Development and Validation of a Novel 13-loci STR Multiplex Method for *Cannabis sativa* DNA Profiling

Rachel Houston, BS; Sheree Hughes-Stamm, PhD; David Gangitano, PhD

Department of Forensic Science
Sam Houston State University
Huntsville, TX, USA

69th Annual AAFS, New Orleans, 2017

Sam Houston
State University

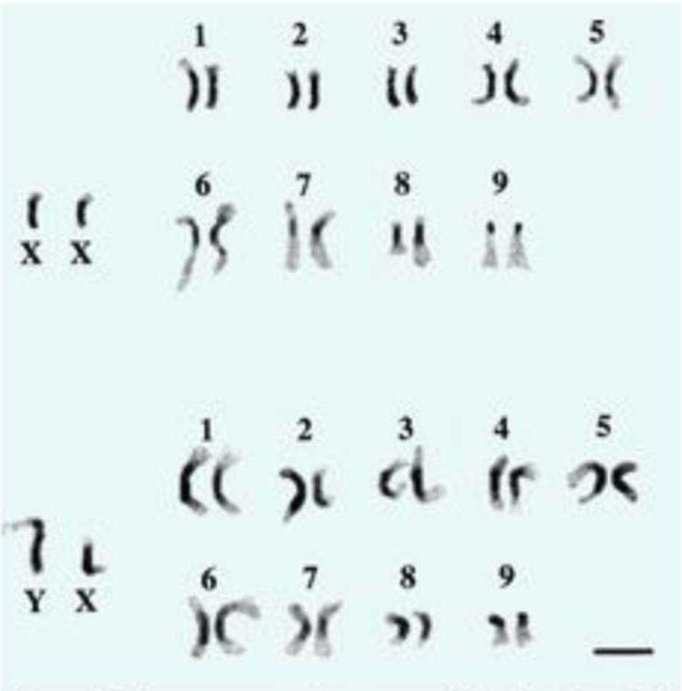
FSF Emerging Forensic Scientist Award
Paper Presentation

Disclosure

- There is no real or apparent conflicts of interest related to the content of this presentation
- Products used:
  - DNeasy® Plant Mini Kit
  - Type-IT® Microsatellite kit
  - SYBR™ Green Master Mix
  - Big Dye Direct® Cycle Sequencing Kit
  - Centri-Sep™ purification columns

Marijuana Background

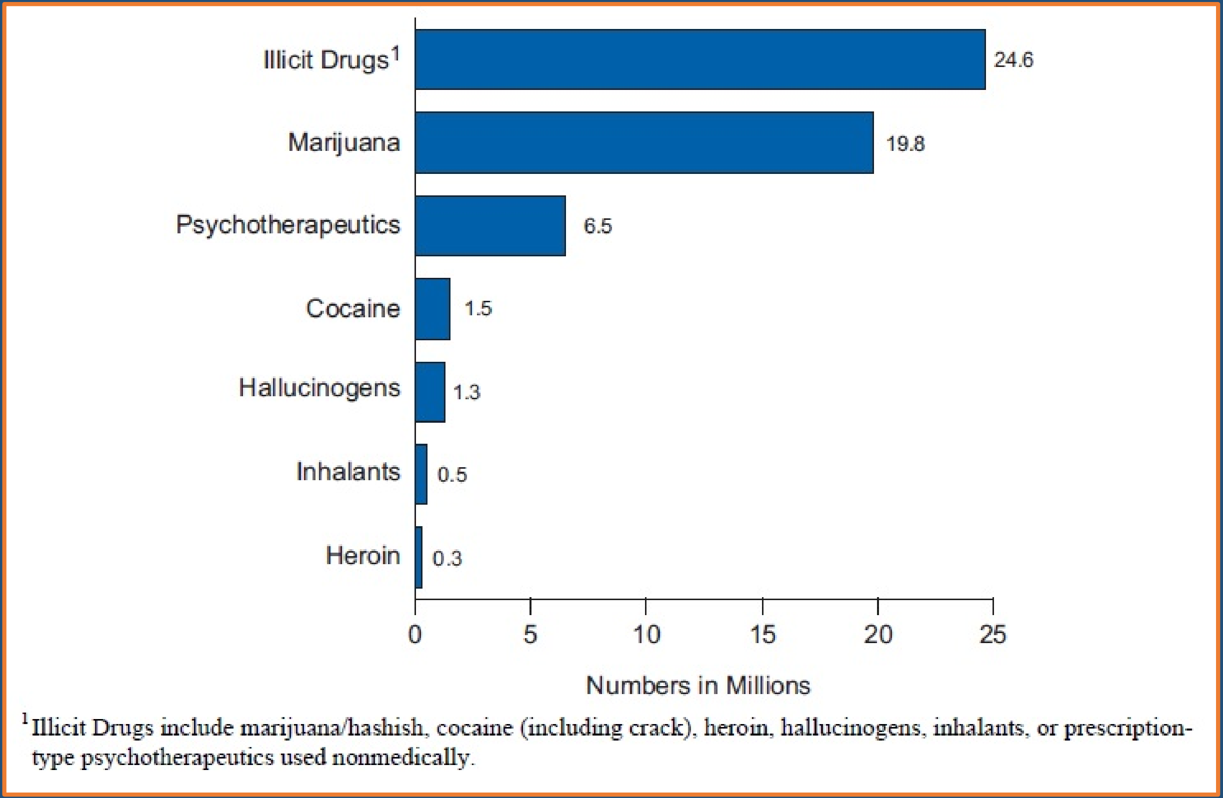
- Family: Cannabaceae
- Genus: *Cannabis*
- Species: *Cannabis sativa*
- Diploid genome (2n = 20)
  - 9 pairs of autosomes
  - Pair of sex chromosomes



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

Significance

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

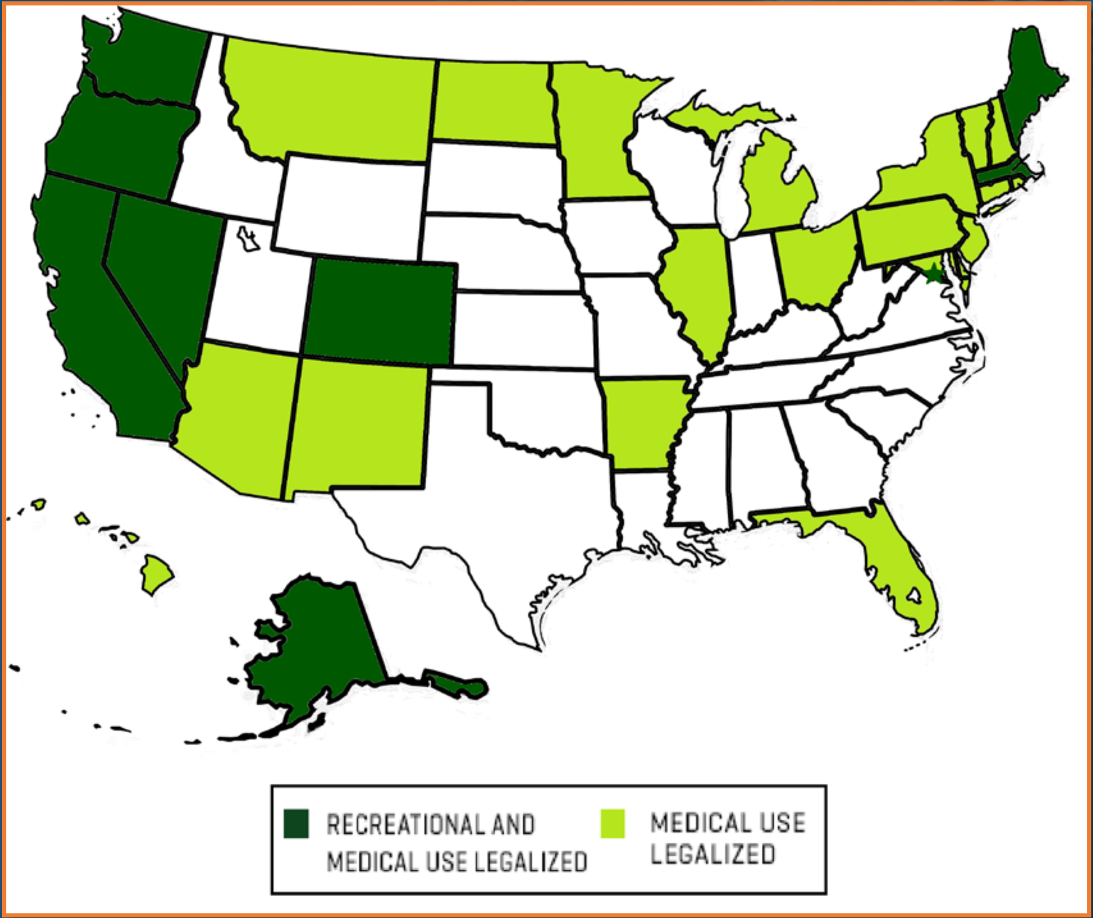


[1] Illicit Drugs include marijuana/hashish, cocaine (including crack), heroin, hallucinogens, inhalants, or prescription-type psychotherapeutics used nonmedically.

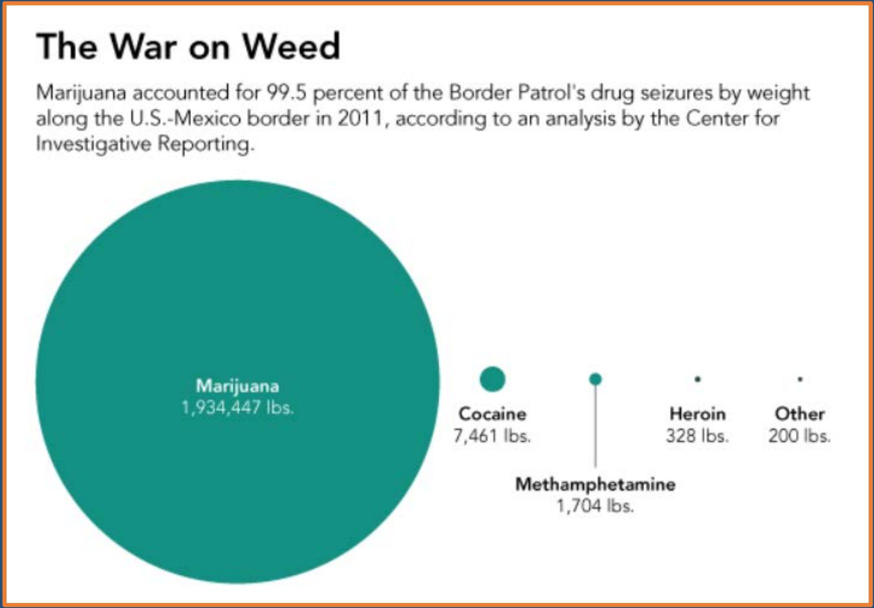
Statistics of Drug Use in United States (U.S. Department of Health and Human Services, 2013)

Marijuana Legalization

- Recreational use:
  - 8 states & D.C.
- Medical use:
  - 20 states



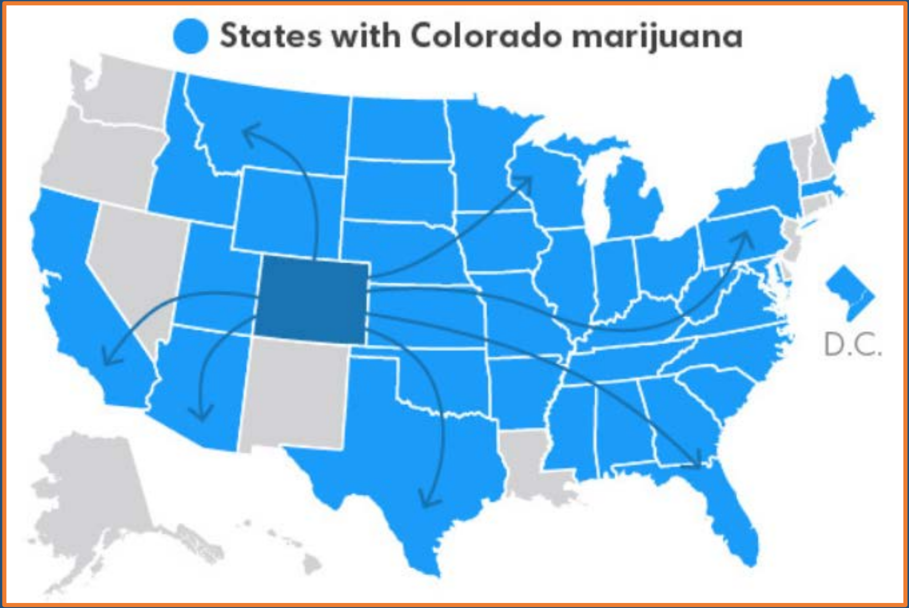
Illegal Trafficking



The War on Weed

Marijuana accounted for 99.5 percent of the Border Patrol's drug seizures by weight along the U.S.-Mexico border in 2011, according to an analysis by the Center for Investigative Reporting.

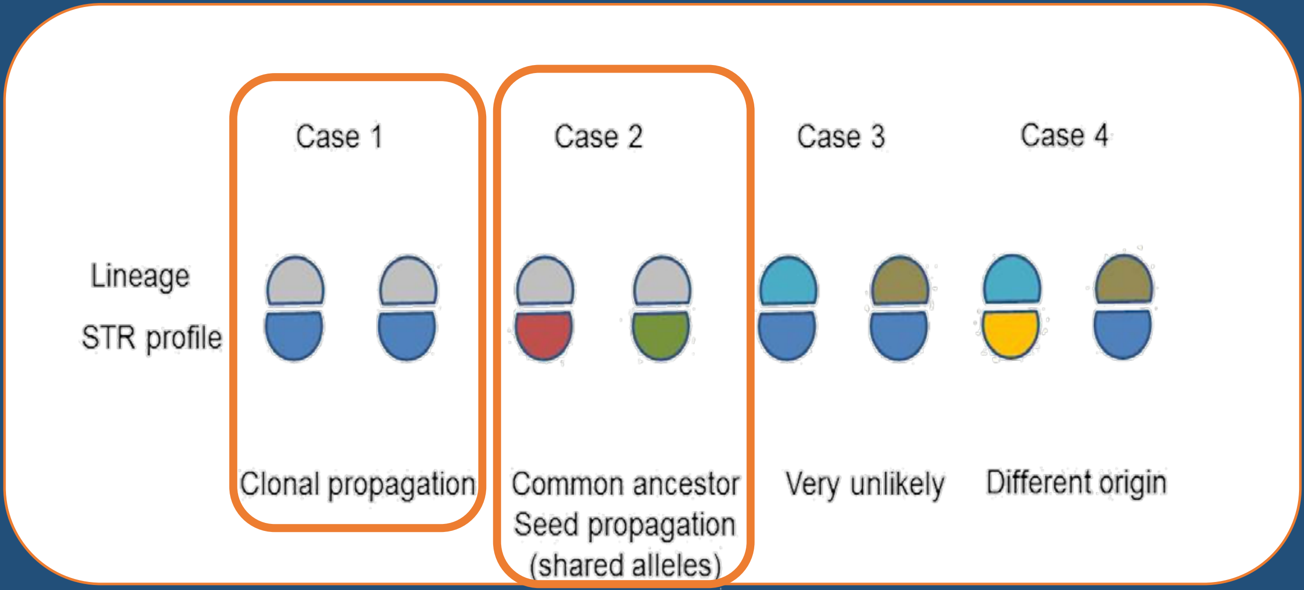
http://www.huffingtonpost.com/2013/06/21/marijuana-accounts-for-va_n_3480127.html



USA Today; El Paso Intelligence Center, National Seizure System, as of March 20, 2015.

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



DNA Based Individualization

- Polymorphic STR markers first described (Gilmore and Peakall (2003); Alghanim and Almirall (2003); Hsieh et al. (2003))
- Marijuana DNA STR multiplex and database (Howard et al. (2008))
- CS1 marker study (Miller Coyle et al. (2003))
- 15 loci - STR tool (Köhnemann et al. (2012))
- Proposed new tetranucleotide markers (Valverde et al. (2014))
- Previous research: 13 loci – STR tool (Houston et al. (2016))

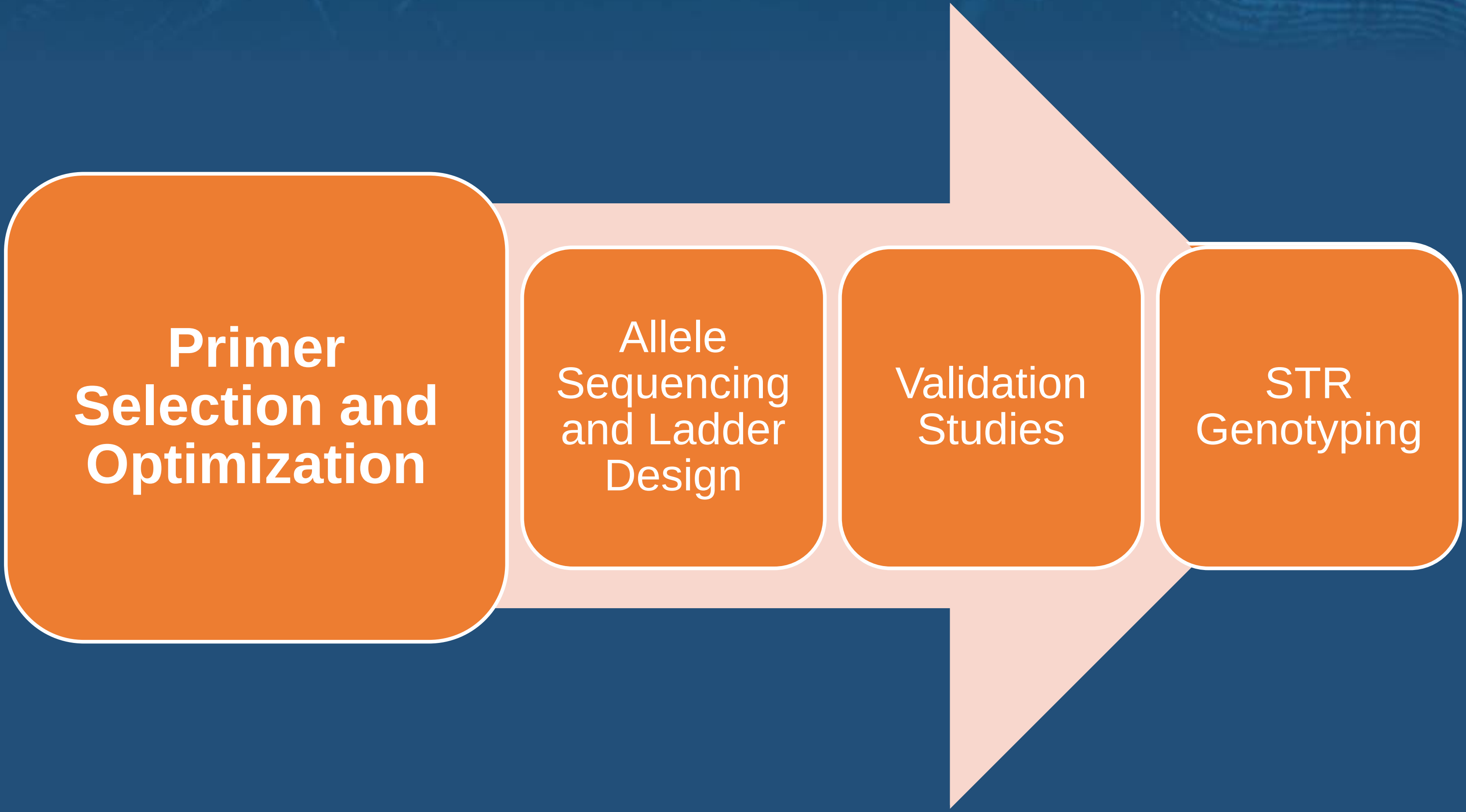
Improvements Upon Previous Research

- Based upon previous STR multiplex:
  1. Discard poorly performing loci
  2. Incorporate six new tetranucleotide markers
  3. Optimization
  4. Developmental validation
  5. Internal validation

Materials and Methods

- Sampling (3 cases – 101 samples) – **Reference Population**
- DNA Extraction (DNeasy® Plant Mini Kit)
- DNA Quantitation (real-time PCR)
- 13 STR Multiplex
- Validation Studies
- Statistical Analysis

13 STR Multiplex



Primer Selection and Optimization

Allele Sequencing and Ladder Design

Validation Studies

STR Genotyping

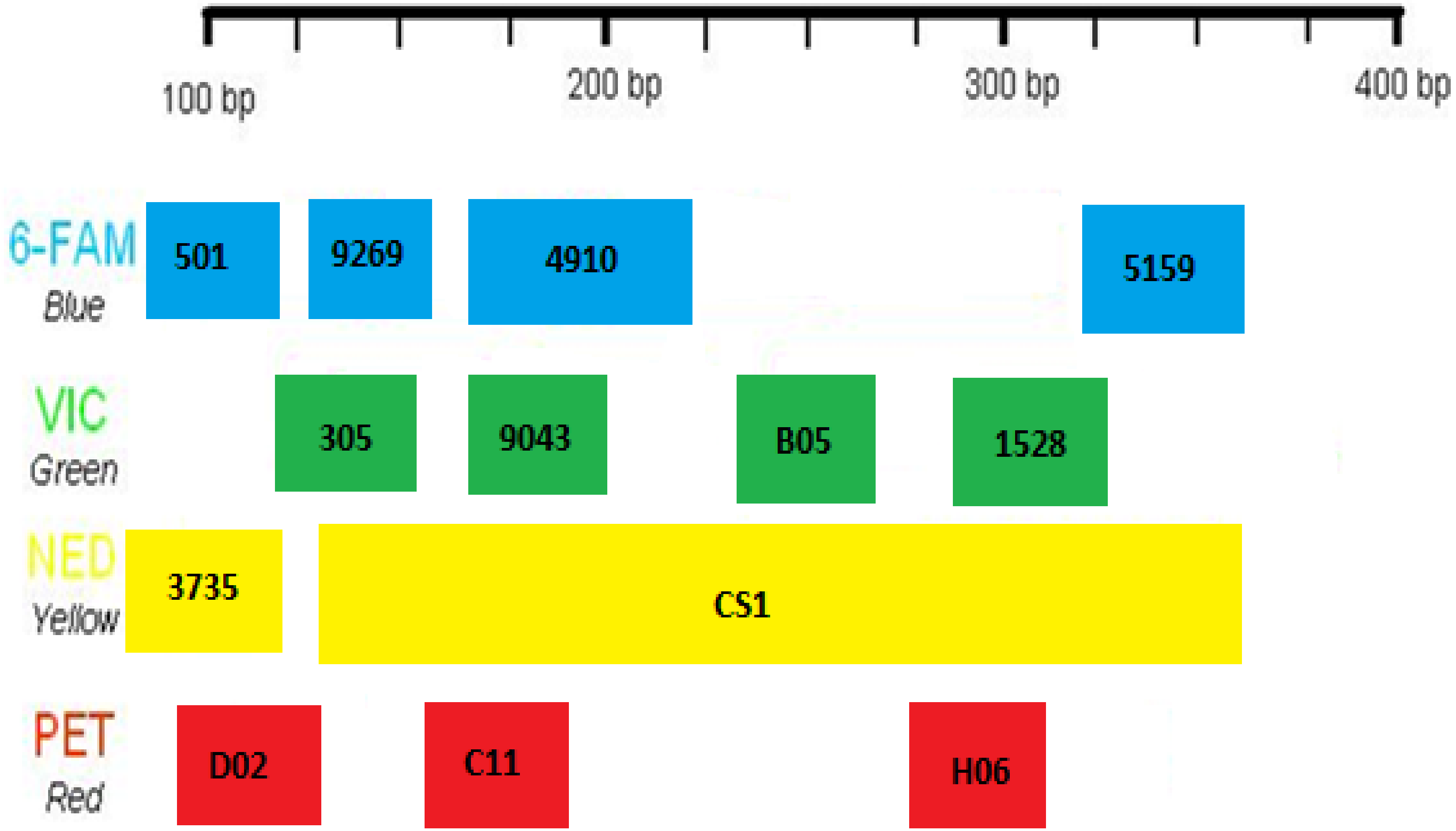
Primer Selection

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

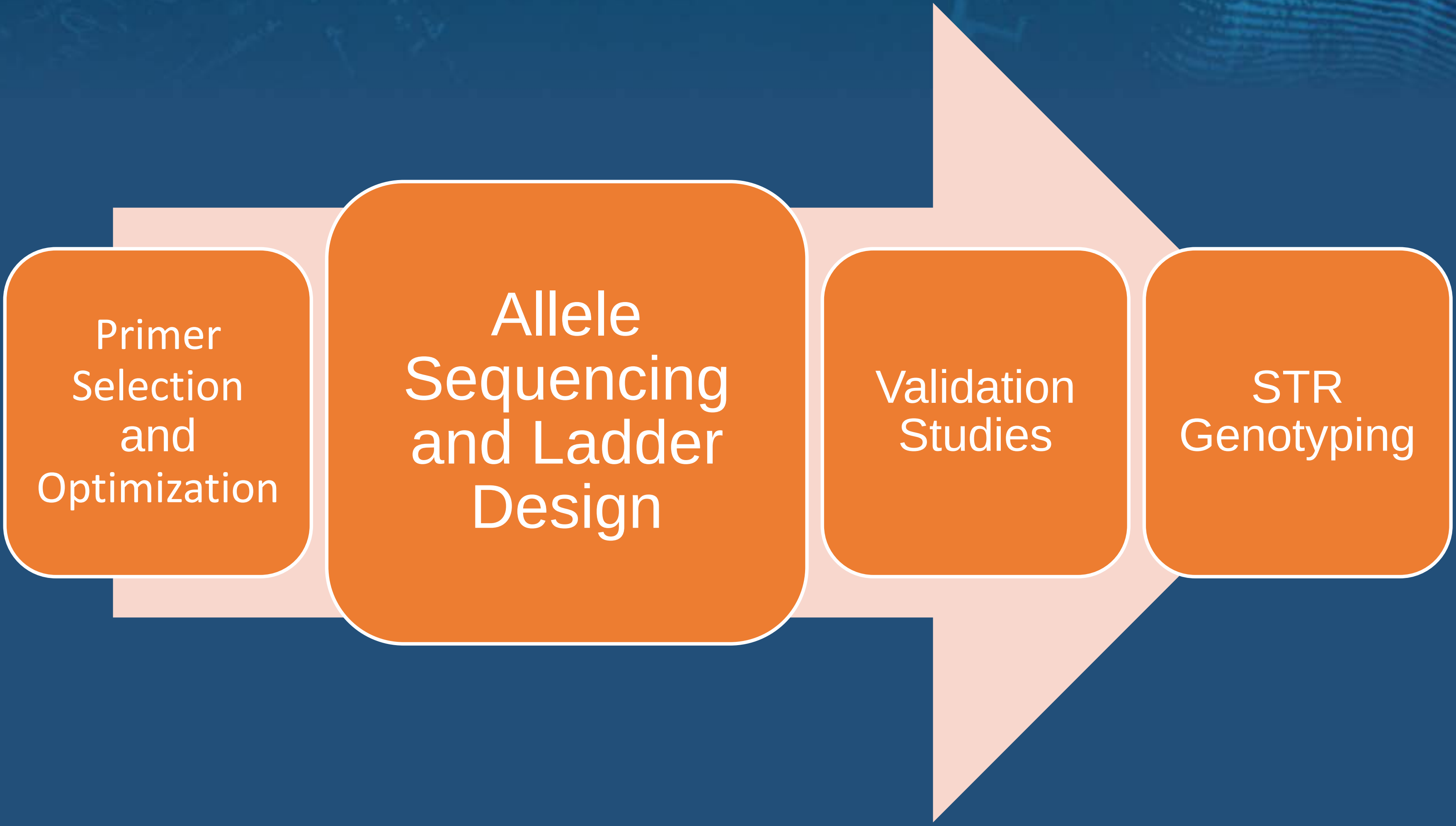
Multiplex Optimization

- Multiplex Manager Software v1.2:
  - 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



Primer Selection and Optimization

Allele Sequencing and Ladder Design

Validation Studies

STR Genotyping

Sequencing/Allelic Ladder

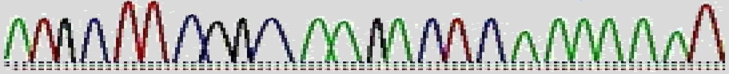
2 – 8 alleles per marker

Big Dye Direct® Cycle Sequencing Kit

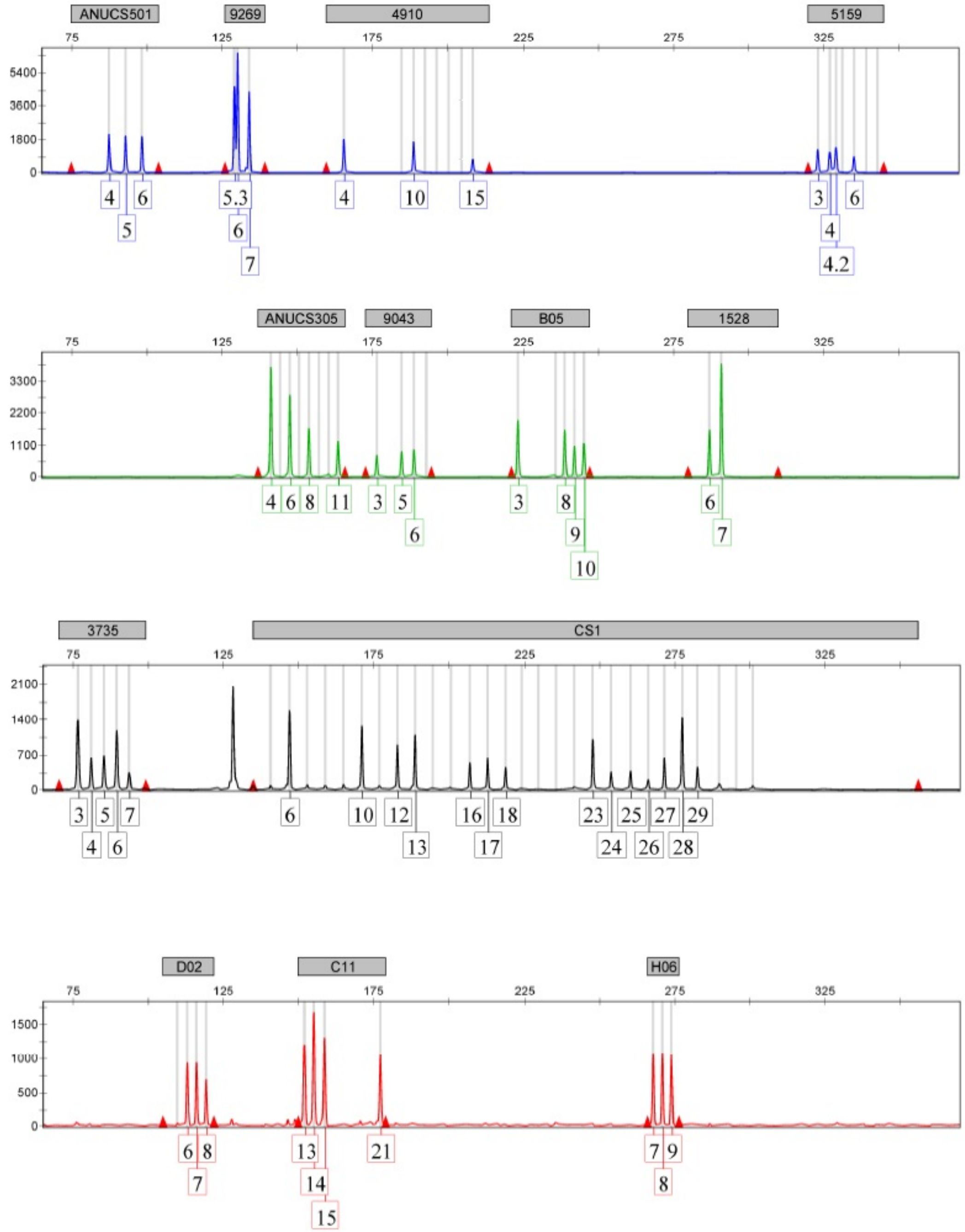
Centri-Sep™ purification columns

Geneious Pro Software

Allelic Ladder Design



Allelic Ladder



13 STR Multiplex

Primer Selection and Titration

Allele Sequencing and Ladder Design

Validation Studies

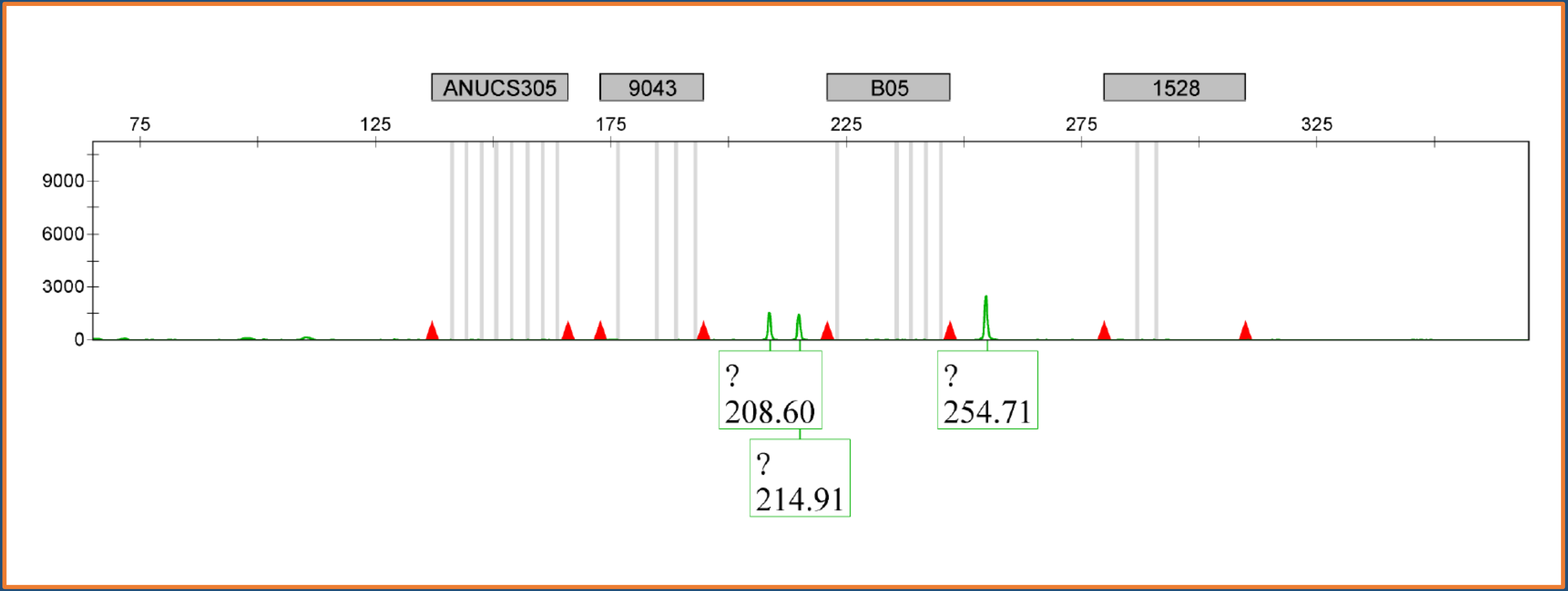
STR Genotyping

Validation Studies

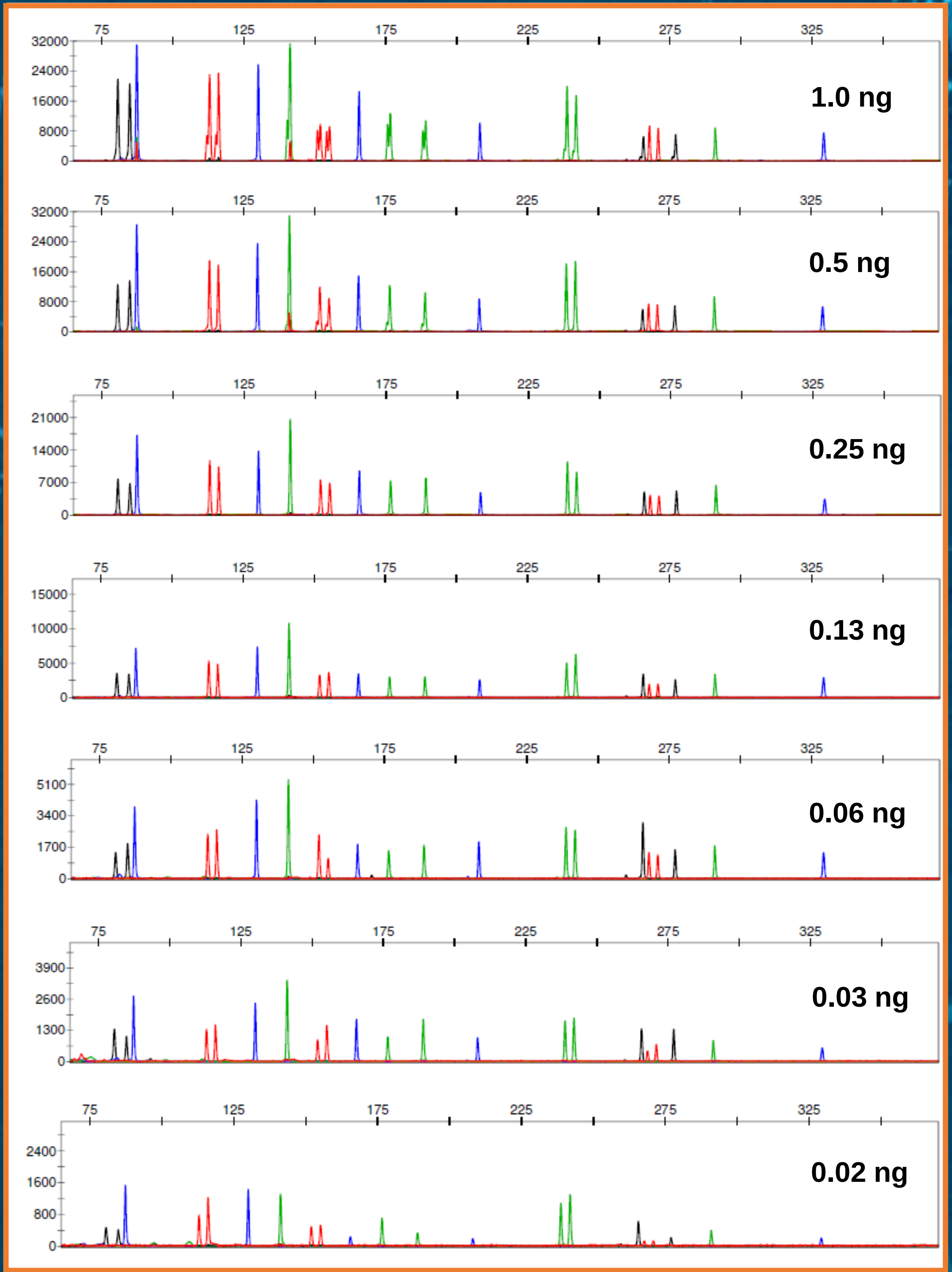
- **Developmental Validation (SWGDAM):**
  - Species specificity
  - Sensitivity/Stochastic effects
  - Precision and accuracy
  - Concordance study
- **Internal Validation:**
  - Stutter ratio
  - Peak height ratio
  - Inter-loci balance

Species Specificity

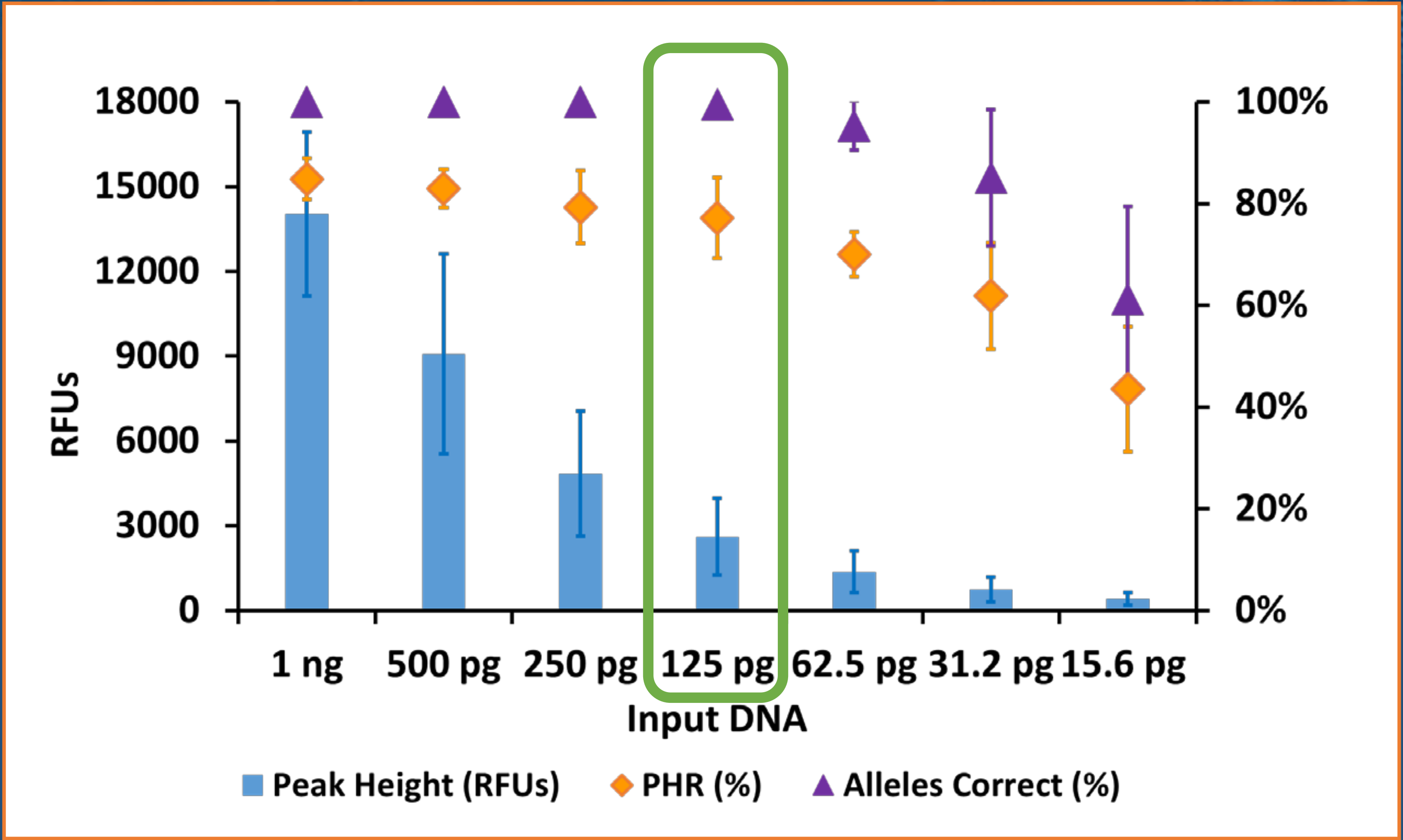
- Cross-reactivity observed in one species:
  - *Humulus lupulus* (Hops)
  - Generated non-specific peaks (previously reported)



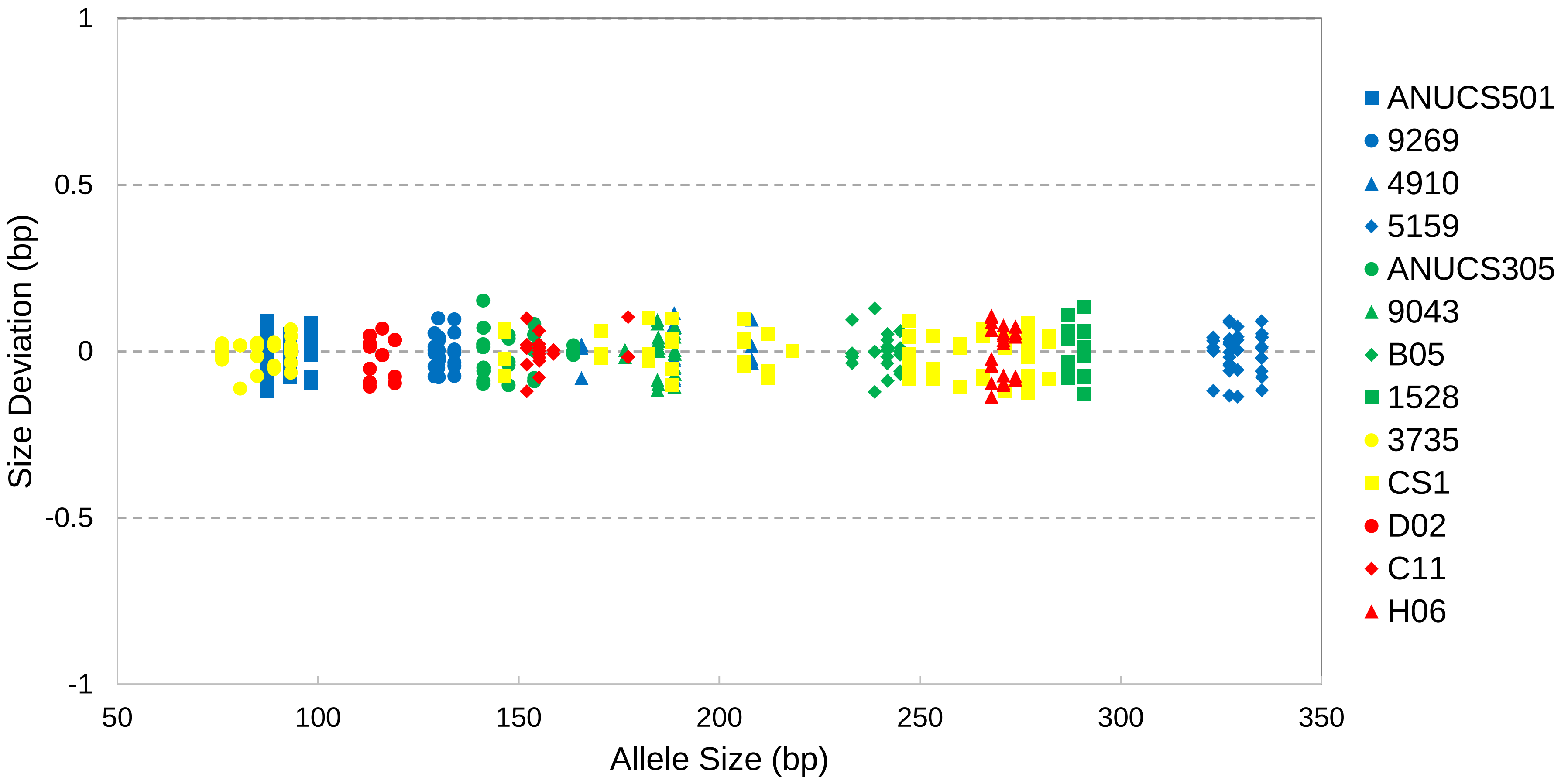
Sensitivity



Sensitivity Cont.



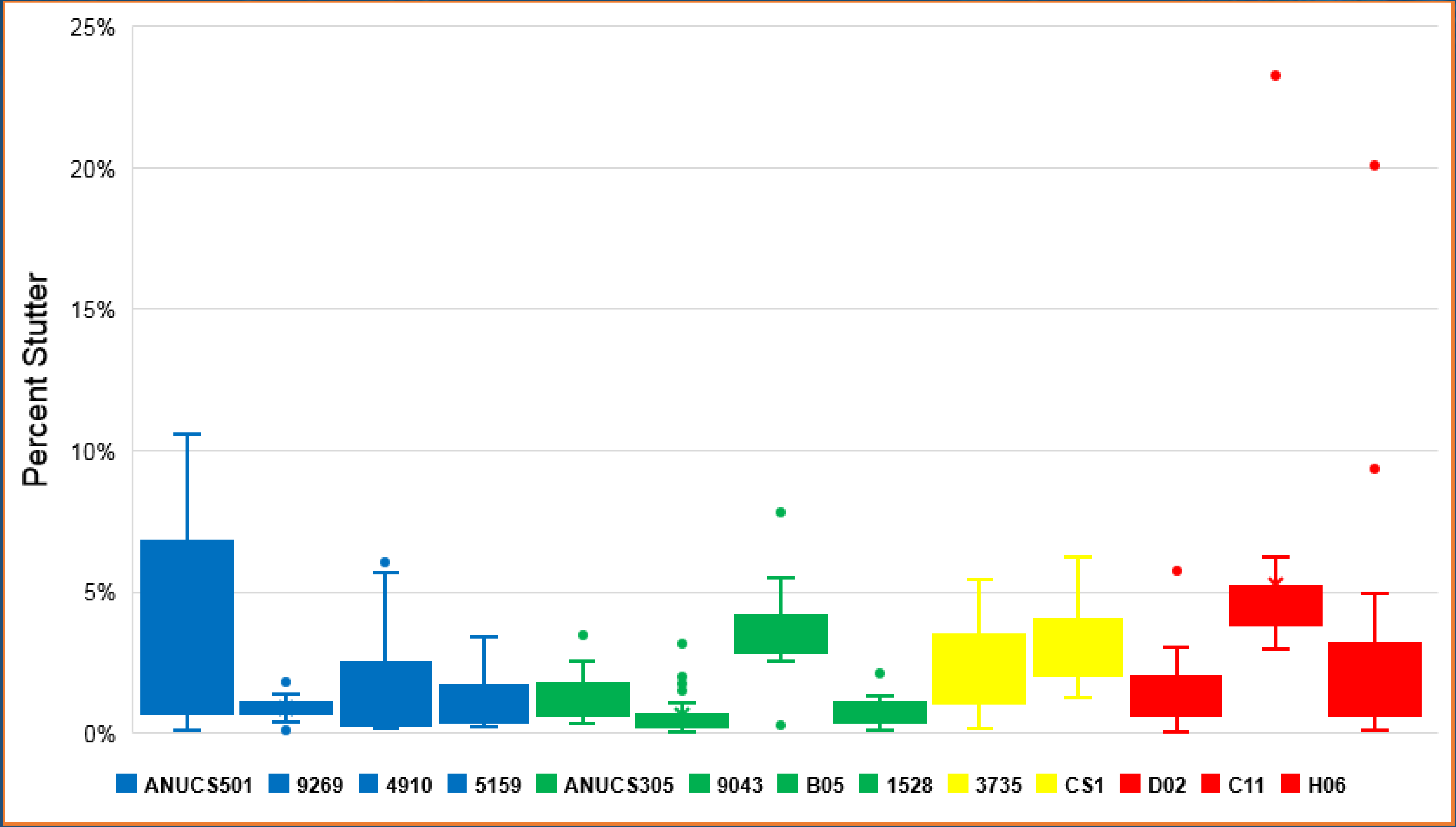
Precision & Accuracy



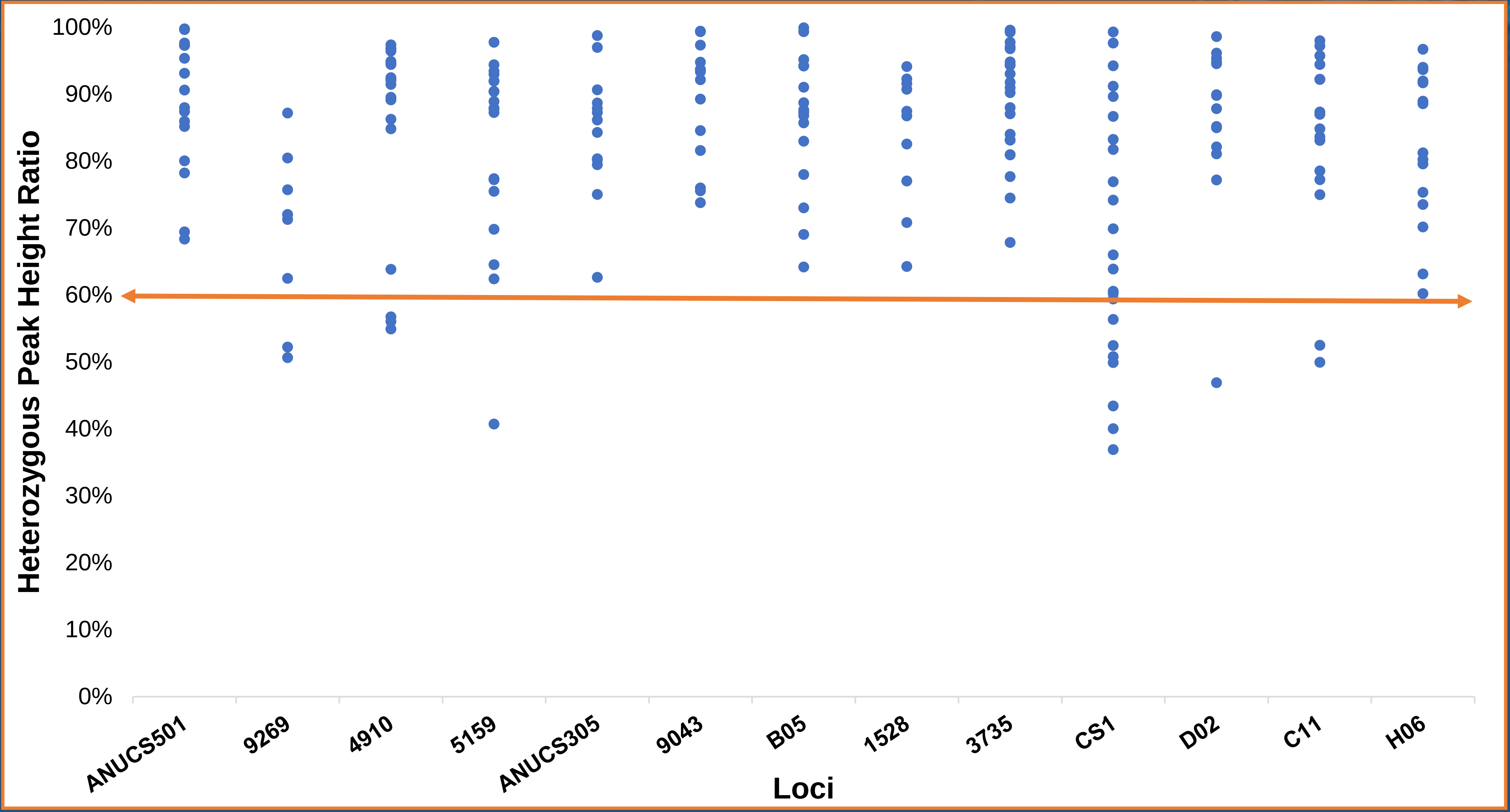
Concordance

- 100% concordance with loci previously amplified:
    1. ANUCS305
    2. ANUCS501
    3. B05
    4. D02
    5. H06
    6. C11
    7. CS1

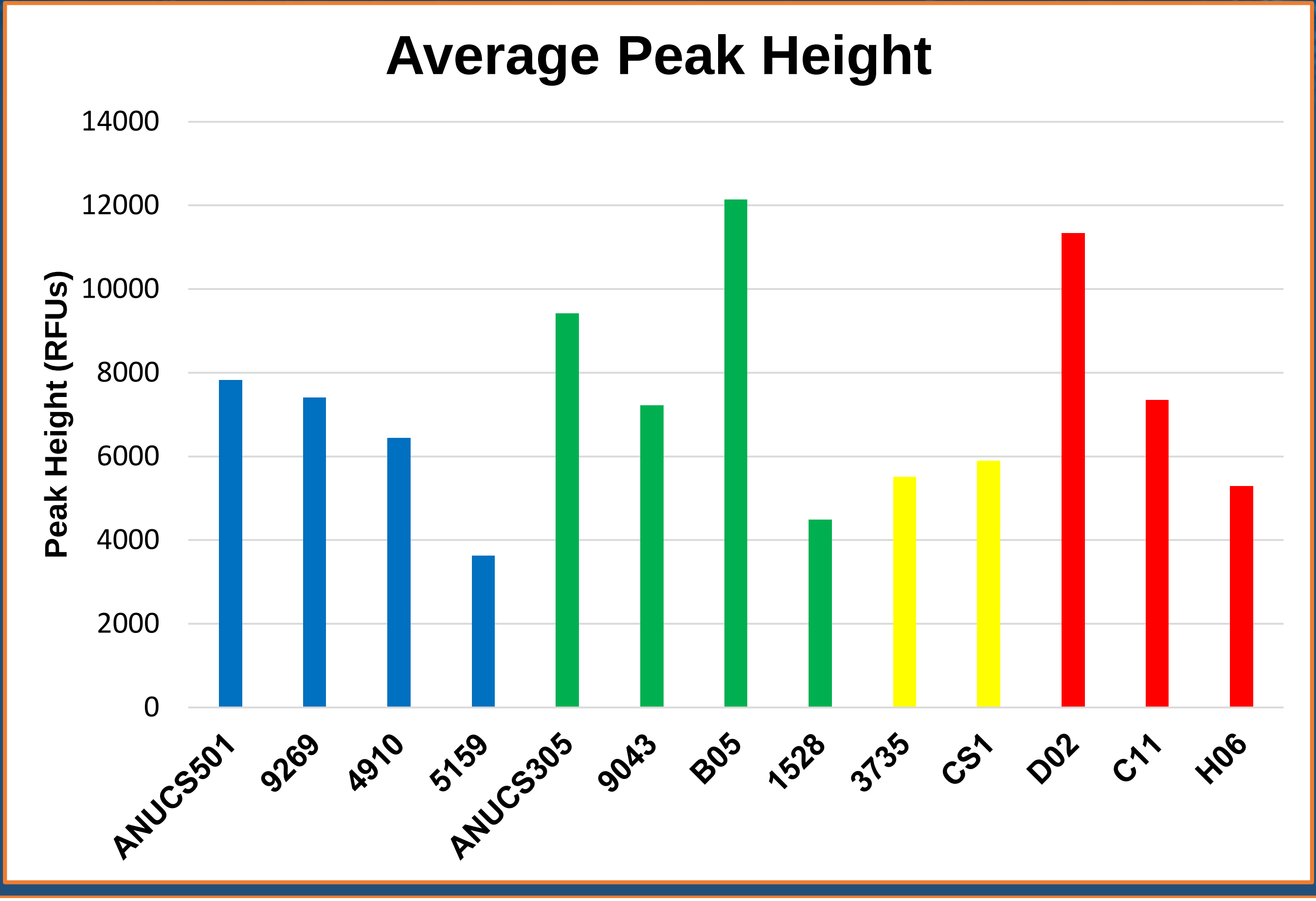
Stutter


Percent Stutter
25%
20%
15%
10%
5%
0%
ANUCS501
9269
4910
5159
ANUCS305
9043
B05
1528
3735
CS1
D02
C11
H06

Peak Height Ratio

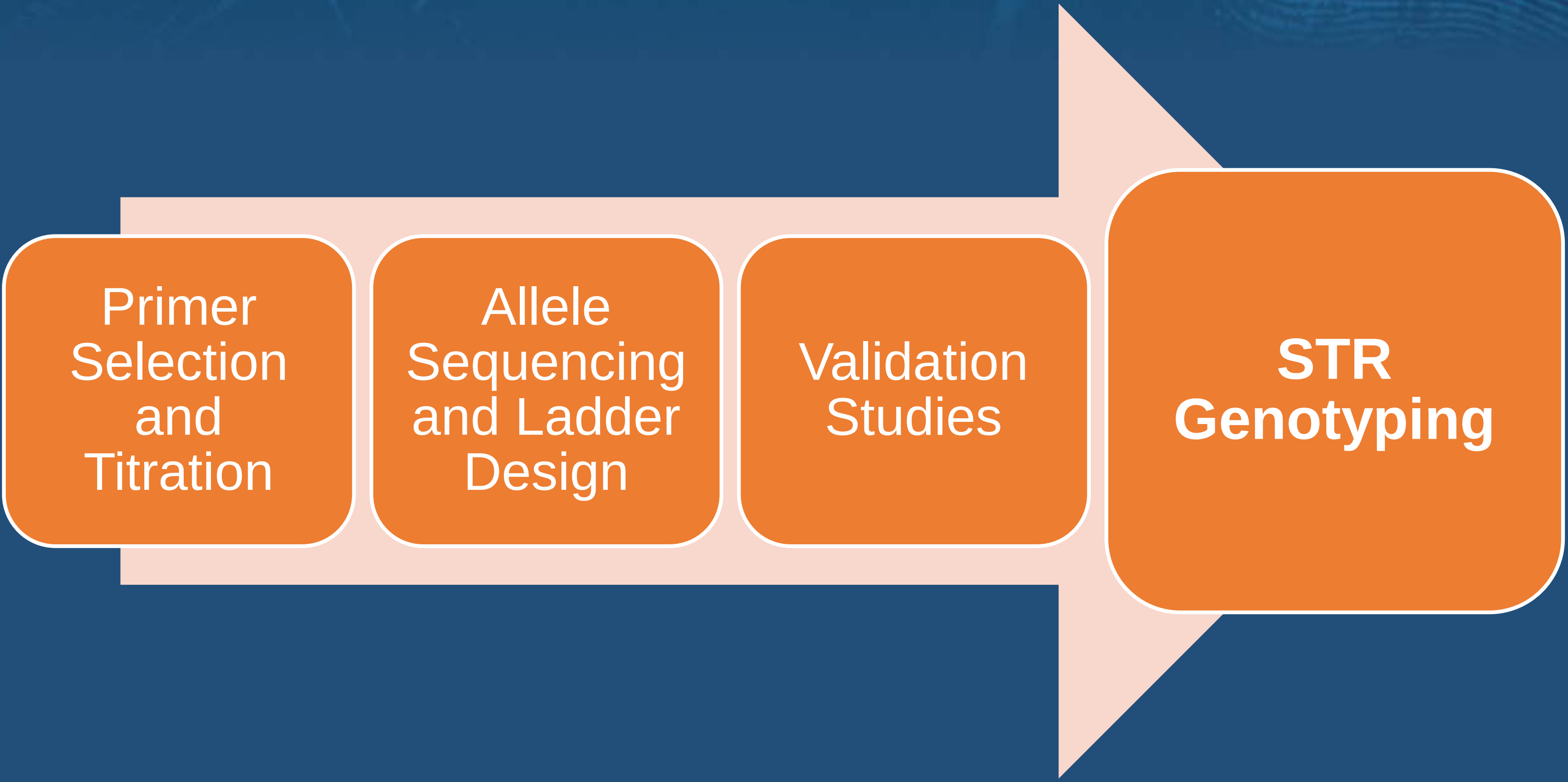


Inter-loci Balance



Inter-loci balance range: 0.50 (5159) – 1.671 (B05)

13 STR Multiplex



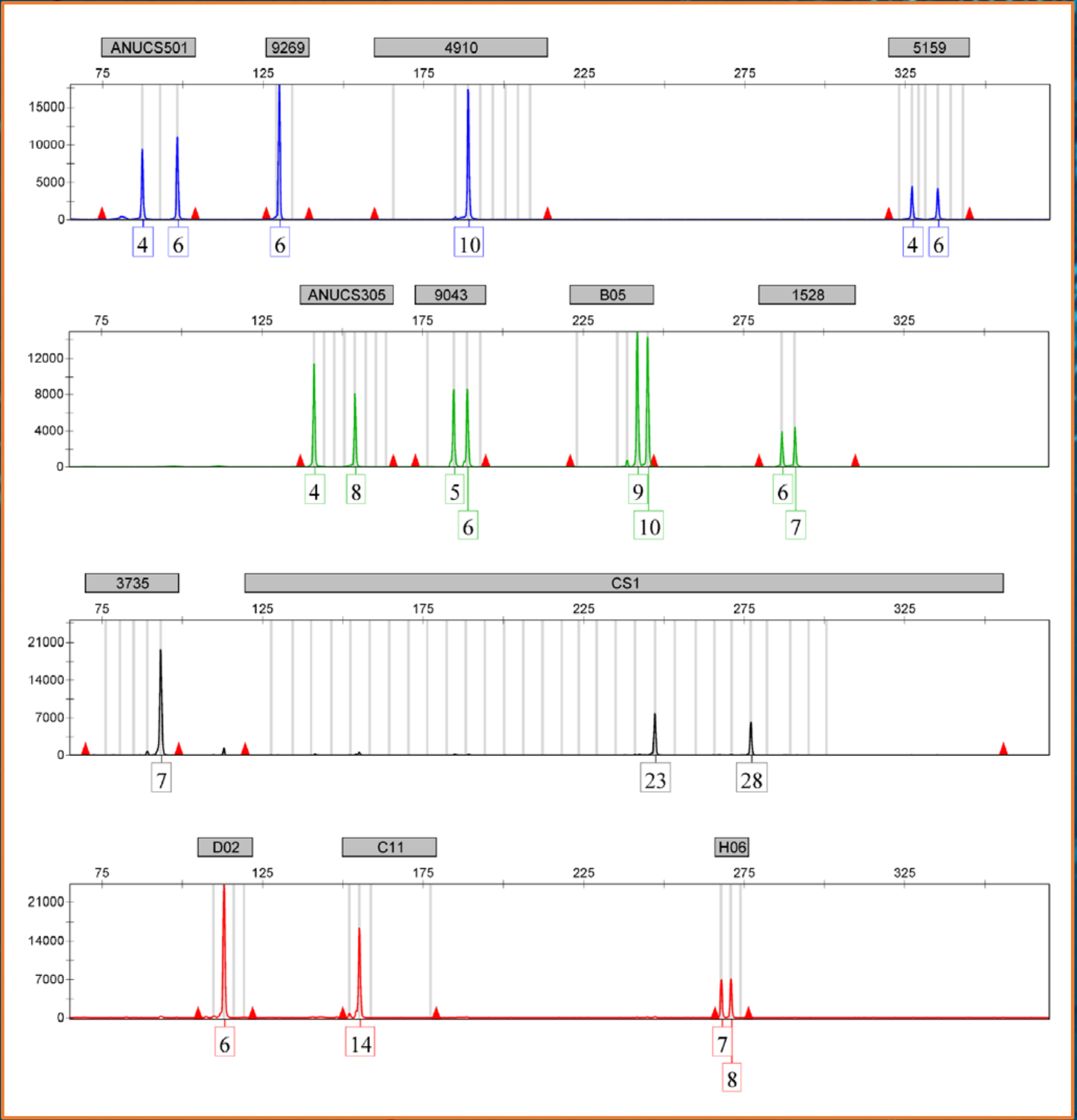
Primer Selection and Titration

Allele Sequencing and Ladder Design

Validation Studies

STR Genotyping

Positive Control



STR Results

- All samples (N=101) successfully amplified:
  - Mixtures (N=5) discarded
  - 2 duplicate genotypes within same seizure found
- 95 distinguishable DNA profiles:
  - 100% full profiles

Statistical Analysis

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

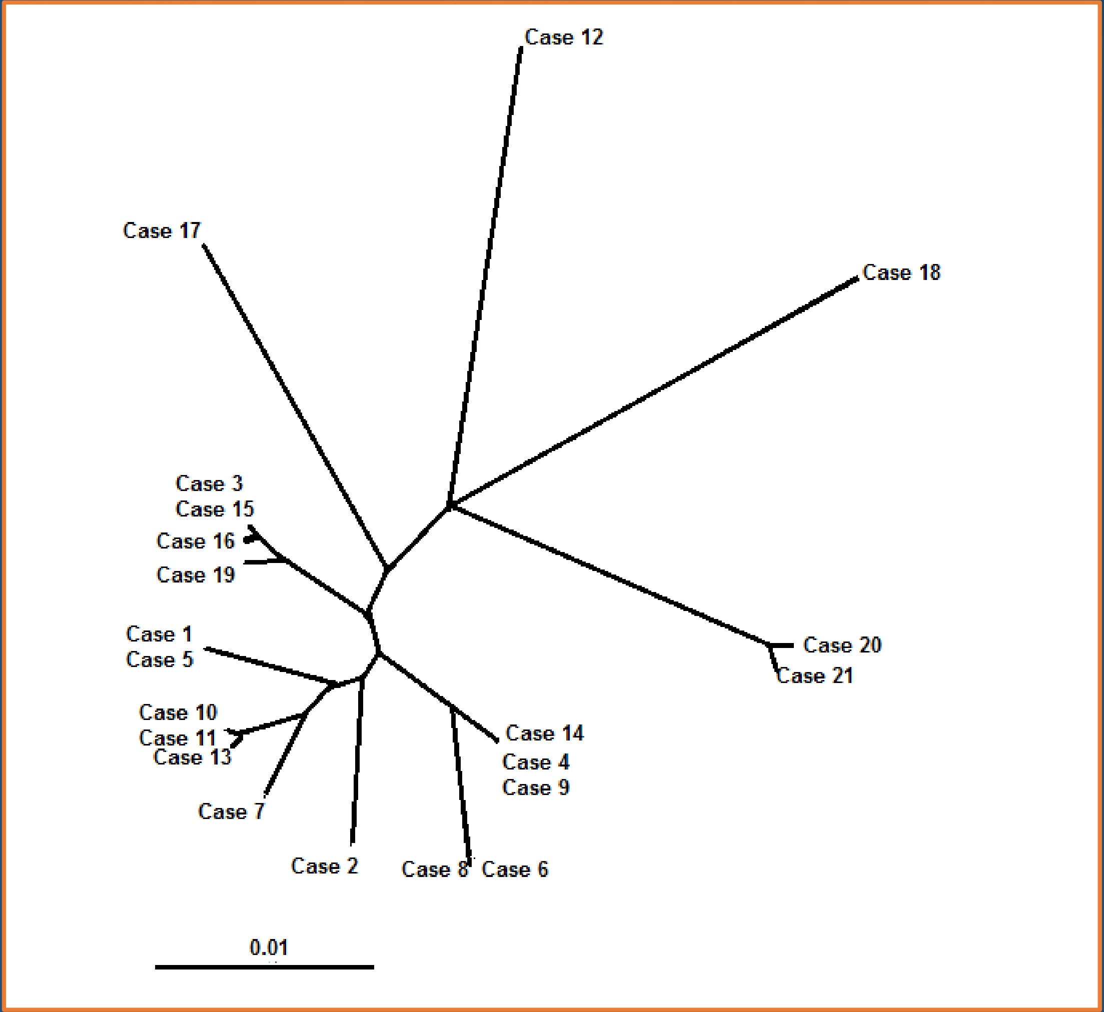
Conclusions

1. High quality profiles with template input as low as 125 pg
2. Negligible cross-reactivity with the 13 STR markers
3. STR success rates improved from previous multiplex (100% vs. 64%)
4. No departures from Hardy-Weinberg
5. One departure from Linkage Equilibrium detected due to genetic drift
6. 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

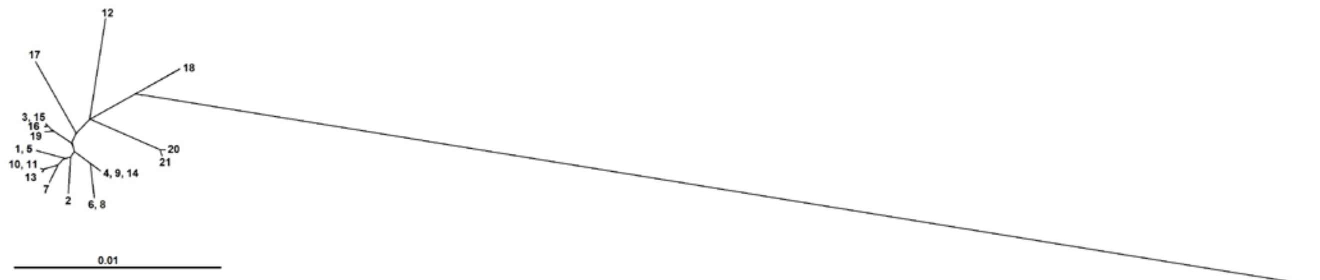
Phylogenetic Analysis



Genetic Distance = F_{st}

Phylogenetic Analysis

Genetic Distance = F_{st}



References

1. Sakamoto K, Akiyama Y, Fukui K, Kamada H, Satoh S. Characterization; Genome Sizes and Morphology of Sex Chromosomes in Hemp (*Cannabis sativa* L.). Cytologia. 1998 Oct; 63: 459 – 64.
2. S. Gilmore and R. Peakall. Isolation of microsatellite markers in *Cannabis sativa L.* (marijuana). Molecular Ecology Notes (2003) 105-7.
3. Alghanim HJ, Almirall JR. Development of microsatellite markers in Cannabis sativa for DNA typing and genetic relatedness analyses. Anal Bioanal Chem. 2003 Aug;376(8):1225-33.
4. Hsieh HM, Hou RJ, Tsai LC, Wei CS, Liu SW, Huang LH, Kuo YC, Linacre A, Lee JC. A highly polymorphic STR locus in *Cannabis sativa*. Forensic Sci Int. 2003 Jan 9;131(1):53-8.
5. Howard C, Gilmore S, Robertson J, Peakall R. Developmental validation of a *Cannabis sativa* STR multiplex system for forensic analysis. J Forensic Sci. 2008 Sep;53(5):1061-7.
6. Miller Coyle H, Shutler G, Abrams S, Hanniman J, Neylon S, Ladd C, Palmbach T, Lee HC. A simple DNA extraction method for marijuana samples used in amplified fragment length polymorphism (AFLP) analysis. J Forensic Sci. 2003 Mar;48(2):343-7.
7. Köhnemann S, Nedele J, Schwotzer D, Morzfeld J, Pfeiffer H. The validation of a 15 STR multiplex PCR for Cannabis species. Int J Legal Med. 2012 Jul;126(4):601-6.
8. Valverde L, Lischka C, Erlemann S, de Meijer E, de Pancorbo MM, Pfeiffer H, Köhnemann S. Nomenclature proposal and SNPSTR haplotypes for 7 new Cannabis sativa L. STR loci. Forensic Sci Int Genet. 2014 Nov;13:185-6.
9. Houston R, Hughes-Stamm S, Gangitano D. Evaluation of a 13-loci STR multiplex system for Cannabis sativa genetic identification. Int J Legal Med. 2016; 130:635-47.

Acknowledgements

- Sam Houston State University:
  - Dr. David Gangitano
  - Dr. Sheree Hughes-Stamm
- Funding: NIJ GRF (2015-R2-CX-0030)

Sam Houston
State University

FSF Emerging Forensic Scientist Award
Paper Presentation

Questions?

Rachel Houston

rmh034@shsu.edu

Sam Houston State University