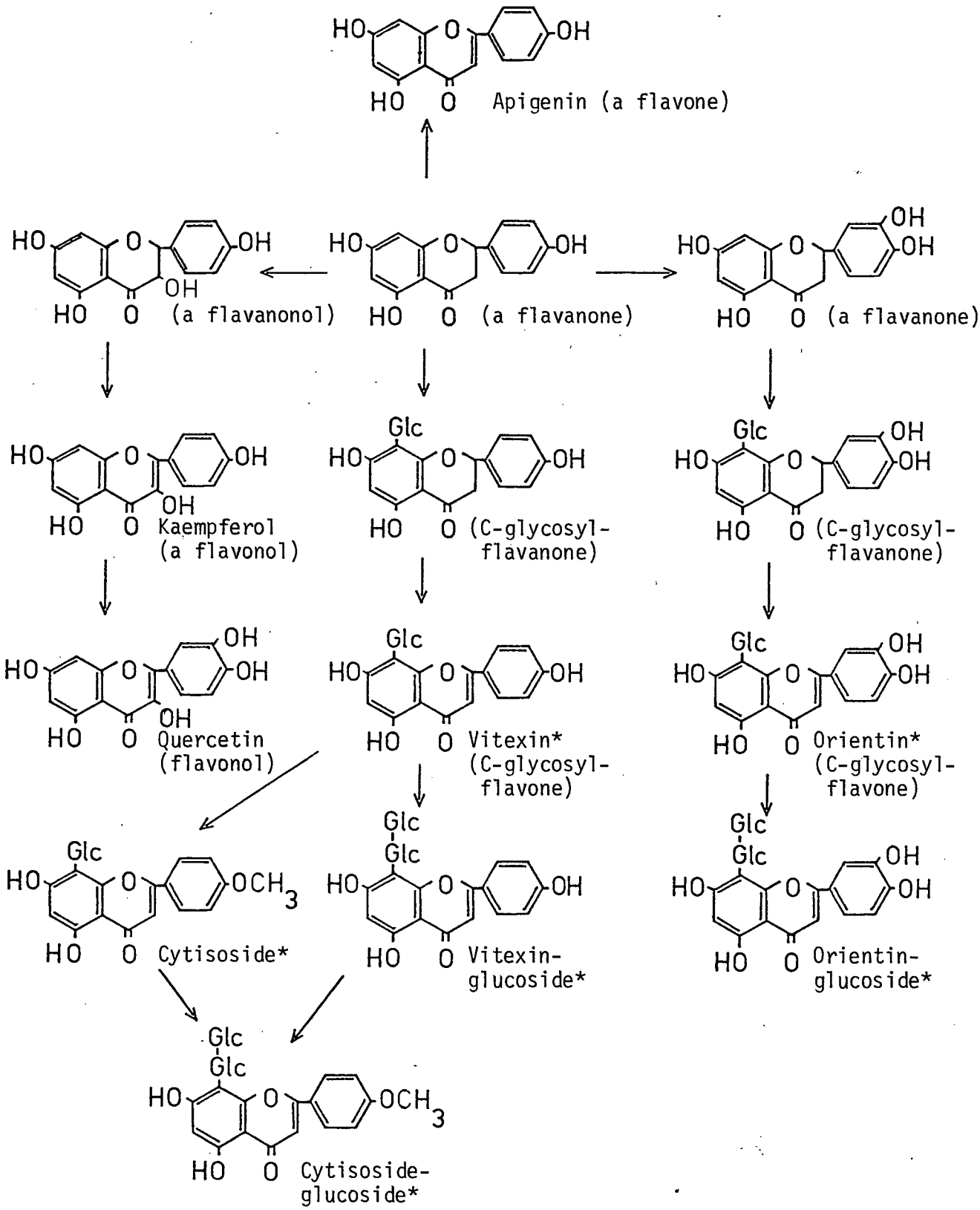


Figure 6. Biosynthetic relationships of some of the flavonoids of Cannabis sativa L. (Only those compounds marked * were found in this study).

V. NUMERICAL ANALYSIS

Data collected from 2-D maps were analyzed by several numerical techniques to determine any groupings which might be present. Sneath and Sokal (1973) stated that two important aims of numerical taxonomy are repeatability and objectivity, and suggested that both ordination and classification techniques be used in a taxonomic study. Data from Table VI were subjected to three types of numerical analyses by a computer, namely, clustering, principle components analysis (PCA), and canonical analysis (CA) (see Seal, 1964, for descriptions of PCA and CA). Cluster analysis was performed using the program UBC CGROUP, which is based on the method of Ward (1963). Principle components analysis was done using a program developed by Dr. G.E. Bradfield, Botany Department, UBC. The program for canonical analysis was taken from the MIDAS package of statistical programs. All analyses were done on an IBM 370/168 computer at the University of British Columbia.

In addition to the three types of computer analysis, the characters in Table VI were converted to standard normal deviates using a desk calculater. The standard normal deviate for each compound was calculated according to the following formula:

$$\text{Standard normal deviate of compound } i = \frac{x_i - \bar{x}_i}{s_i}$$

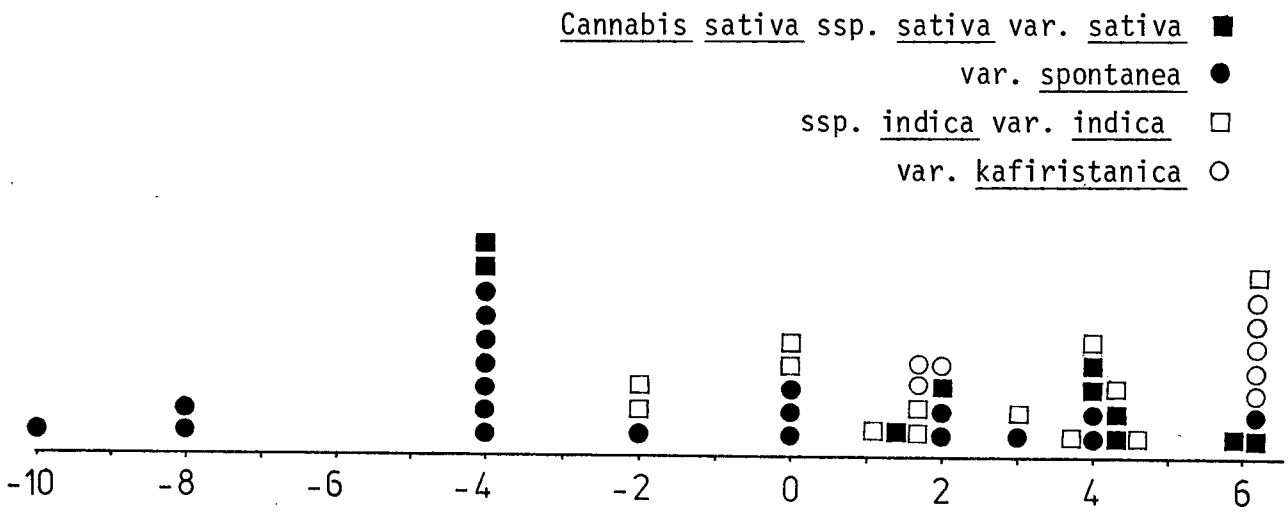
where x_i = original observation (presence or absence) of compound i

$\bar{x}_i$ = the mean of compound i (see Table VI)

s_i = the standard deviation of compound i (see Table VI).

For each plant, the values determined were summed, and the number obtained was located on a single axis. Thus, plants which possessed identical flavonoid profiles were grouped together (see Figure 7).

Figure 7. Standard Normal Deviates of Raw Data



Clustering methods such as CGROUP determine a hierarchical structure within a set of OTU's by minimizing an overall estimate of variation within groups. Results of this method are depicted in a dendrogram. The horizontal axis in the dendrogram indicated the degree of dissimilarity between individuals or groups of individuals which are defined by vertical lines (see Figure 8). Because many groups of individuals had identical flavonoid profiles, their dissimilarity measure was zero. A fundamental aspect of the clustering methods is that, a priori, all characters have equal weight, i.e. each character contributes equally to any decision on relationships (Sneath and Sokal, 1973).

A second method of analysis, PCA, gives an ordination which results in the placement of OTU's in A-space (attribute space of several dimensions). The principle axes are linear combinations of original variables and summarize the major dimensions of variation. Each individual OTU was plotted along six axes (see Figures 9 - 13). The first axis represented the dimension that accounted for the greatest amount of variation (27.13%). The second axis accounted for 14.47% of the variation and so on (see Table VII).

Table VII. Variation contained in each of the PCA axes.

AXIS	% TOTAL	CUMULATIVE TOTAL
1	27.13	27.13
2	14.47	41.60
3	12.73	54.33
4	12.36	66.69
5	9.76	76.45
6	8.46	84.91

The third type of analysis, canonical analysis, is closely related to discriminant analysis; a method which determines the numerical relationships between preassigned groups of individuals. In the results, a group is defined by the co-ordinates of its centroid and the range of variation contained in the population. This technique was done with three different initial groupings of data.

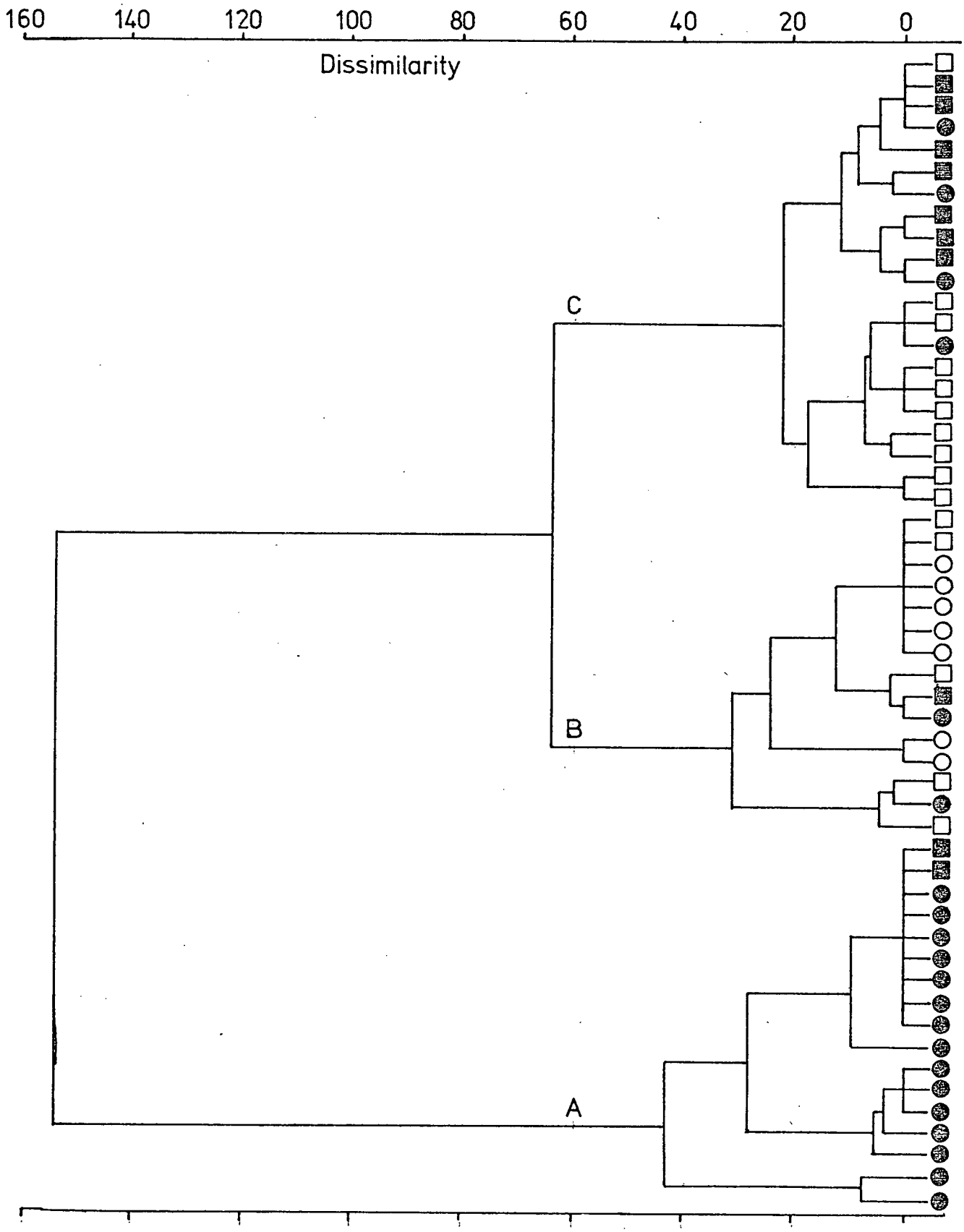


Figure 8. Results of clustering method of analysis.
Symbols represent Cannabis sativa ssp. sativa var. sativa ■
var. spontanea ●
ssp. indica var. indica □
var. kafiristanica ○

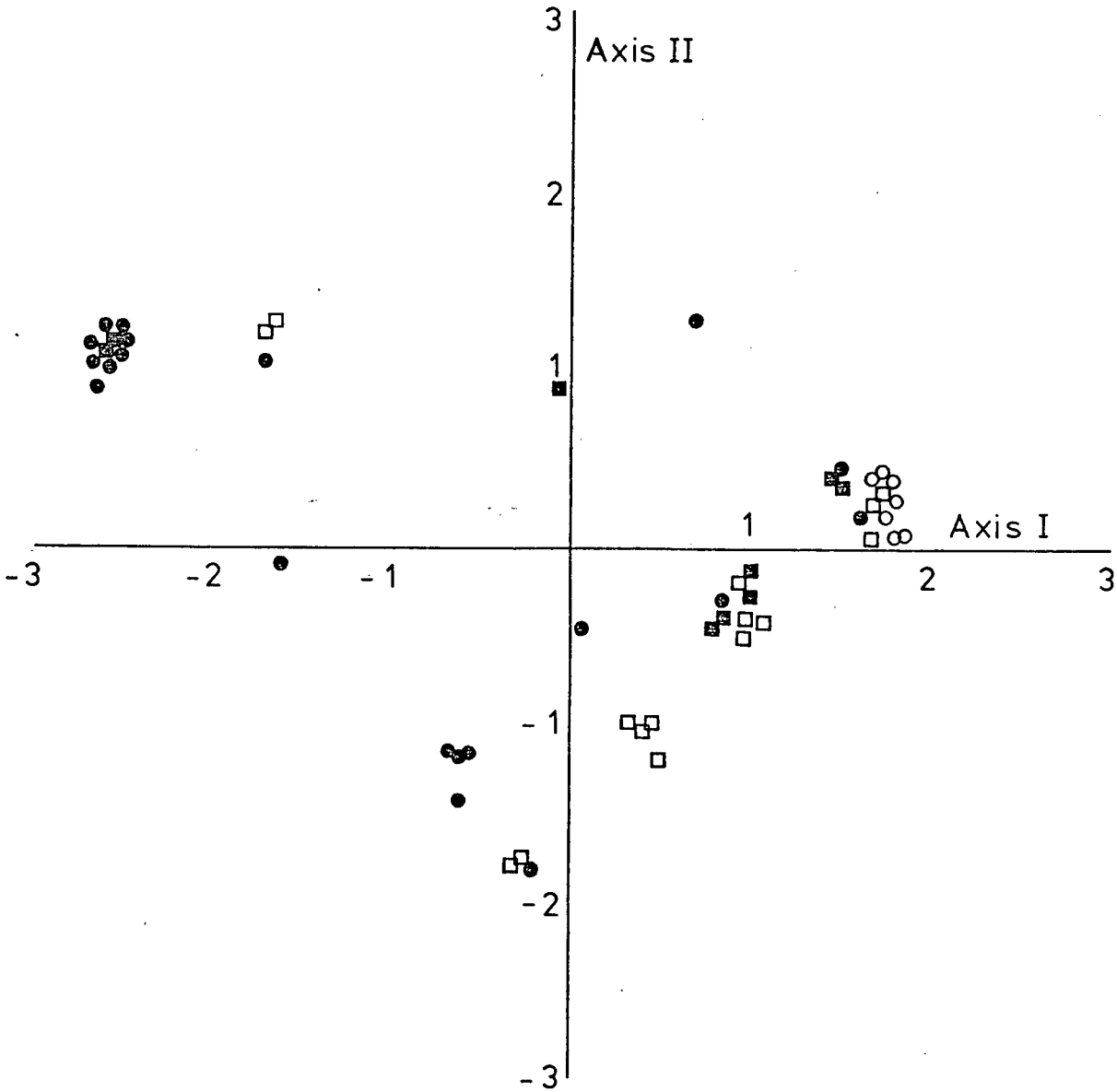


Figure 9. Results of Principle components analysis (Axis 1 vs. 2)

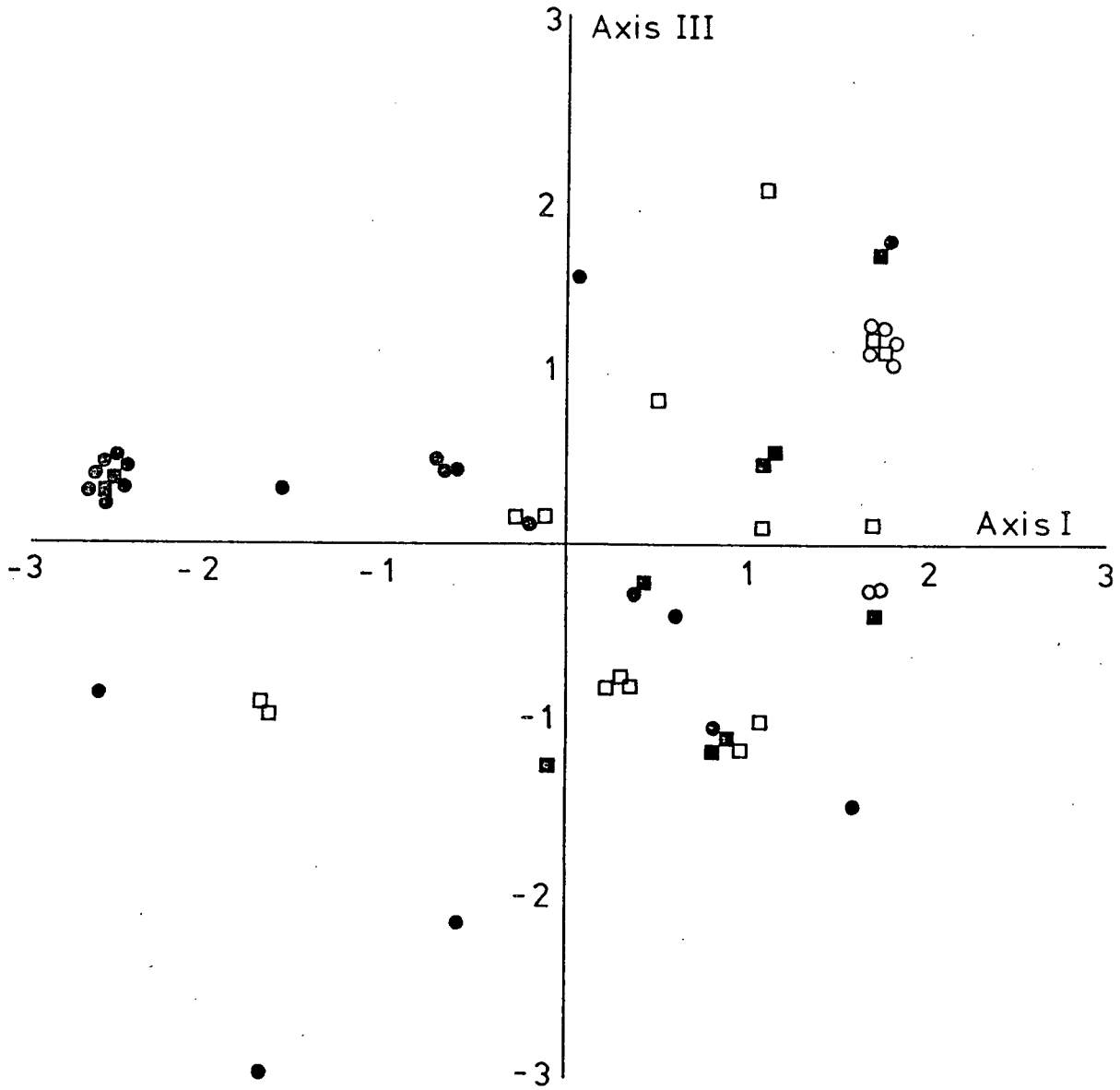


Figure 10. Results of principle components analysis (Axis 1 vs 3)

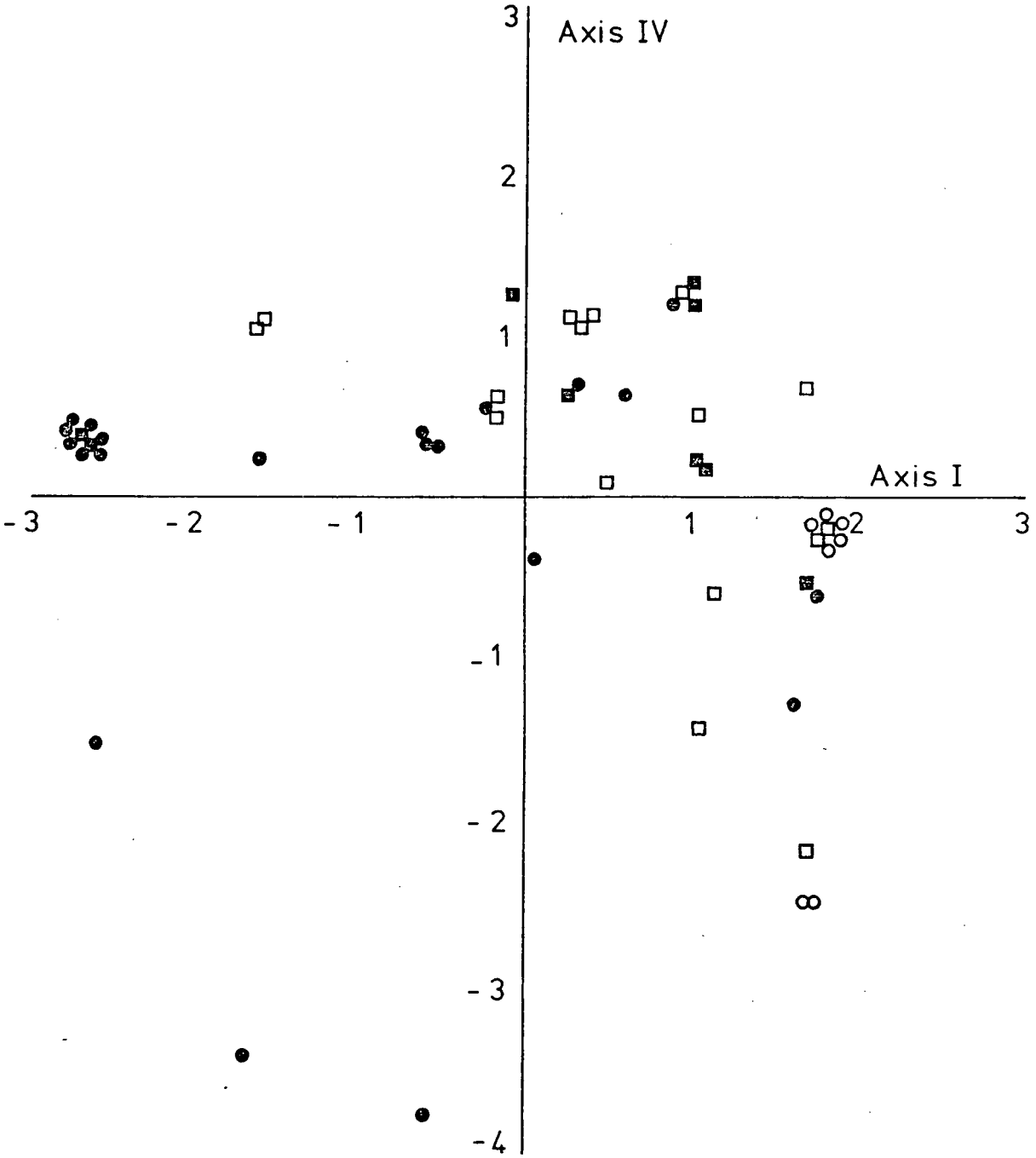


Figure 11. Results of principle components analysis (Axis 1 vs. 4)

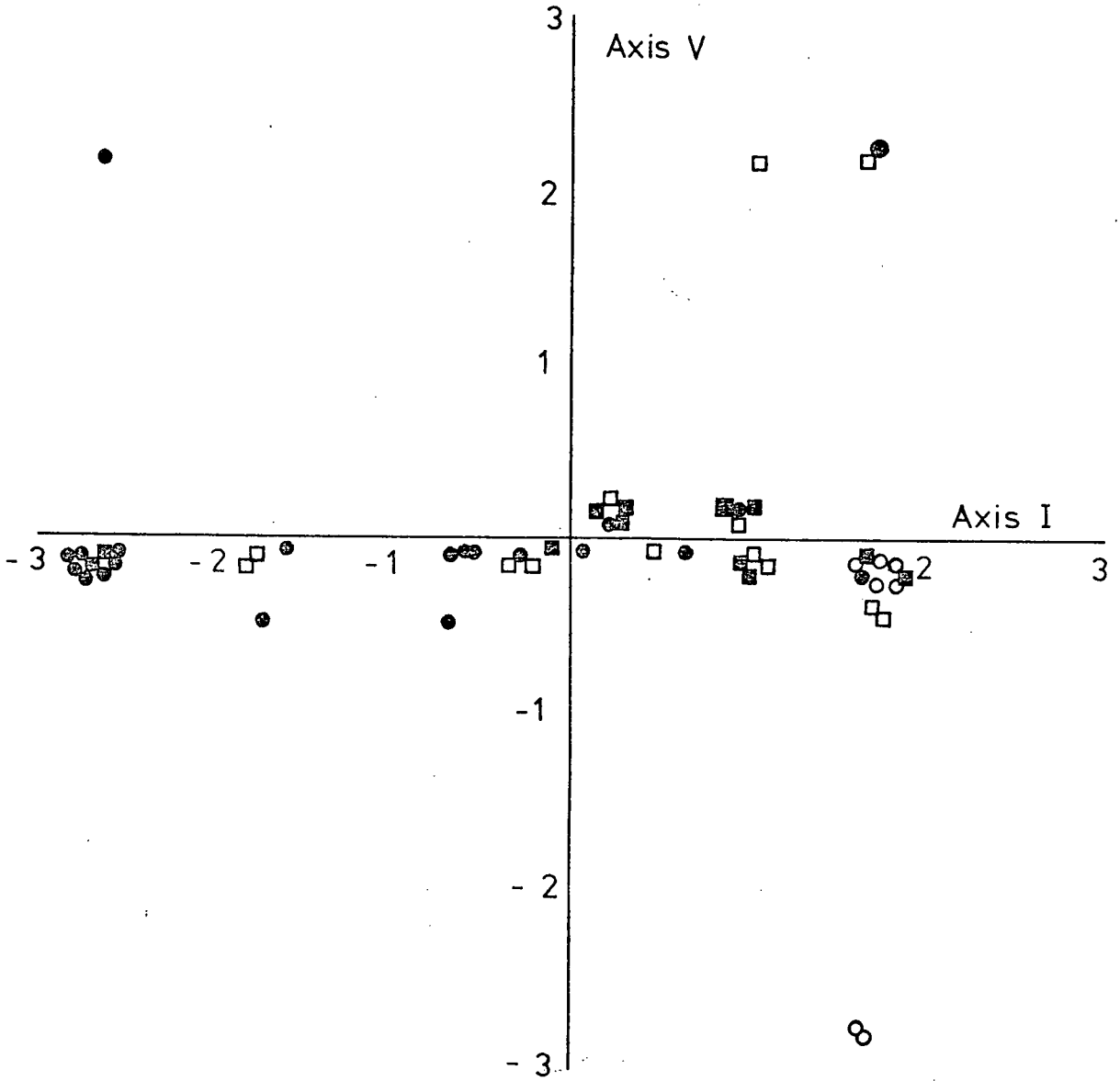


Figure 12. Results of principle components analysis (Axis 1 vs. 5)

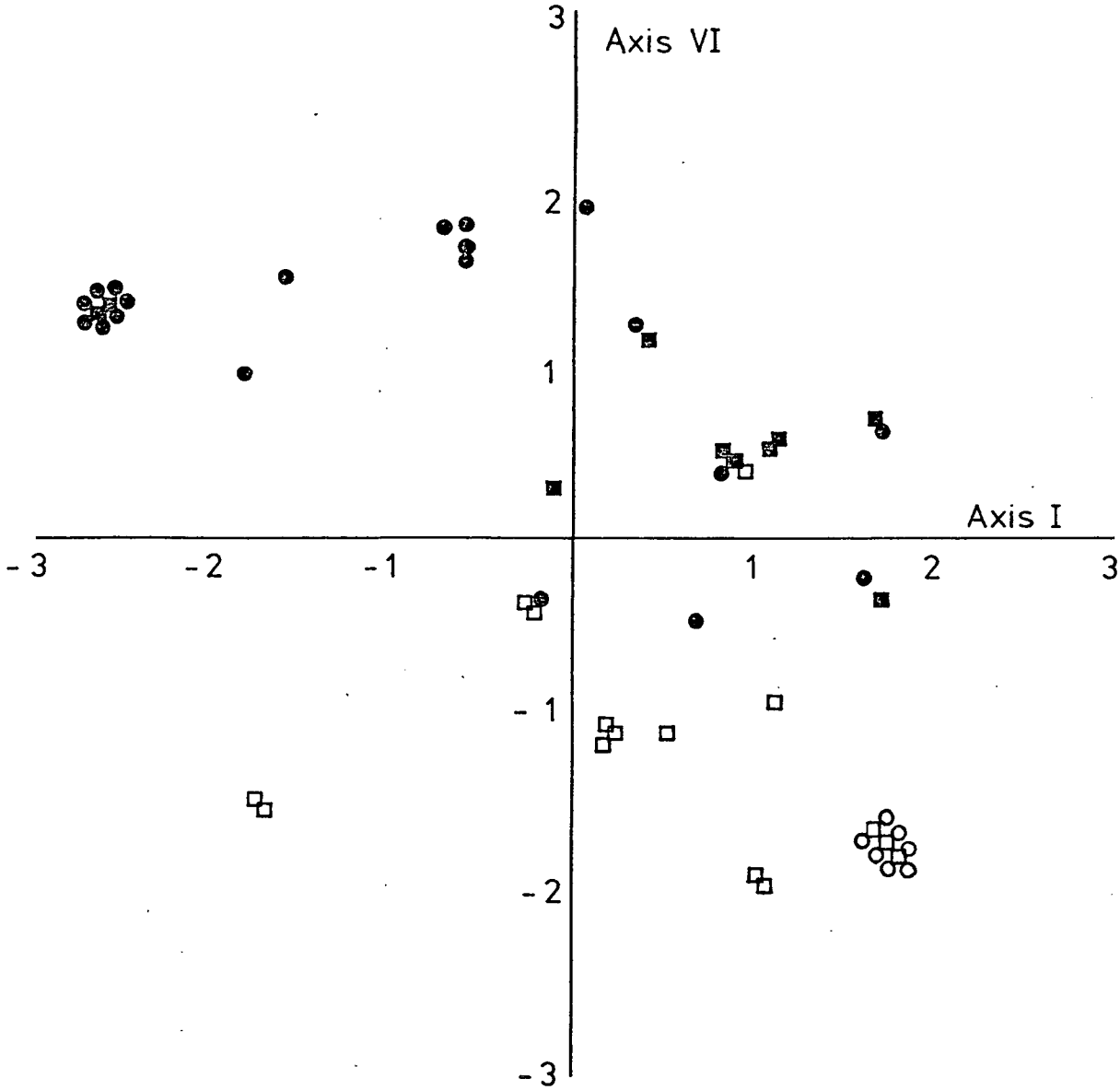


Figure 13. Results of principle components analysis (Axis 1 vs. 6)

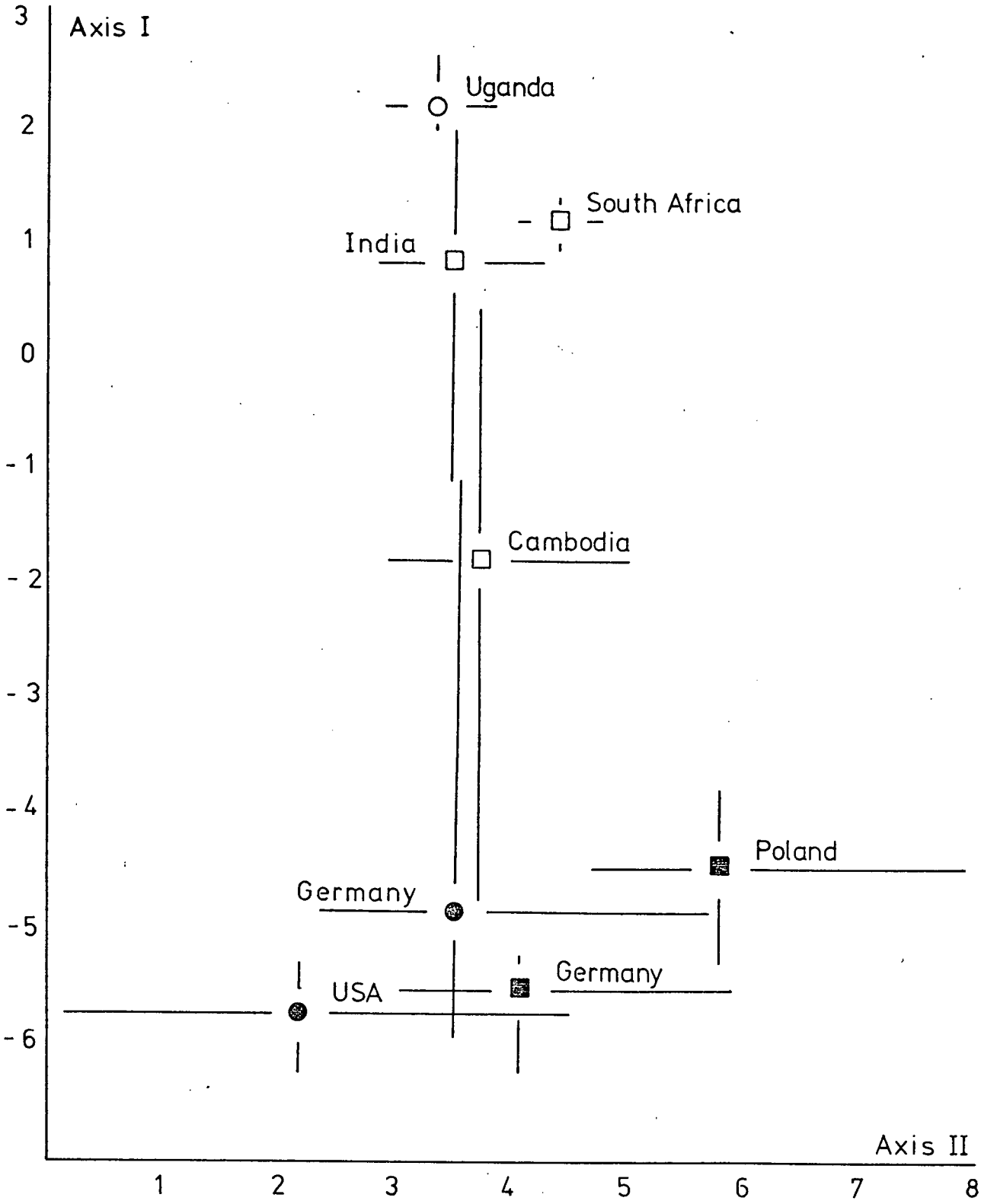


Figure 14. Results of canonical analysis (populational)

1. Nine groups were established on the basis of country of origin of seeds (see Table I). Of the eight canonical axes calculated in this analysis, only the first two, representing 93.49% of the variation are presented in the results (Figure 14).

2. The data were arranged into three groups according to Schultes' taxonomic scheme. In this case, the results were displayed on two canonical axes, the first representing 74.73% of the variation and the second 25.27%. Results of this analysis are shown in Figure 15.

3. Four groups were assigned according to the taxonomy of Small and Cronquist (1976) and results are presented in Figure 16. The first canonical axis represented 84.97% of the variation and, with the second axis, accounted for a cumulative total of 93.17%.

RESULTS:

The calculation of standard normal deviates is a simple way of assessing variation in data. The results did not reveal any consistent patterns of similarity, since there were no areas of large numbers of individuals separated by areas of few individuals (see Figure 7).

Clustering techniques group individuals by bringing together those with the greatest similarity. Three main divisions were noticeable in the data (see Figure 8). Group A were clearly separated from the rest and contained escaped fibre cultivars (●) and two fibre cultivars (■). The second division separated group B, which consisted of all four types of plant, and group C, which contained the majority of the cultivated plants.

In principle components analysis, emphasis is placed on separation of dissimilar individuals, rather than grouping similar individuals. Sneath

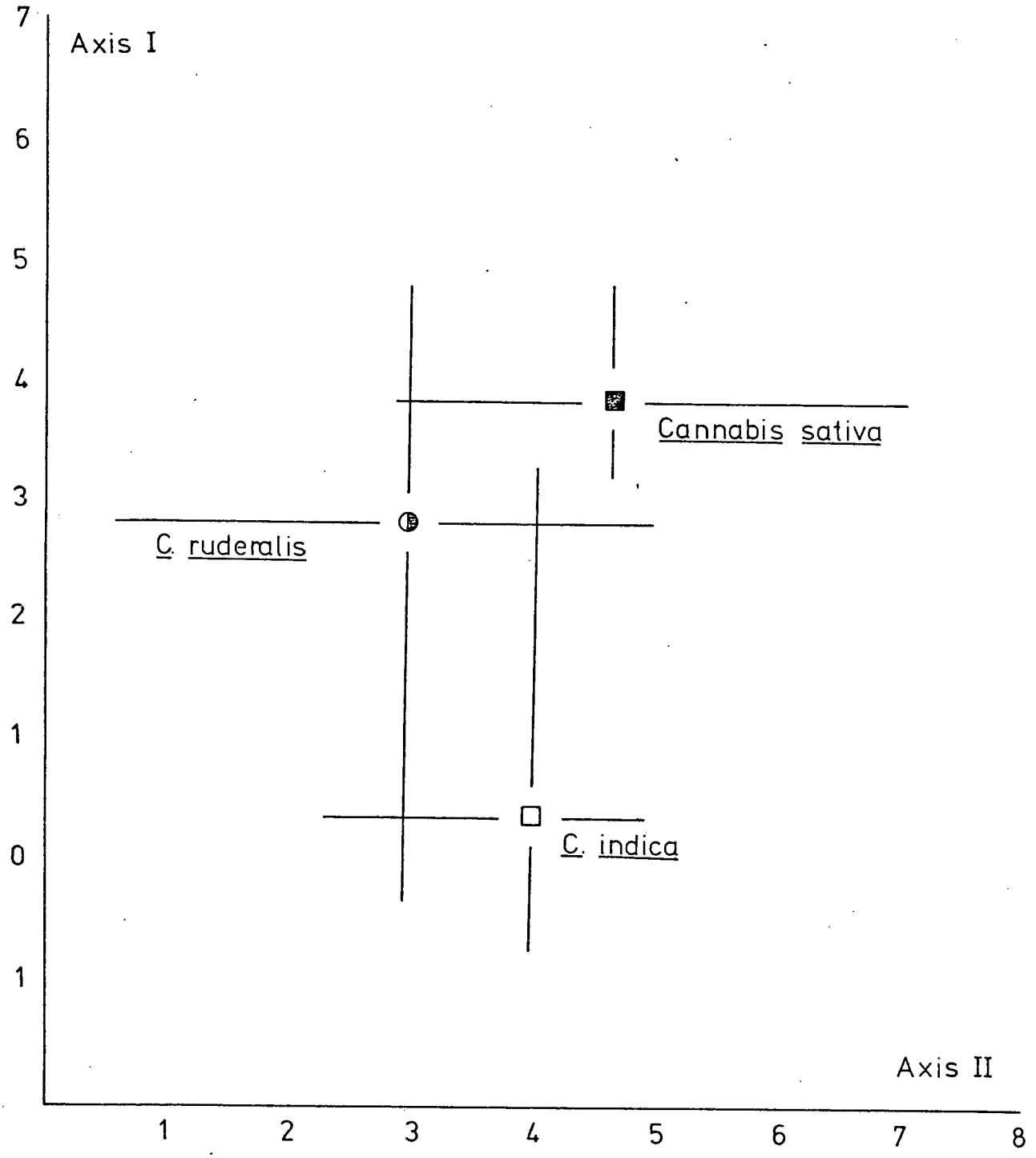


Figure 15. Results of canonical analysis (Schultes' taxonomic groups).

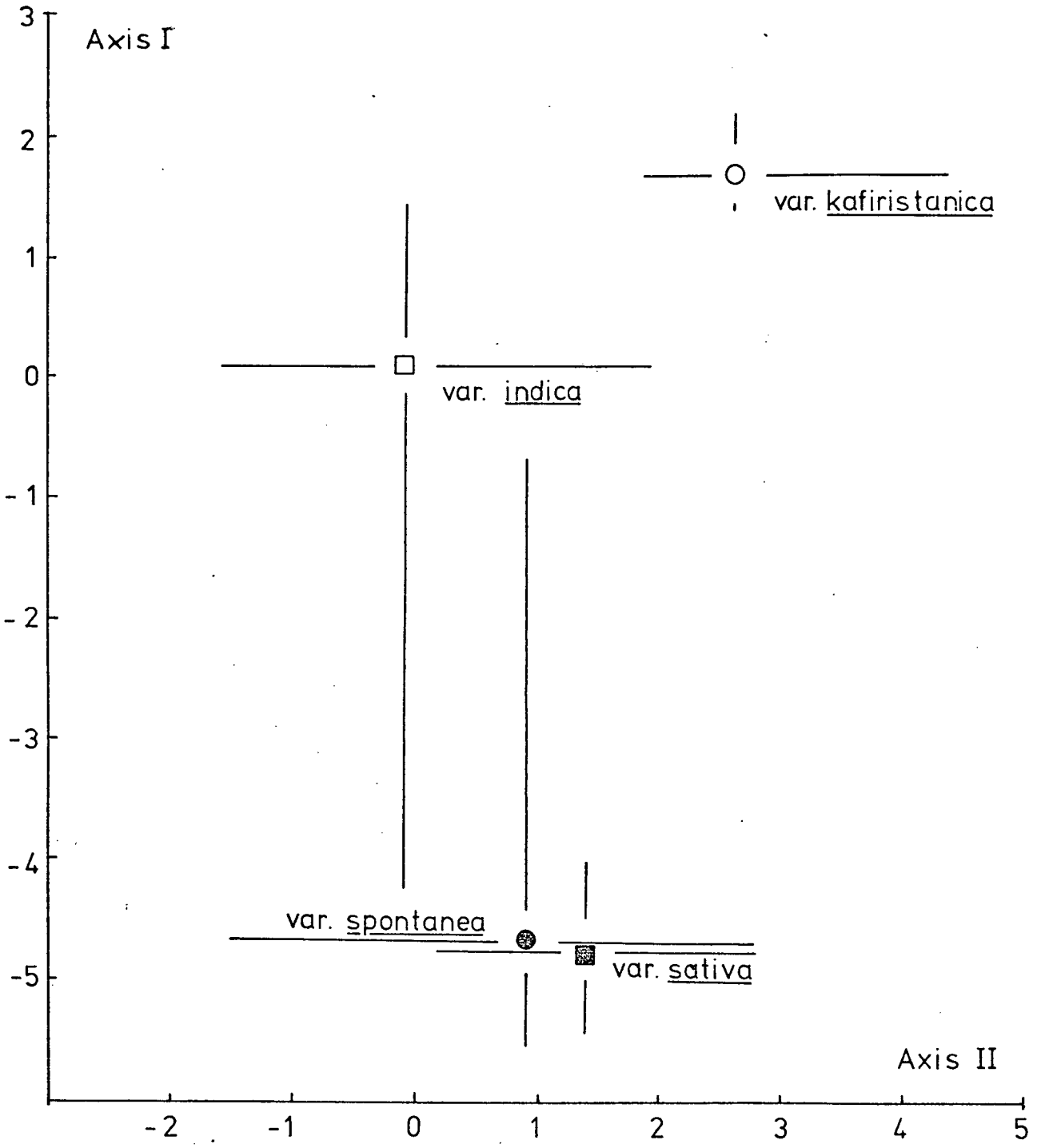


Figure 16. Results of canonical analysis (Small and Cronquist varieties)

and Sokal (1973) stated that, for this reason, PCA tends to represent large differences accurately but is less efficient in describing close neighbours. When data were plotted on axes 1 and 2 (see Figure 9), it was apparent that, although no difinitive groups appeared, escaped fibre plants and two cultivated fibre plants maintained some integrity, as they did in classification (Group A of Figure 8). Escaped drug plants were clustered with cultivated drug plants but were very near both escaped and cultivated fibre plants. When individuals were plotted on axes 1 and 3 (see Figure 10), the distribution became more scattered, but the escaped and cultivated drug-plant group were still obvious. Axes 4 and 5 (when plotted against axis 1) failed to separate any distinct groups. Axis 6, which represented 8.46% of the variation, produced a division of the data which is most consistent with the taxonomy of Small and Cronquist (1976). All but four fibre plants were located above axis 1 while all but one drug plants were located below axis 1 (see Figure 13).

Canonical analysis results are presented according to the three different initial groupings. In Figure 14, nine populations are represented by the centroid (mean) of that population and the range of variation (——). Escaped drug plants (Uganda) were located at the other extreme of the continuum, while fibre plants were located at the other extreme (USA, Germany and Poland). Cultivated drug plants (South Africa, Cambodia and India) were distributed between these extremes. When Schultes' taxonomic criteria were applied to the data, resultant groupings in canonical analysis (see Figure 15) revealed drug plants (*Cannabis indica* Lam.) and fibre plants (*C. sativa* L.) as the extremes of dissimilarity while wild plants (*C. ruderalis* Janis.) were distributed within the ranges

of the other two taxa. Application of Small and Cronquist's taxonomic scheme arranged the plants into four groups (varieties) as shown in Figure 16. In these results, var. kafiristanica was separated from other varieties. The range of variation in var. indica overlapped the range of variation in var. spontanea while var. sativa was completely contained in the range of var. spontanea.

VI. DISCUSSION

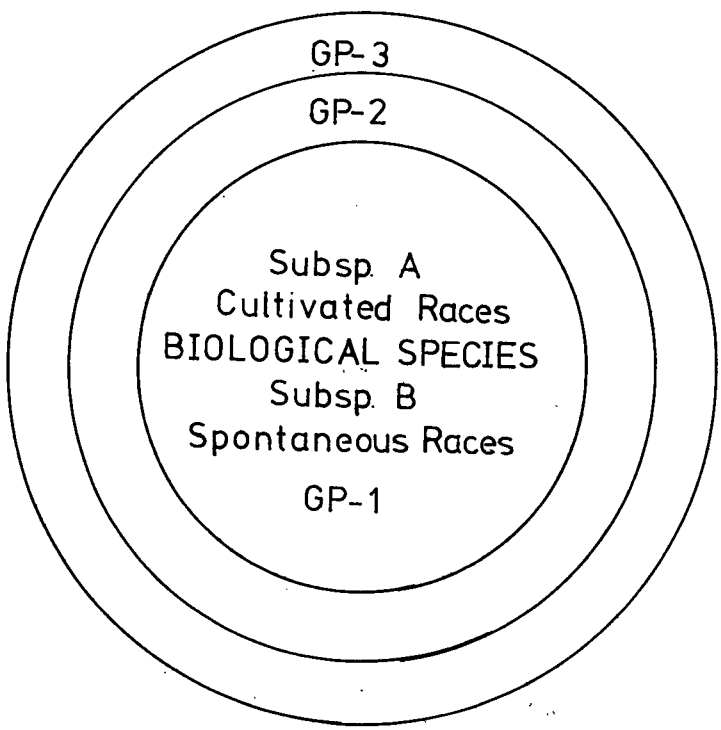
The taxonomic debate on Cannabis hinges on species delimitation in cultivated plants, a problem that has never been resolved. Harlan (1970) stated "the methods of classical taxonomy seem to fail altogether and inevitably result in the establishment of dozens of epithets for races that are fully compatible when crossed." He cited Snowden's treatment of Sorghum in which 52 species of cultivated Sorghum were given formal names. All of these taxa were fully compatible on hybridization.

Harlan (1971) suggested that cultivated plants which belong to one biological species should be placed in a single species, with wild and weedy races separated from cultivated races at the subspecific level. According to Mayr (1970), a biological species consists of "groups of potentially interbreeding natural populations which are reproductively isolated from other such groups". While this concept cannot be applied in some situations, all tested members of the genus Cannabis have been found to belong to one biological species. They have been shown to exhibit normal chromosome pairing during meiosis, and no reduction in pollen stainability of F_1 hybrids, indicating a lack of sterility barriers (Small, 1972).

Harlan and de Wet (1971) have proposed a new terminology for cultivated plants. Their "primary gene pool" (GP-1) consists of those members of a biological species that cross easily, form fertile hybrids with good chromosome pairing, and show normal gene segregation. This biological unit includes both spontaneous (wild and weedy) races in one

subspecies and cultivated races in a second subspecies. "Secondary gene pools" (GP-2) contain all species that can cross with GP-1 but hybrids resulting from such a cross suffer from a lack of fertility. "Tertiary gene pools" (GP-3) include plants from other species or genera that may be artificially crossed with GP-1 but produce hybrids that are anomalous, or sterile hybrids, or hybrids that have lethal genes (see Figure 17). Since there are no members of the genus Cannabis that possess sterility barriers, they can be included in a single biological species or GP-1.

Figure 17. Taxonomic scheme for cultivated plants (Harlan and de Wet, 1971).



Harlan and de Wet (1971) defined a race as "a biological unit with some genetic integrity, which originated in some geographic region at some time. It is not as clearly separable as the species but has a distinct cohesion of morphology, geographical distribution, ecological adaptation and breeding behaviour". The use of their taxonomic guidelines would result in the inclusion of all Cannabis members in a single species, with wild and weedy races in one subspecies and cultivated races in another subspecies. Small and Cronquist (1976) follow traditional taxonomic guidelines to divide the genus into subspecies according to their geographic distribution, and then adhere to the Harlan and de Wet scheme for the separation of the varieties.

Flavonoid analysis of the material in the present study indicated a highly variable group of plants, with no distinct taxa among them. Numerical classification of data by clustering resulted in separation of a group (A) consisting of plants comprised primarily of escaped fibre cultivars (see Figure 8). This group corresponds to those plants which contained very little flavonoid material. The second major group (B) consists of a heterogeneous assemblage of all four types of plants, but composed mainly of escaped drug plants. A third group (C) contained mainly fibre and drug cultivars, which were separated into sub-groups within C.

The first two axes of PCA exhibit several closely-knit groups of individuals, but no trends of areas of concentration are definite. The only axis which separated the plants according to Small and Cronquist's taxonomy was the sixth, but it represented only 8.46% of the variation.

Sneath and Sokal (1973) suggest that discriminant analysis is most useful for distinguishing between very close clusters which partly overlap. Since classification and PCA indicated several closely related groups, canonical analysis was also performed on the data. When data were grouped on the basis of country of origin, plant profiles were distributed along a continuum from wild drug plants (var. kafiristanica) to fibre plants, both wild and cultivated. Cultivated drug plants (var. indica) formed the bridge (see Figure 14). To ascertain which of the two taxonomic schemes was best supported by this method, data were combined according to both schemes. Canonical analysis of plants according to Schultes' taxonomy (1974) resulted in the cultivated taxa being positioned on opposite sides of the wild taxa (see Figure 15). Neither taxa is separated from Cannabis ruderalis Janis. and distribution along the axis of greater variation is continuous. The results of canonical analysis of plants assigned to Small and Cronquist's varieties (1976) showed wild drug plants and fibre plants, both wild and cultivated, formed the extremes of a continuum, with cultivated drug plants in the middle.

The scaling technique used demonstrated the high degree of variation in the material studied, since no easily discernible peaks were formed (see Figure 7). Results of an analysis of two species showed two concentrated areas with no overlap (Maze, unpublished).

VII. CONCLUSIONS

Flavonoid analysis of Cannabis sativa L. provided some insight into the structure of the species. Although highly variable, all plants belonging to the genus Cannabis exhibit enough similarities to warrant their inclusion in a single species. It is true that different types of plants can be recognized, but a single generation of random mating could obliterate any noticeable groups. Because it is dioecious, Cannabis sativa L. is an obligate outbreeder. Possession of this type of breeding system ensures a constant exchange of genetic material and increased hybrid vigor but, because of it, phenotypes often change.

The material studied represented nine different populations originating from well-separated countries. As their history was not known, it was impossible to assess the degree of introgression between the samples before they were obtained. It was assumed, however, that the seed sample represented 'pure' lines of Cannabis in their respective countries.

The use of several different numerical techniques failed to elucidate any consistent trends or groups in the data; therefore, the present study does not support a division of the genus into several species. The association between plant types does indicate that the weedy races are more closely related to their respective cultivated races, than they are to each other. For example, members of var. kafiristanica are usually (but not always) distributed near members of var. indica. Variety spontanea tends to be well separated from var.

sativa in classification and PCA analysis, but it is indistinguishable from var. sativa in canonical analysis. The wide range of variation evident in both var. indica and var. spontanea indicate the plasticity of their flavonoid content. Canonical analysis of original populations (i.e. country of origin) arranged the plants in a triangular fashion with var. kafiristanica at the apex and var. spontanea and var. sativa as the base. Members of var. indica occur in the centre of this triangle. Flavonoid profiles tend to support Small and Cronquist's conclusions that var. sativa and var. spontanea are closely related, as are var. kafiristanica and var. indica. Still, no concise groups can be outlined in canonical analysis results.

Intraspecific variation of flavonoids in Cannabis sativa L. tends to support the monotypic theory for the genus as proposed by Small and Cronquist (1976). Trends seem to occur in a bidirectional manner, reflecting selection which has influenced the genus. That selection is the result of man's choosing drug and fibre cultivars.

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