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Innovative molecular approaches for *Cannabis sativa* DNA typing

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UNT HEALTH SCIENCE CENTER

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Role of funding

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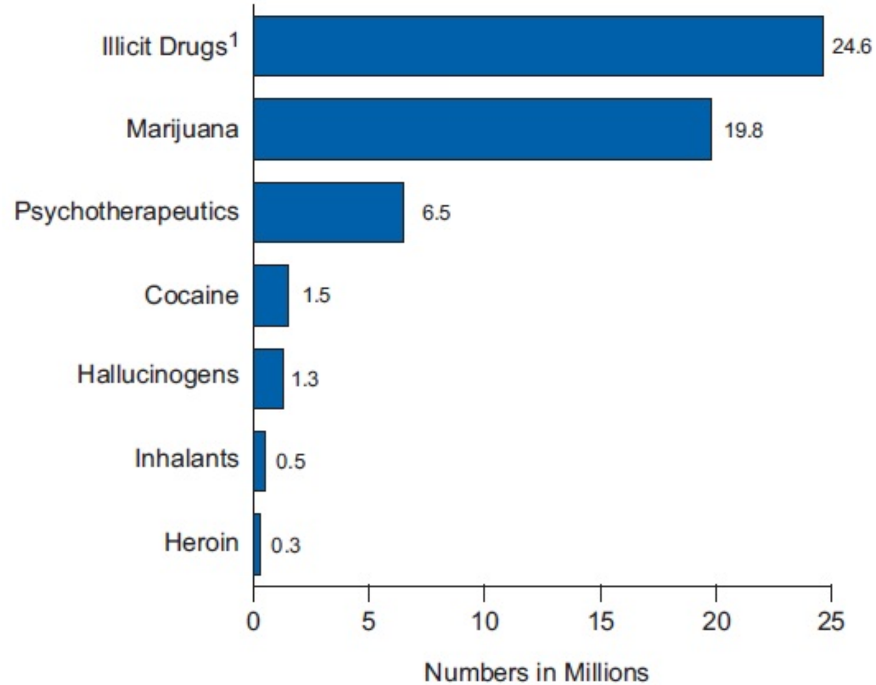
Significance

- Marijuana is the most commonly used illicit drug in United States

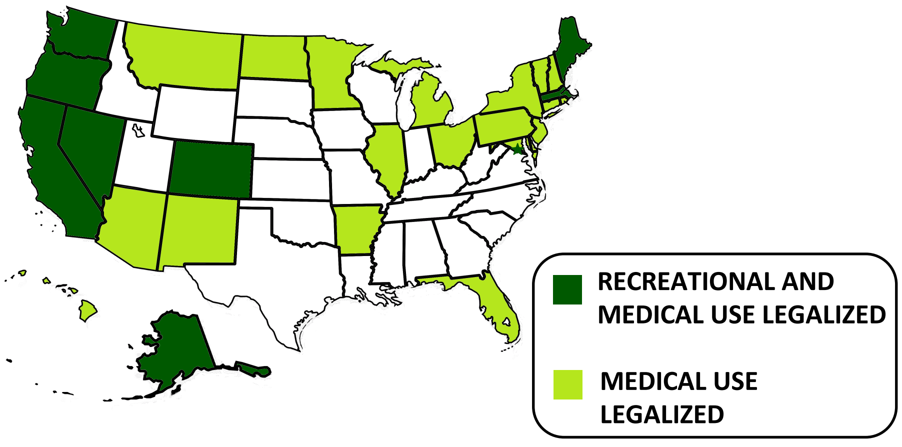


[1] Illicit Drugs include marijuana/hashish, cocaine (including crack), heroin, hallucinogens, inhalants, or prescription-type psychotherapeutics used nonmedically.

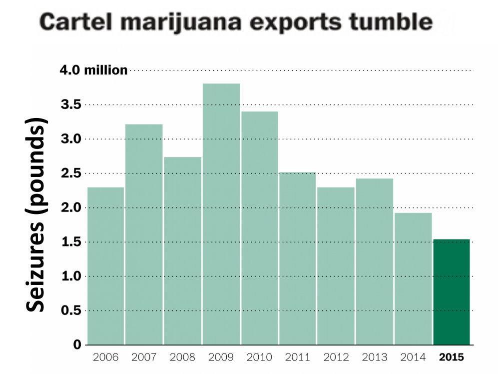
Statistics of Drug Use in United States (U.S. Department of Health and Human Services, 2013)

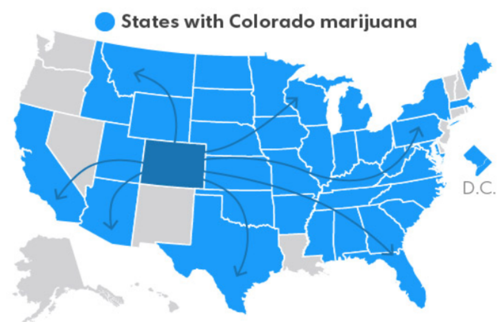
Statement of the problem

Marijuana legalization



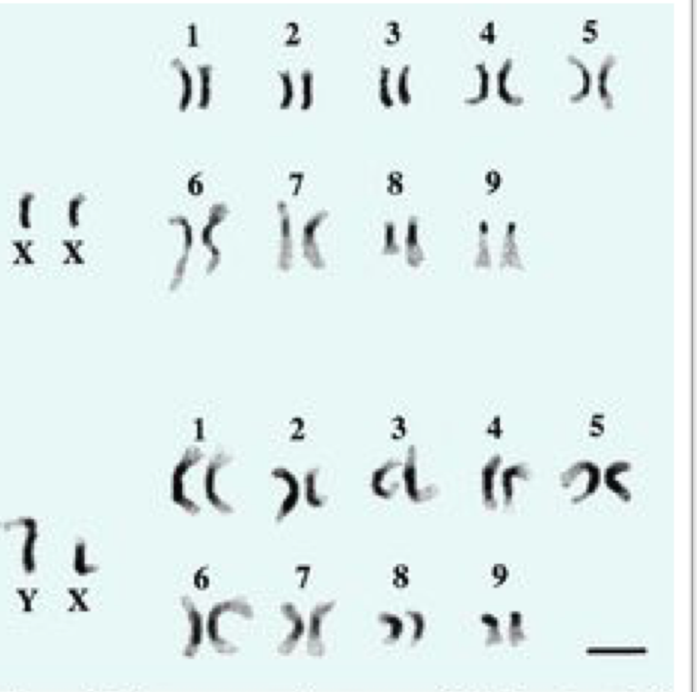
Illegal trafficking





Marijuana background

- Family: Cannabaceae
- Dioecious plant
- Diploid genome: 2n = 20
  - 9 pairs of autosomes
  - Pair of sex chromosomes
- Draft genome complete[1]
  - 818 Mb for female (haploid)
  - 843 Mb for male (haploid)

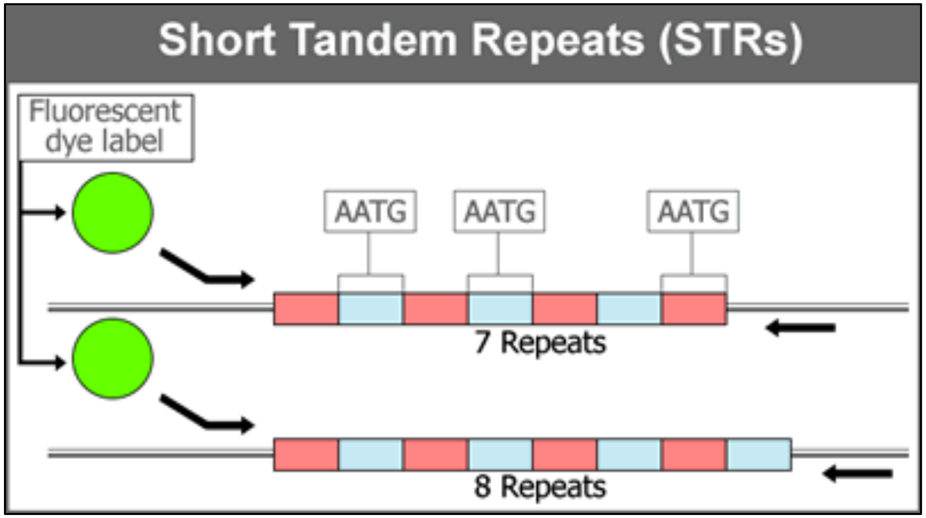


Cannabis Chromosome Karyogram by Sakamoto, et al.
The Japan Mendel Society, 1998

[1]van Bakel, Stout et al. 2011)

DNA based individualization

- **Autosomal short tandem repeat (STR)**
  - Gold standard in human identification
- Chloroplast DNA (cpDNA)
- Mitochondrial DNA (mtDNA)



http://rosalind.info/media/microsatpcr2.gif

Limitations

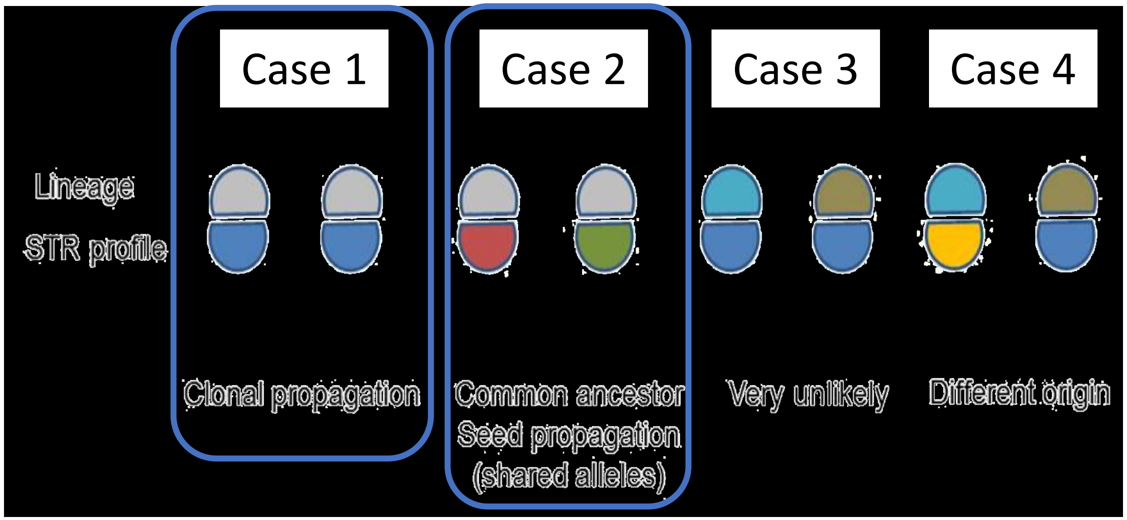
- Did not follow ISFG-SWGDAM recommendations:
  1. Avoiding the use of dinucleotides
  2. Relevant population and forensic parameters in a representative homogeneous population of *C. sativa*
  3. Real-time PCR quantitation
  4. Sequenced allelic ladders for accurate designation of alleles and inter-laboratory profile sharing

SWGDAM Scientific Working Group DNA Analysis Methods

iSFG International Society for Forensic Genetics

Purpose and goals

- Provide forensic DNA community a comprehensive analytical tool to genetically identify *C. sativa* samples:
  1. Presence of clones
  2. Association between group of samples



Materials and methods

- Sampling (4 sources)
- Extraction (DNeasy® Plant Mini Kit)
- Quantitation
- 13 STR Multiplex
- Validation Studies
- Phylogenetic Analysis

STR genotyping

Extraction
DNeasy® Plant Mini Kit

Quantitation (SYBR™ Green)
StepOne™ Real Time PCR Instrument

PCR (Type-It™ Microsatellite Kit)
Eppendorf® MasterCycler® Gradient

Capillary Electrophoresis
ABI 3500 Genetic Analyzer

Data Analysis
Genemapper® v 4.1

DNA quantitation

- Development of real-time qPCR system using SYBR™ Green with marijuana specific primers (ANUCS304) (Howard et al. (2008))
- Validation studies according to SWGDAM guidelines 9.4 and 9.5
  - Reproducibility and precision
  - Sensitivity
  - Species specificity

13 STR multiplex

Primer Selection and Optimization

Allele Sequencing and Ladder Design

Validation Studies

STR Genotyping

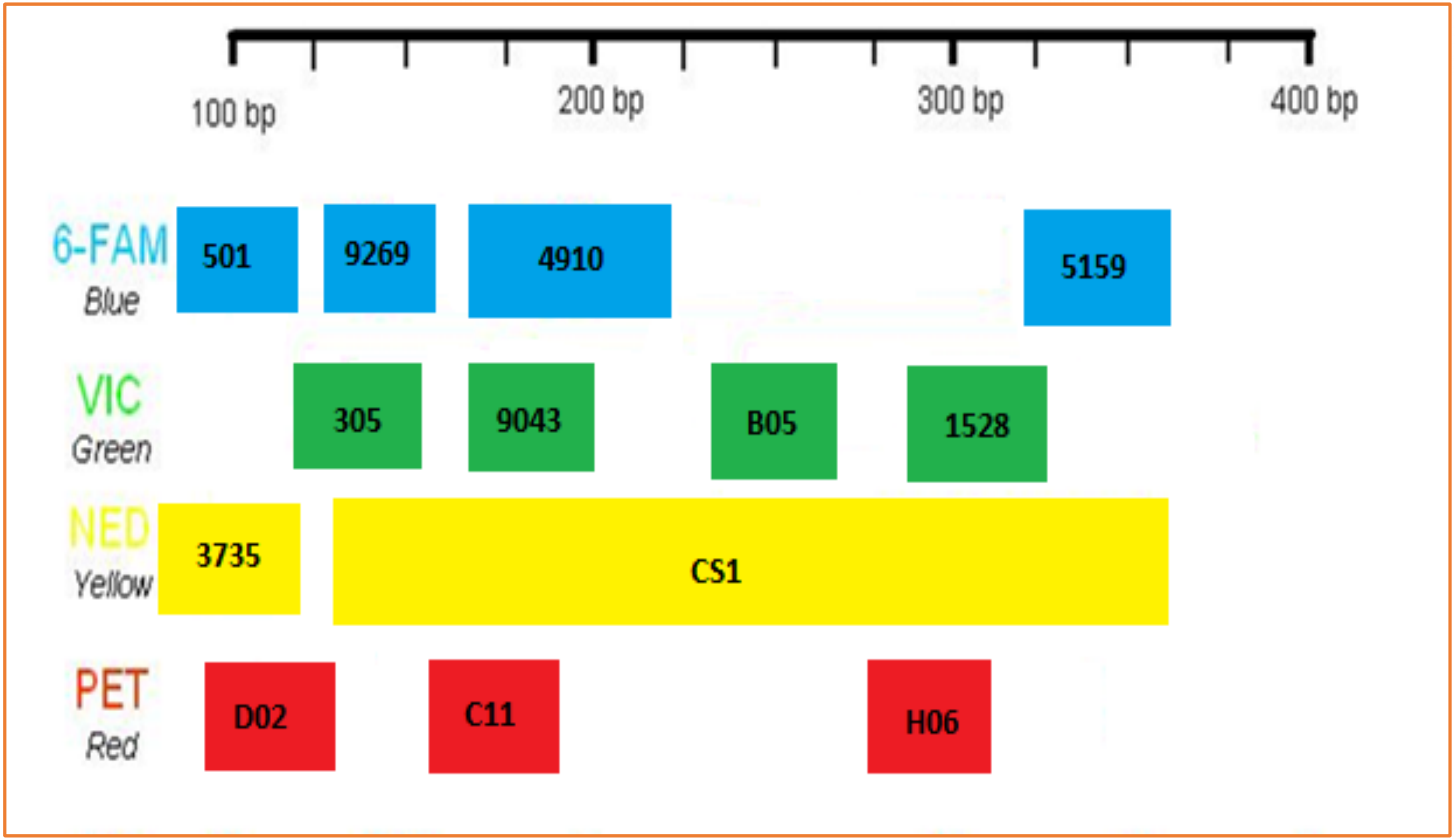
Primer selection

Type	Marker
Dinucleotide	
Trinucleotide	ANUCS305, B05, D02, C11, H06
Tetranucleotide	**9269, 4910, 5159, 9043, 1528, 3735**
Pentanucleotide	ANUCS501
Hexanucleotide	CS1

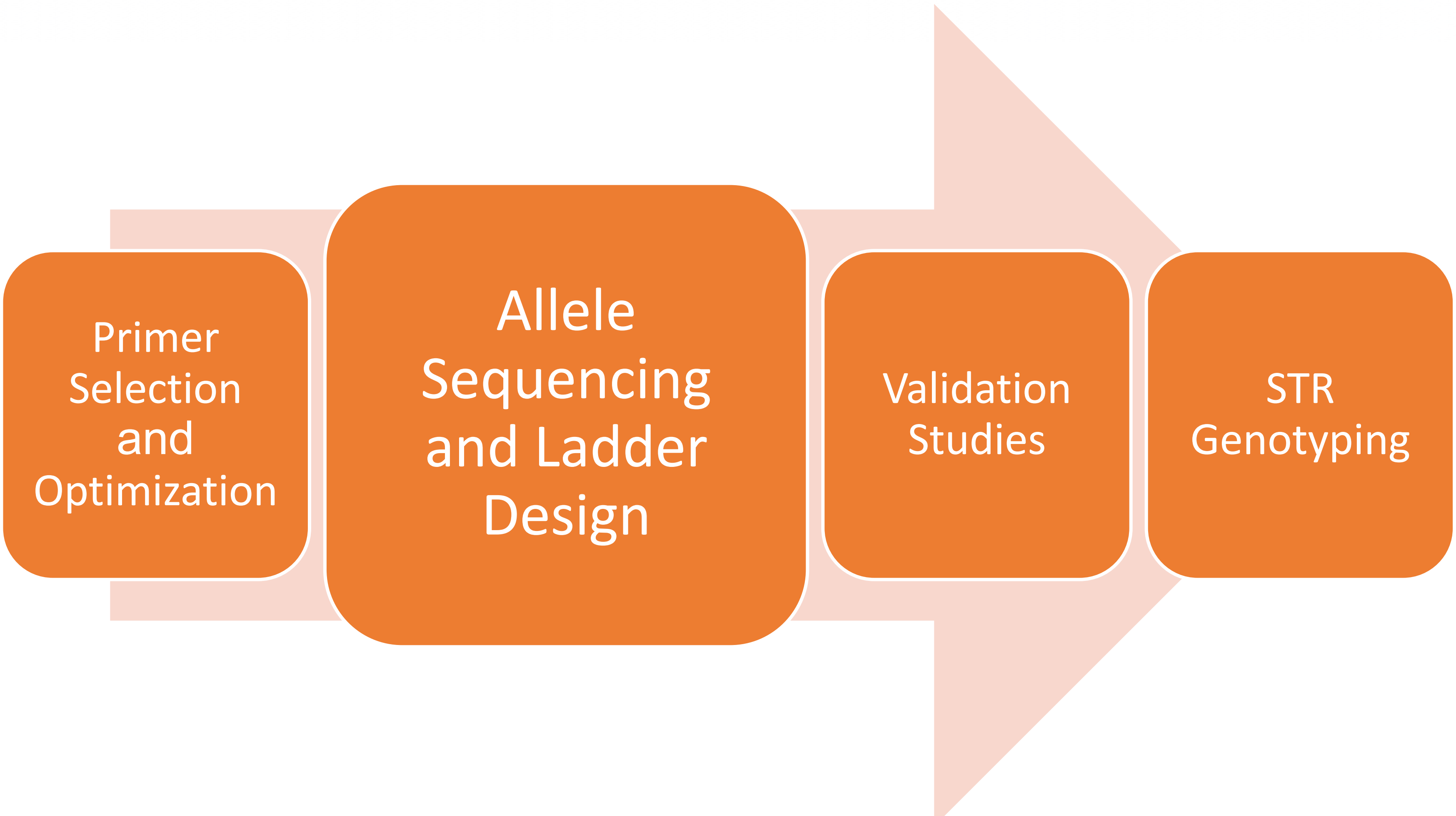
Multiplex optimization

- Multiplex Manager Software v 1.2:
  - Evaluate primer-primer interactions
  - Optimal loci layout
- Annealing temperature determination:
  - Individual loci: 65 ˚C – 55 ˚C
  - Multiplex annealing temperature: 57 ˚C
- Primer titration and cycle number:
  - Type-It™ Microsatellite PCR Kit (Qiagen)

Final 13-plex



13 STR multiplex



Sequencing/allelic ladder

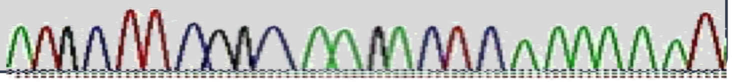
Two to eight alleles per marker

Big Dye Direct® Cycle Sequencing Kit

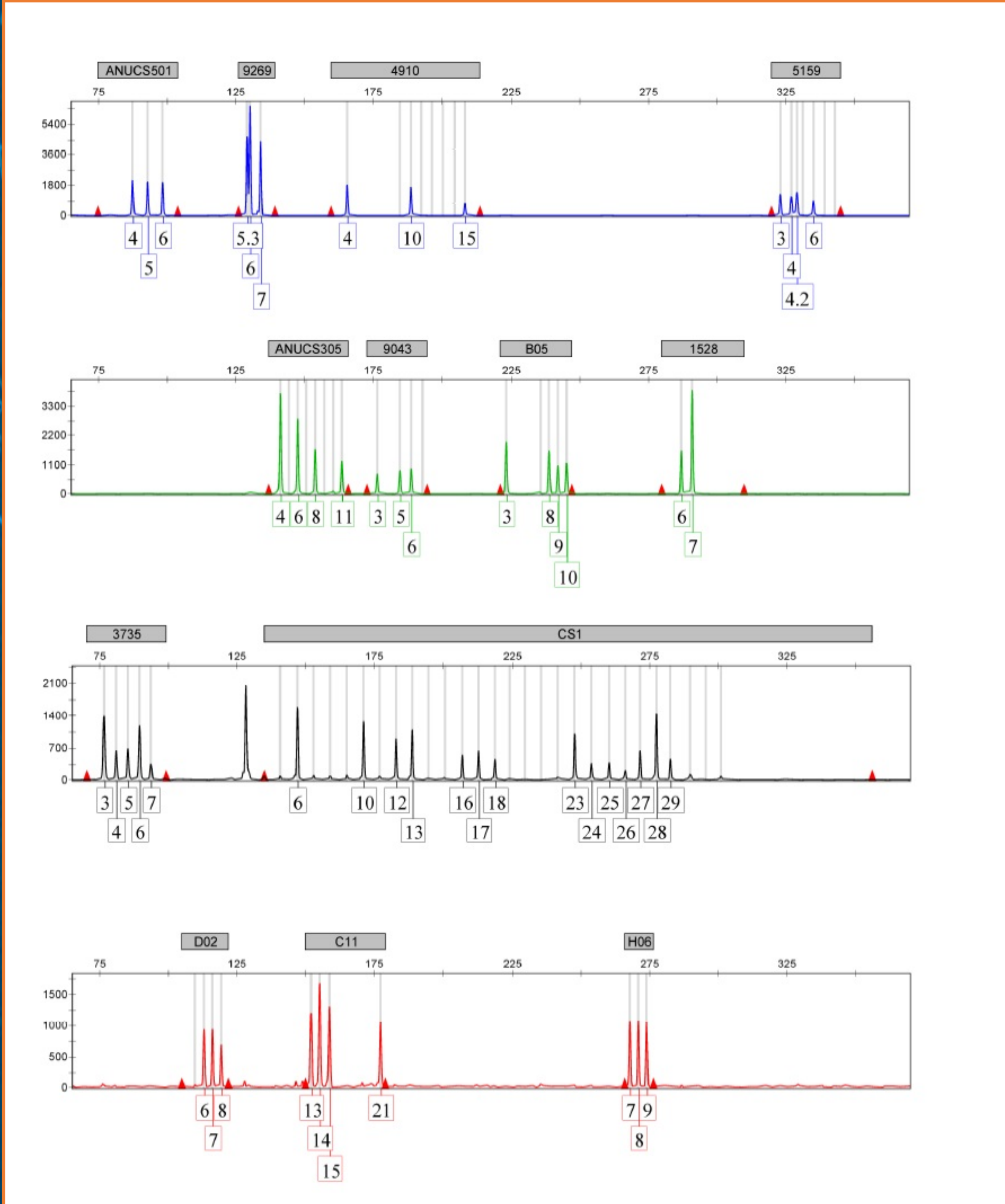
Centri-Sep™ purification columns

Geneious Pro Software

Allelic ladder design



Allelic Ladder



13 STR multiplex

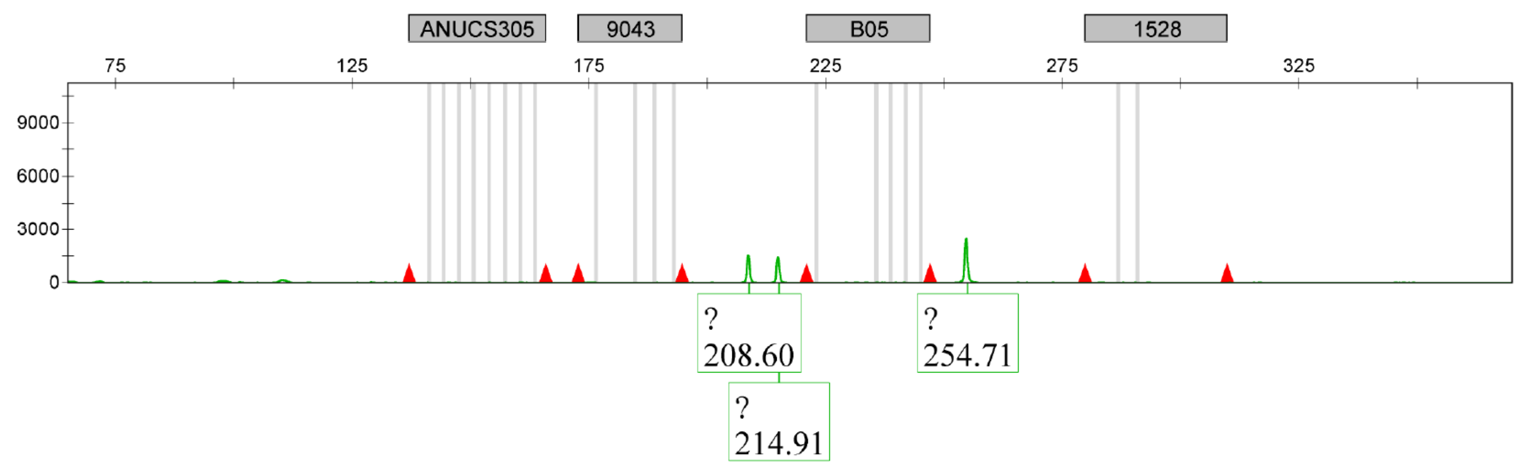
- Primer Selection and Titration
- Allele Sequencing and Ladder Design
- **Validation Studies**
- STR Genotyping

Validation studies

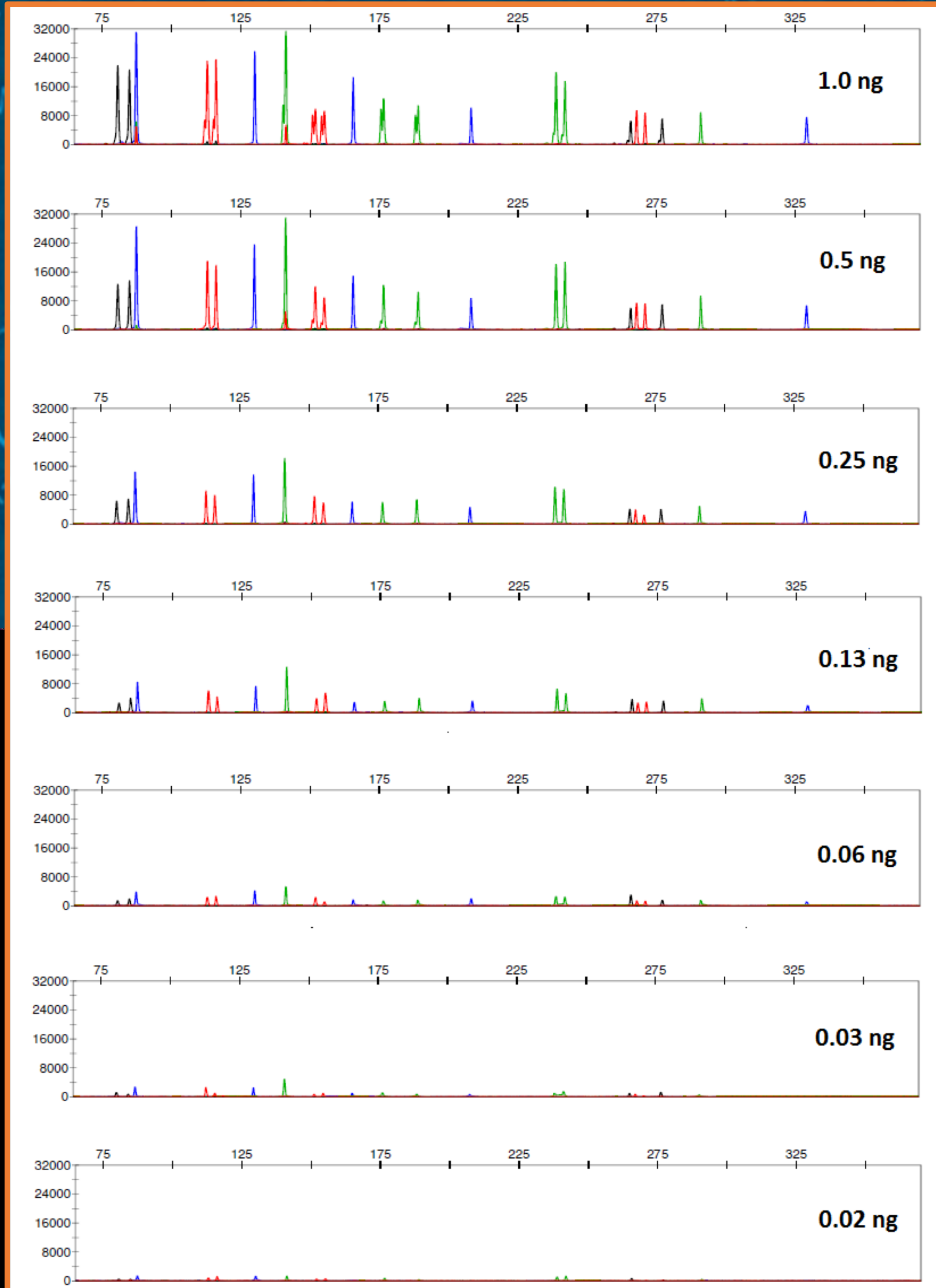
- **Developmental Validation (SWGDAM):**
  - Species specificity
  - Sensitivity/stochastic effects
  - Precision and accuracy
- **Internal Validation (SWGDAM):**
  - Stutter ratio
  - Peak height ratio
  - Inter-loci balance

Species specificity

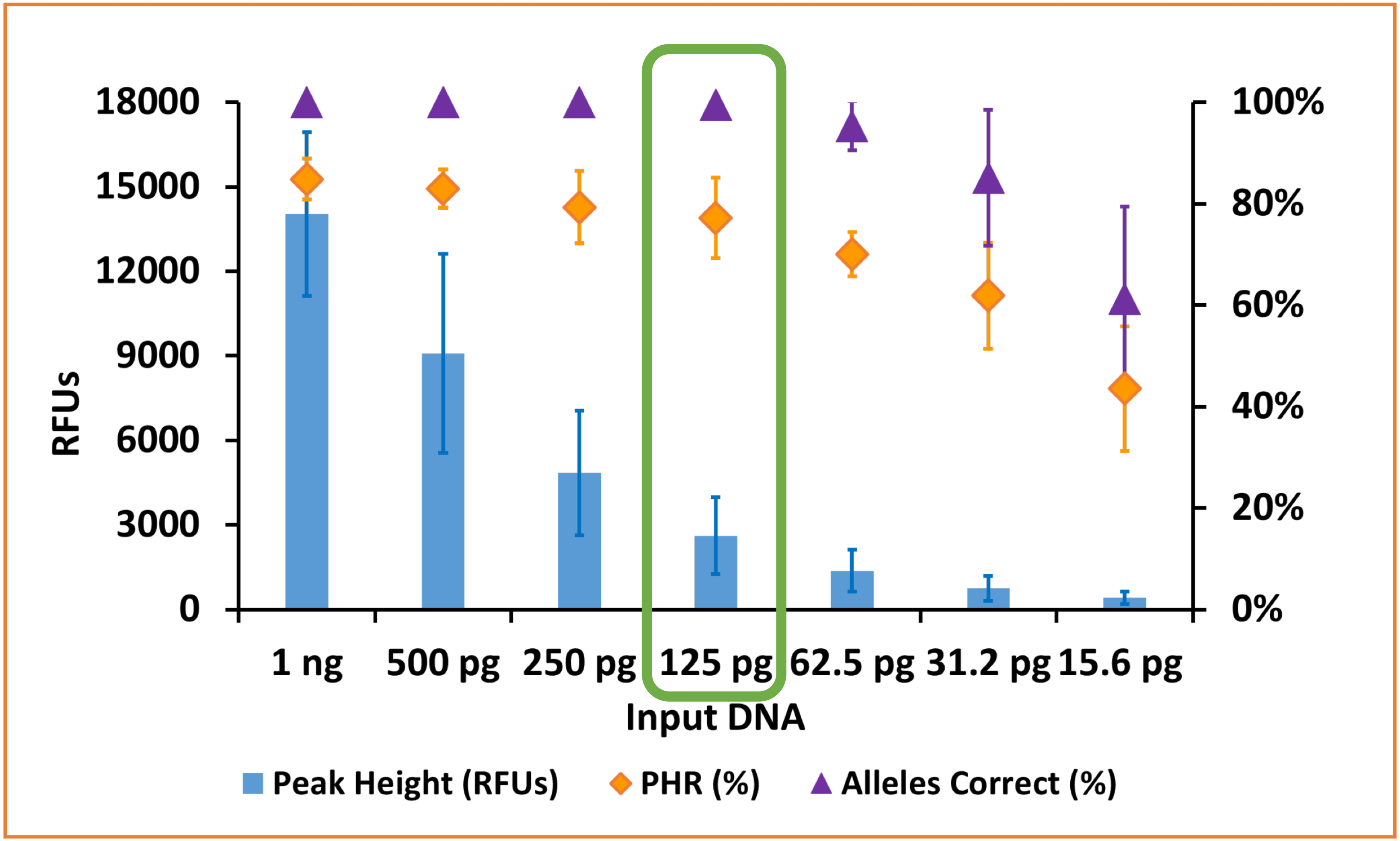
- Cross-reactivity observed in one species:
  - *Humulus lupulus* (Hops)
  - Generated non-specific peaks



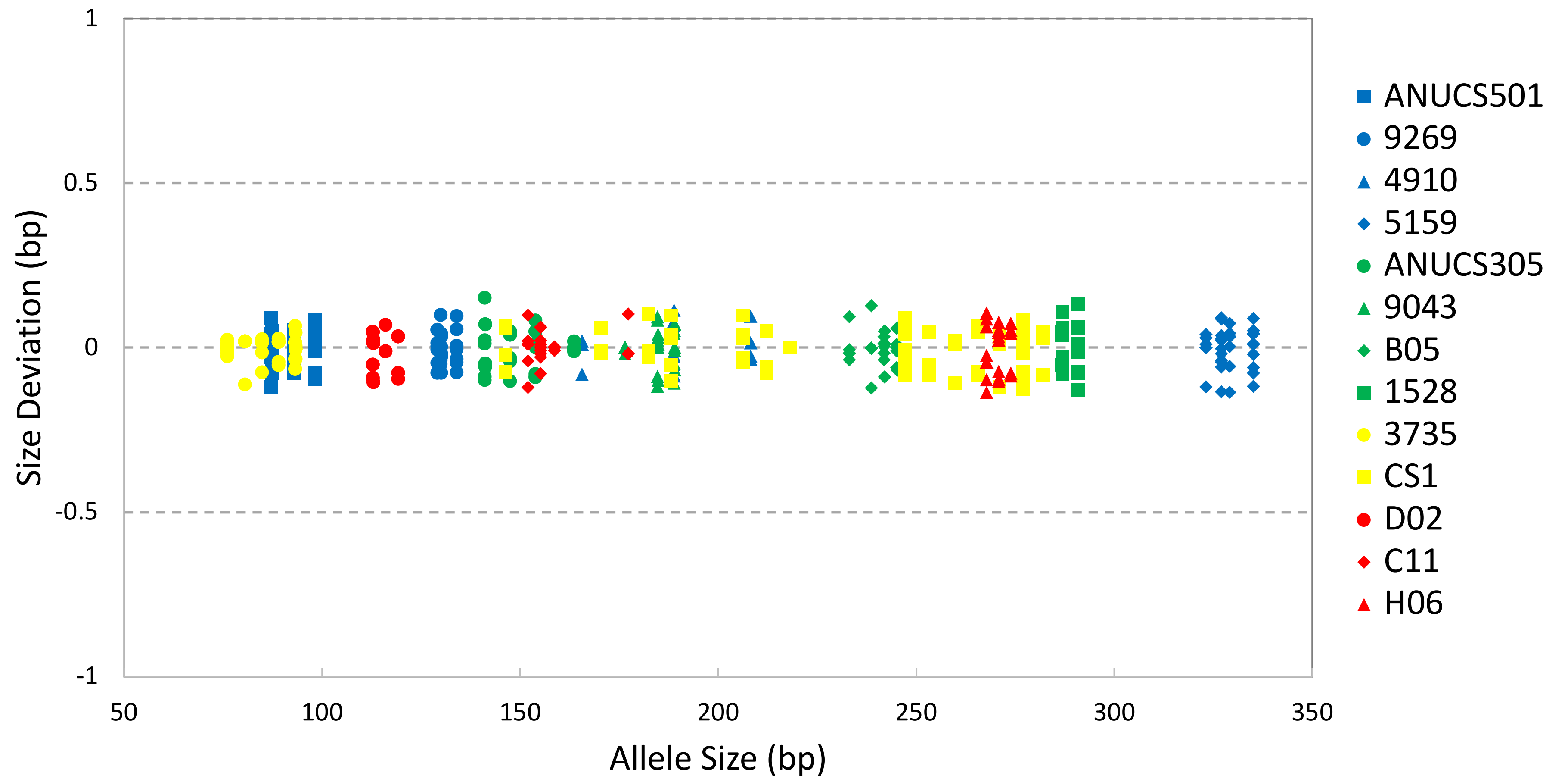
Sensitivity



Sensitivity (cont.)

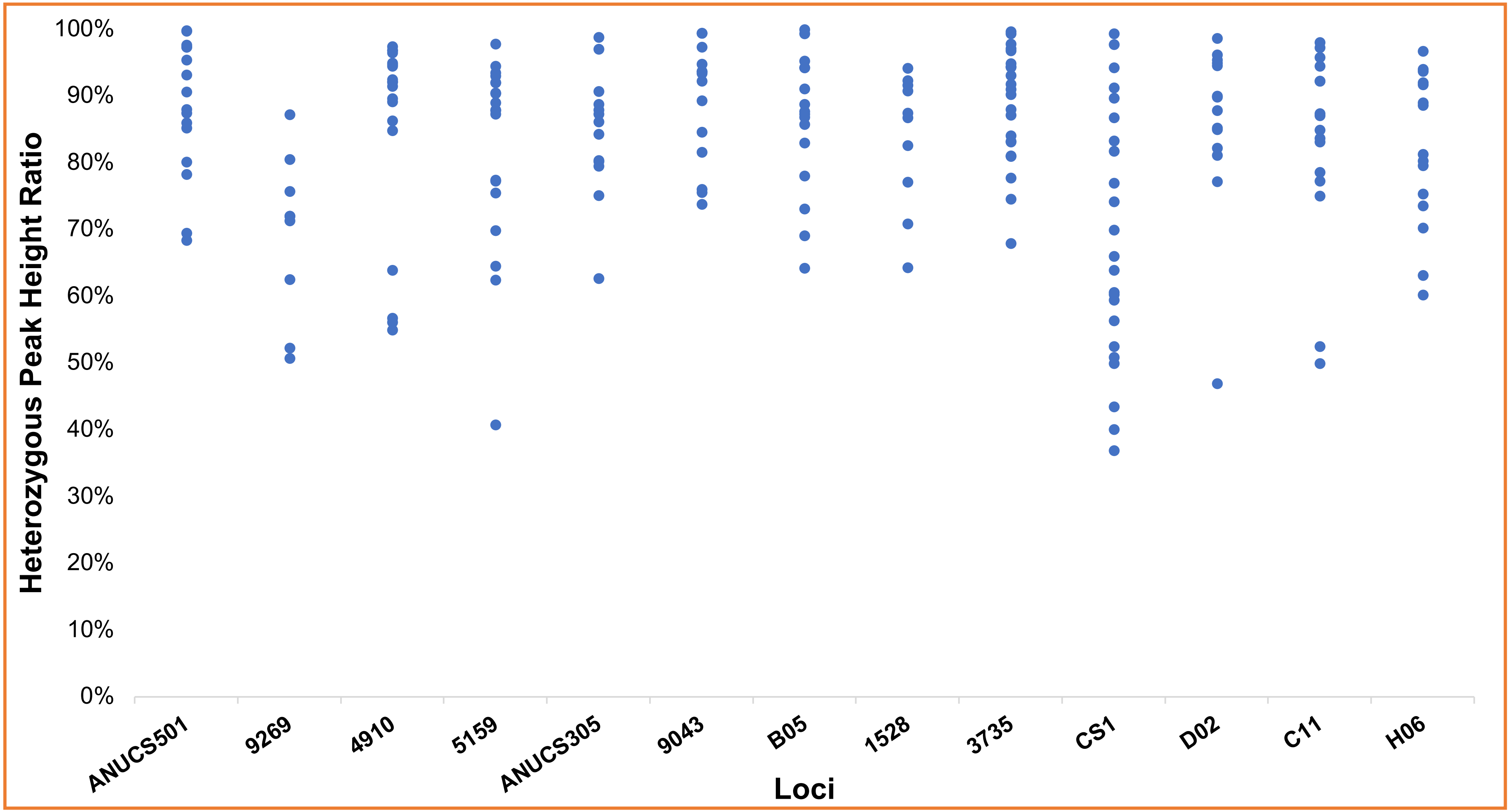


Precision & accuracy



Stutter

Peak Height Ratio



13 STR multiplex

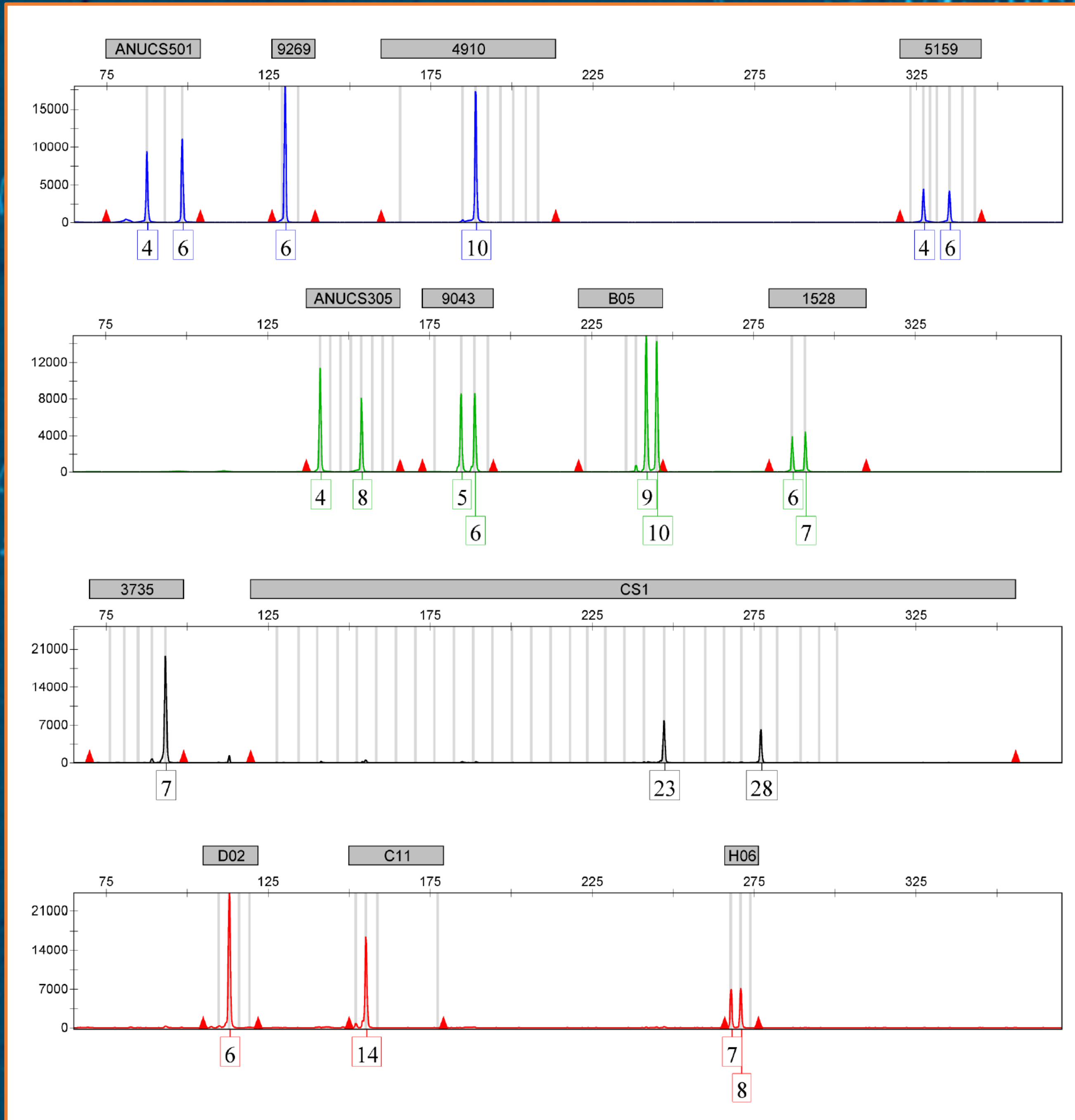
Primer Selection and Titration

Allele Sequencing and Ladder Design

Validation Studies

STR Genotyping

Positive Control



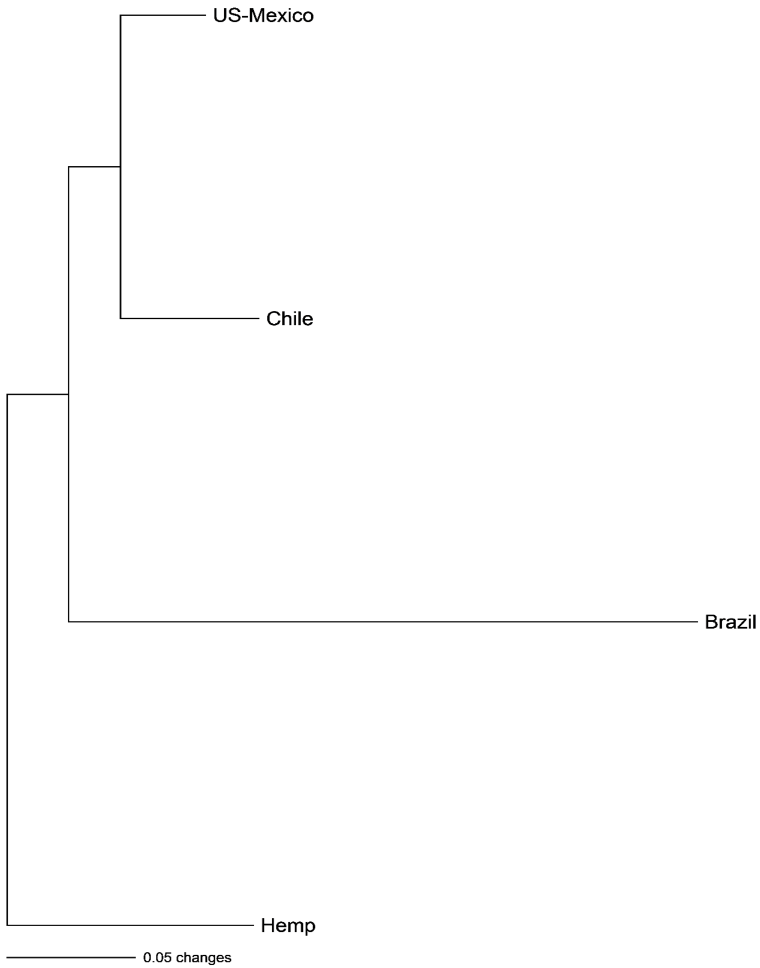
Autosomal – genotypes

Source	Sample Number	Partial Profiles	Mixed Profiles	Full Profiles	Unique Genotypes	Duplicate Genotypes
US-Mexico	422	23	32	367	326	18 (41 samples)
Brazil	8	2	0	6	6	0
Chile	50	18	0	32	4	7 (28 samples)
Hemp	30	9	1	20	20	0
	510	**52**	**33**	**425**	**356**	**25 (69 samples)**

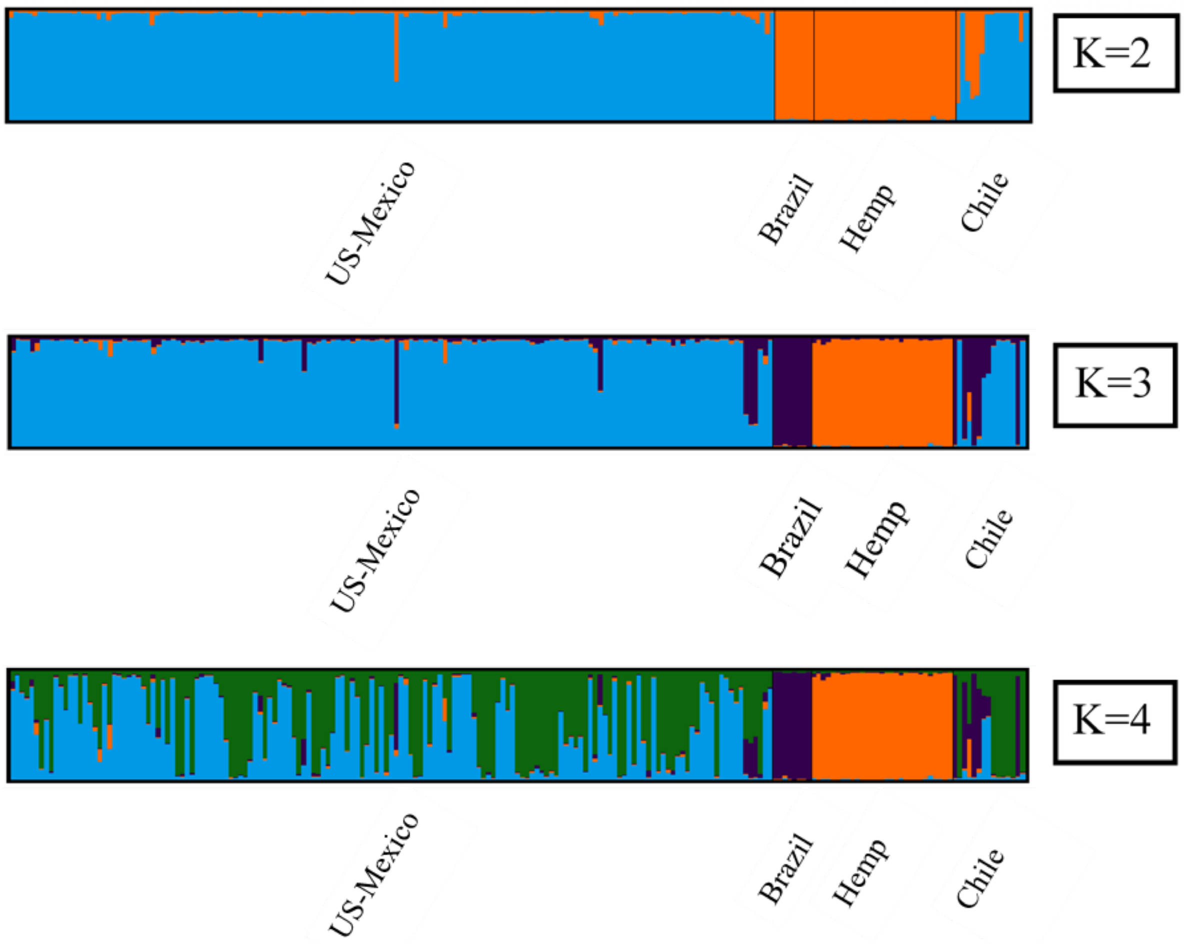
- Duplicates from same seizure or same plant
- Hypothesized that 9 out of 10 Chilean seizures clonally propagated

Autosomal – phylogenetic analysis

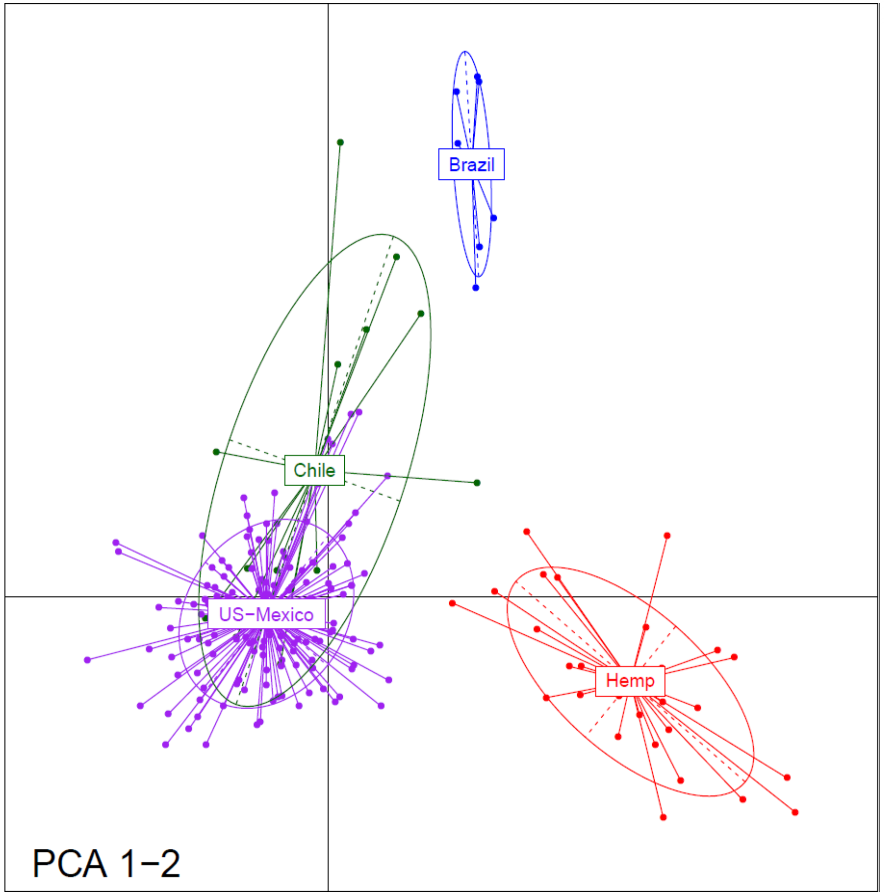
- 9 seizures (N = 157 samples) formed a reference population for US-Mexico
  - $F_{ST} = 0$



Autosomal – Bayesian clustering



Autosomal – PCA



- Genetic relatedness between drug types
- Distinction from fiber type

Statistical analysis of reference database

- Population genetic statistics and parameters of forensic interest:
  - Hardy-Weinberg Equilibrium (GDA)
  - Linkage Equilibrium (GDA)
  - Allele Frequencies (PowerStats v 1.2)
  - Random Match Probability (PowerStats v 1.2)
  - Power of Discrimination (PowerStats v 1.2)

Combined power of discrimination of the multiplex is 1 in 55 million

Potential impact

- Provide the forensic community with a genetic tool for identification of *C. sativa* samples:
  1. Authenticate legal cannabis products
  2. Link cases (as intelligence tool)
  3. Link and identify illegal growers/distributers
- Complement previously established profiling programs for intelligence purposes for organizations, such as Homeland Security/CBP and DEA

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Short Communication

Developmental and internal validation of a novel 13 loci STR multiplex method for *Cannabis sativa* DNA profiling

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ORIGINAL ARTICLE

Nuclear, chloroplast, and mitochondrial data of a US cannabis DNA database

Rachel Houston [1] · Matthew Birck [2] · Bobby LaRue [1] · Sheree Hughes-Stamm [1] · David Gangitano [1]

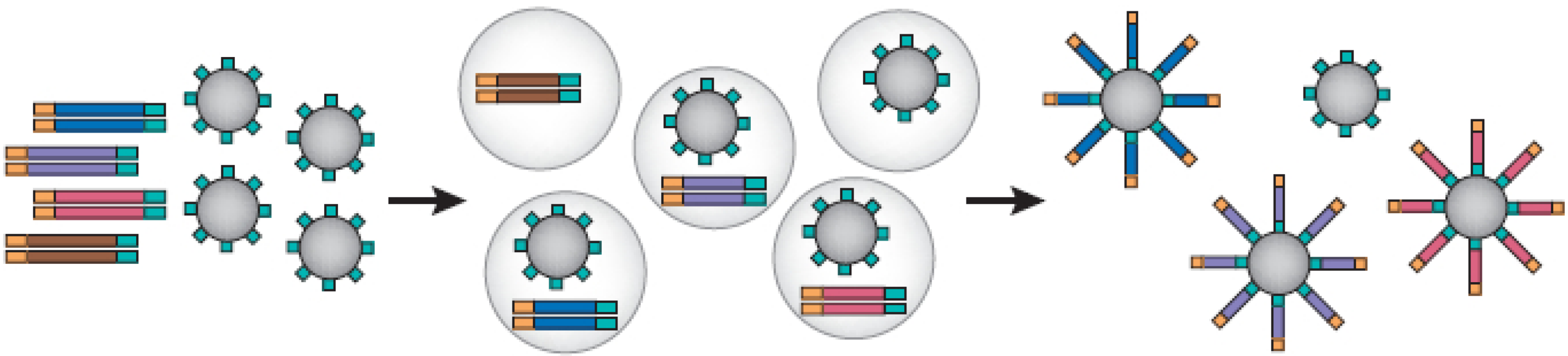
Received: 9 November 2017 / Accepted: 9 February 2018 / Published online: 20 February 2018

Massively parallel sequencing of 12 autosomal STRs in *Cannabis sativa*

SeqWeed: The Next Generation

Why massively parallel sequencing (MPS)?

- High throughput and multiplex capability
- Greater power of discrimination
- Degraded DNA
- Mixture interpretation



Source	N
US-Mexico	8
Brazil	2
Chile	3
Hemp	3

N = 16 samples

Extract & Quantify DNA

Targeted Amplification

Custom PCR assay with Type-It™ Microsatellite Kit (Qiagen)

Library Preparation

Ion Plus Fragment Kit

Clonal Amplification/Templating

Sequencing

Data Analysis

STR panel

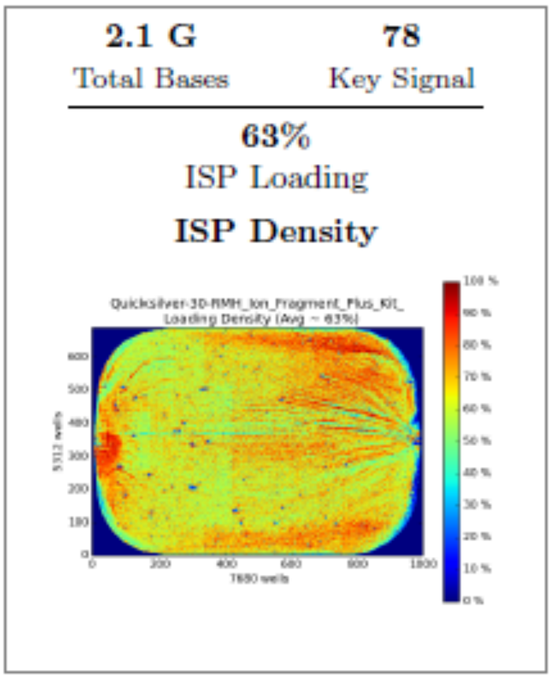
- 12plex STR panel:
  - ANUCS501
  - 9269
  - 4910
  - 5159
  - ANUCS305
  - 9043
  - B05-CANN1
  - 1528
  - 3735
  - D02-CANN1
  - C11-CANN1
  - H06-CANN2
- Analyzed with:
  - STRait Razor v3
  - Excel workbook
- Compared data to genotypes generated on 3500 Genetic Analyzer
- Evaluation of sequence variants within alleles

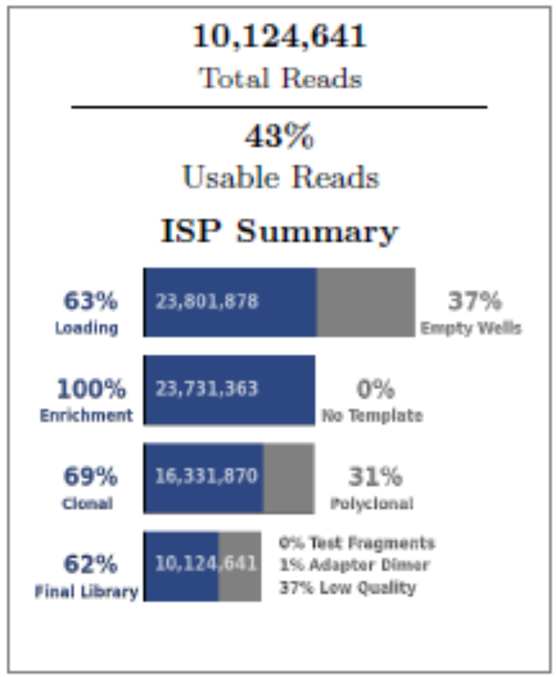
Data sorting and analysis

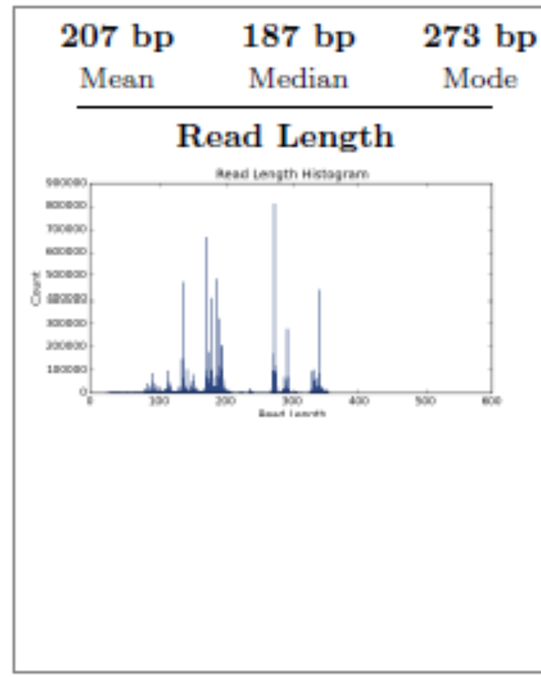
- Sequencing data generated on Torrent Suite Software (v.5.2.2)
- FASTQ files exported to bioinformatics tool (STRait Razor v3)
  - Customizable
  - Extract and parse STR data
- STRait Razor Excel workbook
  - Collate and visualize data

Sequencing data

Torrent Suite Software:

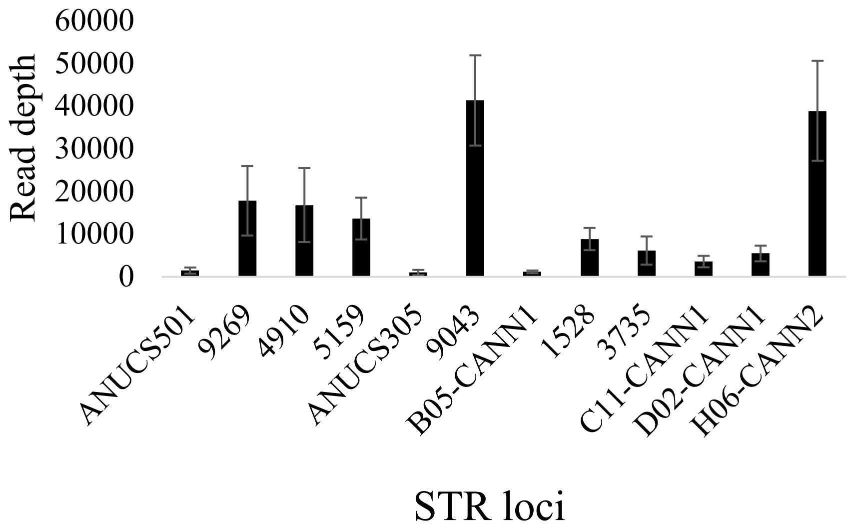






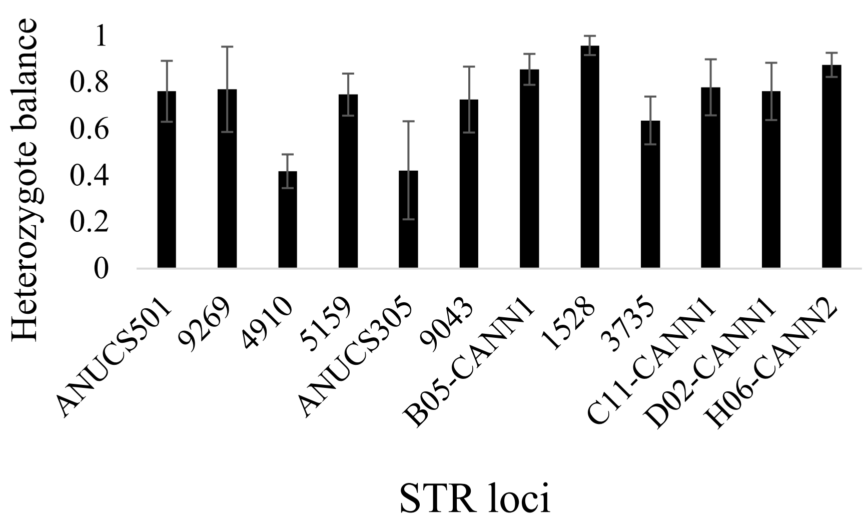
Read depth and heterozygote balance

Read Depth



- Average: 13,000x
- Range: 1,001 – 41,378x

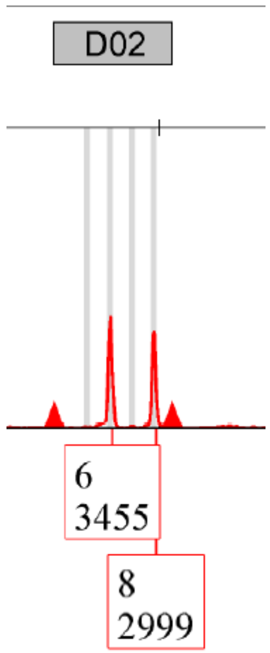
Heterozygote balance

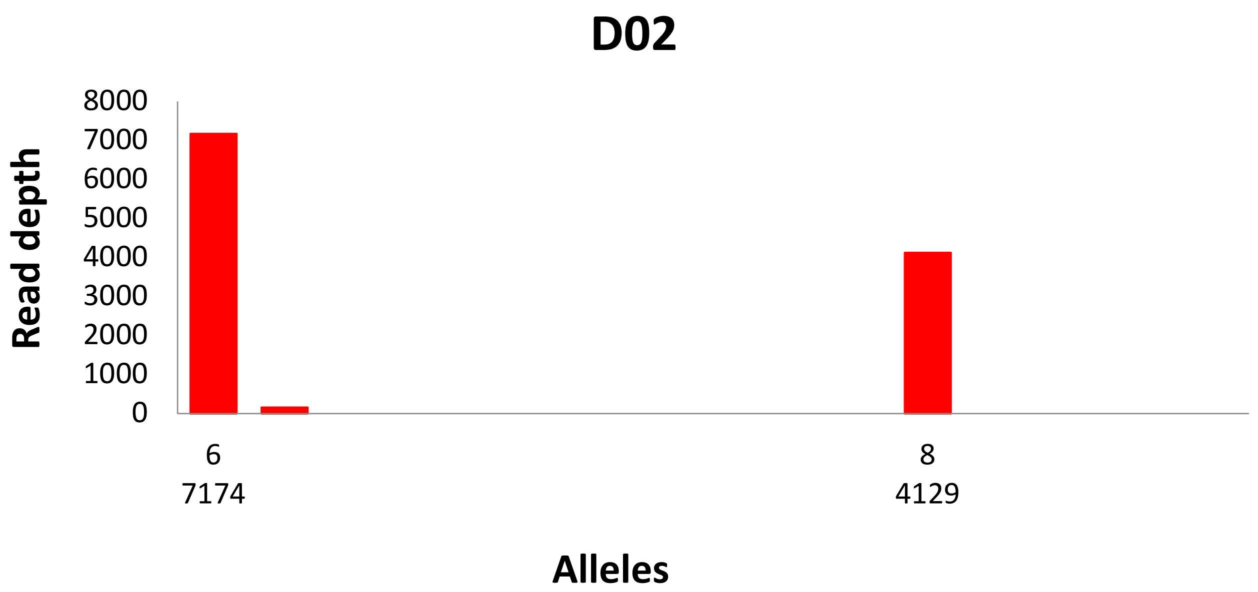


- Average: 0.73 ± 0.16

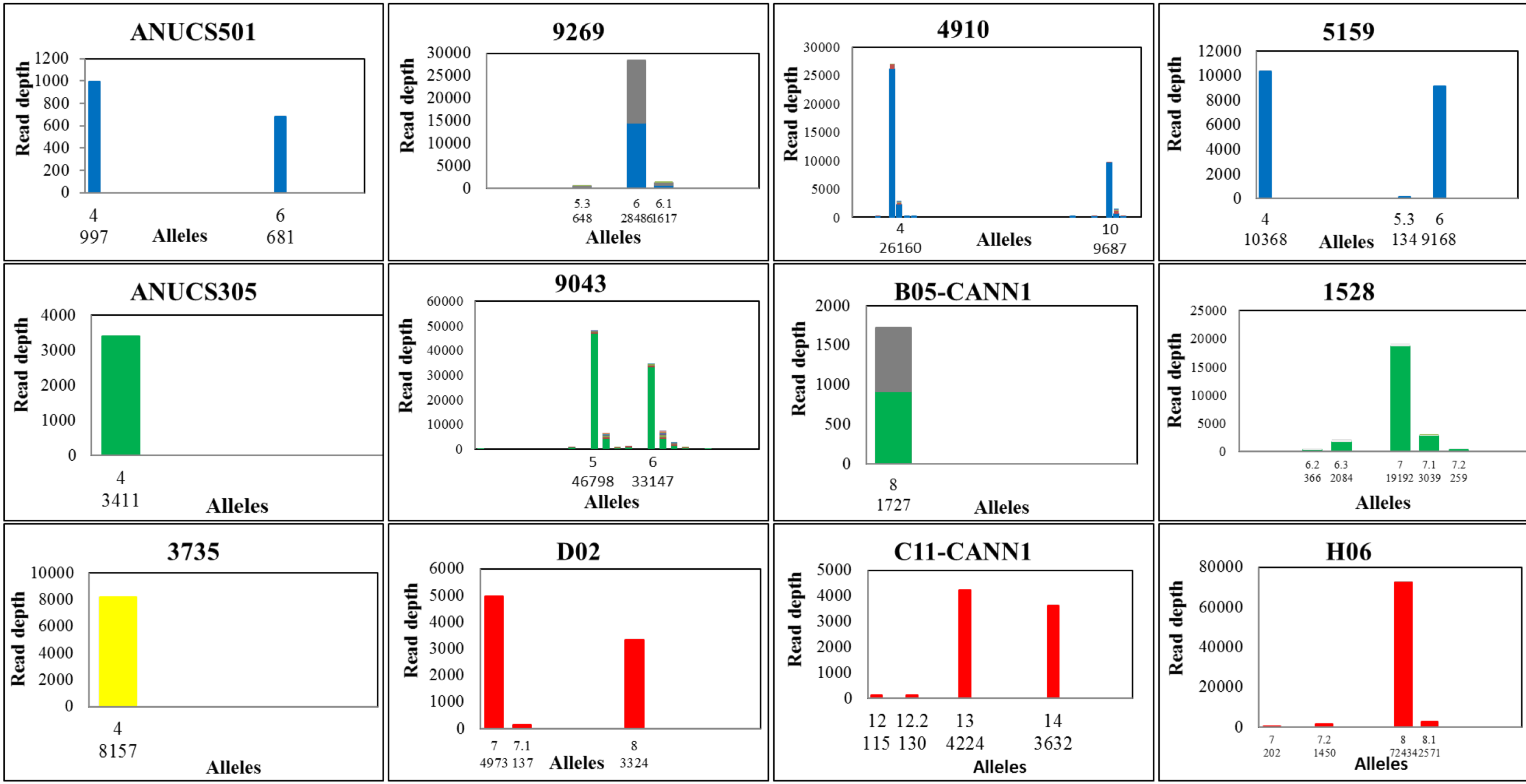
STRait Razor analysis

- Comparable methods of visualization for CE and MPS platforms





Example histogram



STR results

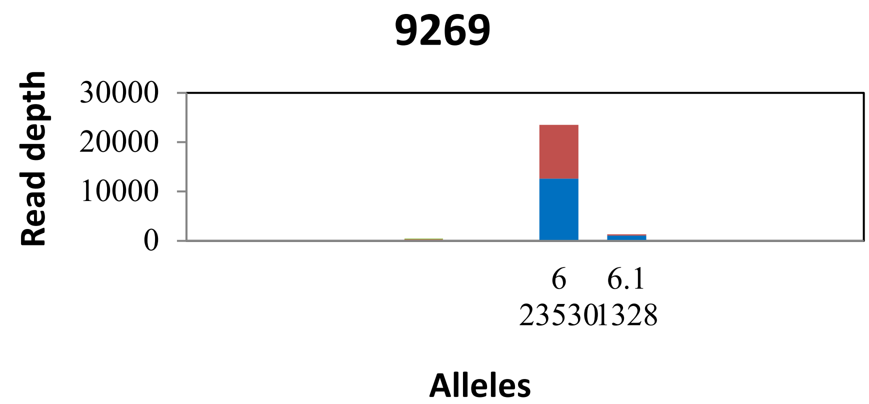
Sample	A501	9269	4910	5159	A305	9043	B05	1528	3735	D02	C11	H06
1	4,4	6,6	4,10	4.2,6	4,6	6,6	8,8	6,7	4,4	6,8	13,14	8,8
2	4,6	6,6	4,10	6,6	4,6	5,5	8,9	6,7	5,7	7,7	13,13	7,8
3	6,6	5.3,6	4,10	4.2,6	4,8	5,6	8,9	6,6	6,7	6,7	13,13	8,9
4	4,4	6,6	10,10	6,6	4,8	3,5	8,9	7,7	3,6	6,7	14,14	7,8
5	4,6	6,6	4,10	4,6	4,4	5,6	8,8	7,7	4,4	7,8	13,14	8,8
6	4,4	6,6	4,4	3,3	6,8	3,6	8,8	7,7	3,5	6,6	13,13	8,9
7	4,4	5.3,5.3	5,5	6,6	4,4	5,5	7,8	7,7	6,6	8,8	14,14	8,8
8	2,4	4,6	5.3,5.3	6,7	8,8	5,6	8,9	7,7	8,8	6,6	14,14	8,8
9	4,6	6,6	4,10	4,4.2	4,8	3,3	8,8	7,7	4,7	6,6	13,14	7,8
10	6,6	6,6	4,4	4,4.2	4,8	3,6	8,8	7,7	4,7	6,7	14,14	8,8
11	4,6	6,6	10,10	6,6	8,8	5,6	8,9	7,7	5,6	7,7	13,14	7,8
12	4,6	6,6	4,10	4.2,6	4,8	3,3	8,8	6,7	3,5	6,7	14,15	8,8
13	4,6	6,6	4,10	4.2,6	4,8	3,3	8,8	6,7	3,5	6,7	14,15	8,8
14	2,4	6,6	10,10	6,6	7,9	6,6	8,9	7,7	4,6	5,6	16,16	8,8
15	2,4	6,6	10,10	6,7	6,9	5,5	9,9	7,7	4,6	6,6	16,16	8,8
16	4,4	5.3,5.3	5,5	6,6	4,4	5,5	8,8	7,7	6,6	8,8	14,14	8,8

All genotypes in concordance with CE data

Isoalleles detected

Sequence variants

- 8 loci where nominal allele differentiated by sequence
  - 9269
  - 5159
  - ANUCS305
  - 9043
  - B05-CANN1
  - 1528
  - D02-CANN1
  - C11-CANN1



Nominal Allele	String sequence	Read depth
6	CTTTCTATCAGTATCTATAAAATATTTACAAGA AAAGAGCATT [ATCA] $[ATAA]_5$	12580
6	CTTTCTATCAGTATCTATAAAATATTTACAAGA AAAGAGCATT $[ATAA]_6$	10950

Conclusions

- Successful proof of concept
- Identified sequence variants (isoalleles)
- Illustrates a workflow for integrating any PCR multiplex into MPS workflow
  - Library prep
  - Bioinformatics
- Future research:
  - Expand number of loci
  - Gao et al. identified 56 polymorphic STRs

Acknowledgements

- NIJ Award Number #2015-R2-CX-0030
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- Dr. Sheree Hughes-Stamm
- Team DNA!

Questions

Rachel Houston

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