

1 Genomic and Chemical Diversity in *Cannabis*

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## 25 **Abstract**

26 Plants of the *Cannabis* genus are the only producers of phytocannabinoids, terpenoid compounds  
27 that strongly interact with evolutionarily ancient endocannabinoid receptors shared by most  
28 bilaterian taxa. For millennia, the plant has been cultivated for these compounds, but also for  
29 food, rope, paper, and clothing. Today, specialized varieties yielding high-quality textile fibers,  
30 nutritional seed oil or high cannabinoid content are cultivated across the globe. However, the  
31 genetic identities and histories of these diverse populations remain largely obscured. We  
32 analyzed the nuclear genomic diversity among 340 *Cannabis* varieties, including fiber and seed  
33 oil hemp, high cannabinoid drug-types and feral populations. These analyses demonstrate the  
34 existence of at least three major groups of diversity, with European hemp varieties more closely  
35 related to narrow leaflet drug-types (NLDT) than to broad leaflet drug-types (BLDT). The BLDT  
36 group appears to encompass less diversity than the NLDT, which reflects the larger geographic  
37 range of NLDTs, and suggests a more recent origin of domestication of the BLDTs. As well as  
38 being genetically distinct, hemp, NLDT and BLDT genetic groups each produce unique  
39 cannabinoid and terpenoid content profiles. This combined analysis of population genomic and  
40 trait variation informs our understanding of the potential uses of different genetic variants for  
41 medicine and agriculture, providing valuable insights and tools for a rapidly emerging, valuable  
42 legal industry.

43

## 44 **Significance Statement**

45 Despite millennia of cultivation and current widespread use across the globe, *Cannabis* is the  
46 only multi-billion dollar crop for which the genetic identities and origins of most varieties are  
47 unknown. As legalized cultivation of hemp and high-cannabinoid types continues to grow

48 rapidly in the US and other countries, the need for a better understanding of the diversity and  
49 evolution of the species has increased. Through analyzing the genomes of 340 hemp, drug and  
50 feral *Cannabis* individuals, we found significant evidence for at least three major genetic groups.  
51 Importantly, each group produces distinct phytochemical profiles. Our results improve the  
52 understanding of genetically and chemically diverse *Cannabis* strains currently cultivated, and  
53 provide a roadmap for developing improved varieties.

54

## 55 **Introduction**

56 Plants of the genus *Cannabis* (Cannabaceae; hemp, drug-type) have been used for thousands of  
57 years for fiber, nutritional seed oil and medicinal or psychoactive effects. Archaeological evidence  
58 for hemp fiber textile production in China dates to at least as early as 6,000 years ago (1), but  
59 possibly as early as 12,000 years ago (2), suggesting *Cannabis* was one of the first domesticated  
60 fiber plants. Archeological evidence for medicinal or shamanistic use of *Cannabis* has been found  
61 at Indian, central-Asian and middle-eastern sites (3), further illustrating the widespread extent of  
62 *Cannabis* utilization throughout human history. A central Asian site of domestication is often cited  
63 (4), although genetic analyses suggest two independent domestication events may have occurred  
64 separately (5).

65 *Cannabis* plants are usually annual wind-pollinated dioecious herbs, though individuals  
66 may live more than a year in subtropical climates (6) and monoecious populations exist (7). The  
67 taxonomic composition of the genus remains unresolved, with two species (*C. indica* and *C. sativa*)  
68 commonly cited (8), although *C. ruderalis* is sometimes proposed as a third species that contains  
69 northern short-day or auto-flowering plants (9). Monospecific treatment of the genus as *Cannabis*  
70 *sativa* L. is also common (10) and various alternative nomenclature schemes (e.g. *Cannabis sativa*

71 subsp. *indica* var. *kafiristanica*) are sometimes referenced (4). Even though an extensive  
72 monograph on the genus has recently been published (11), limited genetic and experimental data  
73 leaves the questions of taxonomy unresolved (12, 13).

74 The geographical and ecological range of *Cannabis* is unusually broad, with cultivated  
75 populations growing outdoors on every continent except Antarctica in a wide range of  
76 environments from sub-arctic to temperate to tropical, and from sea level to over 3,000 meters  
77 elevation (14, 15). Feral or wild populations are also found as far north as the edge of the Arctic  
78 Circle in Eurasia, but are most common in well drained soils of temperate continental ecosystems  
79 in Eurasia and North America, while tropical populations are absent or rare (14). Perhaps  
80 unsurprising, given this diversity of habitats, the species contains extensive phytochemical  
81 diversity, particularly in cannabinoid and terpenoid profiles (5, 16), and also shows extensive  
82 diversity of morphological and life-history characteristics, further fueling debate regarding the  
83 taxonomic status and origins of *Cannabis* domestication.

84 One distinctive feature of the *Cannabis* genus is the production of a tremendous diversity  
85 of compounds called *cannabinoids*, so named because they are not produced at high levels in any  
86 other plant species (17). Cannabinoids are a group of at least 74 known C<sub>21</sub> terpenophenolic  
87 compounds (18, 19) responsible for many reported medicinal and psychoactive effects of *Cannabis*  
88 consumption (20). Some estimates for the total number of phytocannabinoids range to well over a  
89 hundred (21), though this number includes breakdown products as well as compounds found at  
90 extremely low levels. The plants produce a non-psychoactive carboxylic acid form of these  
91 compounds, with heating required to convert cannabinoids into the psychoactive decarboxylated  
92 forms. Interestingly, these compounds have pronounced neurological effects on a wide range of  
93 vertebrate and invertebrate taxa, suggesting an ancient origin of the endocannabinoid receptors,

94 perhaps as old as the last common ancestor of all extant bilaterians, over 500 MYA (22). The plant  
95 compounds thus produced have the potential to affect a broad range of metazoans, though their  
96 ecological functions in nature are not well understood. Indeed, suggested roles for these  
97 compounds include many biotic and abiotic defenses, such as suppression of pathogens and  
98 herbivores, protection from UV radiation damage, and attraction of seed dispersers. These  
99 hypotheses about the selective benefits of cannabinoid production remain speculative, as none  
100 have been conclusively verified to date. We do know more, however, about the more recent  
101 evolution of the plants under human cultivation.

102 High delta-9-tetrahydrocannabinolic acid (THCA) (23) content has been selected for in  
103 many strains due to its potential to be converted to delta-9-tetrahydrocannabinol (THC), which has  
104 potent psychoactive (24), appetite-stimulating (25), analgesic (26) and antiemetic (27) effects.  
105 These effects are mediated through interactions with human endocannabinoid CB1 receptors found  
106 in the brain (28), and CB2 receptors, which are concentrated in peripheral tissues (29). Other THC  
107 receptor binding locations are hypothesized as well (30). After several decades of accelerated  
108 clandestine cultivation technique and breeding improvements, some modern strains can now yield  
109 dried un-pollenated pistillate inflorescence material that contains over 30% THCA by dry-weight  
110 (31). However, other cannabinoids may also be present in high concentrations. In particular, high  
111 cannabidiolic acid (CBDA) plants were historically used in some hashish preparations(32) and are  
112 presently in high demand as an anti-seizure therapy (33). In contrast with THC, which acts as a  
113 partial agonist of the CB1 and CB2 receptors, CBD does not have as strong psychoactive  
114 properties, and instead has antagonist activity on agonists of the CB1- and CB2-receptors (34).  
115 Thus, the two most abundant cannabinoids produced in *Cannabis* have, to some degree, opposing  
116 neurological effects.

117 THCA and CBDA are alternative products of a shared precursor, CBGA (35). A single  
118 locus with co-dominant alleles was proposed to explain patterns of inheritance for THCA to CBDA  
119 ratios (7, 36). However more recent quantitative trait loci (QTL) mapping experiments (37),  
120 expression studies (38) and genomic analyses (10) paint a more complex scenario with several  
121 linked paralogs responsible for the various THCA and CBDA phenotypes. Other cannabinoids  
122 such as cannabigerol (CBG) (39), cannabichromene (CBC) (40) and delta-9-  
123 tetrahydrocannabivarin (THCV) (41) demonstrate pharmacological promise, and can also be  
124 produced at high levels by the plant (42–44). Additionally, *Cannabis* secondary metabolites such  
125 as terpenoids and flavonoids likely contribute to therapeutic or psychoactive effects (2), such as  $\beta$ -  
126 myrcene, humulene and linalool proposed to produce sedative effects associated with specific  
127 strains (45).

128 In this study, plants that produce low levels of total cannabinoids are herein referred to as  
129 hemp, while high cannabinoid producing varieties are described as drug-type strains. Legal  
130 definitions often use a maximum THCA threshold to delineate hemp from drug-types, thus some  
131 high CBDA producing strains are categorized as hemp. However this definition ignores the  
132 broader traditional usage of hemp for fibers or seed oils and historical presence of CBDA-  
133 producing alleles in some drug-type populations (32). Additionally, hemp strains have a distinct  
134 set of growth characteristics (46), with fiber varieties reaching up to 6 meters in height during a  
135 growing season, exhibiting reduced flower set, increased internodal spacing and lower total  
136 cannabinoid concentration per unit mass compared to drug-type relative. Despite the widespread  
137 prohibition of drug-type *Cannabis* cultivation from the 1930s to present (47), hemp cultivation  
138 and breeding continued in parts of Europe and China through this period, and experienced a brief  
139 comeback during World War II in the USA through the Hemp for Victory campaign. Studies to

140 date have found hemp varieties are genetically distinct from drug-type strains (10), though  
141 interestingly Hillig (5) found broad leaflet southeastern Asian hemp landraces to be more closely  
142 related to Asian drug-type strains than to European hemp strains.

143 *Cannabis* has a diploid genome ( $2n = 20$ ), and an XY/XX chromosomal sex-determining  
144 system(48). The genome size is estimated to be 818 Mb for female plants and 843 Mb for male  
145 plants (49). Currently, a draft genome consisting of 60,029 scaffolds is available for the Purple  
146 Kush (PK) drug-type strain from the National Center for Biotechnology Information. Additional  
147 whole genome data is available from NCBI for the Finola and USO31 hemp strains. Various  
148 reduced representation genome, gene and RNA sequence data are also available from NCBI.  
149 Presently *Cannabis* is the only multi-billion dollar legal crop without a sequence-based genetic  
150 linkage or physical genome map. Indeed, the first genetic map for the species, using AFLP and  
151 microsatellite markers, was only recently published, providing for the first time, quantitative trait  
152 mapping of cannabinoid content and other traits (37).

153 Initial studies of *Cannabis* genetic diversity examined either many samples with few  
154 molecular markers (5) or whole genome wide data for relatively few samples types (10). Sawler  
155 et al. (50) recently published a survey of *Cannabis* genomic diversity, using a reduced genomic  
156 representation strategy to evaluate 81 marijuana (drug-type) and 43 hemp strains. The aim of this  
157 present study is to assess the genomic diversity and phylogenetic relationships among 340 total  
158 *Cannabis* plants that have distinct phenotypes, and that were described *a priori* by plant breeders  
159 as various landraces, *indica*, *sativa*, hemp and drug-types, as well as commercially available  
160 hemp and drug-types with unclear pedigrees. We have combined data from existing sources and  
161 generated new data to create the largest sample set of *Cannabis* genomic sequence data  
162 published to date. These data and analyses will continue to facilitate the development of

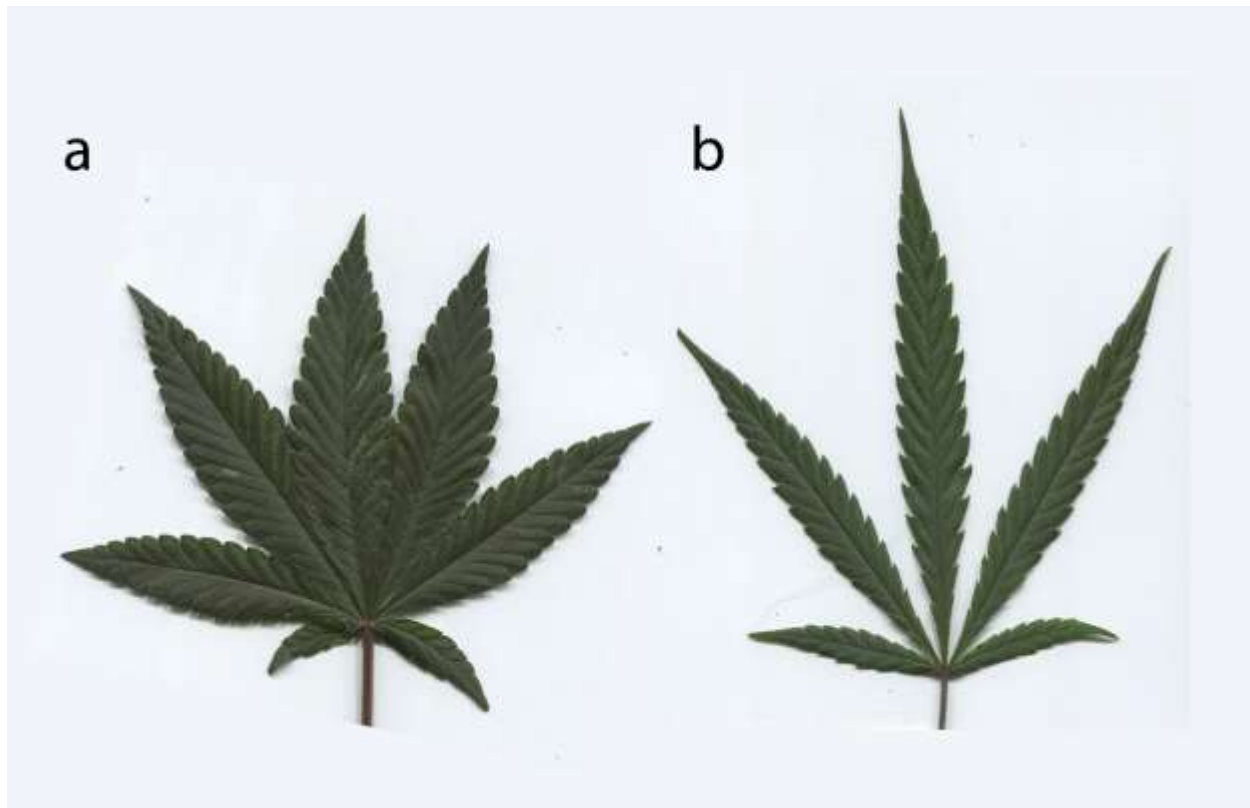
163 modernized breeding and quality assurance tools, which are lacking in the nascent legal  
164 *Cannabis* industry.

165

## 166 **Results and Discussion**

167 **Sequencing and SNPs.** Summary information and raw sequencing libraries are publically  
168 available from the NCBI short read archive (accessions pending). Detailed information about all  
169 samples can be found in Dataset S1 and examples of wide and narrow leaflet forms are shown in  
170 Figure 1. Of the 466,427,059 non-ambiguous base pairs in the PK reference, 77,810,563 bps  
171 were removed due to excess self-similarity ( $\geq 97$  % identity and  $\geq 500$  bps length, Figure S1).  
172 After this filter, the total single copy portion the PK reference within the combined coverage  
173 levels for all 67 WGS samples of 326x – 401x, a 95% Poisson confidence interval around a 362x  
174 mean, was 71,236,365 bps (Figure S1). After quality (Q), genotype quality (GQ), allele  
175 frequency (AF), missing data, biallelic and ambiguous base filters, the following SNP counts  
176 remained: 491,341 WGS, 2,894 GBS (this study), SNPs 4,105 GBS Sawler (50). Forty-five  
177 SNPs overlapped both GBS datasets, and the WGS samples.





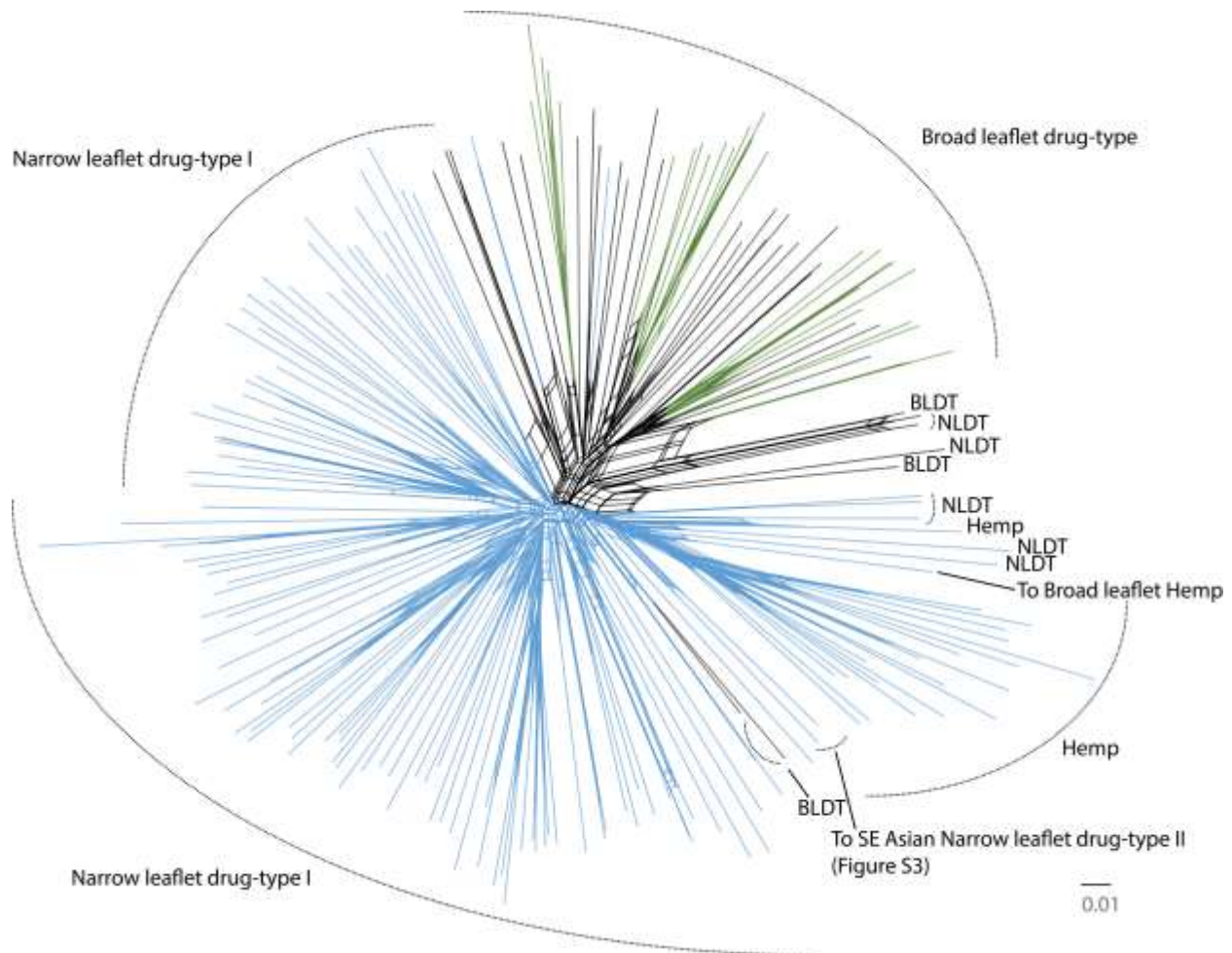
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179 Figure 1. Example of broad leaflet type (a, R4) and narrow leaflet type (b, Super Lemon Haze)  
180 strains. Photograph credits: D. Vergara.

181

182 **Phylogenetic Relationships.** Bifurcating trees are commonly used to model mutation driven  
183 divergence and speciation events. Whole genome wide sequence datasets include information  
184 about recombination, hybridization, and gene loss or genesis events, some of which may be  
185 incongruent with one and other (51). Phylogenetic networks can represent incompatible  
186 phylogenetic signals across large character matrices in a visually informative manner. Figure 2  
187 contains 195 *Cannabis* samples including WGS and GBS data, and shows that all European  
188 hemp strains form a distinct clade, separated from drug-type strains by a consistent band of  
189 parallel branches. Broad leaflet drug-type strains clustered with purported Afghan Kush landrace  
190 samples (Dataset S1 and Figure S3), while narrow leaflet drug-type strains appear to contain

191 several groups with only faint visible distinctions between them, perhaps influenced by the  
192 inclusion of hybrid strains in the analysis.



194 Figure 2. Phylogenetic neighbor network of a 2,894 SNP alignment from the single-copy portion  
195 of the *Cannabis* genome. Clade names on the periphery were inferred via FLOCK (where  $K \geq 3$   
196 was most likely). Colored branches indicate fastStructure population membership of  $\geq 70\%$   
197 assignment (where  $K=2$  was most likely). NLDT = Narrow Leaflet Drug-Type and BLDT =  
198 Broad Leaflet Drug-Type. To SE Asian NLDT II points to Dr. Grinspoon and Somali Taxi Cab  
199 samples. To Broad Leaflet Hemp points to a Chinese hemp sample. A high-resolution version of  
200 this figure that includes each sample name is available from:  
201 [https://figshare.com/articles/Cannabis\\_Tree/1585470/4](https://figshare.com/articles/Cannabis_Tree/1585470/4)

202

203 We found significantly more heterozygosity in drug-type strains than in hemp varieties  
204 (31 % v 22 %,  $p < 0.001$ , two-tailed Mann-Whitney U-test, Table 1). This likely reflects the

205 widespread hybridization of strains in North America during the transition to indoor cultivation  
206 of drug-types starting in the 1970s (52), as well as the extensive reliance on clonal propagation  
207 for indoor commercial cultivation, which does not require trait stable seed stock. Conversely,  
208 fiber and seed oil hemp are grown on multi-acre scales that have necessitated the stabilization of  
209 agronomically important traits in seed stocks, likely leading to reduced heterozygosity at some  
210 loci.

### 211 Group Genetic Information

	Mean Within Distances	Heterozygosity %
Hemp	0.195	0.22*
All Drug-types	0.244	0.31*
NLDT	0.237	0.32
BLDT	0.221	0.30
	Mean Between Distances	F <sub>ST</sub>
Hemp v. All Drug-types	0.273	0.098530
Hemp v. NLDT	0.269	0.091679
Hemp v. BLDT	0.281	0.10131
NLDT v. BLDT	0.258	0.036156

212

213 Table 1. Summary of genetic distance, heterozygosity and F<sub>st</sub> information for major *Cannabis*  
214 groups. \* = significantly different (p < 0.001, two-tailed Mann-Whitney U-test).

215

216 **Population Structure.** To determine the statistical likelihood of various population scenarios  
217 represented in our samples, we first applied the FLOCK model to our data set of 195 GBS and  
218 WGS *Cannabis* samples, which is an iterative reallocation clustering algorithm that does not  
219 require non-admixed individuals to make population assignments (53). Using the K-partitioning  
220 method suggested by the authors (53), we determined that  $K \geq 3$ , after testing K values of one to  
221 eight (Table 2 and peripheral population names in Figure 2). FLOCK was able to assign all  
222 samples to one of the three populations, although it does not calculate admixture proportions.  
223 Sample population assignments were largely consistent with the known history of these samples,

224 and appear visually consistent with MDS analysis (Figure S2). For example all fiber and seed oil  
 225 hems were assigned to an exclusive population, with the exception of sample AC/DC, a high  
 226 CBDA producing variety, with likely hybrid hemp origins (Figure 2, Table 2).

227 Sample Names

A-train	Original_Sour_Diesel	C36	H11	Schemp
Afghan_Kush	Phantom_Cookies	C37	H5	Skunk_#1
Afghan_Kush	Platinum_OG	Canna_Tsu	Harlequin	Somali_Taxi_Cab
Afghan_Kush	Purple_Kryptonite	Cannatonic	Hash_Plant	Spectrum-11
Afghan_Kush	Purple_Kush	CBD_Diesel	Hawaiian	Spectrum-14
Afghan_Kush	Purple_Urkle	CBD_Shark_F-6	Holy_Grail	Super_Lemon_Haze
Boss_Hogg	R4	CBD-0	Holy_Grail_Kush	Sweet_Afghani_Delicious
Bubba_Kush	San_Fran_Valley_OG_Kush	CBD18	Jack_47	Sweet_Skunk
Char_Tango	Screaming_Haze	Charlottes_Web	Jack_Flash	Tangerine_Haze
Chem_91'	SFV	Cheese_Quake	Jack_Herer	Train_Wreck
Chem91	Skywalker_OG	Cherry	Jack_Herrer	Trainwreck
Chemdawg	Snowcap	Cherry_Afghan	Jack_Herrer	Violator_Kush
Chocolate_Kush	Snowcap	Chocolope	Jack_Skellington	White_Cookies
Crippd_Out_Cookies	Sour_Diesel	Chocolope	Juicy_Fruit	White_Widdow
Cript_out_Cookies	Sour_Patch_Kush	Colombia_Rio_Negro	Lebanese	White_Widow
Dead_Head_OG	Sour_Willie	Critical_Kush	Lemon_Skunk	Wonder_Woman
Dog_Walker	Sshrek	Critical_Kush	Liberty_Haze	XJ_13
Flo	The_Sauce	Critical_Mass	Lions_Tabernacle	AC/DC
Girl_Scout_Cookie_#6	The_Sauce	Dr_Grinspoon	Low_Ryder	AZ_Star_#1
Girl_Scout_Cookies	Tora_Bora	Durban_Poison	LSD	Carmagnola
Girl_Scout_Cookies	WAF_B	Durban_Poison	Mad_Cow	Carmagnola
Goast_Train_Haze	4-Jack	Easy_Sativa	Mango_Stomper	Carmagnola
Grapefruit	Afghan_Mango	Exodus_Cheese	Maui_Waui	Carmagnola
Guido_OG	Afghan_Mango	G13	Mazar	Carmagnola
Headband	Afghan_Mango	G13_Haze-31	Medical_Mass	Carmagnola
Hindu_Kush	Alaskan_Thunderfuck	Gin	Melon_Gum	Chinese_hemp
Kandy_Kush	Appalachian_Mad_Sun	Girl_Scout_Cookies	Mexican_E	Dagestani_hemp
King_Chem	Auto_AK47	Glass_Slipper	MO	EuroOil_2
King_Louie_Cookies	B-5	Golden_Goat	Nuclear_Fruit	Feral_Kansas
Kool_Aid_Kush	BC_HQ	Grand_Daddy_Purps	Otto	Feral_Nebraska
Kosher_Kush	Black_Cherry	Grape_AK-47	Peaches_and_Cream	Feral_Nebraska
Kosher_Kush	Black_Jack	Grape_Ape	Pineapple	Finola
Kosher_Kush_#1	Blue_Cheese	Grape_Ape	Pineapple_Express	J7
Kunduz	Blue_Dream	Grape_Kush	Pink_Lady	J20
Larry_OG	Blue_Dream	Grape_Kush	Pre-98_Bubba_Kush	J28
Medibud	Blue_OG	Green_Crack	Purps	Kompolti_1
OG_18	Blueberry_DJ	Green_Crack	R4	Kompolti_2
OG_Kush_1	Bubble_Gum	Green_Mandarine	Red_Purps	Sievers_Infinity
Old_Skool_OG	Bubble_Gum_XL	Green_Poison	Rocky_Mountain_Blueberry	US031

228

229 Table 2. Sample names and FLOCK assignment to three groups, represented with different cell  
230 colors. Green are BLDT, blue are NLDT and yellow are hemp.

231

232           Additionally we applied the admixture model based Bayesian clustering method of  
233 fastStructure to the same 195 samples (54). The most likely population structure analysis of  $K=2$   
234 (Figure 2, Dataset S1), shows consistent separation between BLDT and NLDT and hemp strains.  
235 Some hemp and NLDT strains were each assigned with near 100% population membership to the  
236 same population (Figure 2, light blue samples, Dataset S1), despite the clear separation  
237 visualized in the tree and statistically significant mean between-group genetic distance measured  
238 (Table 1). The separation of BLDT and NLDT strains into fastStructure populations was stable  
239 when hemp samples were excluded from the analysis (Dataset S1). Sawler et al. (50) used  
240 fastStructure to delineate hemp from drug-types as the major division of *Cannabis* diversity, and  
241 found two drug-type sub-groups within their samples when hemp types were excluded from the  
242 analysis. Likewise using a smaller dataset, Lynch (55) found support for  $K=3$ , consisting of two  
243 separate drug-type populations and hemp types, using the original Structure implementation (56)  
244 and the Evanno method to select the best value of  $K$  (57). However, we caution that despite  
245 many claims for the availability of “landrace genetics” (strains) from *Cannabis* producers,  
246 breeders and seed sellers, these may or may not represent non-admixed individuals (52)—a  
247 situation that can be problematic for the Structure and fastStructure approaches (56).

248           The GBS samples from Sawler et al. (50) appear to contain an additional divergent  
249 NLDT clade, with likely SE Asian origins (Supplementary Figures 3 and 4), that did not emerge  
250 from our main analyses. Due to very limited overlap between sequence fragments from the two  
251 GBS datasets, which results from using different restriction enzymes, we were required to re-  
252 analyze the Sawler data in combination with only our 67 WGS samples. A connection was made

253 across the two GBS analyses to this SE Asian NLDT group through two WGS samples (Dr.  
254 Grinspoon and Somali Taxi Cab, Figure 2, Supplementary Figures 3) that were included in both  
255 sets of GBS analyses. Although only 45 SNPs overlapped between both types of GBS data and  
256 the WGS data, a phylogeny of this limited alignment also supports the existence of an additional  
257 distinct SE Asian NLDT clade (Figure S4). Collectively these analyses lend support to a total  
258 lower bound of four *Cannabis* populations, although clearly more extensive sampling with  
259 consistent sequencing is required to fully access standing biogeographic diversity.

260

261 **Tests of Tree Models.** To test hypotheses of tree-like evolution for the three genetic groups, we  
262 first applied the three-population test for admixture (58), and found no evidence for admixture in  
263 any of the pairwise comparisons (positive  $f$  statistic values). Next we constructed maximum  
264 likelihood trees based on the aggregate SNP frequencies for the three genetic groups and  
265 simulated a variety of ‘migration’ events (0-10), but no simulation produced non-zero migration  
266 graph edges (Figure 3).  $F_{ST}$  analysis shows little divergence among lineages for most loci, but a  
267 substantial number of highly-divergent regions are unique to each clade (Figure 4). This  
268 reinforces the importance of using many, high-quality, single-copy regions of the genome, rather  
269 than smaller numbers of loci that could lead to less resolution or even misleading results.

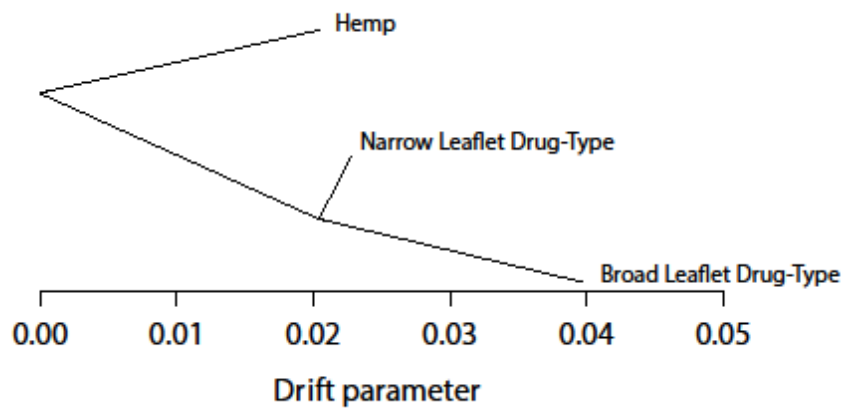
270 Although lore (52), Figure 2 and Figure S2 strongly suggest at least some individuals have  
271 hybrid origins, these tree models for the overall SNP frequencies of the population groups  
272 inferred by FLOCK (Table 2) imply each group contains strong genetic signals from ancestral  
273 biogeographic gene pools.

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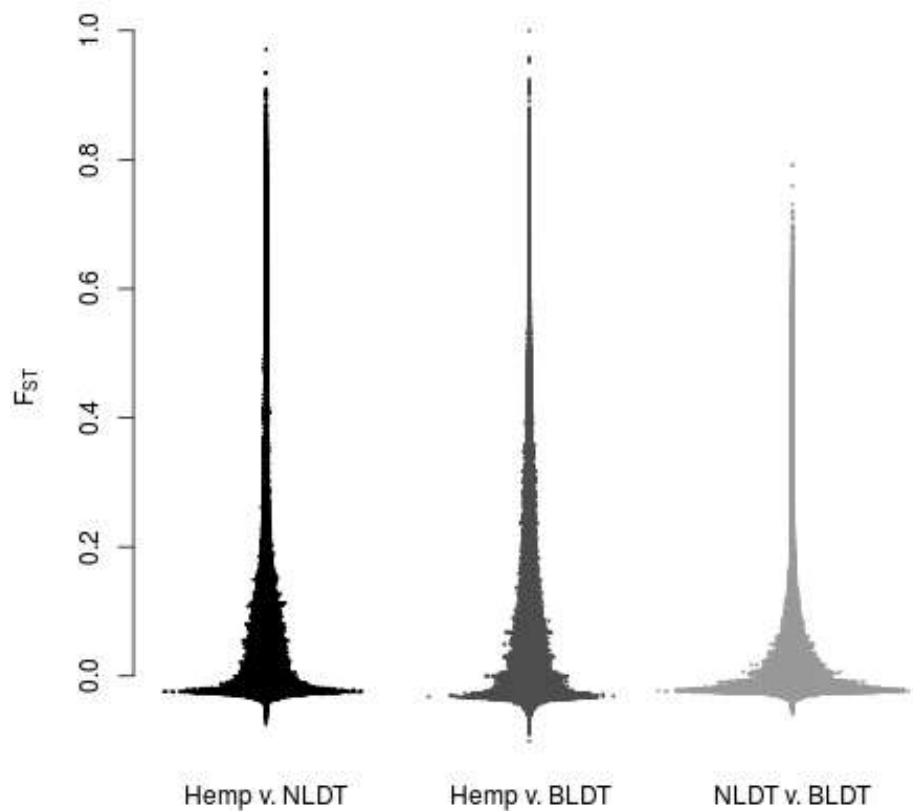
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278

279 Figure 3. Maximum likelihood tree of three *Cannabis* populations. We found no evidence for  
280 extensive admixture or deviations from this tree model.

281



282

283 Figure 4. Distribution of Weir-Cockerham  $F_{ST}$  estimates for each population comparison. Each  
284 population pair has some portion of segregated sites.

285

286 Additional *Cannabis* diversity likely remains to be sampled. Notably absent from all  
287 genome sequence datasets published to date are putative *C. ruderalis* (59) samples. These are  
288 short weedy plants, with free shattering inflorescences found widely from northern Siberia,  
289 through central Asia and into Eastern Europe (60). Whether these populations represent  
290 ancestral, pre-domesticated wild *Cannabis*, more recent feral escapes or some combination of  
291 both remains unclear. Even though we were not able to sample putative *C. ruderalis* populations,  
292 Finola is an early maturing seed hemp strain from Finland with purported northern Russian  
293 landrace ancestry (52), and Low Ryder and Auto AK-47 are auto-flowering drug-type strains



294 with possible *C. ruderalis* heritage included in our samples (Table 2). Our analyses found Finola  
295 fits within the hemp group while Low Ryder and Auto AK-47 are close relatives of each other  
296 within the NLDT group (Figure S3). Further genomic analyses are required to determine the  
297 extent to which *C. ruderalis* populations are genetically distinct from hemp and drug-type  
298 groups, and whether they may in fact harbor an ancestral wild-type gene pool from which  
299 European hemp varieties were domesticated (5, 16).

300 Broad leaflet Asian hemp is also underrepresented, although we included one putative  
301 Chinese hemp sample that occupies an area between the core hemp and BLDT populations  
302 (Table 2, Figure 2 and Figure S2). Hillig's (5) analysis of alloenzymes concluded that Asian  
303 hemp strains were more similar to Asian drug-type strains than they were to narrow leaflet  
304 European hemp. Likewise, Gao et al. (61) found genetic dissimilarity between European hemp  
305 and Chinese hemp, using microsatellites, and showed at least several distinct groups of hemp  
306 occur across the vast geography of Asia. Overall, Asian and European hemp strains appear  
307 dissimilar genetically, possibly reflecting independent domestication events (60).

308 One major complication obscuring the understanding of *Cannabis* diversity and history is  
309 the lack of information about the native range or ranges of *Cannabis*. In addition to divergent  
310 breeding efforts and human-vectored transport of seeds, the tendency of *Cannabis* is to escape  
311 into feral populations wherever human cultivation occurs in temperate climates (62). This,  
312 coupled with wind pollination biology and no known reproductive barriers, makes the existence  
313 of pure wild native *Cannabis* populations unlikely. The weedy tendencies of *Cannabis* are  
314 exemplified by the mid-western USA populations of feral hemp that flourish despite the  
315 eradication efforts by the Drug Enforcement Agency, which have for decades totaled millions of  
316 plants removed per year. A comprehensive evaluation of *Cannabis* diversity, which includes

317 feral and wild Eurasian populations, is required to ascertain if the levels of divergence and gene  
318 flow are consistent with one or more origins of domestication (5). Even if these extant  
319 populations are highly admixed with modern varieties, their study promises to offer insight into  
320 *Cannabis* ecology and evolution, given how different the selective regime of the feral setting is  
321 compared to that of agricultural fields. Considering the similar debates regarding the timing and  
322 origins of *Oryza* domestication that remain as of yet unresolved (63), *Cannabis* requires  
323 substantially more work to unravel its complicated relationship with humans.

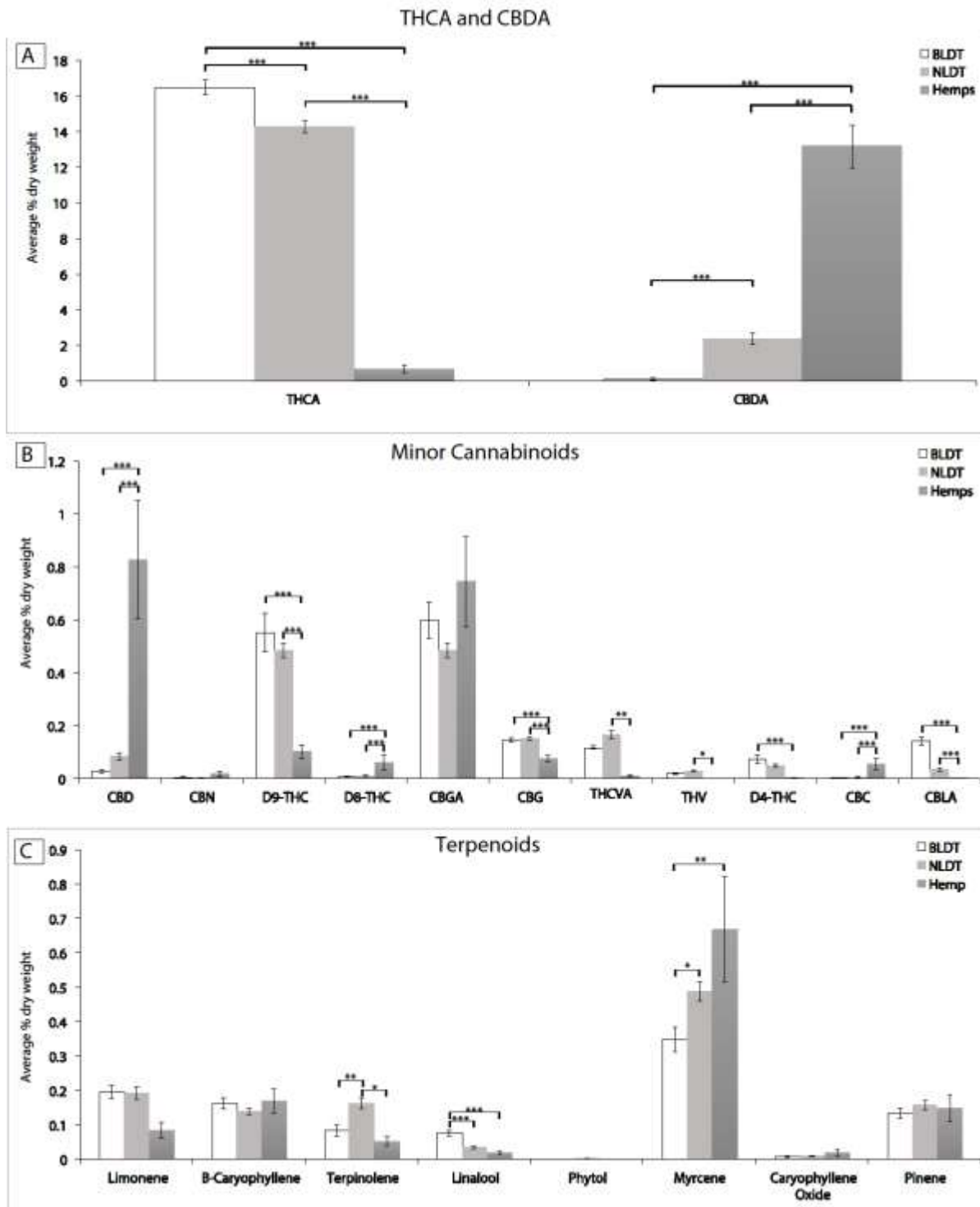
324 'Indica' and 'sativa' are commonly used terms ascribed to plants that have certain  
325 characteristics, often related to leaflet morphology and the perceived effects of consuming the  
326 plant (8). However these names are rooted in taxonomic traditions dating to Linnaeus who first  
327 classified the genus as monotypic (*Cannabis sativa*) based on hemp specimens from Virginia and  
328 Europe (64). Lamarck subsequently designated *Cannabis indica* to accommodate the shorter  
329 stature potent narrow leaflet drug-type plants from the Indian subcontinent (65). Although  
330 currently the term 'indica' is typically used to refer to BLDTs, this biotype from the Hindu-Kush  
331 mountains (14) was not clearly documented until a 1929 survey of Afghani agriculture by  
332 Vavilov (66). This absence of historical documentation until the 20<sup>th</sup> century, a very narrow  
333 geographic range, and some evidence for a broader NLDT gene pool (Table 1, Supplementary  
334 Figures 3 and 4), suggest a separate and more recent origin of the BLDT clade. This origin could  
335 represent a domestication event of a wild or feral BLDT population, or perhaps hybridization  
336 events between NLDT and BLDT populations. Final resolution of *Cannabis* taxonomy will  
337 require complete assessment of standing global genetic diversity and experimental evaluation of  
338 reproductive compatibility across all major genetic groups (67), in conjunction with  
339 morphological circumscriptions. Given the current absence of evidence for reproductive barriers,

340 and overall limited genetic distances between hemp and drug-type strains analyzed in this study  
341 we suggest continued monotypic treatment of plants in this genus as *Cannabis sativa* L. is  
342 warranted.

343 **Cannabinoid and Terpenoid Diversity.** THCA and CBDA are the most abundant cannabinoids  
344 produced by the majority of strains on the North American market today (Figure 5a), and both  
345 compounds show an impressive range of medicinal potential (33, 68), although  
346 endocannabinoid-based therapy trials have a history of significant rates of study withdraws and  
347 adverse effects (69). Historical breeding efforts have resulted in mostly high THCA plants that  
348 produce strong intoxicating effects when consumed, and that synthesize only very low levels of  
349 alternative cannabinoids (Figure 5b). High CBDA plants have only recently become more  
350 available in North America over the last several years in response to demand. Interestingly, these  
351 high CBDA-producing plants form several clusters within the both the NLDT and BLDT groups,  
352 as well as within the hemp group (Dataset S1), but rarely reach equivalent quantities total  
353 cannabinoid production as those found in high THCA plants (Figure 4a). The minor  
354 cannabinoids that are commonly assayed, CBGA, CBCA, THCVA and CBDVA are also of  
355 interest, despite strains producing high levels of these compounds being largely unavailable for  
356 research currently (70). With at least 74 cannabinoids identified in *Cannabis*, modernized  
357 genetic and breeding techniques are required to diversify and optimize *Cannabis* varieties.  
358 Efforts should also be made to document and preserve feral, wild and heirloom populations that  
359 can serve as reservoirs of cultural and genetic diversity.

360 Aromatic terpenoids impart many of the characteristic fragrances to *Cannabis*, and  
361 possibly contribute to the effects of consumption (2). Terpenoids are synthesized in many plant  
362 species, and play a role in relieving various abiotic and biotic stresses through direct and indirect

363 mechanisms (71). Our analysis of strains sharing common genetic groups shows that each group  
364 has a distinct terpenoid profile (Figure 5c and Figure S5). We found NLDTs to contain  
365 significantly more  $\beta$ -myrcene and  $\alpha$ -terpinolene than BLDTs, although interestingly the two  
366 hemp strains for which we analyzed chemical data for had significantly more  $\beta$ -myrcene than  
367 either drug-type group (Figure 5c). Similarly Hillig (72) found NLDTs to yield significantly  
368 more  $\beta$ -myrcene than Afghani BLDTs, yet European hemp and un-cultivated accessions labeled  
369 as *C. ruderalis* contained the highest levels. Hillig also reported that Afghani BLDTs contained  
370 the highest levels of guaiol and eudesmol isomers, which we did not measure, although we found  
371 BLDTs contained more linalool than NLDTs or hemp. Understanding the ecological functions  
372 and evolutionary origins of terpenoids and cannabinoids in *Cannabis* could improve therapeutic  
373 potential, and possibly reduce the need for pesticide application during cultivation.



374

375

376 Figure 5. Average percentage of mass for dried and un-pollinated female flowers of *Cannabis*  
 377 genetic groups. (a) THCA and CBDA cannabinoids (b) Minor cannabinoids (c) Terpenoids.  
 378 THCA = delta-9-tetrahydrocannabinolic acid. CBDA = cannabidiolic acid. CBD = cannabidiol.

379 CBN = cannabinol. D9-THC = delta-9-tetrahydrocannabinol. D8-THC = delta-8-  
380 tetrahydrocannabinol. CBGA = cannabigerolic acid. CBG = cannabigerol. THCVA =  
381 Tetrahydrocannabivarin carboxylic acid. THCV = Tetrahydrocannabivarin. D4-THC = delta-4-  
382 tetrahydrocannabinol. CBC = cannabichromene. CBLA = cannabicyclolic acid.

383

384 **Conclusions.** *Cannabis* genomics offers a window into the past, but also a road forward.

385 Although historical and clandestine breeding efforts have been clearly successful in many  
386 regards (21, 31), *Cannabis* lags decades behind other major crop species in many other respects.  
387 Developing stable *Cannabis* lines capable of producing the full range of potentially therapeutic  
388 cannabinoids is important for the research and medical communities, which currently lack access  
389 to diverse high-quality material in the USA (73).

390 In this paper we extended the initial *Cannabis* genome study (10), by re-mapping WGS  
391 and GBS sequence reads to the existing PK draft scaffolds, to understand diversity and  
392 evolutionary relationships among the major lineages. Although hybridization of cultivated  
393 varieties (52) and human transport of seeds across the globe was hypothesized to have obscured  
394 much of the ancestral genetic signal (13), we found significant evidence for apparent ancestral  
395 signals in genomic data derived largely from modern cultivated varieties (Table 2, Figures 2 and  
396 3). Re-analysis of previously published GBS data (50) provides additional limited evidence for a  
397 fourth group (Supplementary Figures 4 and 5). Interestingly, unique cannabinoid and terpenoid  
398 profiles were associated with three of the genetic groups, lending support to their validity,  
399 despite the limitations of our sampling scheme. Overall, we hope the publicly available data and  
400 analyses from this study will facilitate the continued research on the history of this controversial  
401 plant and the development of the agricultural and therapeutic potential of *Cannabis*.

402

403

404 **Materials and Methods**

405 **Sample collection.** DNA was obtained from numerous sources, including a variety of breeding  
406 and production facilities. The strain names, descriptions and putative origins used in this paper  
407 were recorded from the providers of the DNA and sequence data (Dataset S1). For data not  
408 previously published, DNA extractions were performed using the Qiagen DNeasy Plant Mini Kit  
409 (Valencia, CA) according to the manufacturer's protocol.

410

411 **Whole genome shotgun (WGS) sequencing.** 60 samples were sequenced using standard  
412 Illumina multiplexed library preparation protocols for two 2 x 125 HiSeq 2500 lanes and one 2 x  
413 150 NextSeq 500 run. Sequencing efforts were targeted for approximately 4-6x coverage of the  
414 *Cannabis* genome per sample.

415

416 **Genotype-by-Sequencing (GBS).** 182 samples were sequenced on two 1 x 100 HiSeq 2500  
417 lanes, following a multiplexed library preparation protocol described previously (74).

418

419 **Publicly available data.** We obtained three WGS datasets available from NCBI (10) and  
420 received seven additional WGS datasets from Medicinal Genomics Corporation  
421 ([www.medicinalgenomics.com](http://www.medicinalgenomics.com)). GBS data for 143 samples from Sawler et al. (50) were also  
422 included in this study.

423

424 **Sequence Processing, Alignment and SNP calling.** Trimmomatic (75) was used to trim any  
425 remaining adaptor sequence from raw fastq reads and remove sequences with low quality regions  
426 or ambiguous base calls using the following settings:

427 ILLUMINACLIP:IlluminaAdapters:2:20:10 LEADING:20 TRAILING:20  
428 SLIDINGWINDOW:5:15 MINLEN:100. Trimmed raw reads from the 67 total WGS samples  
429 were then aligned to the only publicly available draft genome of PK (JH226140-JH286168)  
430 using the Burrows-Wheeler Alignment tool (BWA mem) (76). Chloroplast and mitochondrial  
431 regions were excluded. We collated the individual alignments to produce a single variant call  
432 format table (.vcf) for all samples using samtools mpileup -uf | bcftools view -bvcg (77). We  
433 filtered the vcf table to include only high quality informative SNP sites using vcftools (78), bash  
434 and awk with the following vcf parameters: Q (>200), GQ (>10), AF1 (.1 - .9), biallelic sites  
435 only and no ambiguous bases. Next, data filters were applied through plink (79) to require that  
436 individuals have a minimum 50% informative sites and that sites each have data for minimum  
437 20% of samples. Finally we used an estimate of expected coverage for the single copy portion of  
438 the genome based on the estimated genome size and number of reads being aligned. This was  
439 adjusted empirically based on total coverage level (across all WGS samples) per SNP site  
440 (Figure S1) and bounded by a 95% Poisson confidence interval (mean 362x coverage). Further  
441 removal of repetitive content was achieved by aligning the PK reference to itself with BLASTN  
442 and removing all sites that were within regions of  $\geq 97\%$  identity for at  $\geq 500$  bp alignments.  
443 These aforementioned processing, alignment and SNP calling procedures were then performed  
444 separately on the 182 GBS samples generated for this study and the 143 GBS samples previously  
445 published (50), resulting in three vcf tables and filtered SNP sets. GBS SNPs were additionally  
446 required to have a minimum of 5x coverage per sample. Due to limited overlap between the SNP  
447 sites produced by the two GBS libraries, most downstream analyses were performed separately  
448 for each GBS library along with its corresponding set of WGS SNPs. Code used for these  
449 analyses is available at <https://github.com/KaneLab>.



450

451 **SNP Analyses.** To visualize genetic relationships, divergence, and ancestral hybridization  
452 among lineages, a phylogenetic neighbor network was inferred using simple p-distance  
453 calculations (51). Heterozygosity counts and Multidimensional Scaling (MDS) analyses were  
454 calculated with Plink (79). Average within and between group genetic distances, and a 45 SNP  
455 alignment neighbor joining tree based on p-distances, were calculated with MEGA6 (80).  
456 Population structure inferences were made through FastStructure (54) and FLOCK (53). Tests  
457 for reticulation within the trees and admixture between populations were performed in TreeMix  
458 (81)  $F_{ST}$  estimates were calculated with vcftools (78).

459

460 **Chemical Analyses of Genetic Groups.** The cannabinoid and terpenoid information  
461 (chemotype) for a portion of the strains in the genome analysis were generated by Steep Hill  
462 Labs (<http://steephill.com/>). Only strains with matching data in the genomic analysis were  
463 analyzed, for a total of 112 individuals from 17 strains from the BLDT group, 278 individuals  
464 from 35 unique strains from the NLDT group, and 33 individuals from two strains of hemp, for a  
465 total of 423 individuals in this analysis (Dataset S1). This chemotype analysis was performed  
466 using high performance liquid chromatography (HPLC) with Agilent (1260 Infinity, Santa Clara,  
467 CA) and Shimadzu (Prominence HPLC, Columbia, MD) equipment. Between 400 and 600  
468 milligrams of each sample was extracted into methanol, diluted and analyzed by HPLC. A  
469 mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in methanol was  
470 used with a gradient starting at 72% methanol and ending at 99% methanol. Terpenoid standards  
471 were purchased from Sigma-Aldrich (St. Louis, MO). Cannabinoid standards were purchased  
472 from Cerilliant (Round Rock, TX), RESTEK (Bellefonte, PA) and Lipomed (Cambridge, MA).

473 A C18 column from RESTEK (Raptor ARC-18, Bellefonte, PA) or Phenomenex (Kinetex C18,  
474 Torrance, CA) was used. Concentrations of cannabinoids without commercially available  
475 standards were estimated using published absorptivities (82). The chemotype data analyzed for  
476 this research includes 13 cannabinoids and eight terpenoids. Each compound was quantified  
477 using a linear calibration curve. Analytes were measured as percent mass in sample and not  
478 corrected for moisture content.

479 We performed a one-way ANOVA for each cannabinoid and terpenoid separately, with  
480 the group (NLDT, BLDT, and hemp) as the predictor variable. We used Bonferroni corrections  
481 for multiple comparisons. We also implemented a Principal Component Analysis (PCA) with  
482 `prcomp` function in base R, and `car` was used to visualize 95% confidence ellipses for each group  
483 ([www.R-project.org](http://www.R-project.org)). Individuals with missing data values for any cannabinoid or terpenoid  
484 were removed. After removing the individuals with missing values, we had a total of 351  
485 individuals: 94 BLDT, 229 NLDT, and 28 hemp.

486

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494

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496 samples. KHW generated DNA sequencing libraries. RCL, NCK and SBT performed  
497 bioinformatics analyses. KdC, DPL and TCR generated chemical data. DV and SBT performed  
498 chemical data analyses. RCL, DV and NCK wrote the paper.

499

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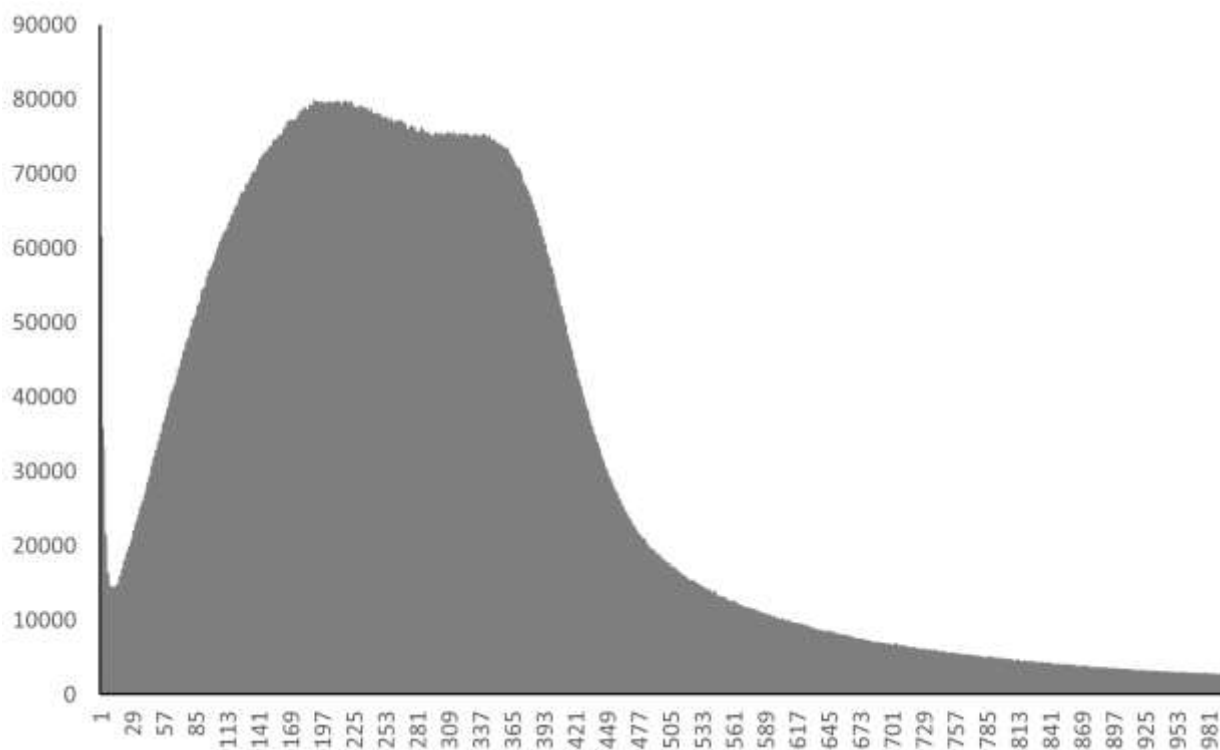
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679 **Supplementary Information**

680 a)



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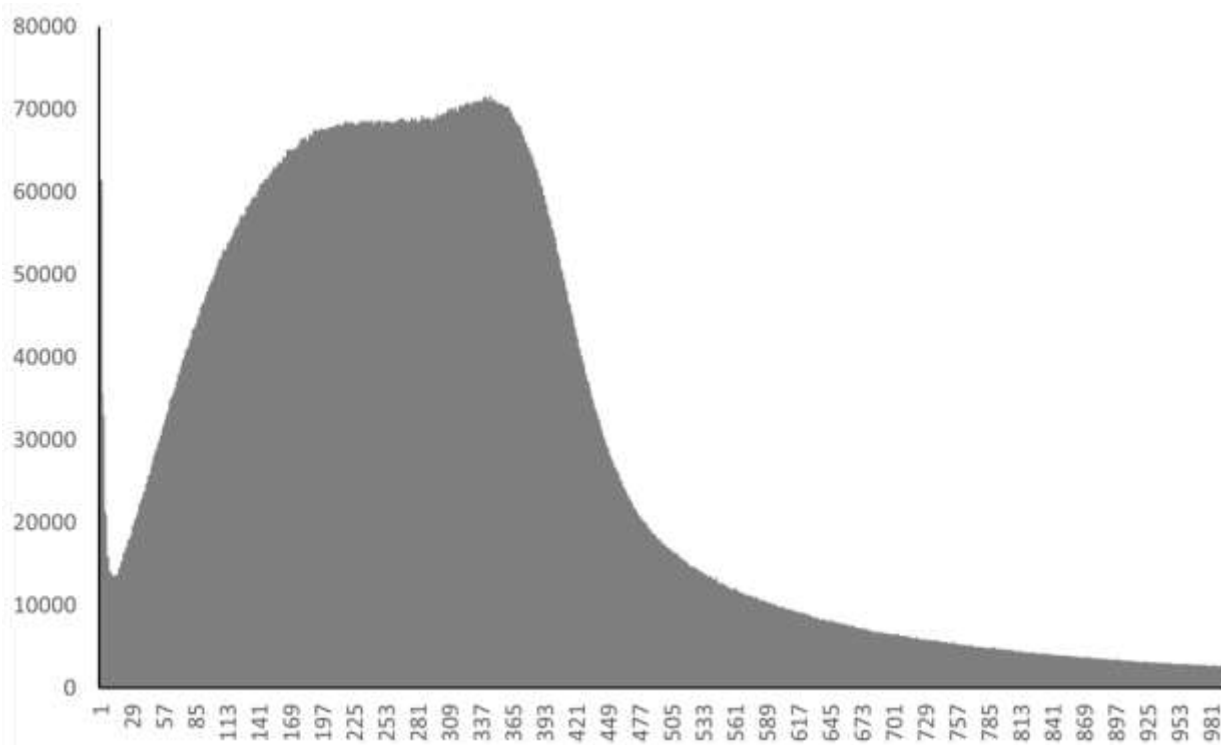
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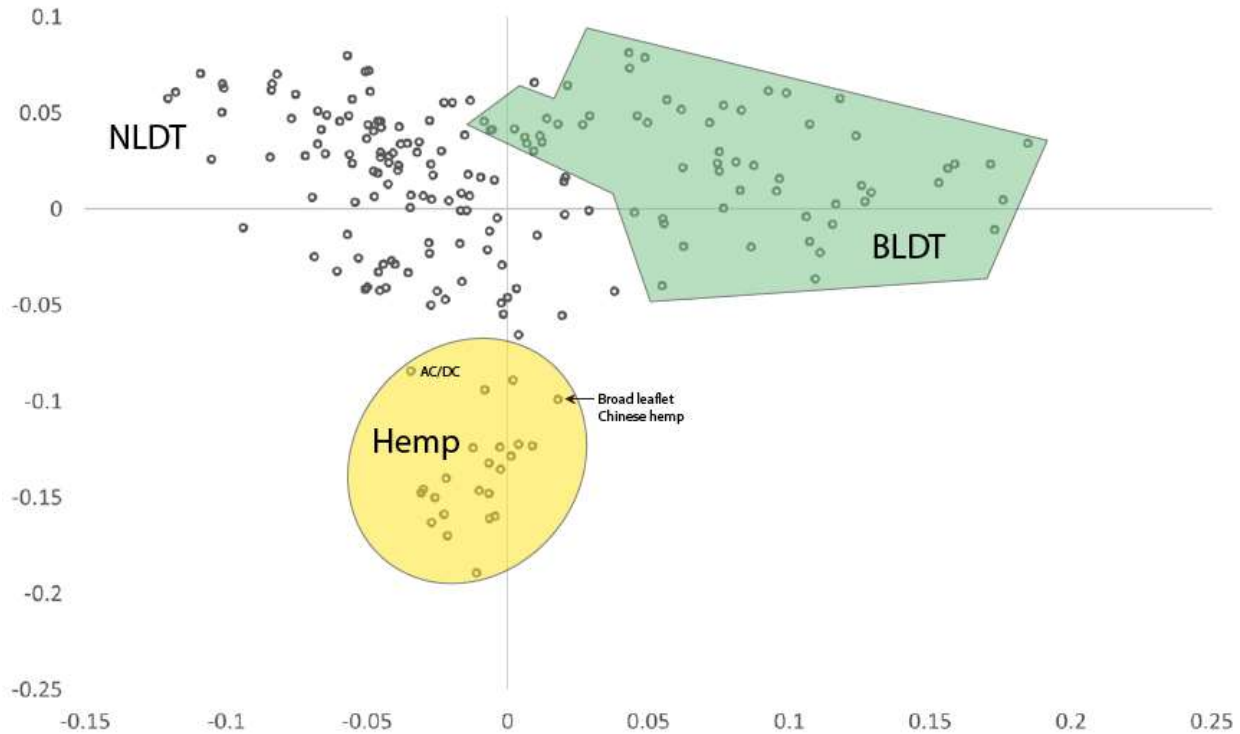
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699 Figure S1. Histograms of WGS read depths at variant loci. a) before PK reference self-  
700 similarity filter. b) after self-similarity filter.

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707 Figure S2. Multidimensional Scaling plot of GBS and WGS SNPs. Hemp, NLDT and BLDT  
708 group assignments were made by FLOCK.

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712 Figure S3. Phylogenetic neighbor network of WGS samples combined with Sawler GBS SNPs  
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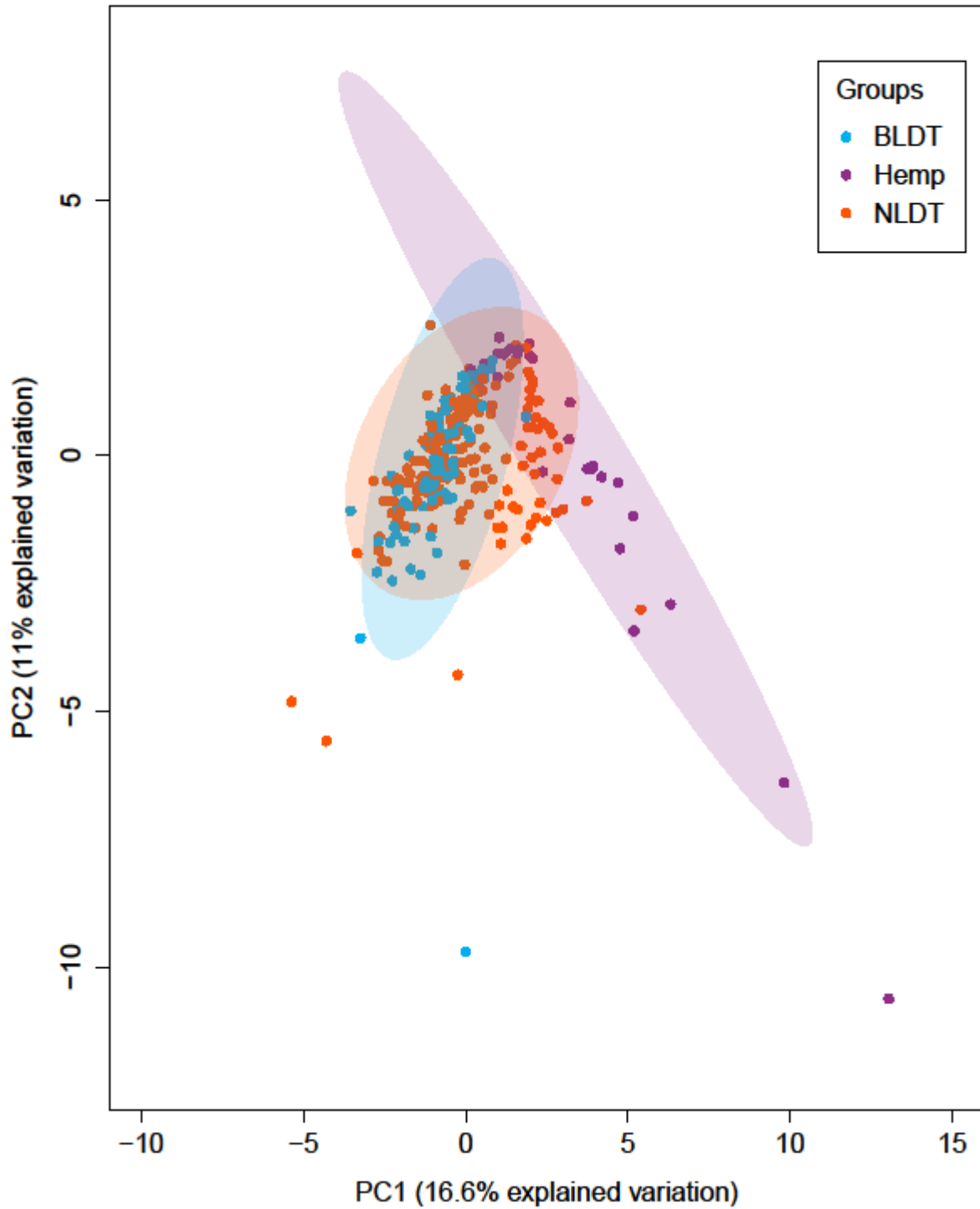
717 Figure S4. Neighbor joining tree from 45 SNP alignment of 289 GBS and WGS samples in  
718 separate high resolution pdf.

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724 Figure S5. Principal Components Analysis of cannabinoid and terpene profiles colored by  
725 FLOCK derived genetic groups.