Research review

Recent advances in *Cannabis sativa* genomics research

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Summary

Cannabis (*Cannabis sativa* L.) is one of the oldest cultivated plants purported to have unique medicinal properties. However, scientific research of cannabis has been restricted by the Single Convention on Narcotic Drugs of 1961, an international treaty that prohibits the production and supply of narcotic drugs except under license. Legislation governing cannabis cultivation for research, medicinal and even recreational purposes has been relaxed recently in certain jurisdictions. As a result, there is now potential to accelerate cultivar development of this multi-use and potentially medically useful plant species by application of modern genomics technologies. Whilst genomics has been pivotal to our understanding of the basic biology and molecular mechanisms controlling key traits in several crop species, much work is needed for cannabis. In this review we provide a comprehensive summary of key cannabis genomics resources and their applications. We also discuss prospective applications of existing and emerging genomics technologies for accelerating the genetic improvement of cannabis.

Introduction

Cannabis sativa L. (cannabis), a member of the Cannabaceae family, is one of the world's oldest domesticated crops (Bradshaw *et al.*, 1981; Long *et al.*, 2017). It is believed to have originated in Central Asia, from where its cultivation rapidly spread throughout Asia and Europe. Nowadays legal and illegal cannabis cultivation occurs globally (Van Bakel *et al.*, 2011).

The exact number of species comprising the *Cannabis* genus is controversial. Some claim the genus consists of three species that display distinct phenotypic differences; namely *C. sativa* L., *C. indica* Lam (Lamarck) and *C. ruderalis* (Sawler *et al.*, 2015; Clarke & Merlin, 2016; Henry *et al.*, 2020). The alternative, and perhaps most accepted, viewpoint is that *Cannabis* is a monotypic genus consisting of a single species, *Cannabis sativa* L. (referred to as cannabis hereafter) (Small & Cronquist, 1976). Cannabis has a diploid genome ($2n = 20$) consisting of nine autosomes and a pair of sex chromosomes (X and Y) (Braich *et al.*, 2019; McKernan *et al.*, 2020). It is predominantly dioecious, meaning a plant is either a male or a female, with estimated haploid genome sizes of 843 Mb

and 818 Mb for male and female plants, respectively (Van Bakel *et al.*, 2011). Despite the presence of defined sex chromosomes, environmental factors such as reduced photoperiod and low temperature, and foliar applications of chemicals such as silver nitrate and the ethylene hormone inhibitor silver thiosulfate induce pollen production in female flowers, leading to the production of 'feminised seeds' (Ram & Sett, 1982; Kaushal, 2012; Lubell & Brand, 2018). This technique has been exploited as a useful tool in cannabis breeding (for example, selfing or crossing female plants) and in generating populations for dissection of the genetic bases of important traits.

Cannabis can be classified as fibre-type (hemp or industrial hemp) and drug-type (medicinal cannabis or marijuana) based on usage and cannabinoid content; fibre-type plants contain < 0.3% Δ^9-tetrahydrocannabinol (THC) whereas drug-type plants contain > 0.3% THC. Both have been exploited by humans for various applications since 8000 BCE (Srinivasababu, 2014). For instance, the stalk of hemp is an important fibre source whilst oil extracted from its seeds is used in several food and nonfood applications (Clarke & Merlin, 2016). More recently, there have been

applications of hemp in construction, geotextiles, cosmetics, as a food product and as a therapeutic agent (Piluzza *et al.*, 2013). Historical medicinal and recreational usage of cannabis has been reported, particularly the use of marijuana for its mood-altering narcotic properties. Consequently, marijuana is the most cultivated, trafficked and abused illicit drug in the world (Sawler *et al.*, 2015). Its prolonged usage has been associated with detrimental health outcomes, such as impaired cognitive development and psychomotor performance, leading to chronic health conditions (Andre *et al.*, 2016). Hence, there is an urgent need to conduct evidence-based research to safeguard purity and quality of products, and to better understand the mode of action of cannabinoids for therapeutic applications.

Cannabis plants grown for medicinal and recreational end-uses are generally shorter, have thinner stems, more branches and a higher density of floral tissues than industrial hemp plants. Cultivars also can be discriminated by their cannabinoid profile, also termed chemotype (Piluzza *et al.*, 2013; Clarke & Merlin, 2016). Cannabinoids are secondary metabolites produced in capitate stalked glandular trichomes (Fig. 1), more than 120 of which have been identified (Braich *et al.*, 2019; Kovalchuk *et al.*, 2020). Two of these, THC and CBD (cannabidiol), are highly sought after by cannabis breeders and pharmaceutical industries (Adams *et al.*, 1940; Weiblen *et al.*, 2015; Andre *et al.*, 2016). Precursor synthesis of these cannabinoids occurs from two distinct metabolic pathways; the polyketide pathway and the methylerythritol phosphate (MEP) pathway (Fig. 1) (Kovalchuk *et al.*, 2020). These produce alkylresorcinolic acids, including olivetolic acid (OA) that is specific to cannabis, and geranyl diphosphate (GPP), respectively. CBGA (cannabigerolic acid) is then synthesized from OA and GPP to produce the acidic precursors of THC (tetrahydrocannabinolic acid; THCA) and CBD (cannabidiolic acid; CBDA) (Weiblen *et al.*, 2015). THC is the main psychoactive/ intoxicant in cannabis. It induces sensations of euphoria, anxiety, paranoia and cognitive deficits, and is associated primarily with the narcotic status of cannabis (Boggs *et al.*, 2018). However, THC also has therapeutic benefits as it confers relief from nausea caused by certain anti-cancer treatments and acts as an anti-inflammatory agent (Andre *et al.*, 2016). CBD, which is an isomer of THC, has an analgesic effect, and also is purported to have neuroprotective, anticancer and anti-diabetic properties (Andre *et al.*, 2016). Epidiolex, the first CBD-based product approved by the US Food and Drug Administration, also has been shown to reduce seizures in children with Dravet syndromes (O'Connell *et al.*, 2017; Chen *et al.*, 2019). Industrial hemp and recreational drug chemotypes differ in their THC : CBD ratios. Cannabis plants are frequently classified into three main chemotypes based on this ratio; chemotype I plants (drug-type) exhibit a THC : CBD ratio well beyond 1.0, chemotype II plants have an intermediate ratio of 0.5–2.0 and chemotype III plants (fibre-type) have a ratio well below 1.0 (Aizpurua-Olaizola *et al.*, 2016). Additionally, the DW of THC in mature female inflorescences is used to demarcate cultivars for industrial hemp end-uses such as seed or fibre production (Piluzza *et al.*, 2013).

The prospects of using contemporary breeding technologies to improve cannabis traits for medicinal applications are promising.

However, progress in this area is hampered by several issues. First, the genetics of cannabis is poorly understood and causes incorrect classification of cultivars/strains, with implications for researchers, growers, cannabis users and regulators (Vergara *et al.*, 2016; Welling *et al.*, 2016; Schwabe & McGlaughlin, 2019). Secondly, intensive clandestine breeding practices since the early 1970s have led to a genetic bottleneck and reduction in allelic diversity in marijuana plants (Clarke & Merlin, 2016). Thirdly, restrictions such as the international narcotics conventions and associated legislation have hampered the exchange of cannabis genetic resources and research materials (Welling *et al.*, 2016).

Genomic analyses have facilitated a paradigm shift in the improvement of cultivars of major crop species over the last two decades (Morrell *et al.*, 2012; Yuan *et al.*, 2017). This has been driven by high-throughput sequencing and single nucleotide polymorphism (SNP) marker-based genotyping platforms, as well as the development of high-quality reference genome and transcriptome assemblies. These technologies have increased our understanding of gene content, genomic variation and the genetic basis of complex agronomic traits in multiple plant species (Xie *et al.*, 2015; Xu *et al.*, 2017; Appels *et al.*, 2018; Thomas *et al.*, 2019). The status of cannabis as an emerging, high-value and clinically efficacious crop and the potential of genomics-assisted breeding, coupled with the relaxation of regulations and restrictions, warrant expanded research efforts. In this review we summarize available cannabis genomics resources and report on the application of these tools. We also discuss future applications and emerging genomics technologies relevant to the genetic improvement of cannabis.

Cannabis sativa genomics resources and the discoveries they have enabled

Genome assemblies

De novo assembly of plant genomes remains challenging and the cannabis genome is no exception (Schatz *et al.*, 2012). The cannabis genome is highly heterozygous (estimated at 12.5–40.5%) and contains large amounts of repetitive elements (estimated at 70%) (Van Bakel *et al.*, 2011; Pisupati *et al.*, 2018; Gao *et al.*, 2020; Kovalchuk *et al.*, 2020). Several attempts have been made to assemble this complex genome, as illustrated by publicly available genome and transcriptome assemblies of varying sizes and completeness for 12 different cannabis cultivars (Table 1a,b). Initial efforts relied upon the use of short-read sequencing technology, but these proved computationally challenging. Application of third-generation long-read sequencing technologies such as Single-Molecule Real-Time (SMRT) sequencing (PacBio) and MinION (Oxford Nanopore Technologies) have greatly improved the contiguity of cannabis reference sequences, as has the anchoring of scaffolds using genetic linkage maps coupled with Hi-C data (Grassa *et al.*, 2018; Laverty *et al.*, 2019; Gao *et al.*, 2020; McKernan *et al.*, 2020). This has resulted in the creation of four chromosome-level assemblies for Purple Kush (PK; drug-type), Finola (FN; hemp), JL (wild accession) and CBDRx (cs10; high-CBD) (Grassa *et al.*, 2018; Laverty *et al.*, 2019; Gao *et al.*, 2020).
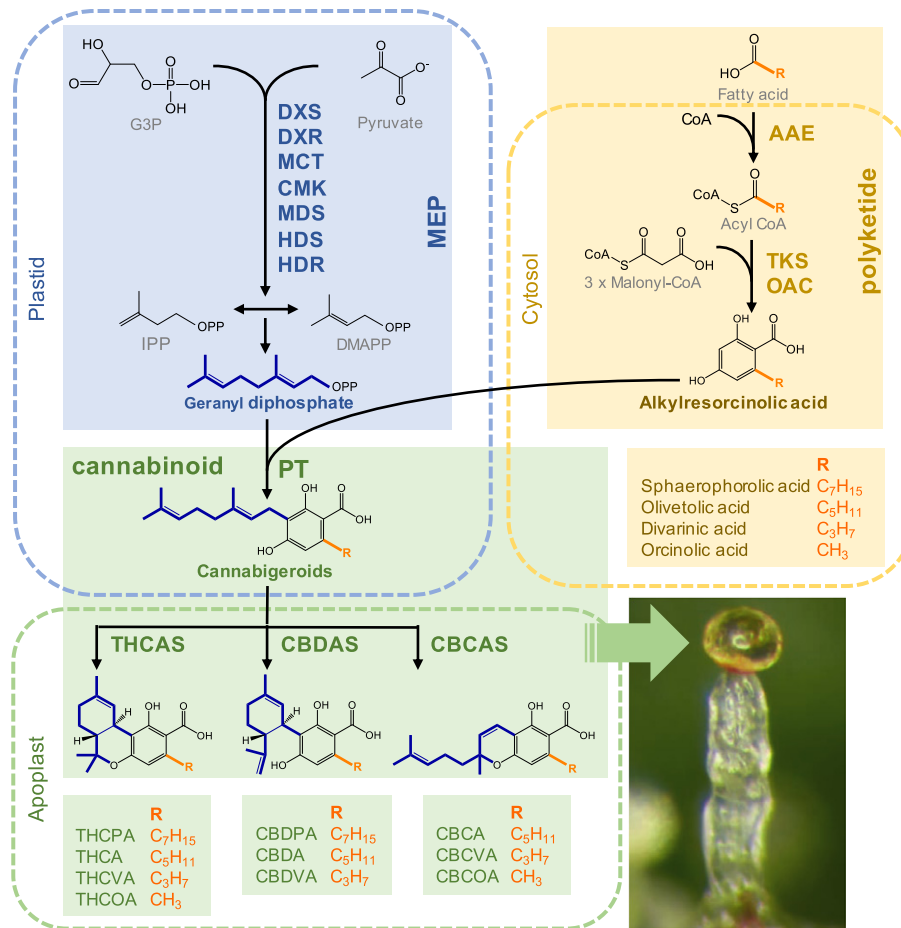
Fig. 1 Schematic diagram of cannabinoid biosynthesis including polyketide and isoprenoid precursor pathways. Precursor pathways are merged by a plastid-localized aromatic prenyltransferase, with alkylresorcinolic acids and geranyl diphosphate intermediates forming cannabigeroids with a linear isoprenyl residue (Gülck & Møller, 2020). Cannabinoid synthesis concludes in the apoplastic storage cavity of glandular trichomes. Here, cannabigeroids are converted to tri- and di-cyclic cannabinoids such as Δ^9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) via stereoselective oxidative cyclisation of the isoprenyl moiety. This occurs enzymatically by the cannabinoid synthases *THCAS, CBDAS* and *CBCAS*. The green arrow indicates location of the extracellular storage cavity of a *Cannabis* stalked glandular trichome; bar, 100 μm. Subcellular locations of cannabinoid and precursor pathway enzymes were predicted with the subcellular location software TargetP-2.0 (http://www.cbs.dtu.dk/services/TargetP/). AAE, acyl-activating enzyme; CBCA, cannabichromenic acid; CBCAS, cannabichromenic acid synthase; CBCOA, cannabiorcichromenic acid; CBCVA, cannabichromevarinic acid; CBDA, cannabidiolic acid; CBDAS, cannabidiolic acid synthase; CBDPA, cannabidiphorolic acid; CBDVA, cannabidivarinic acid; CMK, 4-(cytidine 50-diphospho)-2-C-methyl-D-erythritol kinase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5 phosphate synthase; HDR, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate reductase; HDS, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEP, 2-C-methyl-D-erythritol-4-phosphate; OAC, olivetolic acid cyclase; PT, prenyltransferase (e.g. geranylpyrophosphate:olivetolate geranyltransferase (GOT)); THCA, tetrahydrocannabinolic acid; THCAS, tetrahydrocannabinolic acid synthase; THCOA, tetrahydrocannabiorcolic acid; THCPA, tetrahydrocannabipgorolic acid; THCVA, tetrahydrocannabivarinic acid; TKS, tetraketide synthase.

The cs10 assembly is the most complete and contiguous chromosome-level assembly, comprising 25 302 protein-coding genes (Fig. 2; Maoz, 2020). The current version of this assembly (v.2.0; GenBank acc. no. GCA_900626175.2) has recently been updated with the chromosomes renumbered according to an agreed community standard (Table 2; https://www.ncbi.nlm.nih.gov/assembly/GCF_900626175.2#/st; Maoz, 2020). Earlier this year, the International Cannabis Genomics Research Consortium (ICGRC) proposed that cs10 be used as the reference for cannabis genomics (Maoz, 2020). Cannabis genome assemblies other than cs10 also have much to offer. For instance, the PK, FN and the contig-level Jamaican Lion trio assemblies have been key to confirming findings from earlier, lower-resolution studies as well as

uncovering important biology of the *Cannabis* genus (Van Bakel *et al.*, 2011; Laverty *et al.*, 2019; Prentout *et al.*, 2019; Vergara *et al.*, 2019; Booth *et al.*, 2020; McKernan *et al.*, 2020). Further improvement of these assemblies will assist in fully realising their potential.

Genome assemblies have made enormous contributions to our understanding of the cannabinoid biosynthetic pathways through the underlying synthase genes. In particular, the assemblies have shed light on the inheritance of these genes (Grassa *et al.*, 2018; Laverty *et al.*, 2019; McKernan *et al.*, 2020). Before the availability of genome sequence data, de Meijer et al. proposed a Mendelian inheritance model of chemotype that involved a single locus, *B*, with two co-dominant alleles, B_T and B_D, encoding for *THCAS* and

Table 1 Statistics for the latest *Cannabis sativa* reference genome and transcriptome assemblies.

Genome assemblies

Cultivar (GenBank acc. no.)	Sex	Total sequence length (bp)	Total ungapped length (bp)	Number of scaffolds	Scaffold N50 (bp)	Number of contigs	Contig N50 (bp)	GC content (%)	Number of chromosomes and plasmids	Genome coverage	Sequencing technology	Number of protein-coding genes/transcripts	Reference
cs10/CBDRx[a] (GCA_900626175.2)	Female	876 147 649	736 579 359	221	91 913 889	1052	1959 202	34	10	100×	Oxford Nanopore Technology	25 302 protein-coding genes	Grassa et al. (2018)
Purple Kush[b] (GCA_000230575.5)	Female	891 964 663	891 346 362	6653	60 968 100	12 836	133 904	34	10	79×	PacBio	30 074 transcripts	Laverty et al. (2019)
Finola (GCA_003417725.2)	Male	1009 674 739	1009 380 638	2362	77 135 887	5303	370 471	34	10	97.64×	PacBio	37 689 transcripts	Laverty et al. (2019)
Pineapple Banana Bubba Kush (GCA_002090435.1)	Male	512 174 223	512 174 223	18 355	51 819	18 355	51 819	34	–	72×	PacBio	–	–
LA Confidential (GCA_001510005.1)	Female	595 358 288	595 357 797	311 039	2649	311 039	2649	35	–	50×	454 sequencing	–	–
Chemdog 91 (GCA_001509995.1)	Female	285 932 793	285 527 436	175 088	2250	190 122	2189	33	–	50×	Illumina GAII	–	–
Cannatonic (GCA_001865755.1)	Female	585 823 666	585 823 666	11 110	128 718	11 110	128 718	34	–	10×	PacBio	–	–
Jamaican Lion (female parent)[c] (GCA_012923425.1)	Female	876 735 611	876 735 611	–	–	1599	3283 100	34	–	125×	PacBio Sequel	27 664 genes	McKernan et al. (2020)
Jamaican Lion (male parent)[c] (GCA_013030025.1)	Male	1009 156 132	1009 156 132	–	–	1264	1668 042	34	–	125×	PacBio Sequel	31 591 genes	McKernan et al. (2020)
Jamaican Lion (F1)[c] (JAATIR000000000.1)	Female	999 122 115	999 122 115	–	–	658	1668 042	34	–	–	PacBio Sequel	–	McKernan et al. (2020)
JL[d] (GCA_013030365.1)	Female	812 525 420	811 830 406	483	82 998 198	2978	509 999	34	10	153×	PacBio Sequel	38 382 protein-coding genes	Gao et al. (2020)

Transcriptome assemblies

Cultivar	Sex	Total sequence length (bp)	Total ungapped length (bp)	Total Number of transcripts	N50 length (bp)	Shortest transcript length (bp)	Longest transcript length (bp)	Reference	Source
Purple Kush	Female	33 200 961	33 200 740	30 074	1906	90	12 107	van Bakel et al. (2011)	http://genome.ccbr.utoronto.ca/
Finola	Male	25 682 508	25 682 508	37 689	1280	88	7210	–	http://genome.ccbr.utoronto.ca/

Table 1 (Continued)

Transcriptome assemblies

Cultivar	Sex	Total sequence length (bp)	Total ungapped length (bp)	Total Number of transcripts	N50 length (bp)	Shortest transcript length (bp)	Longest transcript length (bp)	Reference	Source
Cannbio[e]	Male & Female	55 924 982	55 677 217	64 413	1796	201	70 089	Braich et al. (2019)	NCBI (GenBank acc. no. : GIFP00000000.1)

[a]Grassa *et al.* (2018) refer to an earlier version of this assembly in their paper (GenBank acc. no. GCA_900626175.1).
[b]Laverty *et al.* (2019) refer to an earlier version of this assembly in their paper (GenBank acc. no. GCA_0002300575.4).
[c]McKernan *et al.* (2020) refer to earlier versions of these assemblies in their paper (Jamaican Lion, female parent = CoGe ID 55184; Jamaican Lion, male parent = CoGe ID 55360 and Jamaican Lion, F1 = CoGe ID 55567).
[d]Genome annotation not included with this assembly.
[e]The Cannbio assembly was generated using RNA-Seq data from the female and male cannabis cultivars, Cannbio-2 and Cannbio-male, respectively.

CBDAS, respectively (De Meijer *et al.*, 2003). Homozygosity at the B locus led to the production of either THC (drug-type; B_T/B_T) or CBD (fibre-type; B_D/B_D), whilst heterozygous individuals (B_T/B_D) had a mixed THC-CBD chemotype. However, the creation of high-quality genome assemblies supports an alternative, multilocus model whereby *THCAS* and *CBDAS* are two different genes located in close proximity in a retrotransposon-rich region of the cannabis genome (Grassa *et al.*, 2018; Laverty *et al.*, 2019; McKernan *et al.*, 2020). This finding supports other earlier nongenomics studies (De Meijer *et al.*, 2003; Kojoma *et al.*, 2006; Weiblen *et al.*, 2015).

The identification of the less-studied CBCA synthase (cannabichromenic acid synthase; *CBCAS*) gene (also known as inactive *THCAS*) is another notable discovery originating from genomics datasets (Grassa *et al.*, 2018; Laverty *et al.*, 2019; McKernan *et al.*, 2020). This gene catalyses the synthesis of cannabichromene (CBC), which is an emerging target for medicinal cannabis breeding as it is nonintoxicating, can reduce pain sensations and act as an anti-inflammatory agent (Laverty *et al.*, 2019). The functional, chemotype-determining forms of *CBCAS*, *THCAS* and *CBDAS* are highly similar at the nucleotide and amino acid levels (Fig. 3a,b). The fact that long-read sequencing enabled the assembly of such highly similar loci reflects the importance of this technology in resolving complex, repetitive regions in the cannabis genome (Schatz *et al.*, 2012; Michael & VanBuren, 2020). Our survey of the contig-level PacBio genome assemblies of Pineapple Banana Bubba Kush (PBBK) and Cannatonic also supports these findings. We identified a larger number of *THCAS* and *CBCAS* gene loci in these assemblies compared to Chemdog91 and LA Confidential, which were generated using short-read sequencing (Fig. 4; Supporting information Tables S1, S2). It is likely that underestimation of *THCAS, CBDAS* and *CBCAS* loci has occurred in these collapsed and relatively smaller assemblies (<595 Mb). However, the presence of true biological variation among these cultivars cannot be ruled out. For instance, structural variants (SVs) in the form of copy number variations (CNVs) and presence/absence variations (PAVs) are known to affect the gene content of many plant species (Saxena *et al.*, 2014).

The identification of sex chromosomes in the cannabis genome is another notable genomics-driven achievement (Prentout *et al.*, 2019; McKernan *et al.*, 2020). Of the 565 sex-linked genes identified in the PK transcriptome, 363 were mapped to cs10 v.1.0 chromosome 1 (cs10 v.2.0 chromosome 10), indicating that this chromosome pair constitutes the sex chromosomes (Prentout *et al.*, 2019). This enabled the identification of sex-specific molecular markers to aid cannabis breeding. THCA and CBDA are produced at much higher concentrations in the inflorescences of female cannabis plants compared with males, and hence female plants are economically more valuable (Braich *et al.*, 2019; Prentout *et al.*, 2019; McKernan *et al.*, 2020). Having the capacity to identify male and female plants at an early stage enables yield improvement and better management of cannabis crops. Approximately 3500 sex-biased genes have been identified, which are differentially expressed between female and male cannabis plants, with a subset being expressed in the flower buds (Prentout *et al.*, 2019). These genes are not restricted to the sex chromosomes: some are located on the Y-chromosome of male plants and are involved in trichome

development, sex determination, hermaphroditism and photoperiod-independence (McKernan *et al.*, 2020).

Consistent chromosome nomenclature is important when working with multiple genome assemblies of the same species. However, discrepancies exist between the cs10, PK and FN assemblies. Chromosomal mappings performed by NRGene determined that cs10 v.2.0 chromosome 7 (cs10 v.1.0 chromosome 9) corresponds to chromosome 6 of PK and FN (Table 2; Maoz, 2020). Whilst our synteny analyses broadly agree with the findings from NRGene, they also show the lack of synteny between these genomes in some regions as illustrated by breaks in the chromosomal alignments (Fig. 5). These could have occurred due to SVs which would indicate true biological variation between these unrelated chemotypes. Another possibility relates to the more fragmented and less contiguous nature of the PK and FN assemblies relative to the cs10 v.2.0 assembly. Such lack of assembly contiguity can cause an underestimation of the syntenic relationship, causing genomic regions to erroneously appear as absent in one assembly relative to another (Liu *et al.*, 2018).

Our synteny analyses also revealed the presence of multiple *CBDAS, CBDAS-like* and inactive *THCAS* (*CBCAS*) genes on chromosome 6 and unplaced scaffolds of the PK and FN assemblies (Tables S1, S3). We found that the scaffolds harbouring the *CBDAS* gene copies could be best aligned (start to end) against the *CBDAS* gene cluster region (29.63–30.93 Mbp) of cs10 v.2.0 chromosome 7 (Table S3). Likewise, the scaffolds containing the *CBCAS* genes could be best aligned against the inactive *THCAS* gene cluster region (25.82–26.05 Mbp) of cs10 v.2.0 chromosome 7. These findings suggest that the unplaced scaffolds belong to chromosome 6 of PK and FN and that the regions surrounding the candidate genes on these scaffolds share synteny with cs10 v.2.0 chromosome 7. Although these results highlight the syntenic relationship that exists between these various cannabis chemotypes, they also illustrate the difficulty in inferring such a relationship when presented with fragmented assemblies. The continuous improvement of current cannabis assemblies will therefore be required for more accurate comparative genomics analyses within and between species.

Gene expression

Large-scale gene expression studies on cannabis have been instrumental in elucidating cannabinoid metabolism, but application of these approaches to other important traits is limited (Guerriero *et al.*, 2017; Spitzer-Rimon *et al.*, 2019; McKernan *et al.*, 2020). Many enzymes required for the conversion of primary metabolic precursors through to the synthesis of THCA and CBDA were identified by early comparisons between expressed sequence tags from trichome and leaf tissue (Marks *et al.*, 2009; Van Bakel *et al.*, 2011; Stout *et al.*, 2012; Braich *et al.*, 2019; Livingston *et al.*, 2019; Zager *et al.*, 2019). RNA-Seq analysis identified 15-fold increases in cannabinoid pathway genes in flowers of PK compared with FN, although this preliminary investigation lacked a sufficient number of biological replicates for robust statistical analyses (Van Bakel *et al.*, 2011). Tissue and organ comparisons were recently improved, with female and male plants compared as well as various

trichome morphotypes (Braich *et al.*, 2019; Livingston *et al.*, 2019). Gene co-expression network analysis identified functionally relevant cannabis terpene synthases (*CsTPS*) involved in mono- and sesquiterpene accumulation (Zager *et al.*, 2019). The differential expression of *CsTPS* genes among cultivars also has been linked to variation in terpene profiles of these cultivars (Booth *et al.*, 2020). However, the genes underlying the synthesis of many minor cannabinoids and associated expression patterns are less well-defined (Pollastro *et al.*, 2011; Citti *et al.*, 2019; Welling *et al.*, 2019; Basas-Jaumandreu & de las Heras, 2020).

Substantial CNV exists among cannabinoid synthase loci

The CNV of cannabinoid synthases has been reported in the cannabis genome and may have resulted from either natural or artificial selection (Grassa *et al.*, 2018; Vergara *et al.*, 2019; McKernan *et al.*, 2020). Overall, it appears that *CBDAS* exists in significantly larger copy numbers compared to *THCAS* and *CBCAS* (Fig. 4; Tables S1, S2, S4). We also identified a larger number of *CBDAS* gene clusters relative to the other cannabinoid synthases, suggesting the presence of higher sequence variation among *CBDAS* gene copies. A previous report also suggested a more recent evolution of *THCAS* and *CBCAS* genes, which originated from the ancestral gene, *CBDAS*, as a result of gene duplication (Onofri *et al.*, 2015). Additionally, we found the functional copies of *CBDAS* to be highly similar (>99% nucleotide identity) among accessions (Table S1). This also was the case for the functional *THCAS* and *CBCAS* loci, suggesting that intensive breeding practices have been performed to select these desirable cannabinoid synthase loci, which have become less polymorphic as a result. Beyond their similarity at the nucleotide and amino acid sequence levels, studies on the evolution of these genes would shed more light on their origin and functional divergence.

The relationship between cannabinoid synthase CNVs and cannabinoid content

The association between cannabinoid synthases, CNVs and overall cannabinoid yield remains unclear and may, in fact, not be significant (Grassa *et al.*, 2018). Of the five quantitative trait loci (QTL) for total cannabinoid content recently identified, none belong to the cannabinoid synthase gene cluster on cs10 v.2.0 chromosome 7 (cs10 v.1.0 chromosome 9) (Grassa *et al.*, 2018), suggesting that other non-cannabinoid synthase-related loci contribute to cannabinoid yield. Regardless, the functional forms of *THCAS* and *CBDAS* rather than their other copies are essential for the synthesis of THCA and CBDA, respectively. This is supported by our survey of the genome assemblies of the high-THC producing cultivars PBBK and PK. Whilst both assemblies contained similar numbers of *THCAS* and *CBDAS* copies, they also contain a functional *THCAS* copy that bears > 99% nucleotide identity with the THCA-producing gene locus identified by Sirikantaramas and colleagues (GenBank acc. no. AB057805.1; Fig. 4; Table S1) (Sirikantaramas *et al.*, 2004). It is likely that nonfunctional *CBDAS* loci, inferred by the presence of premature stop codons and frameshift mutations, also are present in these
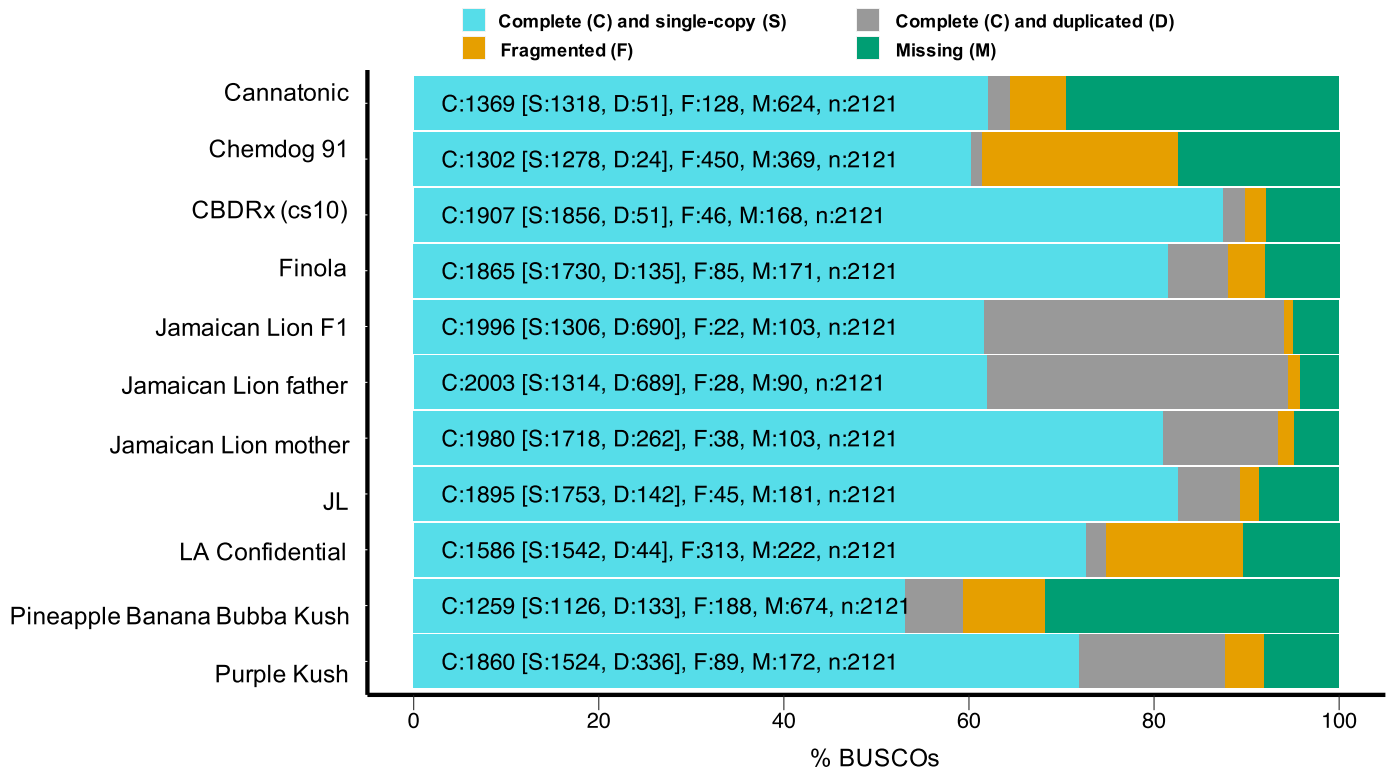
Fig. 2 Benchmarking Universal Single-Copy Ortholog (BUSCO) assessment of the cannabis genome assemblies shown in Table 1(a). The percentages of complete (single-copy and duplicated), fragmented and missing universal single-copy orthologue genes were identified using Busco v.4.02 (Simão *et al.*, 2015). The Jamaican Lion assemblies (female parent, male parent, F$_1$) have more complete BUSCOs on average, but they also harbour a larger number of duplicated BUSCOs, which reflects the fragmented nature of these assemblies.

Table 2 Chromosomal nomenclatures of *Cannabis sativa* genome assemblies highlighting the discrepancies in chromosome numbering among the current assemblies (https://www.ncbi.nlm.nih.gov/assembly/GCF_900626175.2#/st; Maoz, 2020).

cs10 v.2.0 (GenBank acc. no. GCA_900626175.2)		cs10 v.1.0 (GenBank acc. no. GCA_900626175.1)		Finola v.2.0 (latest; GenBank acc. no. GCA_003417725.2)		Purple Kush v.5.0 (latest; GenBank acc. no. GCA_000230575.5)	
Chromosome number	GenBank sequence	Chromosome number	GenBank sequence	Chromosome number	GenBank sequence	Chromosome number	GenBank sequence
1	NC_044371.1	2	NC_044371.1	5	CM011609.1	5	CM010796.2
2	NC_044375.1	6	NC_044375.1	3	CM011607.1	3	CM010793.2
3	NC_044372.1	3	NC_044372.1	4	CM011608.1	4	CM010794.2
4	NC_044373.1	4	NC_044373.1	7	CM011611.1	7	CM010799.2
5	NC_044374.1	5	NC_044374.1	1	CM011605.1	1	CM010790.2
6	NC_044377.1	8	NC_044377.1	2	CM011606.1	2	CM010792.2
7	NC_044378.1	9	NC_044378.1	6	CM011610.1	6	CM010797.2
8	NC_044379.1	10	NC_044379.1	9	CM011613.1	9	CM010798.2
9	NC_044376.1	7	NC_044376.1	8	CM011612.1	8	CM010795.2
10	NC_044370.1	1	NC_044370.1	10	CM011614.1	10	CM010791.2

Chromosome 10 (chromosome 1 in cs10 v1.0) corresponds to the sex chromosome in cs10 v.2.0, Purple Kush v.5.0 and Finola v.2.0.

assemblies (Weiblen *et al.*, 2015). This is probable because CBDAS is a stronger competitor for CBGA than THCAS (Weiblen *et al.*, 2015). However, when the nonfunctional form of *CBDAS* and the functional form of *THCAS* are both present, THCA is produced instead of CBDA (Weiblen *et al.*, 2015). Indeed, we identified two loci in PBBK and PK that share > 96% nucleotide identity with the published nonfunctional *CBDAS* homologues from Skunk #1

(Table S5) (Weiblen *et al.*, 2015). We also identified these loci in Cannatonic and JL, and found that these cultivars also contain one locus which is highly similar (>99% nucleotide identity) to AB057805.1 (Table S1).

It is important to validate hypotheses generated *in silico* to explain chemotypic properties of cannabis cultivars by conducting either *in vitro* or *in vivo* studies. This can be achieved using genetic

approaches such as co-segregation of known chemotypes and enzyme genotypes (Weiblen *et al.*, 2015). For example, the association between functional forms of either *THCAS* or *CBDAS* and chemotype was demonstrated by analysis of an F_2 population derived from crossing hemp and marijuana chemotype plants. The presence of functional *THCAS* or *CBDAS* in F_2 individuals was determined by genotype analysis, then related to the plants' cannabinoid contents (Weiblen *et al.*, 2015).

Short read sequencing creates challenges during CNV and expression analyses of the highly similar cannabinoid synthase loci

The predominant use of short read methods to analyze cannabis transcriptomes may create misleading artefacts resulting from the very high sequence similarity of cannabinoid synthase gene loci. Short reads that originate from paralogous and pseudogenic regions of a genome frequently cannot be distinguished (Ju *et al.*, 2017; Dougherty *et al.*, 2018). These challenges are illustrated in our analysis of trichome transcriptome data from nine medicinal cannabis cultivars generated by Zager *et al.* (2019) (Fig. 6a–c). Using cs10 v.1.0 as the reference, we identified two highly similar inactive *THCAS* loci (>99% nucleotide identity), LOC115697880 and LOC115697886, which were expressed much more highly than the remaining *THCAS-like* and inactive *THCAS-like* copies across all cultivars (Table S6). These loci are more similar to *CBCAS* (>99% nucleotide identity) than to the functional *THCAS* gene, AB057805.1 (>94% nucleotide identity) (Fig. 4; Table S1). It was reported that THCA (>13 % DW on average) was present in these cultivars (Zager *et al.*, 2019). Therefore, we suspect that although the high-THCA cultivars each harbour at least one functional *THCAS* gene copy, the reads that originated from this locus were forced to map to LOC115697880 and LOC115697886 owing to the higher similarity between the reads and these two loci, and the fact that cs10 lacks a functional *THCAS* gene. Whilst our findings agree with our observations above that CNV does not impact the synthesis and accumulation of THCA, they reflect the inadequacy of short reads to differentiate between highly similar loci and highlight the challenges of using an unrelated reference assembly for expression analyses.

Likewise, the higher expression of the *CBDAS* gene, LOC115697762, and higher CBDA content in Canna Tsu (7.76 ± 0.3% DW) relative to the other high-THCA cultivars suggests that only one copy of the *CBDAS* gene is predominantly responsible for CBDA synthesis (Table S6). LOC115697762 bears 100% nucleotide identity with the functional *CBDAS* identified by Taura et al. (GenBank acc. no. AB292682.1; Fig. 4; Table S1) (Taura *et al.*, 2007). Further, the moderate expression of another highly similar *CBDAS-like* gene copy (LOC115696884, 88% nucleotide identity to LOC115697762) in all cultivars could explain the low concentrations (<0.45% DW) of CBDA detected in the high-THCA cultivars (Zager *et al.*, 2019).

Overall, our findings suggest that CNV does not affect cannabinoid content. Recent studies using long-read approaches from PacBio (SMRT isoform sequencing, Iso-Seq) and Oxford Nanopore support this (McKernan *et al.*, 2020; Michael, 2020).

Both concluded that only single copies of the functional *THCAS* and *CBDAS* genes were expressed in the cannabis genome and contribute to the production of THCA and CBDA, respectively (McKernan *et al.*, 2020; Michael, 2020). This finding highlights the strength of long-read sequencing at more accurately identifying and quantifying the expression of paralogous genes (Dougherty *et al.*, 2018).
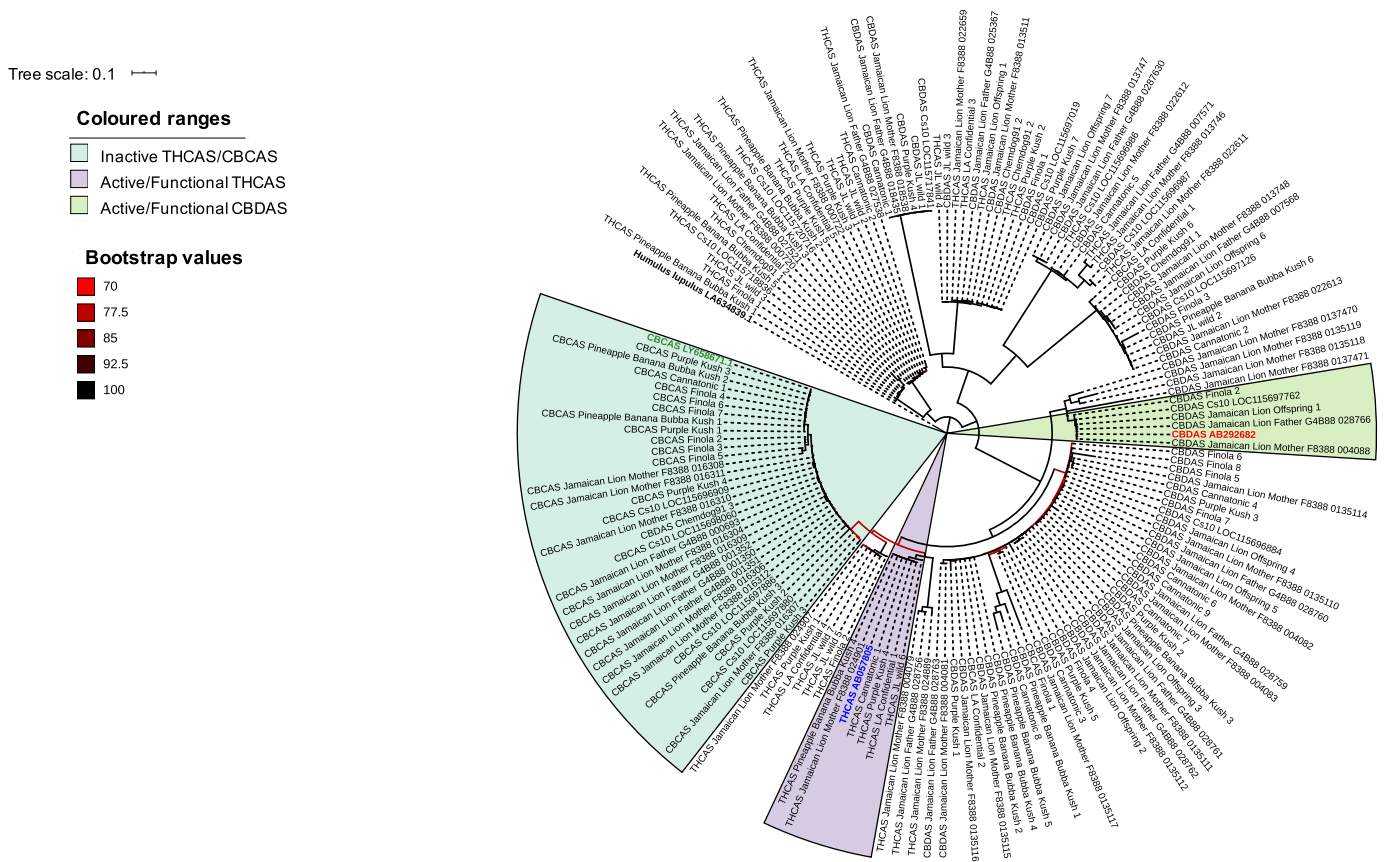
Terpene synthases

Cannabis is a prolific producer of terpenes and these compounds are thought to contribute to the therapeutic efficacy of cannabis preparations via the 'entourage effect', by acting in combination with cannabinoids (Ben-Shabat *et al.*, 1998; LaVigne *et al.*, 2020). However, evidence for the existence of the entourage effect is largely anecdotal and lacks mechanistic explanation, for example whether the effect is additive or multiplicative. Despite their therapeutic potential and similar biosynthetic origin, genetic prediction of terpene composition is challenging. Genomic analysis of 55 *CsTPS* genes suggests a complex genetic background characterized by *CsTPS* nonhomologous gene clusters and tandem duplication events (Allen *et al.*, 2019; Booth *et al.*, 2020; McKernan *et al.*, 2020). Further, the presence of extensive CNVs within the *CsTPS-a* (sesquiterpene synthase) and *CsTPS-b* (monoterpene synthase) gene subfamilies point to their highly diverse nature (Booth *et al.*, 2020). It appears that some members of the *CsTPS-b* gene subfamily can produce sesquiterpene in both cannabis and sandalwood. This indicates that similar selective pressures have occurred on the species-specific monoterpene synthase ancestors to produce these loci (Gao *et al.*, 2012). The diversity of the *CsTPS* genes complicates studies of gene regulation at various levels. Terpene composition can vary between cultivars, tissue types, trichome morphotypes and across development, whilst nonenzy- matic modifications such as the oxidation of β-myrcene cause variation independent of genomic and transcriptomic regulation (Marchini *et al.*, 2014; Aizpurua-Olaizola *et al.*, 2016; Allen *et al.*, 2019; Livingston *et al.*, 2019; Booth *et al.*, 2020). Consequently, future transcriptional studies would need to consider gene- environment interactions, as well as organ, tissue and cell-type specificity.

SNP studies

High-throughput SNP studies have contributed substantially to our understanding of cannabis evolutionary history. Key findings include: the genome-wide distinction between drug and hemp types that resulted from selective breeding; the association between chemotypic identity and variation of loci encoding cannabinoid synthases; and errors in cultivar classi- fication and ancestry by breeders (Van Bakel *et al.*, 2011; Sawler *et al.*, 2015; Lynch *et al.*, 2016; Soorni *et al.*, 2017). These findings are likely to facilitate the development of more accurate diagnostic systems for cannabis germplasm to assist with product compliance, traceability, provenance and con- sumer education (Henry *et al.*, 2020). Domestication and intensive breeding have narrowed the genetic and allelic

(a)

(b)

		CBDAS_AB292682.1	THCAS_AB057805.1	CBCAS_LY658671.1
Percentage identity (nucleotide)	CBDAS_AB292682.1	100	89.8	89.3
	THCAS_AB057805.1	89.8	100	96
	CBCAS_LY658671.1	89.3	96	100
Percentage identity (amino acid)	CBDAS_AB292682.1	100	82.7	81.1
	THCAS_AB057805.1	82.7	100	92.7
	CBCAS_LY658671.1	81.1	92.7	100

Fig. 3 Sequence similarity between the *Cannabis sativa* cannabinoid synthase genes tetrahydrocannabinolic acid synthase (*THCAS*; GenBank acc. no. AB057805.1), cannabidiolic acid synthase (*CBDAS*: GenBank acc. no. AB292682.1) and cannabichromenic acid synthase (*CBCAS*; GenBank acc. no. LY658671.1). (a) Protein sequence alignments of THCAS, CBDAS and CBCAS were performed using Clustal Omega and protein domains were annotated using InterProScan v.5.41-78.0 (Sievers *et al.*, 2011; Jones *et al.*, 2014). The p-cresol methylhydroxylase (PCMH)-type flavin adenine dinucleotide (FAD)-binding domain (residues 77–251, PrositeProfiles: PS51387, InterPro:IPR016166) and berberine and berberine-like domain (residues 480–538 for *THCAS* and *CBCAS*, residues 479–537 for *CBDAS*, Pfam: PF08031, InterPro: IPR012951) are highlighted in red and black, respectively. The FAD-binding domain (residues 81–214, Pfam: PF01565, IPR006094) is not shown. (b) *THCAS* is more similar to *CBCAS* than *CBDAS* at the nucleotide and amino acid levels. It is possible that the presence of *CBCAS* may lead to the production of THCA as a by-product (McKernan *et al.*, 2020).

diversity of cannabis gene pools (Sawler *et al.*, 2015; Soorni *et al.*, 2017). However, these can be expanded by a comprehensive, genomics-based assessment of cannabis germplasm that defines the diversity available. This would in turn help with the identification of heterotic groups for use in crosses to achieve hybrid vigour and hence assist with the creation of elite cultivars (Huang *et al.*, 2015).

Genomics approaches that could be applied in the near-term to improve cannabis traits

Our knowledge of how cannabis traits relate to genotype is currently limited. Many next generation sequencing (NGS)-based tools exist that can rapidly identify genetic variation underlying traits of interest. The available genetic and genomic resources provide an opportunity to apply NGS tools for trait discovery and molecular breeding in cannabis, as has been achieved in other crops (Varshney *et al.*, 2014; Kang *et al.*, 2016; Crossa *et al.*, 2017).

QTL mapping and gene discovery

Low-density molecular markers such as amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) have been employed in cannabis to identify QTLs associated with sex determination and cannabinoid composition (Weiblen *et al.*, 2015; Faux *et al.*, 2016). High-density genetic maps recently developed for cannabis should improve the accuracy of QTL mapping (Grassa *et al.*, 2018; Laverty *et al.*, 2019). Several NGS-based methods are available for high-resolution mapping and interval detection in plants (Schneeberger *et al.*, 2009; Abe *et al.*, 2012; Takagi *et al.*, 2013). A common feature of these methods is that they combine NGS with bulked-segregant analysis to study mutated or natural populations. The methods have been deployed mainly in self-pollinating species with homozygous genomes (Jaganathan *et al.*, 2020). However, recent protocol improvements have allowed their application to heterozygous, outcrossing species, for example to identify loci involved in sex determination, flowering, trichome formation, anthocyanin accumulation and leaf shape in *Dioscorea*

Tree scale: 0.1 ⊢——⊣

Coloured ranges
- ▢ Inactive THCAS/CBCAS
- ▢ Active/Functional THCAS
- ▢ Active/Functional CBDAS

Bootstrap values
- ▮ 70
- ▮ 77.5
- ▮ 85
- ▮ 92.5
- ▮ 100

Fig. 4 Maximum-likelihood phylogenetic tree depicting the relationship among the *Cannabis sativa* cannabinoid synthase genes tetrahydrocannabinolic acid synthase (*THCAS*), cannabidiolic acid synthase (*CBDAS*) and cannabichromenic acid synthase (*CBCAS*). The published nucleotide sequences of the active/functional forms of *THCAS* (GenBank acc. no. AB057805.1), *CBDAS* (GenBank acc. no. AB292682.1), *CBCAS* (GenBank acc. no. LY658671.1) and the paralogues of these genes as annotated in the cs10 v.2.0 and Jamaican Lion (female parent and male parent) assemblies (Supporting Information Tables S1, S2) were aligned against the latest *C. sativa* reference genome assemblies (Table 1) using Blast+/2.2.29 (Altschul *et al.*, 1990). Best hits corresponding to a percentage identity > 98.5%, query coverage > 75% and alignment length = query length ± 100 bp were retained (Tables S1, S2). The nucleotide sequences of these best hits were extracted from each assembly (where applicable) using BEDTools v.2.26.0 (Quinlan & Hall, 2010). TransDecoder v.3.0 was used to predict the longest open reading frame from the extracted regions (https://transdecoder.github.io/). The predicted proteins along with amino acid sequences (complete CDS) of AB057805.1 (gene ID in blue), AB292682.1 (gene ID in red), LY658671.1 (gene ID in green) and the other cannabinoid synthase gene copies annotated in the cs10 v.2.0 and Jamaican Lion (female parent and male parent) genome assemblies were used for multiple sequence alignment using Clustal Omega (Sievers *et al.*, 2011). The phylogenetic tree was reconstructed from these alignments using RAxML v.8.12.12. with 500 bootstrap replicates under the JTT model of amino acid substitution and visualized using Interactive Tree Of Life (iTOL) (Letunic & Bork, 2007; Stamatakis *et al.*, 2008). The tree was rooted with the *Humulus lupulus THCAS* homolog (GenBank acc. no. LA634839.1). Only bootstrap values of > 70% are shown. It is worth noting that all *CBCAS* genes cluster with some the *THCAS* genes reflecting the high sequence similarity between these two cannabinoid synthase genes (Fig. 3).

rotundata (Guinea yam), *Brassica rapa* and *Vitis vinifera* (grapevine) (Tamiru *et al.*, 2017; Demmings *et al.*, 2019; Itoh *et al.*, 2019; Zhang *et al.*, 2020). Similar approaches could be used in cannabis.

Ethyl methanesulfonate (EMS) mutagenesis has been applied successfully to hemp and a protocol exists for cannabis cell culture (Bielecka *et al.*, 2014; Hari, 2020). However, large-scale generation and screening of mutant lines can be a logistical challenge in cannabis owing to its size and the requirement in many jurisdictions to grow it in secure and licensed facilities. Consequently, the exploitation of the cannabis natural diversity provides a better option for mining important genes in the short term (Vergara *et al.*, 2016; Welling *et al.*, 2016). Therefore, the available gene/QTL mapping tools should go beyond simple genetic variations such as SNPs and small insertions/deletions (Indels) and consider SVs

including large Indels, rearrangements and CNVs, all of which have been shown to affect trait diversity in several crops including cannabis (Wang *et al.*, 2016; Chakraborty *et al.*, 2019; McKernan *et al.*, 2020).

Genome-wide association studies (GWAS)

There are only two published cannabis GWAS studies, one of which is ongoing and the other reporting marker-trait association using a limited number of SNPs (B. J. Campbell *et al.*, 2019; Henry *et al.*, 2020; http://multihemp.eu/). The paucity of studies is likely a consequence of the previous limited accessibility of cannabis genetic diversity and insufficient marker density. GWAS relies on linkage disequilibrium (LD) for detecting common genetic variants associated with a trait in natural and experimental populations
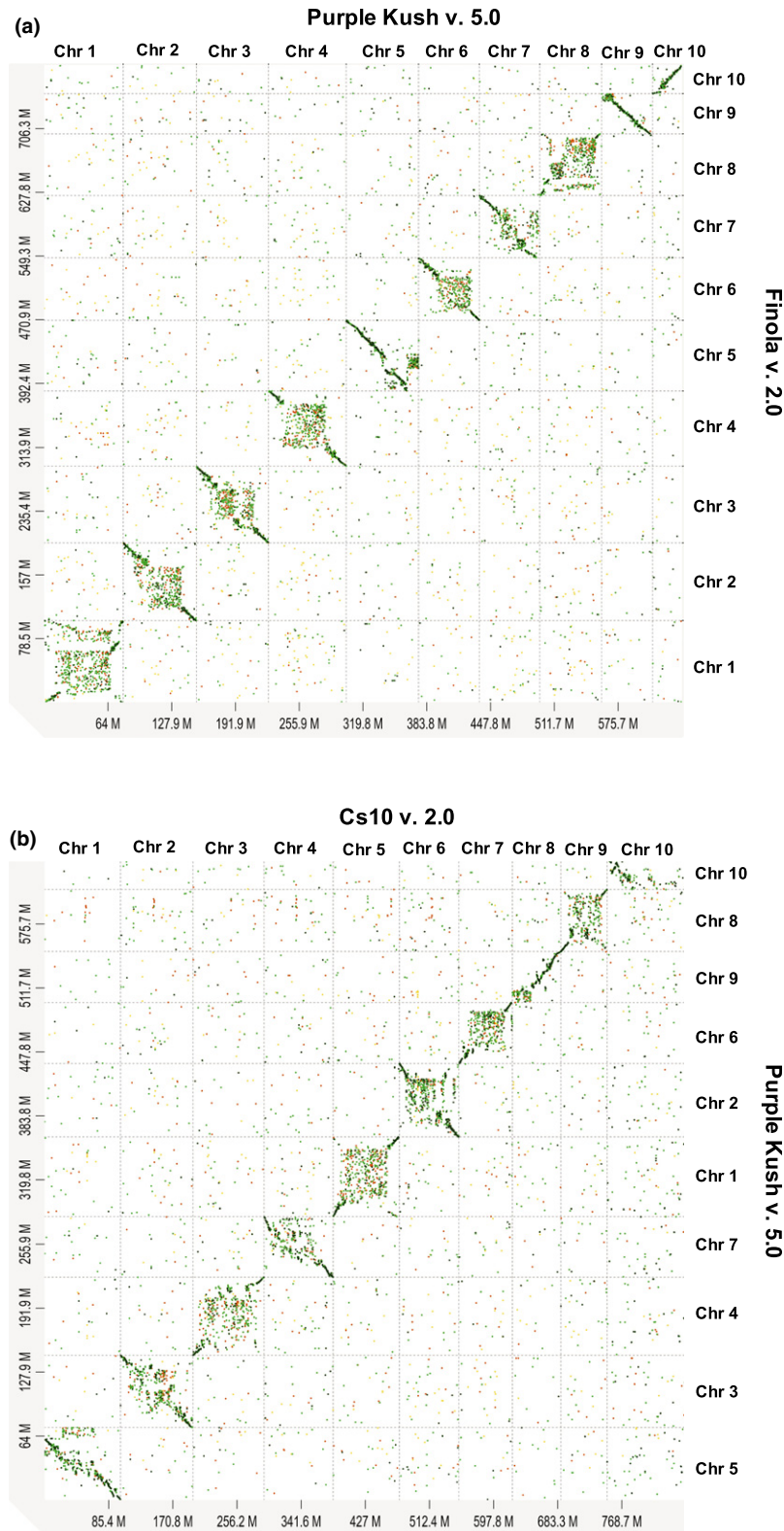
Fig. 5 Dotplots showing the syntenic relationship between genomes of three *Cannabis sativa* cultivars. Pairwise genome alignments for (a) PK v.5.0 (GenBank acc. no. GCA_000230575.5) and FN v.2.0 (GenBank acc. no. GCA_003417725.2), (b) cs10 v.2.0 (GenBank acc. no. GCA_900626175.2) and PK v.5.0 and (c) cs10 v.2.0 and FN v.2.0 were performed using Minimap2 and the alignments were visualized using D-Genies (Cabanettes & Klopp, 2018; Li, 2018) (Supporting Information Table S3). Breaks in the alignment could be due to the presence of structural variants or the less contiguous nature of the PK and FN assemblies. The difference in chromosome orientation between the assemblies also can be seen. Only chromosome-level alignments are shown. PK, Purple Kush; FN, Finola.

Cs10 v. 2.0
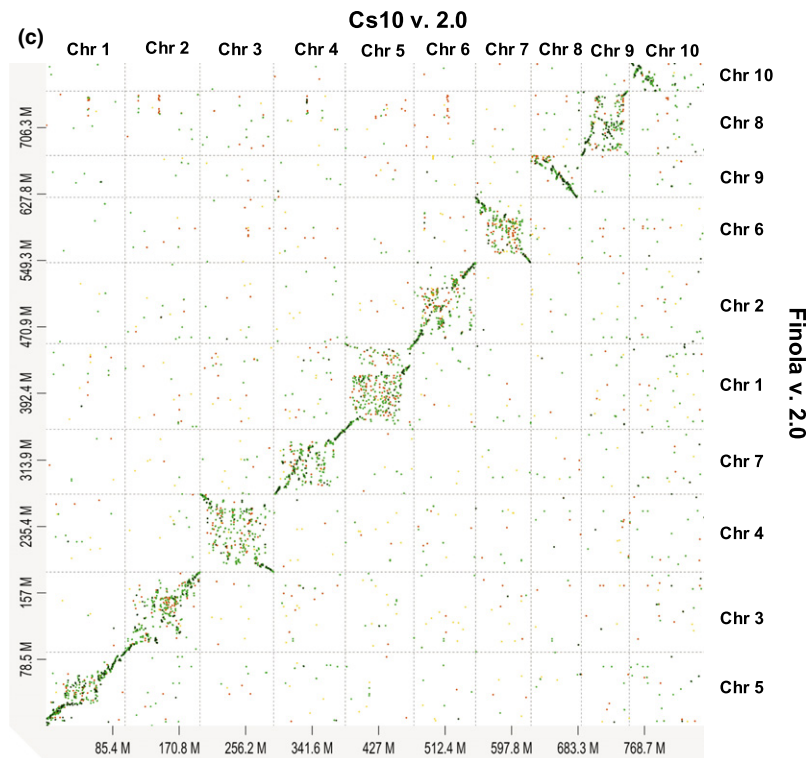
Fig. 5 (Continued)

(Brachi *et al.*, 2011). Consequently, it has been widely deployed in self-crossing species that generally have more extensive LD. Nevertheless, GWAS has been used successfully for genotype–trait association in outcrossing and vegetatively propagated species including date palm, sweet potato and hop (*Humulus lupulus)*, the species most closely related to cannabis (Henning *et al.*, 2015; Hazzouri *et al.*, 2019; Okada *et al.*, 2019). Application of efficient and high-throughput phenotyping systems to cannabis will help GWAS studies owing to the species' high phenotypic plasticity; applicable commercial and open-source solutions already exist (L. G. Campbell *et al.*, 2019). A variant of GWAS termed mGWAS (metabolite-based GWAS) that combines genotyping and metabolic profiling has proved powerful for dissecting the genetic bases of metabolic diversity in plants (Fang & Luo, 2019; Chen *et al.*, 2020). A similar approach could be applied to the cannabinoid and terpene biosynthesis pathways of cannabis.

Genomic selection

Genomic selection utilizes genome-wide marker information to predict the breeding value of genotypes, which is an estimate of the value of a genotype as a parent. It integrates genotypic and phenotypic data from a reference population and uses statistical models to determine the genomic-estimated breeding values (GEBVs) of other individuals for which only genotype information is available. Elite lines with the highest GEBVs are then selected for use as parents in breeding programs. Genomic selection is considered particularly promising for genetic improvement of complex traits controlled by many genes with minor effects (Heslot

et al., 2015; Spindel & McCouch, 2016). The approach has been successfully implemented in breeding programmes for outcrossing heterozygous species such as maize and cassava (Crossa *et al.*, 2017; Elias *et al.*, 2018). It might consequently make a notable contribution to the genetic improvement of complex cannabis traits once marker density, population size, statistical models and accuracy of phenotyping improve.

Emerging genomics technologies with high potential in cannabis research

Phased genome assemblies

The assembly of heterozygous plant genomes remains challenging despite the use of long-read sequencing technology (Michael & VanBuren, 2020). Genome assembly of outcrossing species such as cannabis is particularly challenging because haplotypes consist of various repeating units, short Indels and SVs (Chin *et al.*, 2016; VanBuren *et al.*, 2018). As a result, the majority of near-complete haploid cannabis assemblies are fully unphased, meaning they are a synthetic patchwork of collapsed segments of homologous chromosomes that do not fully capture genome composition (Chin *et al.*, 2016; Grassa *et al.*, 2018; Laverty *et al.*, 2019; Gao *et al.*, 2020). There have been recent efforts to produce partially phased genome assemblies for cannabis (two draft assemblies are now available for the maternal Jamaican Lion cultivar) and hop (var. Cascade) (Padgitt-Cobb *et al.*, 2019; Medicinal Genomics, 2020b). In addition, NRGene recently announced the creation of fully-phased genome assemblies for two proprietary elite
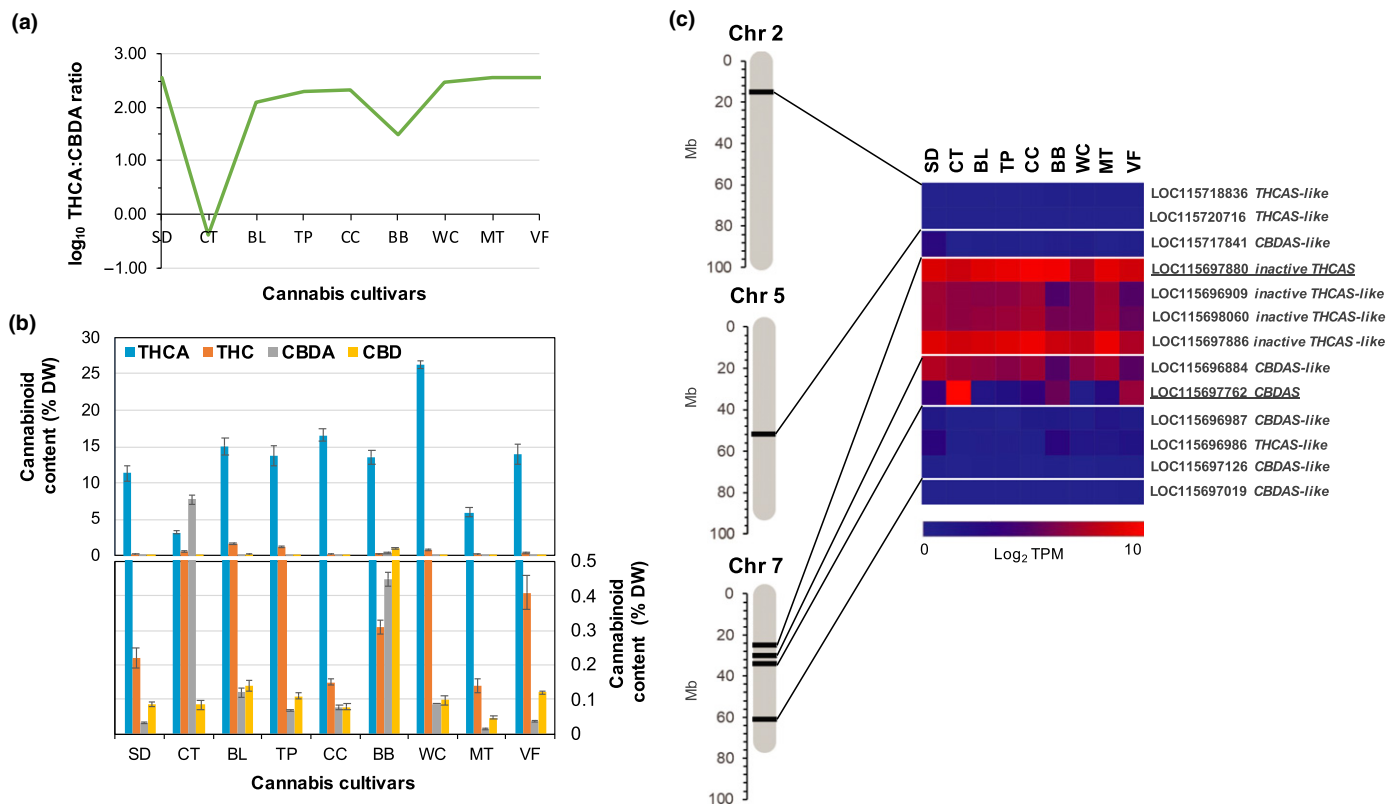
Fig. 6 Cannabinoid synthase gene expression in relation to cannabinoid content and composition in nine high cannabinoid yielding cannabis cultivars (data taken from Zager *et al*., 2019). (a) Tetrahydrocannabinolic acid : cannabidiolic acid (THCA : CBDA) ratio and (b) cannabinoid contents of the cultivars. The lower panel in (b) shows a zoomed-in view of cannabinoid content (% DW) in the range 0–0.5%. (c) Trichome-specific expression patterns of 13 cannabinoid synthase genes from cs10 v.1.0 genome assembly (GenBank acc. no. GCA_900626175.1) in these cultivars. Positions on chromosomes represent one or more cannabinoid synthase locus. The reference *CBDAS* (LOC115697762) and inactive *THCAS* (LOC115697880) loci are underlined. LOC115697762 bears 100% nucleotide identity with the functional *CBDAS* identified by Taura *et al*. (2007) (GenBank acc. no. AB292682.1), whereas LOC115697880 is 99% identical to *CBCAS* (GenBank acc. no. LY658671.1) at the nucleotide level (Taura *et al*., 2007). Of the 13 loci, two (LOC115698060 and LOC115697886) are pseudogenic inactive *THCAS* copies containing in-frame stop codons, whereas the remaining 11 genes produce full-length CDS. Trichome enriched RNA-seq reads previously reported by Zager *et al*. (2019) were accessed from the NCBI Sequence Read Archive (SRA project no. PRJNA498707; Zager *et al*., 2019). The reads were mapped to the *Cannabis sativa* cs10 v.1.0 genome assembly (using HISAT2 v.2.1.0 and sorted by genomic location using SAMTOOLS v.1.9 Li *et al*., 2009; Kim *et al*., 2019). STRINGTIE v.1.3.5 was used to assemble RNA-Seq alignments into potential transcripts and to calculate gene abundances (TPM) (Supporting Information Table S6; Pertea *et al*., 2015). Chromosome numbers have been changed to community standard nomenclature in accordance with cs10 v.2.0. (GenBank acc. no. GCA_900626174.2.). *Cannabis sativa* var. cs10 is associated with a high CBD chemotype. BB, Black Berry Kush; BL, Black Lime; CC, Cherry Chem; CT, Canna Tsu; MT, Mama Thai; SD, Sour Diesel; TP, Terple; TPM, Transcripts per million; VF, Valley Fire; WC, White Cookies. Error bars represent ± 1 SD of the mean metabolite content of each cultivar (*n* = 3).

cultivars using cs10 as the basis for scaffold ordering (Weisshaus, 2020). These assemblies are likely to improve our understanding of haplotype structure and heterozygosity, which is essential for allele-specific analyses of quantitative traits (Chin *et al*., 2016).

Cannabis pangenomics

Plant pangenomics studies conducted over the last five years have demonstrated the inadequacy of a single reference genome in representing the entire genetic diversity of a species (Golicz *et al*., 2016; Hurgobin *et al*., 2018). The creation of a cannabis pangenome promises to shed more light on the extent of gene content variation, as well as forming the basis for cannabis breeding programmes. For instance, the inclusion of diverse genotypes and wild cannabis populations (sometime referred to as *C. ruderalis*), would facilitate the identification of elite marker genes in the dispensable/variable genome of cannabis and drive the process of

heterosis to create resilient and high-yielding cultivars (Tao *et al*., 2019). Additionally, combining gene PAV and pangenome-wide SNPs would lead to a more accurate identification of causal variants associated with traits of interest (Hurgobin & Edwards, 2017).

Independent cannabis pangenome initiatives are being led by NRGene and Medicinal Genomics (NRGene, 2018; Medicinal Genomics, 2020a). Allelic variations and additional genes involved in cannabinoid biosynthesis were identified by comparing the recent fully-phased assemblies from NRGene with existing chromosome-level, unphased cannabis assemblies (Weisshaus, 2020). Conserved genomic regions, as well as variable regions harbouring SVs such as CNVs, PAVs and large rearrangements, which may be implicated in cannabis and hemp breeding, were also identified. The increased accessibility of long-read sequencing will likely encourage the construction of graph-based pangenomes, which are considered to be the future of plant pangenomics studies (Bayer *et al*., 2020). A graph-based pangenome consists of a single,

nonredundant reference that contains SVs from multiple individuals/accessions. It can be visualized as a sequence graph with branches representing accession-specific sequences (Garrison *et al.*, 2018). The first graph-based pangenome of soybean was recently produced using 29 wild and cultivated accessions (Liu *et al.*, 2020). Using this resource and the SVs that they had identified among the accessions, the authors performed a GWAS and identified a 10-kbp PAV, which was associated with seed lustre variation. This type of analysis would be helpful for cannabis as it could be used to determine the SV landscape of this important crop and its association with traits of interest.

Single cell genomics

Transcriptomics has been revolutionized by single cell RNA-Sequencing (scRNA-Seq) because it enables researchers to investigate the complex interplay of molecular regulators and identify active cellular processes at true cellular resolution (Rich-Griffin *et al.*, 2020). Whilst only a handful of scRNA-Seq studies have been conducted in plants to date, they demonstrate that this technology may have great potential in cannabis breeding (Rich-Griffin *et al.*, 2020). For instance, given the specificity of the cannabinoid biosynthetic pathway to the capitate stalked trichomes, a detailed view of gene expression in the cell-types involved in this pathway would assist with the selection of marker genes. An understanding of cell-types which respond most strongly to environmental cues such as biotic or abiotic stresses also would be valuable. Combining this information with single-cell assay for transposable accessible chromatin (scATAC) sequencing data would enable the identification of regulatory regions of the genome that drive cell-type specific expression (Rich-Griffin *et al.*, 2020). Integration of scRNA-Seq with CRISPR/Cas9-based genetic screens would enable more effective selection of cell types for targeted gene manipulation whilst reducing the impact of pleiotropy (Yuan *et al.*, 2018; Shahan, 2019; Marand *et al.*, 2020; Rich-Griffin *et al.*, 2020).

Concluding remarks and future perspectives

Cannabis biology remains poorly understood but the relaxation of legislation, together with the application of existing and emerging genomics technologies, is likely to fuel more scientific research on this fascinating plant. The rapid generation of sequencing data and the creation of additional, fully phased, chromosome-level genome assemblies will enable a more comprehensive assessment of the genetic architecture of important traits and aid in marker discovery. Whilst genomics-assisted breeding will have a pivotal role in increasing the efficiency and precision of cannabis crop improvement, the utility of integrating the other 'omics technologies cannot be overlooked. In line with the overarching goal of the ICGRC, the integration of individual 'omics approaches such as transcriptomics, phenomics, metabolomics and proteomics, among others, and the ongoing development of computational methods will be useful for understanding gene function, biological and metabolic pathways, and regulatory networks underlying traits of interest. Complementing these 'omics approaches with traditional breeding

practices will provide a multifaceted strategy for the improvement of this emerging crop species.

Author contributions

BH and MGL conceived the manuscript; BH and MW conducted genome sequence analyses; BH, MTO and MW wrote the manuscript; BH, MTO, MTW, MGL, MSD, AB and JW revised the manuscript. All authors read and approved the final version.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Summary of best BLAST hits of the functional cannabinoid biosynthesis genes against the latest *C. sativa* reference genome assemblies.

Table S2 Summary of best BLAST hits of other *THCAS-* and *CBDAS-like* gene copies against the latest *C. sativa* reference genome assemblies.

Table S3 Alignment of Purple Kush v.5.0 (GenBank acc. no. GCA_000230575.5) and Finola v.2.0 (GenBank acc. no. GCA_003417725.2) unplaced scaffolds against the cs10 v.2.0 genome assembly (GenBank acc. no. GCA_900626175.2).

Table S4 Cannabinoid synthase genes from cs10 v.2.0 (GenBank acc. no. GCA_900626175.2) and Jamaican Lion (female parent: GenBank acc. no. GCA_012923435.1; male parent: GenBank acc. no. GCA_013030025.1) assemblies.

Table S5 Summary of best BLAST hits of nonfunctional *CBDAS* homologs from Skunk #1 against the latest *C. sativa* reference genome assemblies.

Table S6 Transcript per million (TPM) counts of the 13 cannabinoid synthase genes from the cs10 v.1.0 genome assembly (GenBank acc. no. GCA_900626175.1) in nine medicinal cannabis cultivars from Zager *et al.* (2019).

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