Single molecule sequencing of THCA synthase reveals copy number variation in modern drug-type *Cannabis sativa* L.

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Summary

- Cannabinoid expression is an important genetically determined feature of *cannabis* that presents clinical and legal implications for patients seeking cannabinoid specific therapies like Cannabidiol (CBD).
- Cannabinoid, terpenoid, and flavonoid marker assisted selection can accelerate breeding efforts by offering genetic tools to select for desired traits at an early stage in growth. To this end, multiple models for chemotype inheritance have been described suggesting a complex picture for chemical phenotype determination.
- Here we explore the potential role of copy number variation of THCA Synthase using phased single molecule sequencing and demonstrate that copy number and sequence variation of this gene is common and suggests a more nuanced view of chemotype prediction.

Introduction

The genetics of cannabis chemotype has been extensively studied due to the highly selected THCA and CBDA phenotypes. A co-dominant model of inheritance of THCA and CBDA synthase (THCAS, CBDAS)(de Meijer *et al.*, 2003) has been demonstrated describing a Bt:Bd allele in linkage to the synthase genes(Onofri *et al.*, 2015; Weiblen *et al.*, 2015). In addition to this Bt:Bd allele, single nucleotide polymorphisms in the FAD binding domain of THCAS have been described that impair the function of the enzyme(Sirikantaramas *et al.*, 2004). Cascini *et al.* demonstrated varying copy number of a highly conserved region in THCAS but did not find this assay to be correlative to THCAS expression(Cascini *et al.*, 2012; Cascini *et al.*, 2013). Complimenting de Meijer and Sirikantaramas work, van Bakel *et al*. reported a gene of unknown function (AAE3) that was differentially replicated in drug versus fiber type cannabis(van Bakel *et al.*, 2011).

Whole genome sequencing using 454 and Illumina sequence data demonstrated excessive polymorphic coverage over the single exon THCAS gene(McKernan, https://archive.org/details/SequencingTheCannabisGenome)) but allelic phasing of these important genes has not been possible using short read sequencing and conventional genome assembly technologies. Using bacterial cloning, Onofri *et al*. demonstrated a polyploid status for THCA synthase (THCAS) confirming the putative copy number variation observed with whole genome sequencing. Nevertheless, this polyploid status appeared to be a rare event occurring in a single United States bred cultivar and was not correlated with increased THCA expression. Weiblen *et al(Weiblen et al., 2015).* and Cascini *et al.(Cascini et al., 2012; Cascini et al., 2013)* further supported polyploidy with quantitative PCR and bacterially cloned THCAS DNA but deep sampling of alleles may have been limited by bacterial cloning methods. Here, we apply over 10,000 long-read single molecule sequences per sample in combination with Illumina sequencing methods, to phase the multiple copies of THCA synthase in over a dozen modern medicinal cultivars(Eid *et al.*, 2009). Allelic phasing of this 1.6kb gene adds further support to additional mechanism of polyploidy in chemical inheritance.

Multiple primer pairs were designed to amplify THCAS, and the amplicons were sequenced using Pacific Bioscience's circular consensus sequencing (CCS) method and a size-selected Illumina paired-250bp read system (Table1). Given the 40kb reads of the Pacific Biosciences (PacBio) platform, it is ideally suited for phasing polymorphic genes that are longer than the read lengths accessible by Sanger sequencing. Bacterial cloning and primer walking has been used traditionally to address this, but the methods can be cumbersome, expensive and are susceptible to cloning bias(Metzker, 2010). Single molecule sequencing enables one to deeply sequence a PCR product without bacterial cloning to eliminate this bias. The higher raw error rate of single molecule sequencing is overcome by ligating the target amplicon into a circle and performing 20X rolling circle sequencing(Chaisson *et al.*, 2015). This CCS procedure significantly reduces the stochastic error associated with single molecule detection while providing long phased haplotypes of each copy of the amplified gene. These phased haplotypes were then verified by paired-end Illumina sequencing to confirm pure phase with homozygous alignments. Cultivars that vary in CBDA and THCA content were sequenced to assess the diverse haplotypes present in *cannabis* and to resolve putative pseudogenes from intact open reading frames.

Results

We demonstrate that multiple copies of THCAS with distinct sequences are present in many common dispensary and coffee house cultivars in circulation in 2015 (Figure 1). The ascertainment of THCAS copy number is dependent on primer selection suggesting local divergence in the 5' and 3' untranslated regions (UTRs).

Utilizing two sets of primer pairs (within and external to THCAS), we amplified polyploid copies of THCAS and sequenced them using the Pacific Biosciences CCS method. To mitigate PCR error we required a minimum of 10 independent, but identical sequences from different zero mode waveguides (ZMW) to establish a haplogroup. For each CCS, the molecule was read on the forward and reverse strands 20-30 times to form a circular consensus that was over 99.95% accurate (Table 2).

Once PacBio long reads were error corrected using CCS, Illumina Nextera libraries were generated from size-selected 850 bp fragments derived from the PCR products, and were sequenced with 250 base paired-end reads. The resulting read pairs could be accurately mapped to each PacBio haplogroup to produce homozygous alignments for each allele (Figure 2.). These homozygous alignments were helpful in ascertaining whether all haplogroups were accurately represented in PacBio data and if there were any remaining variants ambiguously mapped. Unphased polymorphic data is difficult to translate into amino acid sequence and obscures accurate assessment of functional alleles.

The THCAS amplicons were initially amplified using the primers described by Onofri *et al.(Onofri et al., 2015)* This primer set spans the start and stop codons of the THCAS gene and thus is incapable of providing sequence information for the first and last 25bp of the gene. Moving the primers out into the flanking non-coding regions (MGC-2130 external primer set) delivered fewer haplogroups for all cultivars tested, presumably due to the divergence of these regions in the different gene copies (Figure 3).

To ascertain the reproducibility of this approach, Chemdog91 was amplified 3 different times and run on 3 different SMRT cells. One sample was run as a single-plex sample on a SMRT cell to afford high enough sampling to ensure no alleles were missed. While each sample consistently sequenced the active haplogroups to high coverage, a few inactive or unknown haplogroups emerged on only 2 of the 3 SMRT cells. Since one of the intermittent haplogroups has a unique variant (S221F genotype) private to Chemdog91, we can rule out contamination from other gDNA being the source of these intermittent haplogroups. We cannot rule out the possibility of other diverged copies of inactive THCAS stochastically amplifying given the PCR reactions were set up independently. Gradient PCR at lower annealing temperatures with the Onofri primers may unveil additional diverged pseudogenes. A few intermittent haplogroups had depressed consensus accuracy and are detectable with appropriate quality filtering tools. As a result of these conditions we do not believe the amplicon subread coverage numbers derived from the Onofri primer sets can be taken as a direct proxy for absolute copy number in the genome. It is possible that some of the high coverage, active THCAS haplogroups may be more than diploid in copy number yet exist as non-diverged sequence in the genome. With the MGC-2130 data being multiplexed, any inter-strain coverage or inferred copy number differences are more likely the result of normalization of barcoded samples prior to multiplexing. Only intra-strain copy number can be assessed with these data.

We segregated the haplogroups based on two genbank accession sequences that have been confirmed as encoding active (Q8GTB6.1 also identical to genbank accession number E33090) and inactive (Q33DQ2.1) THCAS(Sirikantaramas *et al.*, 2004; Kojoma *et al.*, 2006). While these two haplogroups differ by 37 amino acids from each other, several other haplogroups diverged by up to 2 and 6 amino acids from these two reference sequences (Figure 4, Table 3). Other haplogroups were identified that contained termination or frame-shifting variants. These had higher similarity to Q33DQ2.1 than Q8GTB6.1, suggesting an inactive heritage. Phylogenetic trees were constructed that clustered the divergent haplogroups around Active, Inactive, and frame-shifted clusters (Figure 4).

We surveyed CBDA and THCA dominant cultivars seen in Table 1,2 and 3. Cultivars measured to have CBDA-rich chemotypes like WZ_CBD contained 3 haplogroups, one of which was clearly a pseudogene (Figure 1). The other two haplogroups were either identical to, or had 6 amino acid mismatches with the “Inactive THCAS” in genbank (Q33DQ2.1). Since this cultivar has low but measured THCA (0.6% THCA : 20%CBDA), it could be assumed that the 6 amino acid divergence from the inactive form represents a very weakly active THCAS gene. The cultivar was positive for the Bd:Bd genotype described by de Miejer, and if this allele governs the binary expression of CBDAS over THCAS then the expression level of THCAS is uncertain and the impact of the 6 amino acid variants on activity is impossible to infer. Some level of chemotype expression exists and suggests a leaky Bd:Bd expression is responsible for the 0.6% THCA. Of note, this haplogroup failed to amplify with the MGC-2130 external primer set (Table 4).

The 6 amino acid diverged THCAS haplogroup is also found in other THCA dominant cultivars like Chemdog91, however Chemdog91 is a high THCA expressing cultivar and has 5 THCAS copies in total (one with a stop codon, one that is inactive and three that are putatively active). One haplogroup in Chemdog91 is identical in amino acid sequence to the active THCAS sequence (Q8GTB6.1) and the other has a common A250D variant of the active form. Although we did not collect RNA from these samples, Onofri, *et al*. also describe some of these alternative alleles and demonstrates RNA expression(Onofri *et al.*, 2015).

Adding to the observations of Onofri, *et al*., we were able to find THCA producing strains that exclusively held the ambiguous genotypes described in their paper as (1/4 , 2/1 , 2/2 , 2/3). The 1/4 genotype described by Onofri, *et al*. is an A250D variant of the active form. The Cultivar “AK-47” presented with 3 haplogroups of which one had a stop codon, one had an inactive allele (Q33D12.1) and one presented with a single active THCAS haplotype with an A250D variant. We can deduce from this that the A250D allele is the only haplogroup capable of synthesis of the high levels of THCA found in AK-47.

Interestingly, another high THCA strain (Black84) shared this A250D variant but also had the I63L variant described and deemed active by Onofri et al. (Table 3). Only the A250D variant amplified with the MGC-2130 primer set bringing some question to the activity of the I63L variant in our cultivars. Chemdog91, in addition to its Q8GTB6.1 active allele, also has an A250D haplogroup. Both of these haplogroups amplify with the MGC-2130 primer set in Chemdog91 and only the A250D allele amplifies in Black84 suggesting the I63L variant may be inactive (Table 4). Further work is required to characterize the I63L variants functionality.

The other THCA:CBDA hybrid cultivar “Blueberry Essence” has a single haplogroup that amplified with both primers, suggesting the sample had identical maternal and paternal alleles for THCAS sequence. This sequence has a single amino acid variant from the active form (P333R). Considering the structure of the gene, this proline change may alter the activity of the THCAS and explain the 8.11% THCA, 10.8%CBDA expressed in Blueberry Essence(Shoyama *et al.*, 2012). Figure 6 demonstrates a Hybrid Bd:Bd genotype. The difference in Bd:Bd vs Bt:Bd genotypes relies on measuring the magnitude of the 3rd peak in the electropherogram but in our hands the assay did not produce a clear presence-absence result allowing unambiguous differentiation of these two alleles (Figure 6.). Interestingly, the B1080/B1192 primers described in Pacifico *et al*. were confirmed to be the B1180/B1192 genotyping primers utilized in Table 2 of Onofri *et al.* (personal communication G. Mandolino). This forward primer targets a region conserved between active and inactive THCAS while the mid portion of the reverse primer rests on a H494P and A495E variant between active and inactive alleles. Although the 3 prime end of the primer is 12 bases upstream of these primed variants, it is possible but unlikely that this primer set differentiates between active and inactive transcripts of THCAS.

Otto and Sour Tsunami are both CBDA dominant chemotypes but demonstrate 2 and 6 haplogroups respectively. Three of the haplogroups in Sour Tsunami have stop codons. The remaining three haplogroups are exact active and inactive haplogroups with an additional 6 amino acid variant of the inactive haplogroup with unknown activity. Otto has 2 haplogroups, one inactive copy that only amplifies with Onofri primers and another that amplifies with both primer sets and represents a single active Q8GTB6.1 haplogroup with an A411V variant.

Illumina Sequencing of the Bt:Bd alleles

We amplified the Bt:Bd alleles from all of the samples in Table 1 (Figure 6). While 4 of the THCA positive samples demonstrated the 190bp band, 3 did not. A select few of these products were Illumina sequenced to gain a better understanding of the loci the B190:200 primers amplify. Assembling these amplicons produced several contigs per cultivar where the inserts can be mapped to a tandem repeat in CanSat3 Scaffold 19079 (Figure 7.). Only 9-10bp of the 3' end of the B190/B200 primers match the reference at appropriate distances implying a Tm sensitive assay that is likely highly sensitive to salts in various DNA isolations. Of note, the referenced methods in Mandolino et al. recommend 38C annealing temperatures(Mandolino, 1999). We confirmed these alignments with cross comparisons to other public whole genome assemblies with of Chemdog91 and LA Confidential(McKernan, https://aws.amazon.com/datasets/the-cannabis-sativa-genome/). Contig_60162 of LA Confidential has a 100% 159bp alignment of WZ-CBD B190/B200 allele while Chemdog91s contig_96784 has a 148/159bp alignment with many other contigs showing similar homology. Various polymorphisms also exist in the alignments. While this appears to be a repeat, it is also rich in secondary structure notorious for error prone sequencing(Nakamura *et al.*, 2011).

While these are useful markers for tracking chemotype, its unclear by their sequence alone what function they play. Even though THCAS rests on CanSat3 scaffold 19603 and shares little homology to CanSat3 scaffold 19079, one possibility is that the regions are simply in linkage to active and inactive alleles of CBDAS and THCAS and closer markers to the gene may be more predictive in a polymorphic population.

Discussion

Even though absolute copy number does not correlate with CBDA dominant or THCA dominant chemotypes, a more nuanced view of the amino acid alterations in the haplogroups reveals an interesting pattern. All CBDA strains contain at least one haplogroup with similarity to the inactive Q33DQ2.1 haplogroup. The most common second haplogroup in CBDA dominant cultivars is the 6 amino acid diverged form of Q33DQ2.1 (N90F, I266T, L370F, D420G, Y471C, T492S). Additionally, no CBDA dominant strains amplified with perfect Q8GTB6.1 active alleles with the MGC-2130 primers. The only two cultivars to amplify with the MGC-2130 primers had variants (Otto-A411V, and Blueberry Essence-P333R). The other three CBDA cultivars failed to amplify with MGC-2130 primers (WZ-CBD, Sour Tsunami, CannaTsu) despite gradient PCR optimization attempts.

This suggests UTR sequences of various THCAS haplogroups are associated with different chemotypes, and invokes an interest in the linked Bt:Bd allele described by de Meijer *et al.*). Weiblen *et al*. demonstrated this marker is tightly linked to THCAS and CBDAS. Pacifico *et al.* implied these B190/200 markers were imperfect in resolving the Bt:Bd from Bd:Bd alleles but never published the implied and improved B1080/1192 primers(Pacifico, 2006). Although, it is possible that the Bt allele is just in phase with the MGC-2130 primer set and predicts active form THCAS while the Bd allele is in phase with functional CBDAS, the current genome assemblies can only anchor partial B190 or B200 primer sequences (used to amplify the Bt:Bd allele) to a genomic contig (scaffold 19079). Sequencing these Bt:Bd products with Illumina sequencing demonstrated high coverage and high homology between the various sized bands suggesting a recently expanded repetitive element that is hard to assemble (Figure 7).

The UTR based MGC-2130 primers imply the THCAS replicated inactive events did not include 5 or 3' UTRs. Primers that rest 387 & 160 bases back from the start and stop of the gene amplify fewer haplogroups of THCAS presumably due to the genomic divergence flanking the transcribed sequences. 10/13 of the cultivars amplified successfully with the more distant MGC- 2130 primers (WZ-CBD, Sour Tsunami, and CannaTsu failed to amplify). Only one hybrid cultivar demonstrated a single haplogroup with both primers (Blueberry Essence Q8GTB6.1 P333R). The MGC-2130 primers appear to predominantly amplify the active forms and could possibly be used to better inform chemotype prediction. Due to the high polymorphism rate of this species and locus, sampling of additional cultivars with well-measured chemotypes is underway.

These haplogroups help to inform why copy number assays targeting highly conserved regions of THCAS can fail to differentiate active from inactive haplogroups. Cascini *et al.* demonstrate copy number variation in THCAS with qPCR but these primer selections would have failed to amplify only one inactive variant haplogroup in this study (Black84_Onofri-lbc1_C0_P0_NumRs92). A comprehensive sequencing strategy can properly identify active from inactive copies.

Conclusions

The study of the inheritance of THCAS in modern drug type cultivars must consider polyploidy. Even though many of the replication events in THCAS appear to be pseudogenes or inactive forms, partially active forms are emerging and appear to be associated with more the balanced (Bt:Bd) THCA:CBDA cultivars. While de Meijer et al. demonstrate (Jurka & Kapitonov, 2001)chemotype prediction from the B190/B200 primers for the Bt:Bd allele, its precise location in the genome is difficult to uniquely resolve and while it can inform THCA and CBDA chemotype it has not been able to predict the magnitude of THCA or CBDA expression and some ambiguity on Bt:Bd vs Bd:Bd genotyping has been noticed. The sequencing of these alleles demonstrates a repetitive and polymorphic locus that appears difficult to design higher throughput qPCR assays for.

The observation of diverged THCAS genes and copy number variation may provide another tool in refining the chemotype prediction for *Cannabis* in light of previous work. Novel variants have been identified and phased with other variants of interest in THCAS. In light of both copy number variation, amino acid variation, B190-B200 alleles and van Bakels AAE3 observations, it is possible that the highly selected chemotype is experiencing convergent evolution. Of note are the several CWCTTA/TTAGWG "P Instability Factor" (PIF) sequences present in THCAS. While it is unclear if this replication event is transposon driven, the multiple, albeit small, recognition sequence signals in the gene and the repetitive sequence flanking THCAS is of note(Jurka & Kapitonov, 2001; Zhang *et al.*, 2001). Structural variation in the human genome is often attributed to segmental duplication of ancestral alleles followed by Nonhomologous end joining (NHEJ) or non allelic homologous recombination (NAHR). VNTRs and retrotransposition are also known to drive these events(Kidd *et al.*, 2008). While gene duplication of THCAS is common, the exact mechanism driving it remains to be determined.

Methods

Two different amplicons for THCAS were selected. Primers described by Onofri *et al* were utilized but also redesigned as these previously described primers are internal to the gene (5' ATG in the Forward primer is the start codon for the gene) and cannot inform on start codon or N-terminal signal peptide sequence. To multiplex multiple cultivars per SMRT cell, 5 prime dual 16 base pair molecular barcodes were utilized as described by the vendor.

THCAS PCR

PCR was performed with 50ul 2x LongAmp (New England Biolabs- #M0287S), 8ul 10uM each primer, 42ul DNA+ddH20 (30ng total). Plant DNA was extracted with SenSATIVAx according to manufacturers instructions (Medicinal Genomics part #420001). DNA is eluted with 50ul ddH20.

THCAS was amplified from genomic 30ng of genomic DNA using primers Onofri-F (Forward;ATGAATTGCTCAGCATTTTCCTT) and Onofri-R (Reverse; ATGATGATGCGGTGGAAGA). Product was cycled using 98C 30 s followed by 28 cycles of 98C 10 s, 48C 30 s, 72C 90 s followed by a 72C for 5 min.

THCAS was also amplified from genomic 30ng of genomic DNA using primers MGC-2130-F (Forward; ATGGGAACCATAATAAACTATAAAAGTCATT) and MGC-2130-R (Reverse; TATGATTGTCTACACAGTTCTAATGTAGATTTTC). Product was cycled using

94C 30s followed by 28 cycles of 98C 10s, 58C 30s, 65C 150s followed by a 65C for 5 min.

PCR products were purified with equal volume of SenSATIVAx (100ul) followed by two 100ul 70% EtOH washes. Samples were eluted in 25ul ddH20 and quantified with Qubit and Agilent HS chips, normalized and pooled for Pacific Biosciences circular consensus sequencing. DNA barcodes recommended by the manufacturers were used to multiplex sequencing on each SMRT cell. Sequencing was performed by University of Florida service center.

Nextera Libraries for Illumina phase confirmation.

To generate sized shotgun libraries 9ul of THCAS PCR product (1.7ng/ul) was added to 20ul Nextera Buffer with 0.5ul Nextera Enzyme and 0.5ul ddH20. Nextera reaction was driven 7 minutes at 51C. Reaction was quickly purified with 40ul of SenSATIVAx. Tip mixes 10 times and placed on a magnet plate (Medicinal Genomics part#420202) for 10 minutes, removed supernatent and washed with 100ul 70% EtOH twice. After 5 minutes of benchtop room temp drying of residual EtOH the samples were eluted with 25ul ddH20.

Post Nextera end healing or NT-PCR.

To end repair the transposition event a nick translation event was performed prior to initial denaturization (NT-PCR). 31ul of LongAmp 2X master mix (New England Biolabs- #M0287S) was added to 6ul primer+indicies (2ul Index 1, 2ul Index 2, 2ul ILMN primers). 25ul Purified Nextera library DNA (62ul total reaction volume) was used for NT-PCR.

Reactions were cycled with 72C for 3 minutes, 98C for 30 seconds, 98C for 10 seconds, 63C for 30s, 72C for 1minute, Goto step 3 for 14cycles, 10C-hold. After NT-PCR, the sample was purified with 60ul SenSATIVAx added to the 62ul NT-PCR reaction. SenSATIVAx was tip mixed 10 times and placed on a magnet plate for 10 minutes. After separation and supernatant removal the sample was washed twice with 100ul of 70% EtOH. The beads were dried on a magnet plate for 10 minutes and eluted in 25ul ddH20

SAGE size separation

25ul sample was added to 10ul R2 Marker (Sage Sciences) and loaded into a Sage Science Blue Pippin, 1.5% Agarose Dye-Free 40ul EM. Software was set to size cut at 600-800bp in size. 40-60ul of elution was captured in the output port. Optionally QC 1ul on an Agilent 2100 Bioanalyzer High Sensitivity chips. Loaded library according to Illumina V2 instructions protocol with 2x250bp reads.

Bt:Bd Allele Amplification and sequencing

Primers described by de Meijer *et al.* were used named B190F(Forward; B190F TGCTCTGCCCAAAGTATCAA) and B200R (Reverse; CCACTCACCACTCCACCTTT).

Using 5ng input DNA was added to 25uL Q5 polymerase 2X Master Mix (NEB #).

1.25uL B190F Primer (10uM) with 1.25uL B200R Primer (10uM) were mixed with 22.5uL Water. Total volume was brought to 50ul with water + DNA (5ng total).

Genomic amplification

PCR reactions were heated to 98C for 30 seconds and 40 cycles of 98C for 10s, 56C for 30s, 72C for 30s. A final 72C for 5 minutes was performed before 8C hold. PCR products were purified with 75ul SenSATIVAx. After two 100ul 70% EtOH washes, the beads were dried and eluted in 25ul ddH20. Yield was measured with a Qubit and an Agilent HS chip.

PCR product Cloning

DNA libraries were constructed with 250ng DNA using NEB's NEBNext Quick ligation module (NEB # E6056S). End Repair used 3ul of Enzyme Mix, 6.5ul of Reagent Mix, 55.5ul of DNA + ddH20. After End Repair, Ligation was performed directly with 15ul of Blunt End TA Mix, 2.5ul of Ilumina Adaptor (10uM) and 1ul of Ligation enhancer (assumed to be 20% PEG 6000). After 15-minute ligation at 25C, 3ul of USER enzyme was added to digest the hairpin adaptors and prepare for PCR. The USER enzyme was tipmixed and incubated at 37C for 20 minutes. After USER digestion, 86.5ul of SenSATIVAx was added and mixed. The samples were placed on a magnet for 15 minutes until the beads cleared and the supernatent could be removed. Beads were washed twice with 150ul of 70% EtOH. Beads were left for 10 minute to air dry and then eluted in 25ul of 10mM Tris-HCl.

Amplification of libraries

Samples adapted with NEB adaptors were amplified with 25ul of 2X Q5 Polymerase (NEB). 1ul of 25uM i7 Primer and 1ul Universal primer were added. 23ul of Eluted DNA was added to make a 50ul total reaction. 98C for 30s was used to hot start the enzyme, followed by 6 cycles of 98C for 10s, 65C for 30s and 72C for 30s. A final 72C for 5 minutes was performed before 8C hold. PCR products were then purified using equal volumes (50ul) of SenSATIVAx. Samples were run on Qubit and Agilent to gauge their quality and further size selected on a SAGE Blue Pippin 1.2% Agarose dye free system to remove off target amplicons. (100-400bp). Quantified libraries were run on a MiSeq with V2 chemistry using 2x250bp reads.

Analysis

Circular Consensus was achieved using Pacific Biosciences tools for de-multiplexing symmetric barcodes with the RS Long Amplicon Analysis.1 using SMRTanalysis 2.3.0 workflows (http://www.pacb.com/devnet/). Minimum size was selected as 1500 and 2300 for Onofri and MGC-2130 amplicons respectively with a maximum subread of 700. Fasta files were aligned with a CLCbio workstation and confirmed using BLAST to active and inactive accessions numbers (Q8GTB6.1 and Q33DQ2.1). Phylogenetic trees were graphed with CLC bio with E33090 set as the root.

Illumina reads were mapped with BWA-MEM version 0.7.12-r1044 to the E33090 with 600-650bp inserts selected. These reads were subsequently mapped to each PacBio derived haplogroup for each respective cultivar. Reads mapped to PacBio haplogroup references were displayed in the Integrated Genome Viewer or IGV(Robinson *et al.*, 2011). Genbank accession numbers for this manuscript: SRP064442

Author Contributions

KJM- Experimental design, barcoded PCR, Nextera, Sequencing and Manuscript drafting
YH- Barcoded PCR, Nextera, Sequencing, B190, B200 tests
VT- THCAS primer design and PacBio software installment.
SM- Illumina alignments to phased PacBio data and Figure generation
JS- Experimental optimizations and method development
LZ- Experimental optimizations and method development
DS- Manuscript review and drafting.

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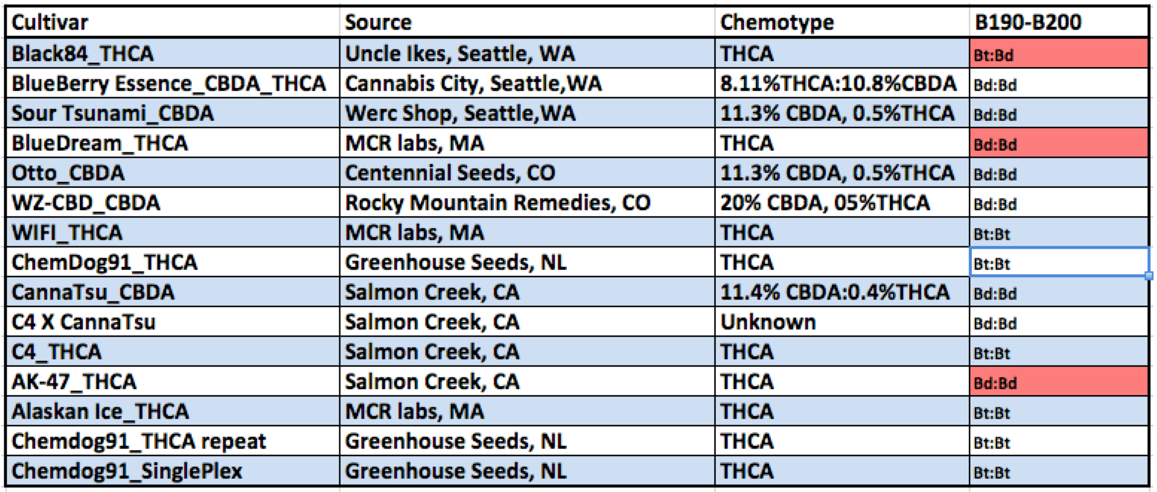
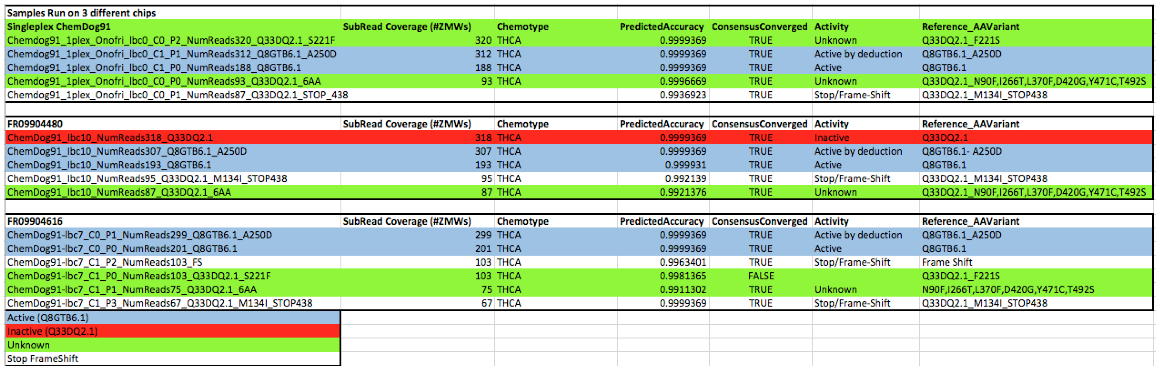


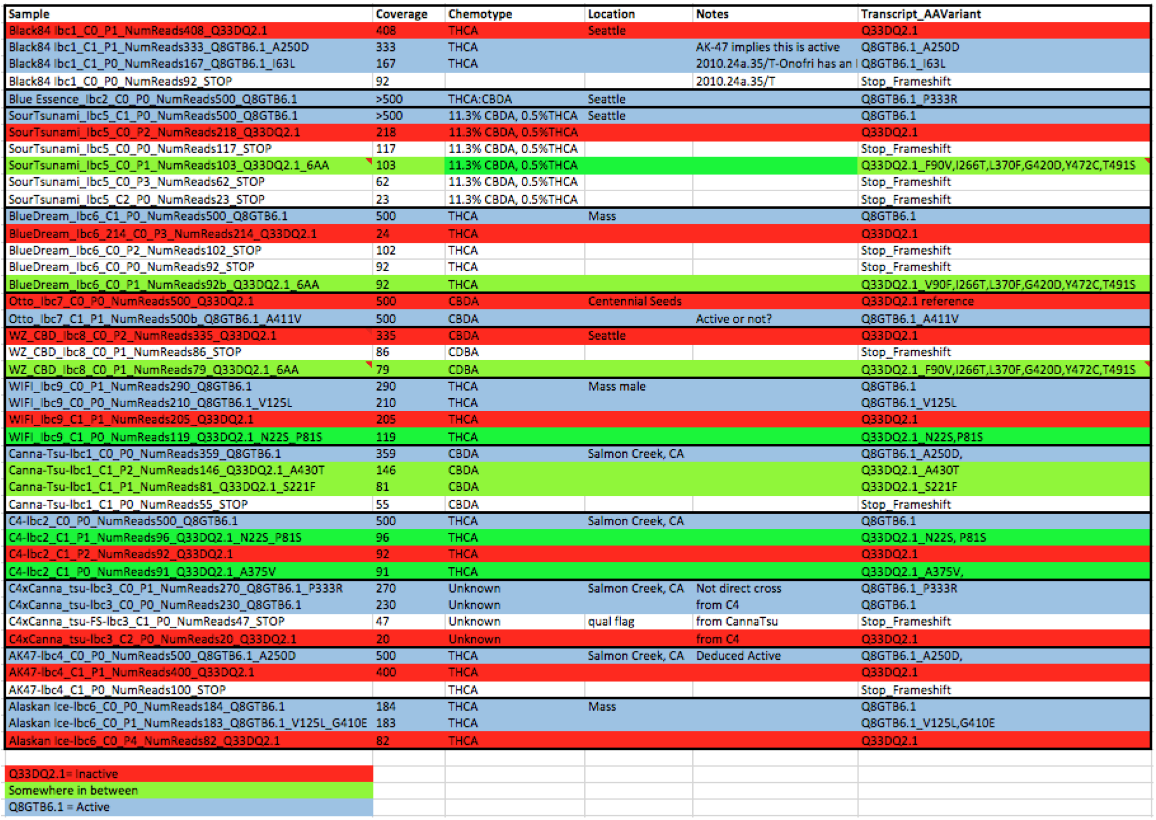
Cultivar	Source	Chemotype	B190-B200
Black84_THCA	Uncle Ikes, Seattle, WA	THCA	Bt:Bd
BlueBerry Essence_CBDA_THCA	Cannabis City, Seattle,WA	8.11%THCA:10.8%CBDA	Bd:Bd
Sour Tsunami_CBDA	Werc Shop, Seattle,WA	11.3% CBDA, 0.5%THCA	Bd:Bd
BlueDream_THCA	MCR labs, MA	THCA	Bd:Bd
Otto_CBDA	Centennial Seeds, CO	11.3% CBDA, 0.5%THCA	Bd:Bd
WZ-CBD_CBDA	Rocky Mountain Remedies, CO	20% CBDA, 05%THCA	Bd:Bd
WIFI_THCA	MCR labs, MA	THCA	Bt:Bt
ChemDog91_THCA	Greenhouse Seeds, NL	THCA	Bt:Bt
CannaTsu_CBDA	Salmon Creek, CA	11.4% CBDA:0.4%THCA	Bd:Bd
C4 X CannaTsu	Salmon Creek, CA	Unknown	Bd:Bd
C4_THCA	Salmon Creek, CA	THCA	Bt:Bt
AK-47_THCA	Salmon Creek, CA	THCA	Bd:Bd
Alaskan Ice_THCA	MCR labs, MA	THCA	Bt:Bt
Chemdog91_THCA repeat	Greenhouse Seeds, NL	THCA	Bt:Bt
Chemdog91_SinglePlex	Greenhouse Seeds, NL	THCA	Bt:Bt

Table1. THCAS was amplified with two primer sets (Onofri and MGC-2130) for each Cultivar and single molecule sequenced with Pacific BioSciences SMRT cell circular consensus sequencing. B190, B200 alleles were measured according to Mandolino *et al.* Red highlighted samples represent B190-B200 Genotype-Chemotype discordant samples.



Samples Run on 3 different chips						
Singleplex ChemDog91	**SubRead Coverage (#ZMWs)**	**Chemotype**	**PredictedAccuracy**	**ConsensusConverged**	**Activity**	**Reference_AAVariant**
Chemdog91_1plex_Onofri_lbc0_C0_P2_NumReads320_Q33DQ2.1_S221F	320	THCA	0.9999369	TRUE	Unknown	Q33DQ2.1_F221S
Chemdog91_1plex_Onofri_lbc0_C1_P1_NumReads312_Q8GTB6.1_A250D	312	THCA	0.9999369	TRUE	Active by deduction	Q8GTB6.1_A250D
Chemdog91_1plex_Onofri_lbc0_C1_P0_NumReads188_Q8GTB6.1	188	THCA	0.9999369	TRUE	Active	Q8GTB6.1
Chemdog91_1plex_Onofri_lbc0_C0_P0_NumReads93_Q33DQ2.1_6AA	93	THCA	0.9996669	TRUE	Unknown	Q33DQ2.1_N90F,I266T,L370F,D420G,Y471C,T492S
Chemdog91_1plex_Onofri_lbc0_C0_P1_NumReads87_Q33DQ2.1_STOP_438			0.9936923	TRUE	Stop/Frame-Shift	Q33DQ2.1_M134I_STOP438
FR09904480	**SubRead Coverage (#ZMWs)**	**Chemotype**	**PredictedAccuracy**	**ConsensusConverged**	**Activity**	**Reference_AAVariant**
ChemDog91_lbc10_NumReads318_Q33DQ2.1	318	THCA	0.9999369	TRUE	Inactive	Q33DQ2.1
ChemDog91_lbc10_NumReads307_Q8GTB6.1_A250D	307	THCA	0.9999369	TRUE	Active by deduction	Q8GTB6.1- A250D
ChemDog91_lbc10_NumReads193_Q8GTB6.1	193	THCA	0.999931	TRUE	Active	Q8GTB6.1
ChemDog91_lbc10_NumReads95_Q33DQ2.1_M134I_STOP438	95	THCA	0.992139	TRUE	Stop/Frame-Shift	Q33DQ2.1_M134I_STOP438
ChemDog91_lbc10_NumReads87_Q33DQ2.1_6AA	87	THCA	0.9921376	TRUE	Unknown	Q33DQ2.1_N90F,I266T,L370F,D420G,Y471C,T492S
FR09904616	**SubRead Coverage (#ZMWs)**	**Chemotype**	**PredictedAccuracy**	**ConsensusConverged**	**Activity**	**Reference_AAVariant**
ChemDog91-lbc7_C0_P1_NumReads299_Q8GTB6.1_A250D	299	THCA	0.9999369	TRUE	Active by deduction	Q8GTB6.1_A250D
ChemDog91-lbc7_C0_P0_NumReads201_Q8GTB6.1	201	THCA	0.9999369	TRUE	Active	Q8GTB6.1
ChemDog91-lbc7_C1_P2_NumReads103_FS	103	THCA	0.9963401	TRUE	Stop/Frame-Shift	Frame Shift
ChemDog91-lbc7_C1_P0_NumReads103_Q33DQ2.1_S221F	103	THCA	0.9981365	FALSE		Q33DQ2.1_F221S
ChemDog91-lbc7_C1_P1_NumReads75_Q33DQ2.1_6AA	75	THCA	0.9911302	TRUE	Unknown	N90F,I266T,L370F,D420G,Y471C,T492S
ChemDog91-lbc7_C1_P3_NumReads67_Q33DQ2.1_M134I_STOP438	67	THCA	0.9999369	TRUE	Stop/Frame-Shift	Q33DQ2.1_M134I_STOP438
🟦 Active (Q8GTB6.1)						
🟥 Inactive (Q33DQ2.1)						
🟩 Unknown						
⬜ Stop FrameShift						

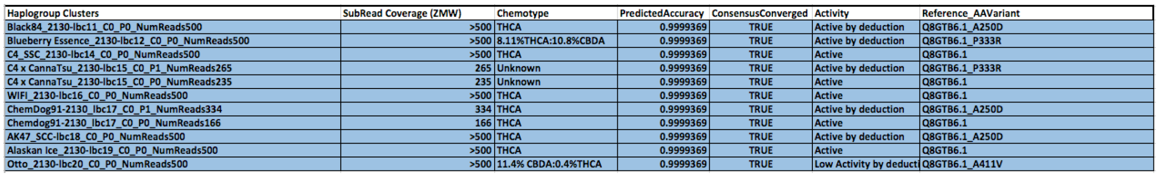
Table2. Triplicate Sample analysis. ChemDog91 was amplified 3 distinct times and sequenced on 3 different SMRT cells to assess reproducibility and sampling. Active forms consistently amplify and sequence across all amplification products. A few private inactive or unknown activity haplogroups amplify inconsistently (Q33DQ2.1- Red and Q33DQ2.1_F221S- Green).



Sample	Coverage	Chemotype	Location	Notes	Transcript_AAVariant
Black84 Ibc1_C0_P1_NumReads408_Q33DQ2.1	408	THCA	Seattle		Q33DQ2.1
Black84 Ibc1_C1_P1_NumReads333_Q8GTB6.1_A250D	333	THCA		AK-47 implies this is active	Q8GTB6.1_A250D
Black84 Ibc1_C1_P0_NumReads167_Q8GTB6.1_I63L	167	THCA		2010.24a.35/T-Onofri has an I	Q8GTB6.1_I63L
Black84 Ibc1_C0_P0_NumReads92_STOP	92			2010.24a.35/T	Stop_Frameshift
Blue Essence_Ibc2_C0_P0_NumReads500_Q8GTB6.1	>500	THCA:CBDA	Seattle		Q8GTB6.1_P333R
SourTsunami_Ibc5_C1_P0_NumReads500_Q8GTB6.1	>500	11.3% CBDA, 0.5%THCA	Seattle		Q8GTB6.1
SourTsunami_Ibc5_C0_P2_NumReads218_Q33DQ2.1	218	11.3% CBDA, 0.5%THCA			Q33DQ2.1
SourTsunami_Ibc5_C0_P0_NumReads117_STOP	117	11.3% CBDA, 0.5%THCA			Stop_Frameshift
SourTsunami_Ibc5_C0_P1_NumReads103_Q33DQ2.1_6AA	103	11.3% CBDA, 0.5%THCA			Q33DQ2.1_F90V,I266T,L370F,G420D,Y472C,T491S
SourTsunami_Ibc5_C0_P3_NumReads62_STOP	62	11.3% CBDA, 0.5%THCA			Stop_Frameshift
SourTsunami_Ibc5_C2_P0_NumReads23_STOP	23	11.3% CBDA, 0.5%THCA			Stop_Frameshift
BlueDream_Ibc6_C1_P0_NumReads500_Q8GTB6.1	500	THCA	Mass		Q8GTB6.1
BlueDream_Ibc6_214_C0_P3_NumReads214_Q33DQ2.1	24	THCA			Q33DQ2.1
BlueDream_Ibc6_C0_P2_NumReads102_STOP	102	THCA			Stop_Frameshift
BlueDream_Ibc6_C0_P0_NumReads92_STOP	92	THCA			Stop_Frameshift
BlueDream_Ibc6_C0_P1_NumReads92b_Q33DQ2.1_6AA	92	THCA			Q33DQ2.1_V90F,I266T,L370F,G420D,Y472C,T491S
Otto_Ibc7_C0_P0_NumReads500_Q33DQ2.1	500	CBDA	Centennial Seeds		Q33DQ2.1 reference
Otto_Ibc7_C1_P1_NumReads500b_Q8GTB6.1_A411V	500	CBDA		Active or not?	Q8GTB6.1_A411V
WZ_CBD_Ibc8_C0_P2_NumReads335_Q33DQ2.1	335	CBDA	Seattle		Q33DQ2.1
WZ_CBD_Ibc8_C0_P1_NumReads86_STOP	86	CDBA			Stop_Frameshift
WZ_CBD_Ibc8_C0_P1_NumReads79_Q33DQ2.1_6AA	79	CDBA			Q33DQ2.1_F90V,I266T,L370F,G420D,Y472C,T491S
WIFI_Ibc9_C0_P1_NumReads290_Q8GTB6.1	290	THCA	Mass male		Q8GTB6.1
WIFI_Ibc9_C0_P0_NumReads210_Q8GTB6.1_V125L	210	THCA			Q8GTB6.1_V125L
WIFI_Ibc9_C1_P1_NumReads205_Q33DQ2.1	205	THCA			Q33DQ2.1
WIFI_Ibc9_C1_P0_NumReads119_Q33DQ2.1_N22S_P81S	119	THCA			Q33DQ2.1_N22S,P81S
Canna-Tsu-Ibc1_C0_P0_NumReads359_Q8GTB6.1	359	CBDA	Salmon Creek, CA		Q8GTB6.1_A250D,
Canna-Tsu-Ibc1_C1_P2_NumReads146_Q33DQ2.1_A430T	146	CBDA			Q33DQ2.1_A430T
Canna-Tsu-Ibc1_C1_P1_NumReads81_Q33DQ2.1_S221F	81	CBDA			Q33DQ2.1_S221F
Canna-Tsu-Ibc1_C1_P0_NumReads55_STOP	55	CBDA			Stop_Frameshift
C4-Ibc2_C0_P0_NumReads500_Q8GTB6.1	500	THCA	Salmon Creek, CA		Q8GTB6.1
C4-Ibc2_C1_P1_NumReads96_Q33DQ2.1_N22S_P81S	96	THCA			Q33DQ2.1_N22S, P81S
C4-Ibc2_C1_P2_NumReads92_Q33DQ2.1	92	THCA			Q33DQ2.1
C4-Ibc2_C1_P0_NumReads91_Q33DQ2.1_A375V	91	THCA			Q33DQ2.1_A375V,
C4xCanna_tsu-Ibc3_C0_P1_NumReads270_Q8GTB6.1_P333R	270	Unknown	Salmon Creek, CA	Not direct cross	Q8GTB6.1_P333R
C4xCanna_tsu-Ibc3_C0_P0_NumReads230_Q8GTB6.1	230	Unknown		from C4	Q8GTB6.1
C4xCanna_tsu-FS-Ibc3_C1_P0_NumReads47_STOP	47	Unknown	qual flag	from CannaTsu	Stop_Frameshift
C4xCanna_tsu-Ibc3_C2_P0_NumReads20_Q33DQ2.1	20	Unknown		from C4	Q33DQ2.1
AK47-Ibc4_C0_P0_NumReads500_Q8GTB6.1_A250D	500	THCA	Salmon Creek, CA	Deduced Active	Q8GTB6.1_A250D,
AK47-Ibc4_C1_P1_NumReads400_Q33DQ2.1	400	THCA			Q33DQ2.1
AK47-Ibc4_C1_P0_NumReads100_STOP		THCA			Stop_Frameshift
Alaskan Ice-Ibc6_C0_P0_NumReads184_Q8GTB6.1	184	THCA	Mass		Q8GTB6.1
Alaskan Ice-Ibc6_C0_P1_NumReads183_Q8GTB6.1_V125L_G410E	183	THCA			Q8GTB6.1_V125L,G410E
Alaskan Ice-Ibc6_C0_P4_NumReads82_Q33DQ2.1	82	THCA			Q33DQ2.1

Q33DQ2.1= Inactive					
Somewhere in between					
Q8GTB6.1 = Active					

Table 3. List of all haplogroups discovered from each cultivar other than Chemdog91 (Table 2). Red highlighting are Inactive haplogroups. Green highlightings are unknown haplogroups and blue highlightings are active haplogroups.



Haplogroup Clusters	SubRead Coverage (ZMW)	Chemotype	PredictedAccuracy	ConsensusConverged	Activity	Reference_AAVariant
Black84_2130-lbc11_C0_P0_NumReads500	>500	THCA	0.9999369	TRUE	Active by deduction	Q8GTB6.1_A250D
Blueberry Essence_2130-lbc12_C0_P0_NumReads500	>500	8.11%THCA:10.8%CBDA	0.9999369	TRUE	Active by deduction	Q8GTB6.1_P333R
C4_SSC_2130-lbc14_C0_P0_NumReads500	>500	THCA	0.9999369	TRUE	Active	Q8GTB6.1
C4 x CannaTsu_2130-lbc15_C0_P1_NumReads265	265	Unknown	0.9999369	TRUE	Active by deduction	Q8GTB6.1_P333R
C4 x CannaTsu_2130-lbc15_C0_P0_NumReads235	235	Unknown	0.9999369	TRUE	Active	Q8GTB6.1
WIFI_2130-lbc16_C0_P0_NumReads500	>500	THCA	0.9999369	TRUE	Active	Q8GTB6.1
ChemDog91-2130_lbc17_C0_P1_NumReads334	334	THCA	0.9999369	TRUE	Active by deduction	Q8GTB6.1_A250D
Chemdog91-2130_lbc17_C0_P0_NumReads166	166	THCA	0.9999369	TRUE	Active	Q8GTB6.1
AK47_SCC-lbc18_C0_P0_NumReads500	>500	THCA	0.9999369	TRUE	Active by deduction	Q8GTB6.1_A250D
Alaskan Ice_2130-lbc19_C0_P0_NumReads500	>500	THCA	0.9999369	TRUE	Active	Q8GTB6.1
Otto_2130-lbc20_C0_P0_NumReads500	>500	11.4% CBDA:0.4%THCA	0.9999369	TRUE	Low Activity by deducti	Q8GTB6.1_A411V

Table4- MGC-2130 primers only amplify active alleles. Active transcript Q8GTB6.1 is the most common haplogroup sequenced. A250D is the second most common haplogroup and is the only haplogroup present in a high THCA expressing cultivar AK-47 and is thus labeled as “Active by deduction”. P333R is also present in Blueberry Essence with 8.11% THCA suggesting Active by deduction Classification. A411V may be low activity or in a cultivar with Bd:Bd genotype.

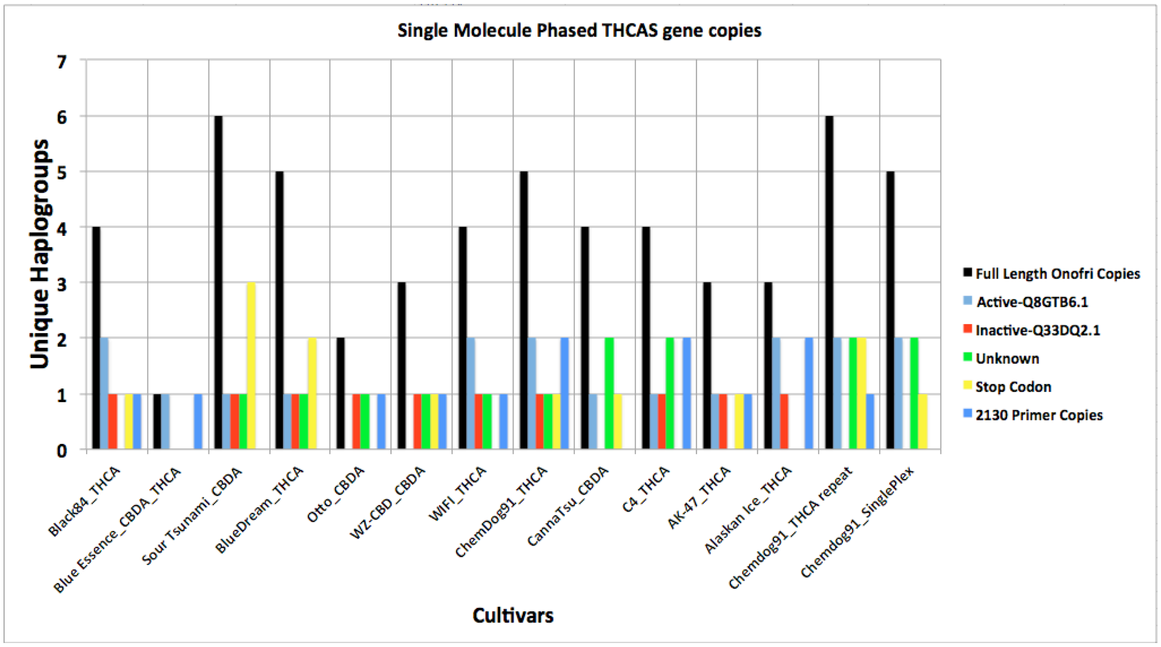


Figure 1. Unique full length THCA Synthase sequences for each cultivar demonstrate diploidy is a rare event in Drug Type cultivars selected for high cannabinoid content. All THCA positive strains have an active form of THCAS with the exception of AK-47 that has an A250D variant of the active form Q8GTB6.1. A250D variants are also present in Black84 as the only possible functional THCAS and as the 2nd THCAS haplogroup in Chemdog91. While it is also present in a CannaTsu high CBDA cultivar, this cultivar does not amplify with the MGC-2130 primers suggesting an MGC-2130 A250D haplogroup is in fact an active THCAS when full length.

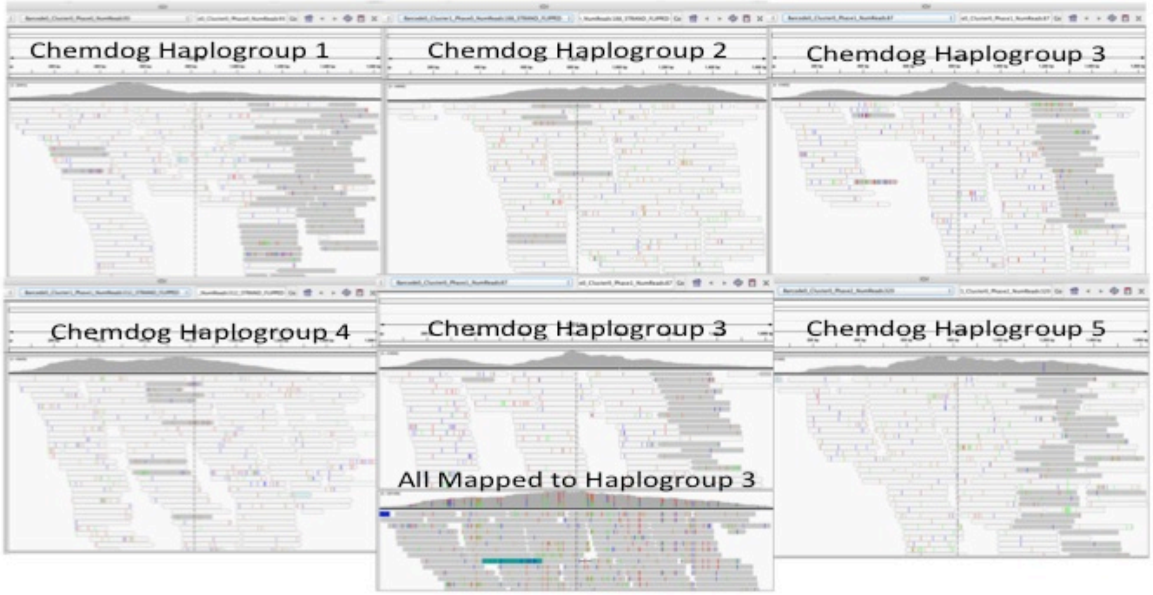


Figure 2. Mapping 2x250 Illumina reads to all of the Phased Pacific Bioscience generated haplogroups compared to mapping them to a single THCAS haplogroup sequence as you might find in CanSat3. 5 haplogroups of Chemdog91 shown in IGV with Illumina reads mapped to a refernce containing all 5 references. The center bottom view "All Mapped to Haplogroup3" represents all of the reads mapped to just Haplogroup 3 demonstrating collapsed polymorphic reads when mapped to the unphased reference sequence of a single haplogroup. Most reads in IGV are labeled with poor mapping scores due to homology between the haplogroups. IGV also filters out alleles under 20% frequency inducing a more detailed dissection of this across more samples in Figure 5.

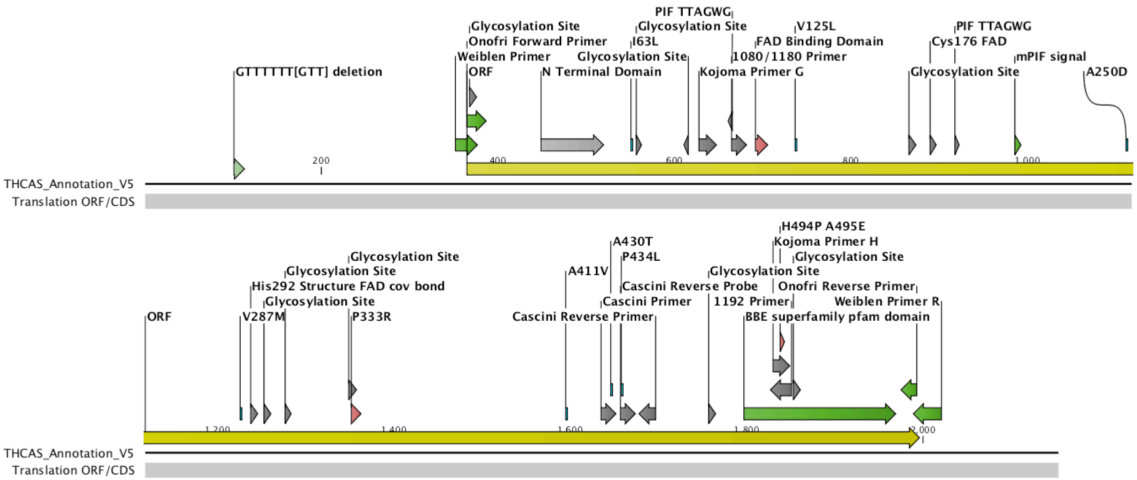


Figure 3. THCA Synthase Annotation.
MGC-2130 Primers bracket the displayed region of interest. Onofri and Weiblen primers are in green. THCAS ORF is in yellow. Various nonsynonmous SNPs (I63L, V125L, A250D, V287M, M289T, P333R, A411V) found in Active THCAS haplogroups are annotated near respective functional groups like Glycosylation sites, FAD binding domains and mPIF signals. Cascini primers and BBE Pfam domains are labeled accordingly.

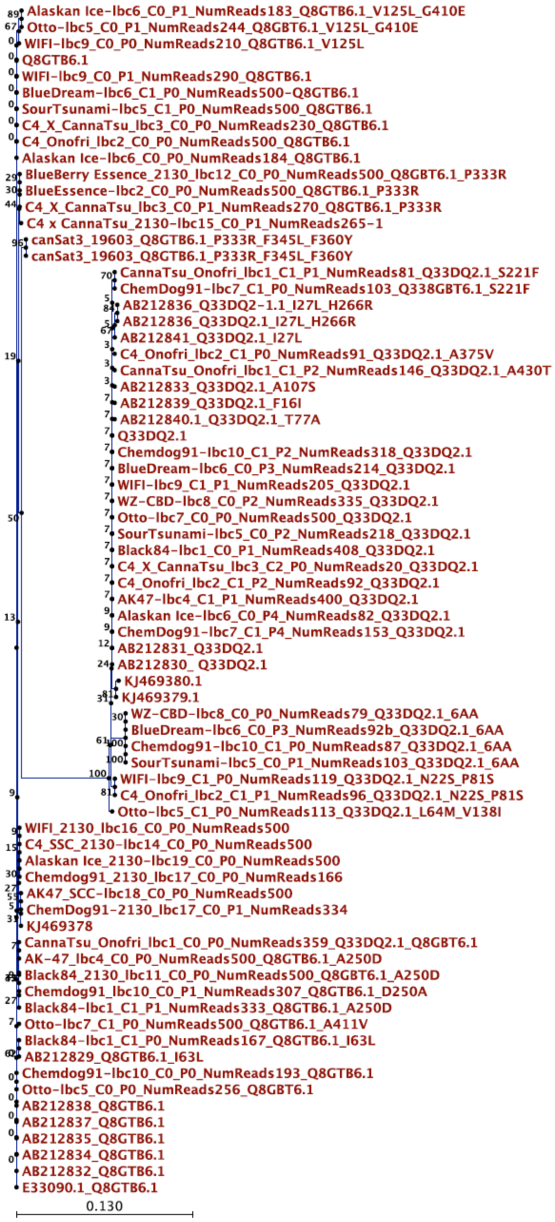

Alaskan Ice-lbc6_C0_P1_NumReads183_Q8GTB6.1_V125L_G410E
Otto-lbc5_C0_P1_NumReads244_Q8GBT6.1_V125L_G410E
WIFI-lbc9_C0_P0_NumReads210_Q8GTB6.1_V125L
Q8GTB6.1
WIFI-lbc9_C0_P1_NumReads290_Q8GTB6.1
BlueDream-lbc6_C1_P0_NumReads500-Q8GTB6.1
SourTsunami-lbc5_C1_P0_NumReads500_Q8GTB6.1
C4_X_CannaTsu_lbc3_C0_P0_NumReads230_Q8GTB6.1
C4_Onofri_lbc2_C0_P0_NumReads500_Q8GTB6.1
Alaskan Ice-lbc6_C0_P0_NumReads184_Q8GTB6.1
BlueBerry Essence_2130_lbc12_C0_P0_NumReads500_Q8GBT6.1_P333R
BlueEssence-lbc2_C0_P0_NumReads500_Q8GTB6.1_P333R
C4_X_CannaTsu_lbc3_C0_P1_NumReads270_Q8GTB6.1_P333R
C4 x CannaTsu_2130-lbc15_C0_P1_NumReads265-1
canSat3_19603_Q8GTB6.1_P333R_F345L_F360Y
canSat3_19603_Q8GTB6.1_P333R_F345L_F360Y
CannaTsu_Onofri_lbc1_C1_P1_NumReads81_Q33DQ2.1_S221F
ChemDog91-lbc7_C1_P0_NumReads103_Q338GBT6.1_S221F
AB212836_Q33DQ2-1.1_I27L_H266R
AB212836_Q33DQ2.1_I27L_H266R
AB212841_Q33DQ2.1_I27L
C4_Onofri_lbc2_C1_P0_NumReads91_Q33DQ2.1_A375V
CannaTsu_Onofri_lbc1_C1_P2_NumReads146_Q33DQ2.1_A430T
AB212833_Q33DQ2.1_A107S
AB212839_Q33DQ2.1_F16I
AB212840.1_Q33DQ2.1_T77A
Q33DQ2.1
Chemdog91-lbc10_C1_P2_NumReads318_Q33DQ2.1
BlueDream-lbc6_C0_P3_NumReads214_Q33DQ2.1
WIFI-lbc9_C1_P1_NumReads205_Q33DQ2.1
WZ-CBD-lbc8_C0_P2_NumReads335_Q33DQ2.1
Otto-lbc7_C0_P0_NumReads500_Q33DQ2.1
SourTsunami-lbc5_C0_P2_NumReads218_Q33DQ2.1
Black84-lbc1_C0_P1_NumReads408_Q33DQ2.1
C4_X_CannaTsu_lbc3_C2_P0_NumReads20_Q33DQ2.1
C4_Onofri_lbc2_C1_P2_NumReads92_Q33DQ2.1
AK47-lbc4_C1_P1_NumReads400_Q33DQ2.1
Alaskan Ice-lbc6_C0_P4_NumReads82_Q33DQ2.1
ChemDog91-lbc7_C1_P4_NumReads153_Q33DQ2.1
AB212831_Q33DQ2.1
AB212830_ Q33DQ2.1
KJ469380.1
KJ469379.1
WZ-CBD-lbc8_C0_P0_NumReads79_Q33DQ2.1_6AA
BlueDream-lbc6_C0_P3_NumReads92b_Q33DQ2.1_6AA
Chemdog91-lbc10_C1_P0_NumReads87_Q33DQ2.1_6AA
SourTsunami-lbc5_C0_P1_NumReads103_Q33DQ2.1_6AA
WIFI-lbc9_C1_P0_NumReads119_Q33DQ2.1_N22S_P81S
C4_Onofri_lbc2_C1_P1_NumReads96_Q33DQ2.1_N22S_P81S
Otto-lbc5_C1_P0_NumReads113_Q33DQ2.1_L64M_V138I
WIFI_2130_lbc16_C0_P0_NumReads500
C4_SSC_2130-lbc14_C0_P0_NumReads500
Alaskan Ice_2130-lbc19_C0_P0_NumReads500
Chemdog91_2130_lbc17_C0_P0_NumReads166
AK47_SCC-lbc18_C0_P0_NumReads500
ChemDog91-2130_lbc17_C0_P1_NumReads334
KJ469378
CannaTsu_Onofri_lbc1_C0_P0_NumReads359_Q33DQ2.1_Q8GBT6.1
AK-47_lbc4_C0_P0_NumReads500_Q8GTB6.1_A250D
Black84_2130_lbc11_C0_P0_NumReads500_Q8GBT6.1_A250D
Chemdog91_lbc10_C0_P1_NumReads307_Q8GTB6.1_D250A
Black84-lbc1_C1_P1_NumReads333_Q8GTB6.1_A250D
Otto-lbc7_C1_P0_NumReads500_Q8GTB6.1_A411V
Black84-lbc1_C1_P0_NumReads167_Q8GTB6.1_I63L
AB212829_Q8GTB6.1_I63L
Chemdog91-lbc10_C0_P0_NumReads193_Q8GTB6.1
Otto-lbc5_C0_P0_NumReads256_Q8GBT6.1
AB212838_Q8GTB6.1
AB212837_Q8GTB6.1
AB212835_Q8GTB6.1
AB212834_Q8GTB6.1
AB212832_Q8GTB6.1
E33090.1_Q8GTB6.1
0.130

Figure 4. Phylogenetic tree of amino acid sequences of THCAS using E33090 as the root. Long pseudogenes were omitted from the tree since they had excessive divergence. The NumReads reflects the number of subreads that support a haplogroup after circular consensus has been achieved. Most haplogroups cluster around Active and Inactive like accessions (Q8GTB6.1 & Q33DQ2.1 accessions). Only Q8GTB6.1 cluster amplify with MGC-2130 primers. Other GenBank Accessions are label as AB#.

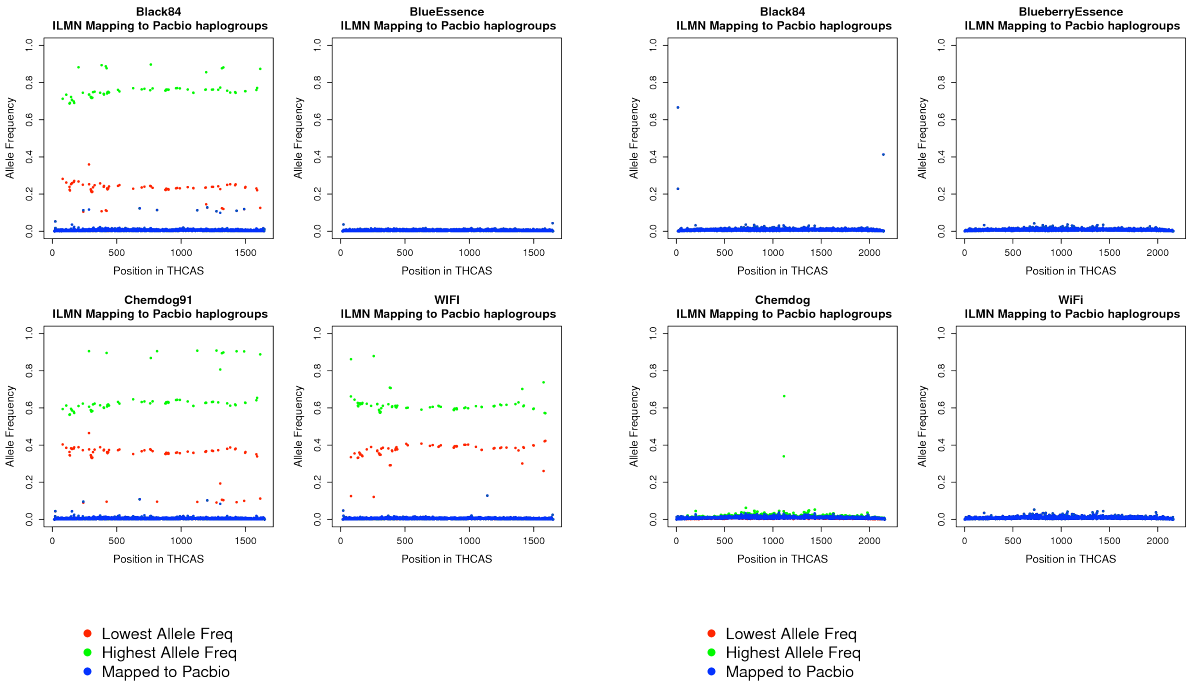


Figure 5. Over 100,000X coverage with Illumina 2x250bp reads derived from Onofri and MGC-2130 primer based amplification of THCAS. The Illumina data is mapped to the Pacbio FASTA files derived from Pacbio LAA analysis. The strain sequence is respectively mapped- for example- *Black84* Illumina Data is mapped to the *Black84* Pacbio FASTA. As an example, if there are 5 haplogroups, the data is mapped 6 times; once to each separate haplogroup and once to all 5 together in one FASTA reference. An allele count is performed on each position of THCAS. A clustal alignment is performed between all the haplogroups so we are able to compare the same position across the multiple reference/bam files. The minimum and maximum non-reference ratio at each position (~1600) is calculated across all the haplogroups (i.e. 5). This is the green and red dots shown. The non-reference ratio for all haplogroups mapped together is also calculated and shown in blue. Left two columns of the chart is Onofri amplification allelic coverage charts. Right 2 columns are MGC-2130 amplicon allelic coverage charts. On the X-axis is THCAS bases 1-1654bp or 1-2130bp respectively. On the Y-axis is Heterozygosity where homozygous genotypes are either 1 or 0 and heterozygous genotypes would display at 0.5. Samples Blueberry essence has been fully sampled with the PacBio depth on both primer sets (all blue homozygous alignments). Samples with red and green dots at equal allele frequency across the amplicon represent PacBio data that may have a remaining haplogroups detected at 100,000 X Illumina sequence but not yet sampled at the PacBio read depth. All 2130-MGC amplicons look fully sampled with phased active alleles.

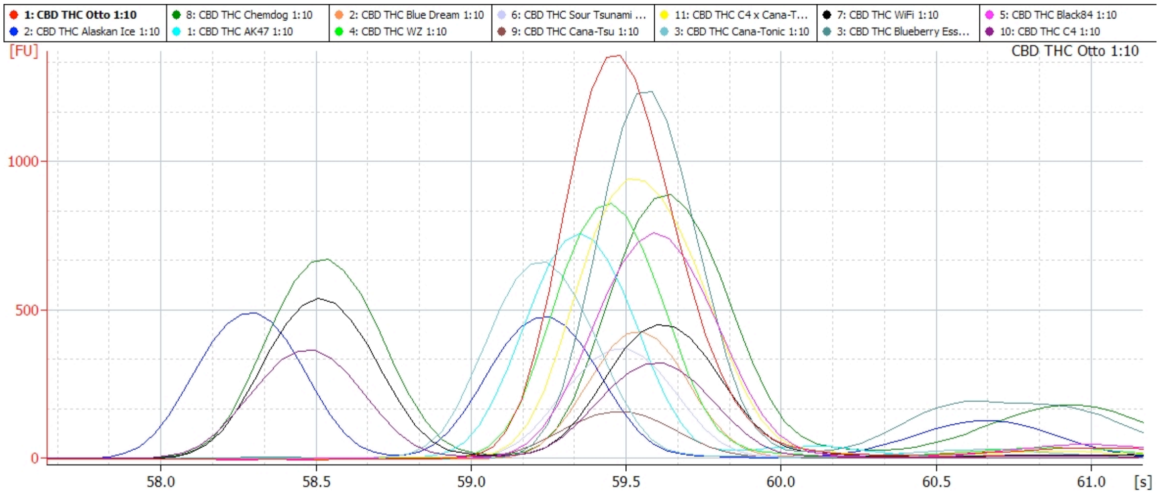
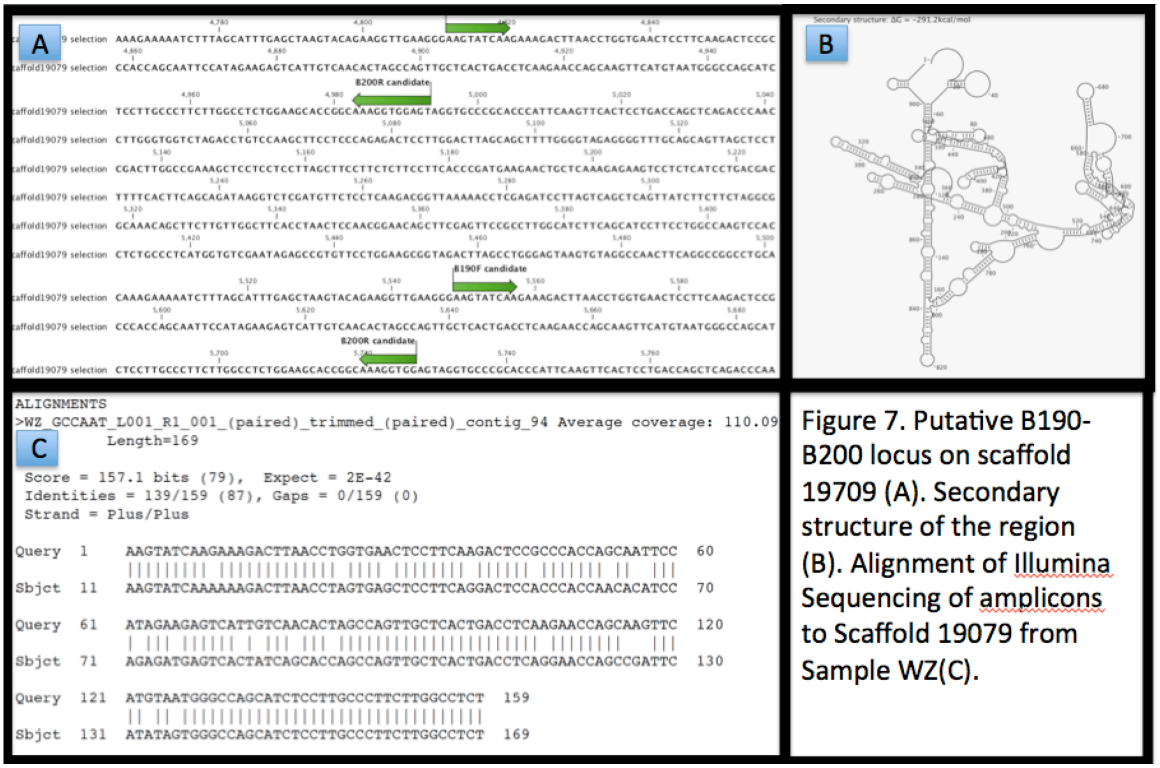


Figure 6. Bt:Bd allele amplified and run on an Agilent High Sensitivity chip. High THCA cultivars (WIFI, Chemdog, C4, Alaskan Ice) replicate a small ~190bp band known as Bt:Bt. High CBDA alleles generate a major 200 bp and a minor 215bp band known as Bd:Bd. Hybrid Bt:Bd alleles generate similar bands as Bd:Bd but with more 215bp product. This later peak is a bit subjective to measure.



```
ca 9 selection        AAAGAAAAATCTTTAGCATTTGAGCTAAGTACAGAAGGTTGAAGGGAAGTATCAAGAAAGACTTAACCTGGTGAACTCCTTCAAGACTCCGC
caffold19079 selection CCACCAGCAATTCCATAGAAGAGTCATTGTCAACACTAGCCAGTTGCTCACTGACCTCAAGAACCAGCAAGTTCATGTAATGGGCCAGCATC
                                                      B200R candidate
caffold19079 selection TCCTTGCCCTTCTTGGCCTCTGGAAGCACCGGCAAAGGTGGAGTAGGTGCCCGCACCCATTCAAGTTCACTCCTGACCAGCTCAGACCCAAC
caffold19079 selection CTTGGGTGGTCTAGACCTGTCCAAGCTTCCTCCCAGAGACTCCTTGGACTTAGCAGCTTTTGGGGTAGAGGGGTTTGCAGCAGTTAGCTCCT
caffold19079 selection CGACTTGGCCGAAAGCTCCTCCTCCTTAGCTTCCTTCTCTTCCTTCACCCGATGAAGAACTGCTCAAAGAGAAGTCCTCTCATCCTGACGAC
caffold19079 selection TTTTCACTTCAGCAGATAAGGTCTCGATGTTCTCCTCAAGACGGTTAAAAACCTCGAGATCCTTAGTCAGCTCAGTTATCTTCTTCTAGGCG
caffold19079 selection GCAAACAGCTTCTTGTTGGCTTCACCTAACTCCAACGGAACAGCTTCGAGTTCCGCCTTGGCATCTTCAGCATCCTTCCTGGCCAAGTCCAC
caffold19079 selection CTCTGCCCTCATGGTGTCGAATAGAGCCGTGTTCCTGGAAGCGGTAGACTTAGCCTGGGAGTAAGTGTAGGCCAACTTCAGGCCGGCCTGCA
                                                           B190F candidate
caffold19079 selection CAAAGAAAAATCTTTAGCATTTGAGCTAAGTACAGAAGGTTGAAGGGAAGTATCAAGAAAGACTTAACCTGGTGAACTCCTTCAAGACTCCG
caffold19079 selection CCCACCAGCAATTCCATAGAAGAGTCATTGTCAACACTAGCCAGTTGCTCACTGACCTCAAGAACCAGCAAGTTCATGTAATGGGCCAGCAT
                                                     B200R candidate
caffold19079 selection CTCCTTGCCCTTCTTGGCCTCTGGAAGCACCGGCAAAGGTGGAGTAGGTGCCCGCACCCATTCAAGTTCACTCCTGACCAGCTCAGACCCAA
```

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ALIGNMENTS
>WZ_GCCAAT_L001_R1_001_(paired)_trimmed_(paired)_contig_94 Average coverage: 110.09
          Length=169

 Score = 157.1 bits (79),  Expect = 2E-42
 Identities = 139/159 (87), Gaps = 0/159 (0)
 Strand = Plus/Plus

Query  1    AAGTATCAAGAAAGACTTAACCTGGTGAACTCCTTCAAGACTCCGCCCACCAGCAATTCC  60
            ||||||||| ||||||||||||| |||| |||||||| |||||| ||||||| ||  |||
Sbjct  11   AAGTATCAAAAAAGACTTAACCTAGTGAGCTCCTTCAGGACTCCACCCACCAACACATCC  70

Query  61   ATAGAAGAGTCATTGTCAACACTAGCCAGTTGCTCACTGACCTCAAGAACCAGCAAGTTC  120
            | ||| |||||| | ||| ||| |||||||||||||||||||||| ||||||||   |||
Sbjct  71   AGAGATGAGTCACTATCAGCACCAGCCAGTTGCTCACTGACCTCAGGAACCAGCCGATTC  130

Query  121  ATGTAATGGGCCAGCATCTCCTTGCCCTTCTTGGCCTCT  159
            || || |||||||||||||||||||||||||||||||||
Sbjct  131  ATATAGTGGGCCAGCATCTCCTTGCCCTTCTTGGCCTCT  169
```

Figure 7. Putative B190-B200 locus on scaffold 19709 (A). Secondary structure of the region (B). Alignment of Illumina Sequencing of amplicons to Scaffold 19079 from Sample WZ(C).

Figure 7. Assembly of Illumina reads from Bt:Bd allele in Cultivar WZ with homology to the B190/B200 partial primer sequences highlighted in green arrows.