Comparison of Hemp Varieties Using Random Amplified Polymorphic DNA Markers

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ABSTRACT

The objective was to study the genetic structure and degree of variability of hemp (*Cannabis sativa* L.) varieties. Six varieties of hemp were analyzed by random amplified polymorphic DNA (RAPD) analysis, using 10 plants per variety. The varieties were a dioecious landrace, a dioecious selection from it, a cross-bred cultivar, a monoecious variety, a drug strain, and an inbred female line. The genetic complexity of each cultivar was investigated by determining the number of bands produced by the primers used, the number of fixed and polymorphic loci, the average allele frequency, and the heterozygosity. A good correlation was found between these parameters and the genetic origin and breeding strategy of each variety. The average polymorphism over all varieties and loci was 97.1%; the single cultivar polymorphism ranged from 31.1 to 85.5%. Heterozygosity ranged from 0.05 (inbred female line) to 0.26 (cross-bred Fibranova). The average heterozygosity calculated over all 102 loci and all plants studied was 0.29. The Fst (Wright's fixation index) value calculated for all loci was 0.48, and only 33.3% of the scored loci had higher values and can be considered informative for cultivar identification. A Fisher's test based on allele frequencies suggested complete differentiation among all varieties, with the exception of the Italian dioecious varieties Carmagnola and CS, for which no discriminating alleles were found. The correlations among the molecular data and the genetic structure of the different cultivars and the consequences in relation to variety discrimination in hemp are discussed.

HEMP is a traditional crop in Europe and Asia. It belongs to the Cannabaceae family, which comprises two genera: *Cannabis* and *Humulus* (hops). Despite hemp's past importance for both the textile and manufacturing industry, its cultivation declined rapidly during the sixties primarily for two reasons: strong competition from cotton (*Gossypium hirsutum* L.) and synthetic fibers and multiple efforts to limit marijuana abuse carried out in virtually all western countries. Hemp varieties used for drug consumption, which have a high content of the psychoactive compound Δ^9–tetrahydrocannabinol (Δ^9–THC), are in many cases not morphologically distinguishable from fiber varieties with a lower, nonpsychoactive level of Δ^9–THC (de Meijer, 1999).

Recently, the need for alternative crops has renewed interest in hemp cultivation for several reasons: (i) the comparatively low chemical fertilizer input required by hemp, (ii) its exceptional disease resistance, and (iii) increased public perception of the value of natural fibers compared with synthetic ones (Montford and Small, 1999). A number of hemp varieties were included in the European Union list as eligible for subsidies, and legislation has been modified in almost all countries to allow cultivation of hemp varieties with a Δ^9–THC content below 0.3%. By 1998 hemp was reintroduced in practically all of Europe (Bócsa and Karus, 1998), though in some countries significant resources are still being employed to prevent hemp diffusion regardless of its Δ^9–THC content (Linacre and Thorpe, 1998).

Because of the continuous variation of most morphological and biochemical traits within the *Cannabis* genus, it would be useful to identify groups on the basis of their use or origin. A nonbiosystematic classification distinguishes wild populations from fiber landraces, cultivars, drug strains, and ornamentals (de Meijer, 1995). Hemp cultivars are characterized by different degrees of genetic variability because of commercial value, reproductive biology (dioecious or monoecious), end use, and different criteria employed in breeding.

Dioecious cultivars (e.g., Italian fiber varieties) are obligate outbred, and the method used to multiply them (open-field pollination) approaches a random-mating condition. This is also true for monoecious cultivars, which are composed of about 50 to 70% monoecious plants and 30 to 50% female plants, with a small number of male plants. The original monoecious trait occurred spontaneously in material from central Russia (de Meijer, 1995) and the presence of male plants in a monoecious stand must be limited to avoid reversion to the dioecious condition. Monoecious plants freely pollinate female and monoecious individuals during multiplication.

Drug varieties are dioecious, but their genetic background probably has been narrowed to keep the Δ^9–THC content high and uniform; drug hemp breeders make extensive use of plants cloned through in vivo propagation, and of full-sib crosses. It is possible to get partial reversion of sex in female plants (Mohan Ram and Sett, 1982), which when self-pollinated leads to the inbred, genetically female lines.

In general, current fiber cultivars include open-pollinated populations, selections, and F1 and F2 hybrids with a high degree of genetic variability. Italy was one of the leading European countries in hemp cultivation and breeding (Bócsa and Karus, 1998). Italian cultivars have the highest fiber quality employed in the textile industry and range from landraces (Carmagnola), or selections from landraces (C.S., Carmagnola Selezionata), to cross-bred populations (Fibranova). In other countries breeding programs are designed to produce monoecious varieties, especially useful for pulp and paper production and for seed yield (e.g., the French cv. Fibrimon and its derivatives). These differences in

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Abbreviations: AFLP, amplified fragment length polymorphism; bp, base pairs; AMOVA, analysis of molecular variance; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; Δ^9–THC, Δ^9–tetrahydrocannabinol.

breeding strategies presumably affected the variability within hemp varieties; however, no in-depth studies on this issue have been performed.

The terms *sativa* and *indica* are often used in different ways by different authors, reflecting marked disagreement on *Cannabis* taxonomy. Small and Cronquist (1976) defined as *C. sativa* ssp. *sativa* all the low Δ^9–THC fiber strains, and as *C. sativa* ssp. *indica* the entire group of Δ^9–THC-rich strains, irrespectively of their morphological phenotypes. Other authors (de Meijer, 1999) prefer to use the term *indica* to discriminate at the subspecific level the hashish plants from the Afghanistan and Pakistan region, characterized by wide leaflets and a densely branched morphotype. On the other hand, subspecies *sativa*, characterized by a slender appearance and narrow leaflets, comprises all fiber hemp populations as well as the marijuana landraces from Nepal, Thailand, South Africa, and elsewhere. Finally, other authors have discriminated between *C. sativa* and *C. indica* species, classifying the plants in one or the other taxon regardless of their psychoactivity, but according only to their morphology (Schultes et al., 1974; Emboden, 1974).

Cultivar discrimination on the basis of phenotypic traits is difficult in most crops, but this has particular consequences in *Cannabis*. The necessity of preventing drug hemp cultivation on one hand, and the desirability of encouraging fiber hemp as a valuable alternative for crop rotation on the other, underlies the immediate need for a fast and reliable method to identify fiber and drug hemp types. Current methods are based on estimates of cannabinoids content in the dry matter of inflorescences and leaves from 500 individual plants by gas chromatography (de Meijer et al., 1992). This is a demanding procedure, though amenable to automation, and experiments are underway to set up faster and equally reliable techniques based on immunoassays (Grassi and Ranalli, 1999). Molecular fingerprinting could be a reliable method to discriminate both cultivar types (fiber and drug), and could also be used to obtain estimates of phylogenetic relationships among taxa identified by different authors.

The approach used for varietial identification in allogamous species depends upon their genetic diversity, which is determined primarily by the breeding methods adopted. Variation can be measured at different scales by different molecular markers—restriction fragment length polymorphism (RFLP), RAPD, microsatellites, and amplified fragment length polymorphism (AFLP). In many cases RAPD makes it possible to determine the overall profile of a bulked sample from several individual plants, and hence the identification of variety-specific DNA bands (Dulson et al., 1998); alternatively, the marker frequencies in a sampled group of individuals belonging to the same variety appear to be suitable indicators for cultivar characterization. Furthermore, RAPD markers have been used previously to assess the existence of different gene pools in this species (Faeti et al., 1996).

The objective of our research was to determine whether there is a correspondence between molecular differentiation measured by RAPD analysis (Williams et al., 1990) and the genetic structure of six different hemp varieties. Furthermore, the possibility of hemp cultivar discrimination using molecular markers and suitable statistical methods was investigated.

MATERIALS AND METHODS

Plant Material

Six hemp varieties were chosen, including four fiber cultivars with low or very low Δ^9–THC content—(i) Carmagnola, a dioecious landrace from northern Italy; (ii) Carmagnola Selezionata (C.S.), a dioecious selection from this landrace (Allavena, 1967); (iii) Fibranova, a dioecious cultivar selected from the progeny of the cross Carmagnola × Bredemann Elite (a German selection; Allavena, 1961); and (iv) Fibrimon, a monoecious cross-bred French cultivar—and two varieties characterized by high levels of Δ^9–THC—(v) Northern Lights, a dioecious drug strain, and (vi) line b92.73.2.13, a high Δ^9–THC and inbred female line, bred through partial reversion and selfing of a female plant.

Seed of the Italian cultivars, Carmagnola, C.S., and Fibranova, were obtained from the collection of the Istituto Sperimentale per le Colture Industriali in Bologna, Italy. Professor G. Venturi (Dept. of Agronomy, University of Bologna, Italy) kindly provided seeds for the Fibrimon variety bred by Fédération Nationale des Producteurs de Chanvre, Le Mans, France. Northern Lights seeds were obtained from the Italian Police, Dept. of Drug Repression. Leaf material for the line b92.73.2.13 was kindly provided by Dr. E. de Meijer, Horta-Pharm B.V., Amsterdam, the Netherlands. This study included four dioecious cultivars, one monoecious cultivar, and one inbred female line.

Leaf samples from individual plants were collected about 20 d after sowing in a greenhouse. The collected material was used to prepare genomic DNA; the $SCAR_{400}$ male-specific marker (Mandolino et al., 1999) was used to select five female and five male plants within the dioecious varieties, Carmagnola, CS, Fibranova, and Northern Lights, and the only male plant included in the study from the monoecious variety, Fibrimon. This partition was also validated by direct inspection of the flowers at maturation. Horta Pharm B.V kindly provided freeze-dried leaf material of the line b92.73.2.13.

DNA Preparation and Polymerase Chain Reaction Analysis

DNA was prepared and quantified as in Mandolino et al. (1999). Bulks of the different varieties were prepared by mixing equal amounts of DNA from single plants.

Polymerase chain reaction (PCR) analysis was performed with the arbitrary decamer primers OPA08 (5′GTGACGTAGG3′), OPA11 (5′CAATCGCCGT3′), OPB06 (5′TGCTCTGCCC3′), OPG04 (5′AGCGTGTCTG3′), and OPH01 (5′GGTCGGAG AA3′), Operon Technologies (Alameda, CA) on DNA of every variety. The PCR conditions and gel electrophoresis were as described elsewhere (Faeti et al., 1996).

The band profile obtained with each primer was analyzed for band presence and frequency in all individuals of each variety in at least two replicate experiments. Bands above 2500 base pairs (bp) and below 300 bp were not considered, because of low reproducibility. Pattern alignment from different gels was performed by the Molecular Analyst software Fingerprinting Plus, version 1.5 (BioRad Laboratories, Hercules, CA), using methods described elsewhere (Forapani et al., 1999).

Statistical Analysis

DNA bands were assumed to indicate a genetic locus with the additional assumptions that marker alleles from different loci do not comigrate and that each locus can be treated as a two-allele system (1 and 0; Lynch and Milligan, 1994).

Collected data were organized as matrixes composed of ones and zeros at each locus. For each individual plant the number of bands was counted, and for each variety the total and average number of bands and the number of loci (total, fixed, and polymorphic) was calculated. Differences in band number between varieties were analyzed using a one-way analysis of variance (SAS, PROC GLM procedure; SAS Institute, 1985). Before running each analysis the assumption of homoscedasticity was checked by Bartlett's test (Sokal and Rohlf, 1981). The TFPGA (Tools for Population Genetic Analysis, version 1.3; Miller, 1999) software was used to calculate—under the Hardy-Weinberg equilibrium assumption—the following parameters: (i) frequency of the recessive (0) allele (and consequently of the dominant one) as the square root of the null (recessive) genotype frequency (Weir, 1996) and (ii) unbiased heterozygous frequency obtained as two times the product of both allele frequencies (Nei, 1978). These parameters were calculated at each locus and averaged over all scored loci, and were also calculated for each of the varieties. TFPGA was also used to obtain the fixation index Fst (Wright, 1969) for each locus and to perform a Fisher's exact test according to Raymond and Rousset (1995) to evaluate cultivar differentiation based on the observed marker frequencies. Their method uses a Markov Chain Monte Carlo simulation to estimate the probability that the observed marker frequency in each variety could be due to chance, that is, whether or not the observed frequencies are significantly different in the different cultivars. AMOVA-PREP (Miller, 1998) was used to transform dominant raw data sets in input files for the program Analysis of Molecular Variance (AMOVA, version 1.55; Excoffier et al., 1992). Because of the occurrence of male-associated markers (Sakamoto et al., 1995; Mandolino et al., 1998), the amount of variation determined by sex linked markers in dioecious cultivars was preliminarily assessed by dividing each dioecious variety in male or female subpopulations; this partition was based on the visual inspection of the male plants, always associated to the male markers. Line b92.73.2.13 is known as pure female from pedigree data and origin and accordingly does not show the male markers observed in the other cultivars. Ninety-seven RAPD markers were used for this analysis. In a subsequent two-level analysis, sex contribution to the overall amount of variability was ignored and intra- and intercultivar variation was determined using the total set of 102 markers in AMOVA 1.55. Before both analyses, Bartlett's test was employed to check homoscedasticity.

Table 1. Number of different random amplified polymorphic DNA (RAPD) patterns obtained with the different primers (10 plants per variety).

	Number of different patterns					
	OPA08	OPA11	OPB06	OPG04	OPH01	All primers
Carmagnola	10	10	9	10	10	10
C.S.	10	10	8	10	10	10
Fibranova	10	10	9	10	10	10
Fibrimon	5	8	10	9	10	10
Northern Lights	6	8	6	8	10	10
b92.73.2.13	2	5	1	1	9	10

RESULTS

Descriptive Statistics and Analysis of Variance

The data set obtained using five primers and 10 plants for each variety included 102 markers, out of which 99 had a variant in at least one plant of one variety. Most primers yielded individual specific patterns, and the combination of the five primers allowed a full discrimination at the individual level for all varieties, including the inbred female line, where the discriminative power of male-associated markers was not present (Table 1).

The range of R^2 values calculated by ANOVA on the band number indicates that the model explains a good proportion of the variation except in two cases: OPA08 and OPB06 (Table 2). The R^2 for all primers considered simultaneously explained 61% of the variability. However, the varieties could not be fully discriminated on the basis of band number either using individual primers or considering all the primers simultaneously. If all the primers are considered simultaneously, the average number of bands per plant (data not shown), and the total number of bands amplifiable (Table 2, numbers in brackets) are lowest for the inbred female line b92.73.2.13, and highest for the three cultivars, Fibranova, Fibrimon, and Northern Lights. Line b92.73.2.13 always showed the lowest standard deviation and the narrowest minimum to maximum range (Table 2). The widest range was found for Carmagnola and the strictly related CS selection, while Fibranova, Fibrimon, and Northern Lights showed similar values.

The data indicated the presence of different degrees of polymorphism within each variety (Table 3). The number of scorable markers was highest for Fibranova and lowest for line b92.73.2.13. The number of fixed loci was very similar for the three Italian varieties, and significantly higher for the French fiber cultivar Fibri-

Table 2. Analysis of variance (ANOVA) results for band number differences between varieties for the different primers separately and cumulatively.†

ANOVA									
Model				Varieties					
Primer	F value	P	R^2	Carmagnola	CS	Fibranova	Fibrimon	N. Lights	b92.73.2.13
OPH01	19.40	0.0001	0.64	ab‡	ab	b	a	b	c
OPA08	5.79	0.0002	0.35	ab	abc	ab	c	a	bc
OPB06	4.35	0.0021	0.29	b	ab	a	ab	ab	ab
OPA11	14.19	0.0001	0.57	d	cd	ab	a	bc	cd
OPG04	19.56	0.0001	0.64	d	cd	b	bc	a	bcd
All primers	16.87	0.0001	0.61	bc (410)	b (433)	a (476)	a (477)	a (477)	c (379)
Range				3–14	4–15	6–14	6–15	6–13	6–9

† Numbers in brackets indicate the total number of bands. The band number range for all primers is shown in the last row.
‡ Different letters (rows) refer to significantly different means at $P = 0.05$ (Tukey test).

Table 3. Statistical parameters relative to the loci identified by random amplified polymorphic DNA analysis, and characterizing the six hemp varieties studied. The data relative to all the varieties cumulatively are shown in the last row.

	Loci					Alleles	
Genotype	Total no.	Fixed	Polymorphic	Percentage	Cv. specific loci	Avg. allele frequency	Avg. heterozygosity
Carmagnola	68	14	54	79.4%	–	0.46	0.20
CS	71	15	56	78.9%	–	0.47	0.20
Fibranova	83	12	71	85.5%	–	0.42	0.26
Fibrimon	62	24	38	61.3%	–	0.64	0.15
Northern Lights	61	26	35	57.3%	3	0.67	0.15
b92.73.2.13	45	31	14	31.1%	5	0.79	0.05
All	102	3	99	97.1%		0.58	0.29

mon, for the drug variety Northern Lights, and for the inbred female line b92.73.2.13. On the other hand, the number and percentage of polymorphic loci showed an opposite trend, with the lowest value (31.1%) for the inbred female line and the highest for the cross-bred cultivar Fibranova (85.5%). Similar values were found for Carmagnola and its derived selection C.S. (79.4 and 78.9%, respectively). A sharp decline in the number of scorable and polymorphic loci was observed for the monoecious fiber cultivar Fibrimon and for the dioecious drug variety Northern Lights (62 loci, 61.3% polymorphism and 61 loci and 57.3% polymorphism, respectively). Finally, the lowest levels of all the parameters characterized the inbred female line b92.73.2.13, where the sex-specific polymorphism was absent. As expected, the highest average allele frequency was found for the line b92.73.2.13 (0.79), while hybrid population Fibranova showed the lowest value (0.42).

Estimates of unbiased heterozygosity for Carmagnola and CS are indistinguishable (0.20), while Fibranova showed the highest heterozygosity (0.26), and Fibrimon and Northern Lights had the same lower value (0.15). Finally, the overall average polymorphism was 97.1% and heterozygosity was 0.29.

Fixed Differences between Varieties, *F*-Statistics, and Test for Population Differentiation

The Fst value between varieties for all the 102 loci considered simultaneously was 0.48 ± 0.03 (99% confidence interval, 10 000 bootstrapped replication). Fst estimates for each locus varied considerably, from a minimum of 0 (for 4 loci), up to the maximum of 1 (for the 8 cultivar-specific bands, Table 3). Out of the 102 loci identified, only 34 (i.e., 33.3%) had Fst values above the average value of 0.48. Markers with Fst above this threshold value are more likely to be informative for variety characterization; therefore, only one-third of all scorable loci can be useful for this purpose. Each of the five primers identified some of these 34 loci.

Fisher's combined probability test applied to marker frequencies resulted in a 0 value in most cases (i.e., full differentiation; data not shown). All cultivars were distinguishable from each other, with the exception of the three Italian varieties, Carmagnola, CS, and Fibranova. The amplification from a bulked DNA sample containing equal amounts of all 10 DNAs belonging to a variety was not a reproducible method for determining cultivar specific RAPD profiles. However, when fixed differences were found (i.e., alleles with Fst = 1), the amplification of a bulked DNA sample seemed to be a reliable method to assess different fingerprints (Fig. 1). Using this approach the drug cultivar Northern Lights and the inbred female line b92.73.2.13 could be differentiated from all the other fiber strains and from one other.

Variance Composition

Variance component analysis was performed by AMOVA (Excoffier et al., 1992). Initially, the differences among individual plants within sexes were analyzed, and therefore, only the four dioecious varieties were considered. This first analysis revealed a small (3.3%) contribution of sex to the total observed variance

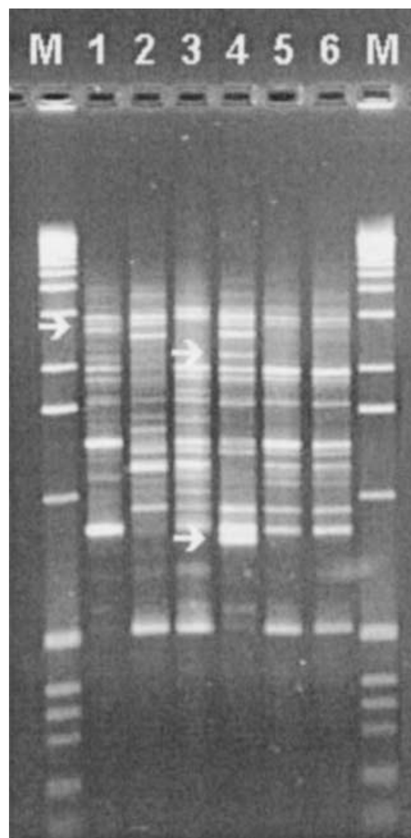

Fig. 1. Amplification by primer OPG04 of six DNA bulks, each composed of equimolar amounts of the DNAs of 10 plants of each variety. Arrows indicate cultivar-specific bands. Lane 1, line b92.73.2.13; Lane 2, Fibrimon; Lane 3, Fibranova; Lane 4, Northern Lights; Lane 5, Carmagnola; Lane 6, CS.

Table 4. Variance composition due to dioeciousness and to variety. The data set for the contribution of sex included only the four dioecious varieties (97 markers, upper part). Analysis of molecular variance (AMOVA) was also performed on all 60 plants belonging to the six varieties (lower part), ignoring the contribution of sex to the total variance (102 markers). Statistics include degrees of freedom (df), mean squares (MS), variance components (VC), percentage of the total variation, and Bartlett's test (*B*).

Source of variation	df	MS	VC	% total	*B*
Male vs. female	1	25.15	0.51	3.3*	0.06ns
Individuals/sex	38	14.94	14.94	96.7	
Variety	5	94.52	8.55	48.8***	6.73***
Individuals/variety	54	8.97	8.97	51.1	

* Significant at the 0.05 probability level.
*** Significant at the 0.001 probability level.

(Table 4, upper part). However, the assumption of variance homogeneity was violated; therefore, the analysis was not meaningful. The between-varieties variance was homogeneous and the contribution to the total observed variance of the differences between varieties and between individuals within varieties was almost the same (48.8 and 51.2%, Table 4). These estimates, however, critically depend upon the cultivars studied. When single pairs of varieties were compared, the proportion of among-cultivar variation changed dramatically, ranging from 12.8 to 76.0% (Table 5). When considering all the varieties cumulatively, the observed variance ranged from a minimum of 40.1% (OPG04) to a maximum of 58.4% (OPH01; data not shown). Primers OPH01 and OPA11 (58.2% among-cultivar variance) were therefore the most informative to highlight differences between cultivars and to minimize individual polymorphism within cultivars.

DISCUSSION

Diversity in *Cannabis*

Cannabis sativa is an obligately outbreeding species, dioecious in nature. This mating system is probably responsible for the extremely high degree of variation found at both the intra- and the intergenotypic level by RAPD analysis. The assumption made, that bands observed correspond to genetic loci, is justified by a parallel study of the heritability of RAPD markers in specific crosses, showing a largely mendelian behavior of DNA bands (data not shown). Our results confirm an exceptionally high degree of polymorphism for this species, as reported by Faeti et al. (1996). In fact every plant of each variety could be easily distinguished with a combination of one or more primers (Table 1). Nonetheless, two out of five primers tested (OPB06 and OPG04) showed no polymorphism in line b92.73.2.13, indicating that the inbreeding strategy, consisting of selfing and progeny selection, has effectively restricted genetic variation in this line.

Table 5. Values of among-cultivar variation (expressed as percentage of the total variation) for each couple of the varieties studied, calculated by analysis of molecular variance (AMOVA) analysis.

	Carmagnola	CS	Fibranova	Fibrimon	N. lights	b92.73.2.13
	%					
Carmagnola	–					
CS	12.8	–				
Fibranova	19.5	18.0	–			
Fibrimon	40.7	42.5	34.5	–		
N. Lights	51.5	46.5	41.1	58.3	–	
b92.73.2.13	65.2	61.2	57.9	76.8	76.3	–

The degree of polymorphism found in hemp is comparable with other outbred, heterogeneous species like tea (*Camellia sinensis* L.), where a survey of 38 genotypes with RAPD markers showed 84% of primers and an average 62% of the identified loci were polymorphic (Wachira et al., 1995). In potato (*Solanum tuberosum* L.), the best probe–enzyme combination tested in a study on discrimination of *4n* varieties and *2n* lines, resulted in 95% polymorphism (Gebhardt et al., 1989), and a similar result (93%) was obtained by RAPD analysis (Forapani et al., 1999). In perennial ryegrass (*Lolium perenne* L.), RAPD analysis showed that diversity within cultivars was higher than between cultivars (Sweeney and Danneberger, 1994); in *Medicago* meiotic mutants from different species, 119 of 125 RAPD fragments identified (95%) were polymorphic (Barcaccia et al., 1994). These data suggest that variability in hemp is of the same magnitude as the other outbred species.

Extensive variation for the total and average number of bands generated by each primer within each variety was found (Table 2). Among the cultivars analyzed, the inbred female line b92.73.2.13 had the lowest band number (total, per primer, or per genotype) and was characterized by a narrower range of variability compared with the other varieties, probably because of the loss of several alleles during the inbreeding-selection cycles. Among the other cultivars no differences in band number suitable for varietal characterization were found, as indicated by the overlapping of the groups identified by Tukey's test (Table 2). On the contrary, the number of loci identifiable and the proportion of fixed to polymorphic loci seemed to be good indicators of differences in genetic structure (Table 3). Fibranova, the Italian cross-bred variety, showed a high level of heterozygosity and heterogeneity, the highest number of loci, the highest percentage of polymorphism, and the lowest average allele frequency. Average heterozygosity was lower for the other two Italian cultivars, Carmagnola and CS, because of the introgression in Fibranova of alleles originating outside the Italian genepool. The low heterozygosity found in Fibrimon and Northern Lights could reflect the strong selective pressure necessary to maintain the main phenotypic characteristics of these varieties (monoecy and high Δ^9–THC, respectively). Finally, the low polymorphism and heterozygosity found for b92.73.2.13 is probably the result of inbreeding to fix the chemotype. The high average heterozygosity (0.29) found considering all loci regardless of cultivars is comparable to other cultivated allogamous species like potato, where the heterozygosity measured by RFLP analysis was about 0.31 (Gebhardt et al., 1989). This suggests the possibility of molecular mapping in F1 progenies, provided that the parental strains are chosen among the most heterozygous populations. We assumed Hardy-

Weinberg equilibrium to estimate allele frequencies and heterozygosity. The conditions necessary for the existence of such equilibrium (diploidy; sexual reproduction; nonoverlapping generations; random mating; large populations; absence of migration, mutations, and selection) is consistent with the hemp multiplication practice. Hemp varieties are in most cases populations, obligately outbred and reproduced by open-field pollination, during which pollen produced by every male flower can freely impollinate every female flower; therefore, it is our opinion that Hardy–Weinberg equilibrium in these conditions is reasonably respected.

The Italian landrace Carmagnola and CS, the selection derived from Carmagnola, were extremely similar for all the genetic diversity parameters measured (Table 3). Furthermore, their within- and among-cultivars variance partitioning was similar. Specifically the among-cultivar difference was smallest for this pair (12%, Table 5) and of the same magnitude of the among-years variation found comparing two different Carmagnola seed lots (15%; data not shown). Although it is possible that this lack of variability is caused by the relatively limited sample size, by no molecular or statistical means could these two materials be distinguished. The question arises whether the CS selection can still be considered a variety separated from Carmagnola, at least based on the seed lot analyzed. The possibility that Carmagnola and CS are actually the same cultivar is also supported by the allele frequency comparison by Fisher′s exact test. In summary:

1. No differentiation was possible between Carmagnola and CS, at least with the scored loci.
2. It is possible to distinguish the Carmagnola/CS pool from the Italian cross-bred Fibranova, although the discrimination between Carmagnola and Fibranova appears more pronounced than the one between CS and Fibranova.
3. All other varieties can be differentiated from each other and from the Italian germplasm. Therefore, it seems that a large number of loci in a sample of 10 individuals per cultivar is sufficient to properly identify at least five out of the six hemp varieties examined.

Loci Distribution

Results of *F*-statistics allowed a more detailed inspection of the most discriminative loci. One possible approach to characterize hemp varieties could be based on the definition of a minimum Fst value above which loci are assumed to be discriminative. This is obviously true for the eight variety specific markers indicated in Table 3 and having a Fst value of 1.00. These markers could be used to discriminate the amplification products of a bulked DNA sample (Fig. 1). However, 26 other markers were found with Fst above 0.48 (average value) and are therefore potentially useful for discriminating single cultivars. Using cumulatively all the markers with Fst >0.48, all the varieties could be fully discriminated, with the exception of the pair Carmagnola–CS, which were characterized by similar allele frequencies. Out of 102 loci, 68 have Fst values <0.48. This is an indication of the low overall discriminating power of the majority of the loci, and it underlines the necessity of examining a wide number of markers despite the high variability found in hemp. This also hints at the existence of a widely shared gene pool in *Cannabis*, with limited loci segregation in different populations.

Variance Composition

Variance component analysis revealed that the proportion of variance attributable to between gender differences within dioecious cultivars is not significant. Instead, interindividual differences within sexes explain most of the observed variation (Table 4, upper part). Even when gender was ignored and all the varieties were considered simultaneously (dioecious, monoecious and female cultivars), 51.2% of the observed variation was explained by interindividual differences within cultivars, and only 48.8% was explained by among-cultivar variation (Table 4, lower part). In summary, with every primer tested, the amount of intracultivar variation is of the same magnitude as the intercultivar variation. This result supports previous findings suggesting the existence of a single, widely shared gene pool with limited genetic separations among groups (de Meijer, 1999). The AMOVA results indicate the presence of a reservoir of variation even within the most selected varieties, which could also explain the extreme adaptability and flexibility of this species. Nonetheless, the proportion of among-cultivar variance changes dramatically depending upon the cultivars examined (Table 5), ranging between 12.8% (Carmagnola–CS, where the variety boundary is faint), up to over 76% (b92.73.2.13 vs. Fibrimon or Northern Lights) in the cases of highly selected, divergent cultivars.

The amounts of intra- and intercultivar variation found in this study and in previous ones (Faeti et al., 1996; Mandolino et al., 1998) appear to be typical of dioecious, outbreeding species, and are comparable with those reported in species like buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.], white clover (*Trifolium repens* L.), and foxglove (*Digitalis obscura* L.), where the proportion of total variation attributable to individual differences within the populations ranged between 73 and 85% (Huff et al., 1993; Gustine and Huff, 1999; Nebauer et al. 1999).

CONCLUSIONS

The low Fst values found for the majority of the tested loci points to the difficulties encountered in hemp cultivar characterization. A wide number of markers need to be scored, and in this respect, AFLP technology seems to be the best option for future germplasm studies. On the other hand, this technology is still costly, and our results have shown the necessity to consider a large number of individuals per cultivar, due to the high variability found even within the most selected materials. Nonetheless, RAPD analysis is already accepted as suitable for the identification of the genetic structure and the geographical origin of *Cannabis* germ-

plasm (Faeti et al., 1996). This study also shows that the sexual phenotype and variety sex ratio do not need to be taken into account during population sampling, as their contribution to cultivar diversity was not significant. However, it is advisable to be aware of the existence of a high number of markers associated with the Y chromosome (Mandolino et al., 1998; Sakamoto et al., 1997, 1998).

Overall uniformity and distinctness, together with stability over time, are of crucial importance for the definition of modern varieties. There is the possibility that the time elapsed since the last period of active breeding and selection (i.e., for Italy and other countries the first half of the sixties) might have caused a change or shift in varietal characteristics; this could be true for CS, found to be not different from Carmagnola in this study. It is somehow paradoxical that until 1996, when hemp cultivation and breeding was resumed in most western European countries, very advanced hemp selection methods were employed mainly in the breeding of drug varieties. The reexamination, with modern technologies, of the hemp germplasm and possibly its marker-assisted recovery from farmers or collectors, can also lead to a revival of hemp breeding, aiming to the introduction of new traits in this crop species.

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Blend Response and Stability and Cultivar Blending Ability in Oat

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ABSTRACT

Genetic diversity in cropping systems can provide buffering against varying environmental conditions. Therefore, cultivar blends may have greater and more stable yields than their pure-line components. Optimization of cultivar blend development requires knowledge of the relative importance of pure-line yield potential, blend response, and cultivar interactions to blend yield. Grain yield and volume weight of oat (*Avena sativa* L.) pure-line cultivars and cultivar blends were measured in eight Iowa environments in order to compare their productivity and stability and to estimate genetic components of blend yields. In one experiment, five early-maturing cultivars were grown as pure lines and as all possible two- and three-way cultivar blends. In a second experiment, ten midseason-maturing cultivars were grown as pure lines and as all possible two-way blends. Grain yield was 3% greater ($P < 0.05$) and volume weight was 1% greater ($P < 0.05$) in blends than in pure lines in the early-maturity experiment; however, pure line and blends did not differ in the midseason-maturity experiment. Blends had more stable ($P < 0.05$) yields than pure lines in the early-maturity experiment only. Modified diallel analysis was used to partition the variation among two-way blends into general yielding ability (GYA) and true general competitive ability (TGCA) of each component genotype, and specific competing ability (SCA) interaction between blend components. General yielding ability variation was significant, whereas variation for neither TGCA nor SCA was significant. Oat genotype responses to blending were sufficiently consistent across blending partners that superior blends can be selected based on pure-line evaluations of early-maturing cultivars.

Oat hectarage in the USA has declined dramatically since 1950 (USDA-National Agricultural Statistics Service, 1998). Inclusion of oat in crop rotations, however, can enhance species diversity on farms and help to reduce weed and insect pests (Liebman and Dyck, 1993), increase soil quality and curb erosion (Gantzer et al., 1991), and stabilize farm incomes (Brummer, 1998). Because oat has value as feed for livestock, in human nutrition, and as a partial remedy for many production problems, methods to increase and stabilize oat grain yields are needed.

To minimize the adverse effects of environmental stresses on yield, plant breeders have attempted to develop cultivars that will perform reliably well across a range of years and sites (Evans, 1993; Allard and Bradshaw, 1964). Yield stability is the result of a crop's buffering capacity, that is, its ability to adapt to variable weather, insect, disease, weed, and soil conditions. Ways to improve a crop's buffering capacity include intra-population interplant buffering through mixing cultivars or genotypes, and individual intraplant buffering through maintenance of heterozygosity (Allard and Bradshaw, 1964). A cultivar blend can capitalize on the principle of intra-population buffering, because a mixture of genetically different plants may have a greater chance of successful adaptation across a range of environments than a genetically homogeneous population.

Smithson and Lenné (1996) reviewed the literature on cultivar blends in many crops and concluded that blends generally yield slightly more than pure lines, but their true benefits lie in disease control and stability. Blending can have significant positive effects on disease control (Mundt et al., 1995; Finkh and Mundt, 1992; Power, 1991), and can reduce yield losses caused by variability in soil quality (Trimble and Fehr, 1983). The usefulness of cultivar blends in oat, however, has not been established definitively. Pfahler (1965) reported that a small sample of cultivar blends had greater yield stability than the component pure lines. Frey and Maldonado (1967) found that cultivar blends had significantly higher yields than their component pure-line cultivars only when in more stressful environments. Shorter and Frey (1979), by contrast, found no difference between blend and pure-line performance.

Comparisons of blends and pure lines can vary among samples of cultivars because of genotypic variation for contributions to blend performance. Gizlice et al. (1989) used a modified diallel analysis to characterize specific genotypic contributions to blend response. In this analysis, variation among blends was partitioned into general blending ability (GBA) and SCA variances. These effects are analogous to the general and specific combining abilities estimated from diallel analyses of single-cross hybrids in maize (*Zea mays* L.; Sprague and Tatum, 1942). Gizlice et al. (1989) demonstrated that if pure-line components are evaluated in the same experiment as the blends, then GBA can be partitioned into two components, GYA and TGCA. The GYA represents the innate yielding ability of a cultivar grown as a pure line, and the TGCA is the additional mean competitive response of a cultivar calculated as the difference be-

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Abbreviations: GBA, general blending ability; GLM, general linear models; GYA, general yielding ability; SCA, specific competing ability; TGCA, true general competitive ability.