Evaluation of a 13-loci STR multiplex System for *Cannabis sativa* genetic Identification

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

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

68th Annual AAFS, Las Vegas, 2016

Sam Houston State University

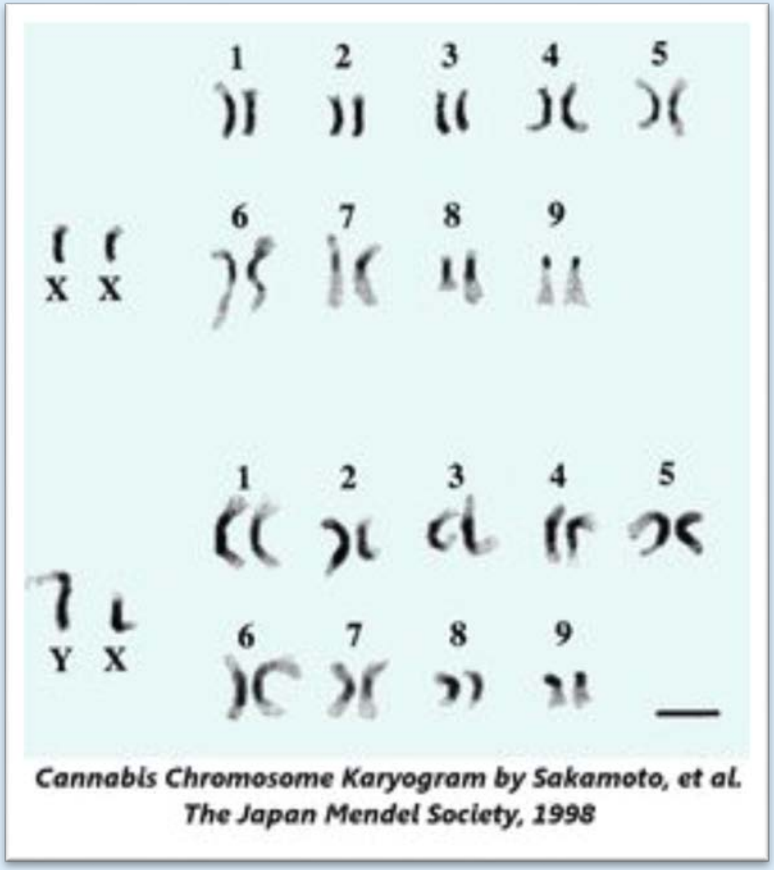
FSF Emerging Forensic Scientist Award
Oral Presentation

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
- The authors declare no competing interest

MARIJUANA BACKGROUND

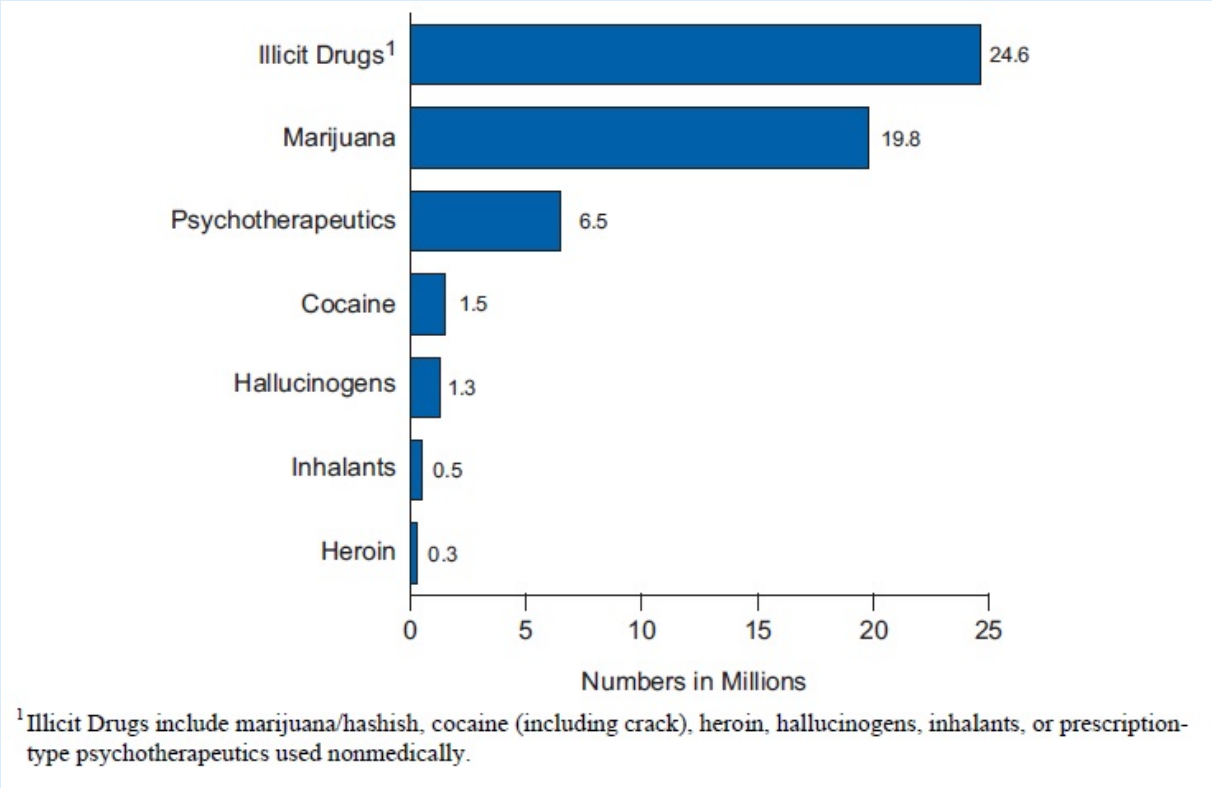
- Plant material from *Cannabis sativa*
- Family: Cannabaceae
- Genus: *Cannabis*
- Species: *Cannabis sativa*
- Diploid genome (2n = 20)
  - 9 pairs of autosomes
  - Pair of sex chromosomes



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

SIGNIFICANCE

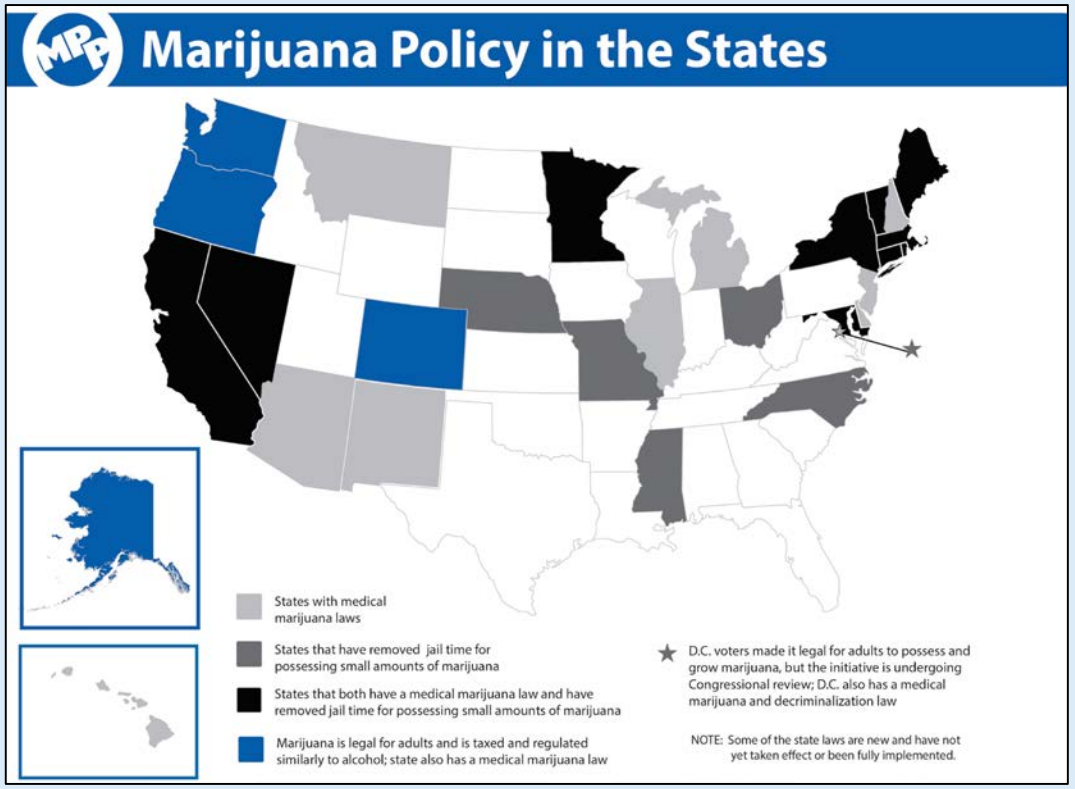
- Marijuana is the most commonly used illicit drug in United States



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

MARIJUANA LEGALIZATION

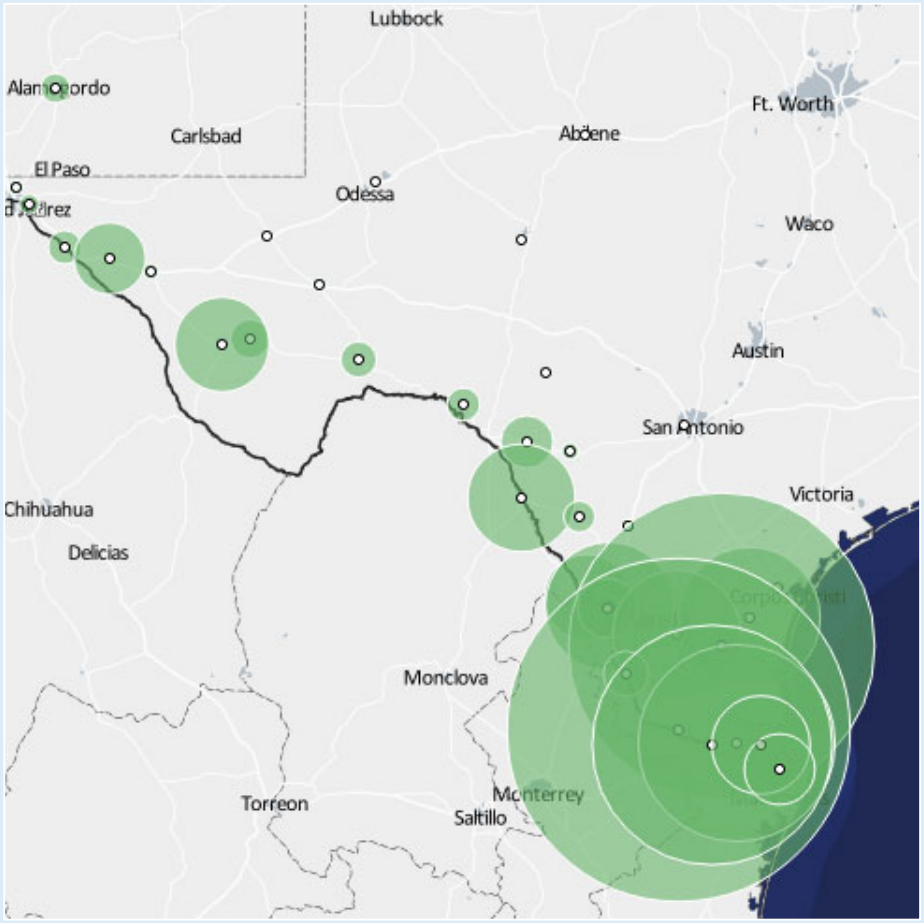
- Legalized for recreational use in:
  - Colorado
  - Washington
  - Alaska
  - Oregon
  - District of Columbia



Marijuana policy in the States (http://www.mpp.org/states/; 2015)

ILLEGAL TRAFFIC AT US – MEXICO BORDER

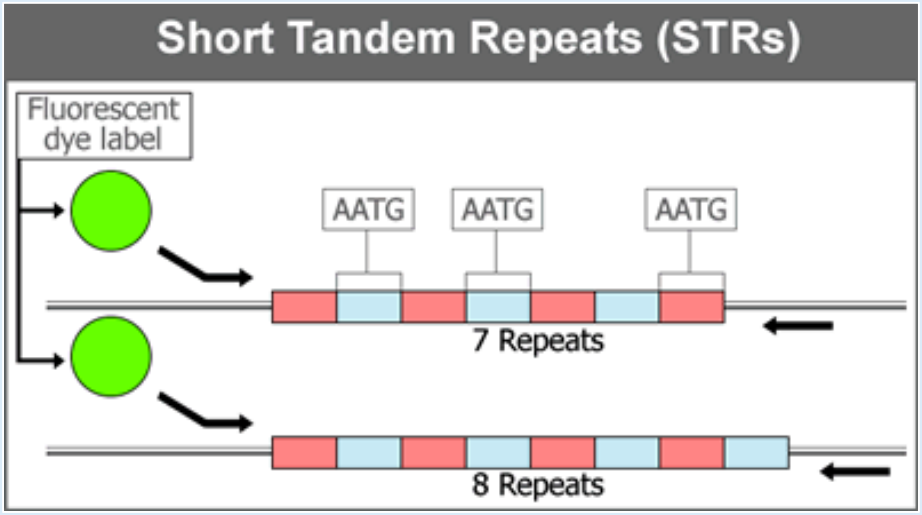
- ~89% drug seizures at border are marijuana
- Serious concern for US Federal government



http://static.apps.cironline.org/border-seizures/img/OGScreenshot.jpg

DNA BASED INDIVIDUALIZATION

- Chloroplast DNA (CpDNA)
- Mitochondrial DNA (MtDNA)
- **Short Tandem Repeat (STR)**
  - Gold standard in human identification



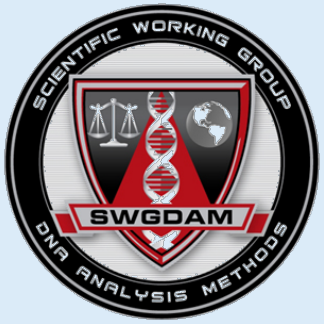
http://rosalind.info/media/microsatpcr2.gif

PREVIOUS STR RESEARCH

- Polymorphic STR markers first described (Gilmore and Peakall (2003); Alghanim and Almirall (2003); Hsieh *et al*. (2003))
- Australia: Marijuana DNA STR multiplex and database (Howard *et al. (2008)*)
- United States: CS1 marker study (Miller Coyle *et al.* (2003))
- Germany: 15 loci - STR tool (Köhnemann *et al. (2012)*); Proposed new tetranucleotide markers (Valverde *et al.* (2014))

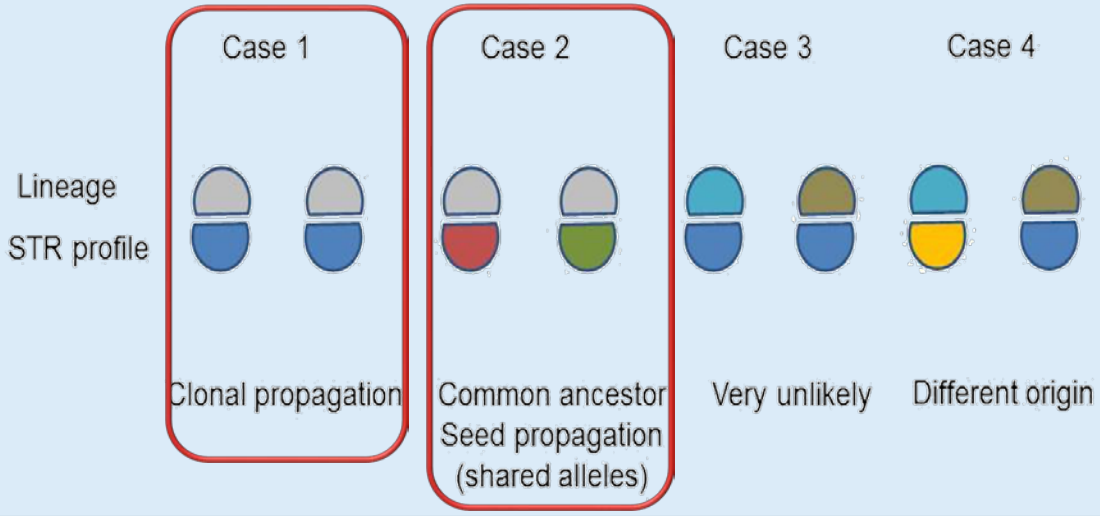
LIMITATIONS

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

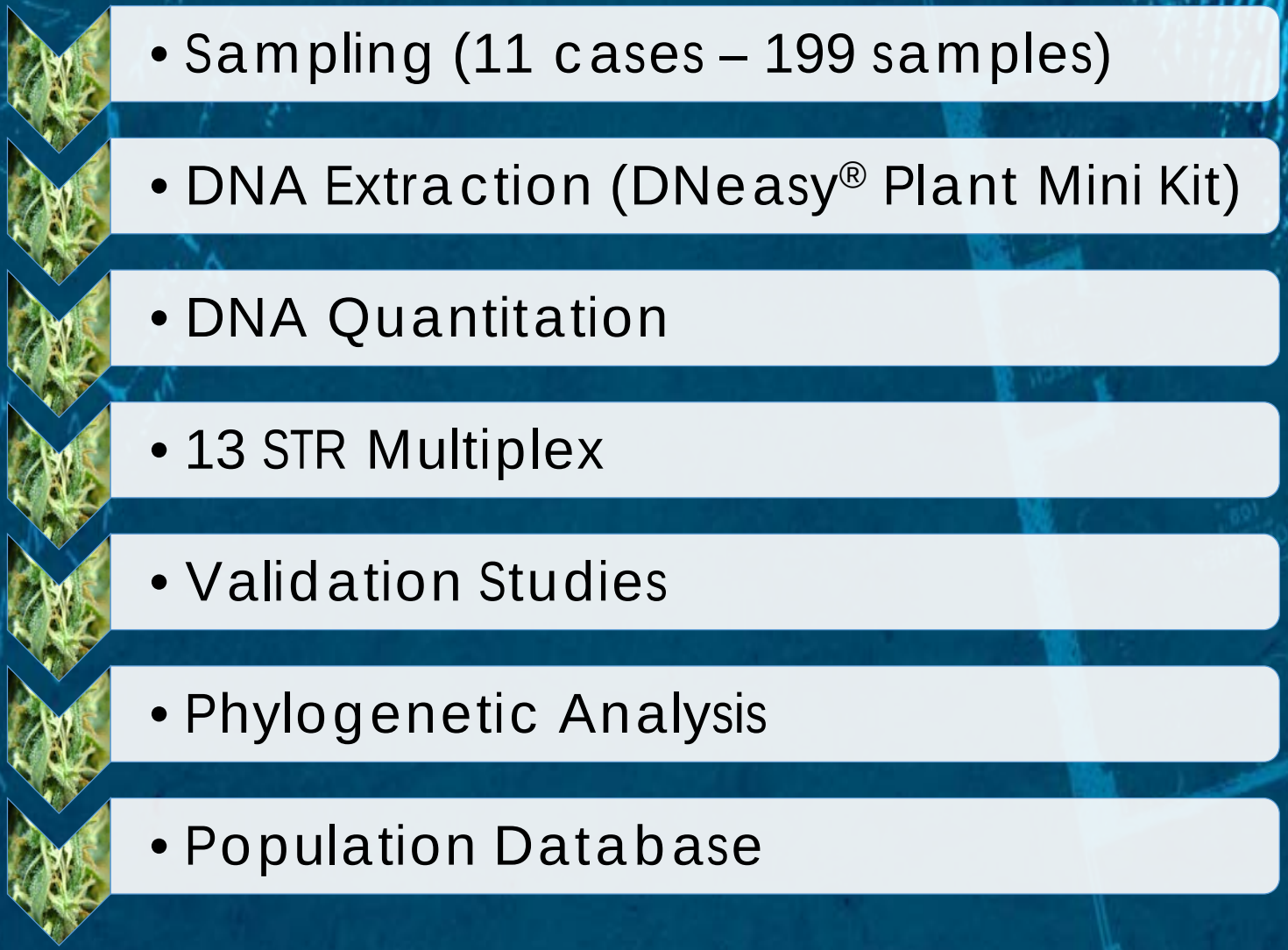


PURPOSE AND GOALS

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



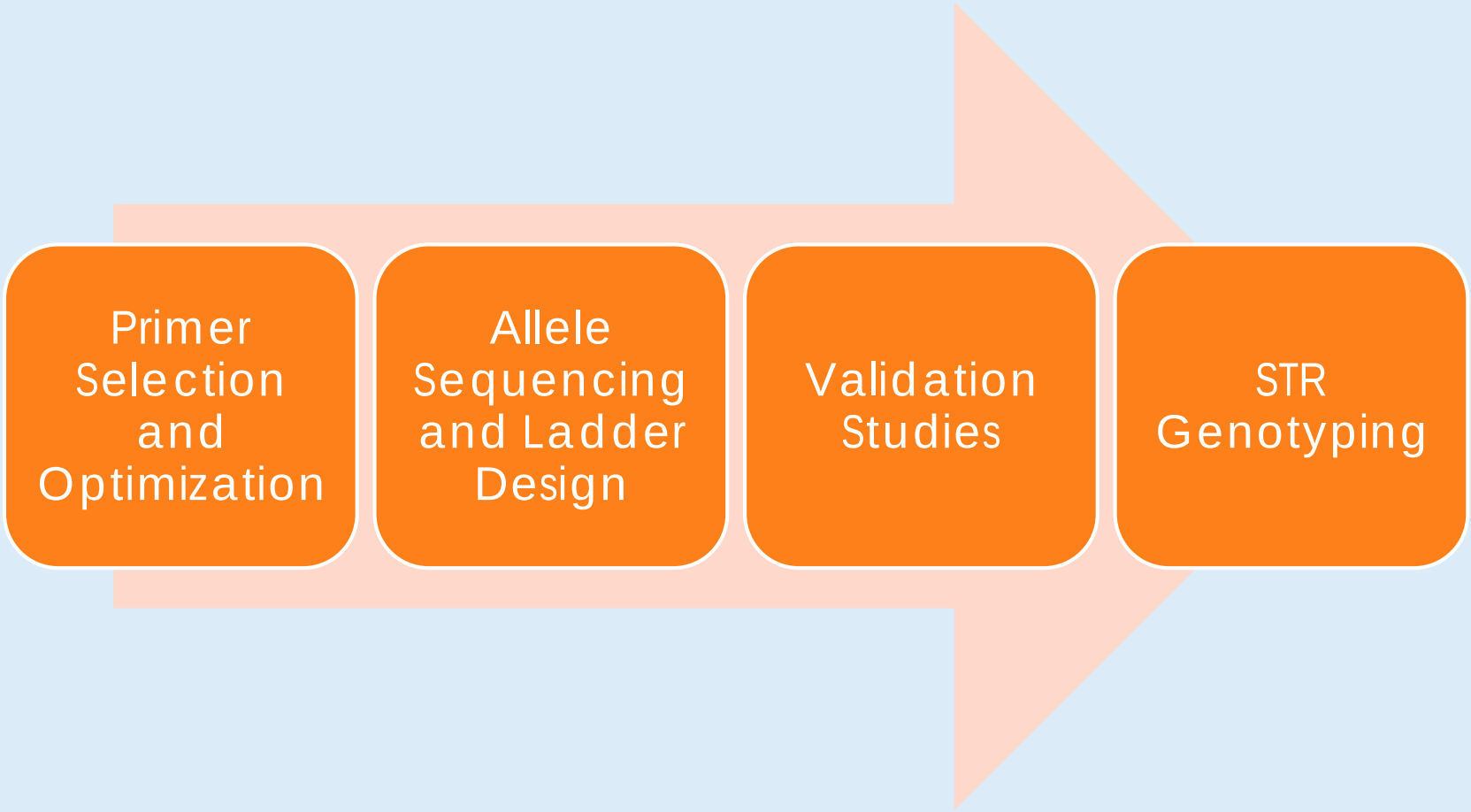
MATERIALS AND METHODS


• Sampling (11 cases – 199 samples)
• DNA Extraction (DNeasy® Plant Mini Kit)
• DNA Quantitation
• 13 STR Multiplex
• Validation Studies
• Phylogenetic Analysis
• Population Database

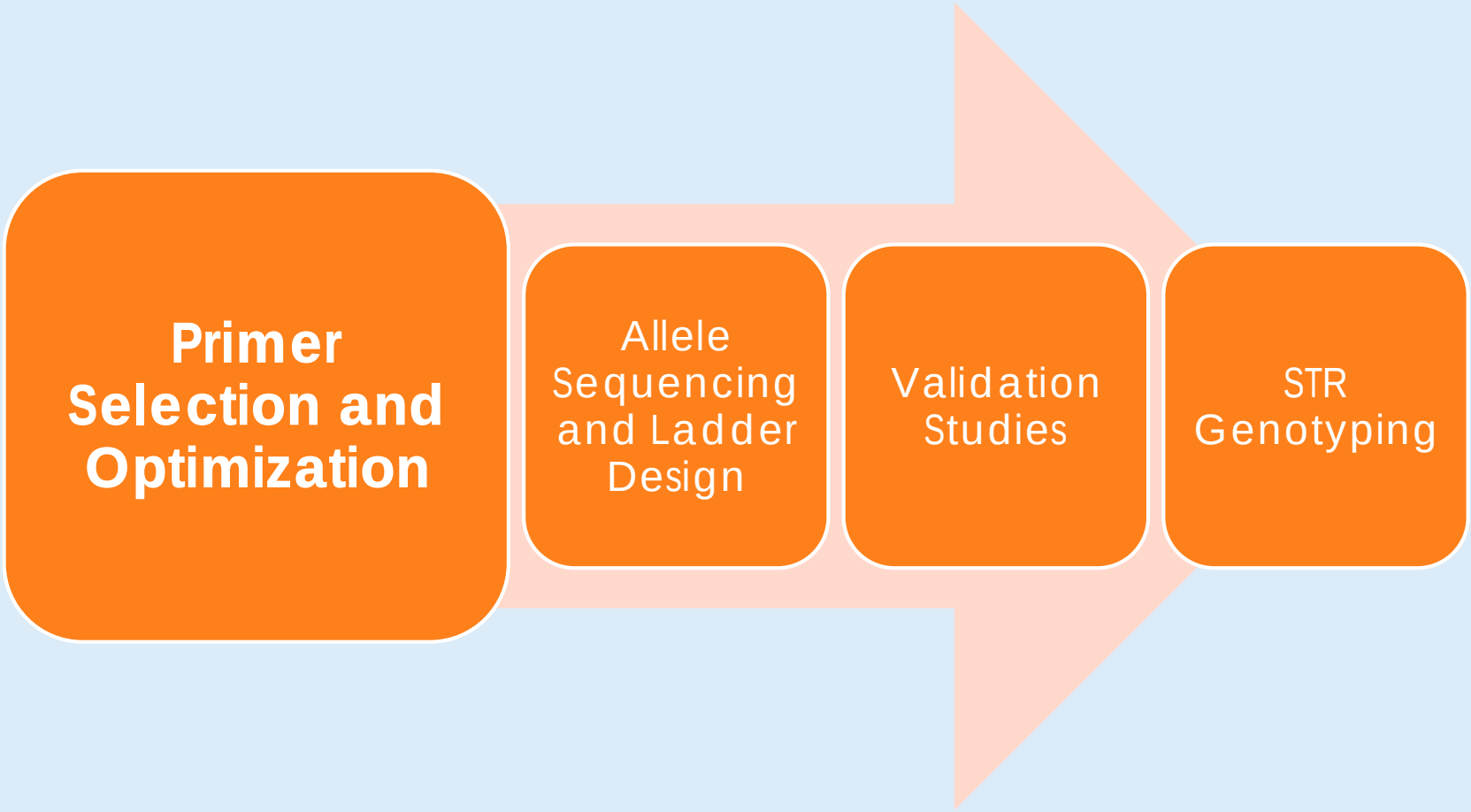
DNA QUANTITATION

- Development of real-time quantification 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



13 STR MULTIPLEX



PRIMER SELECTION

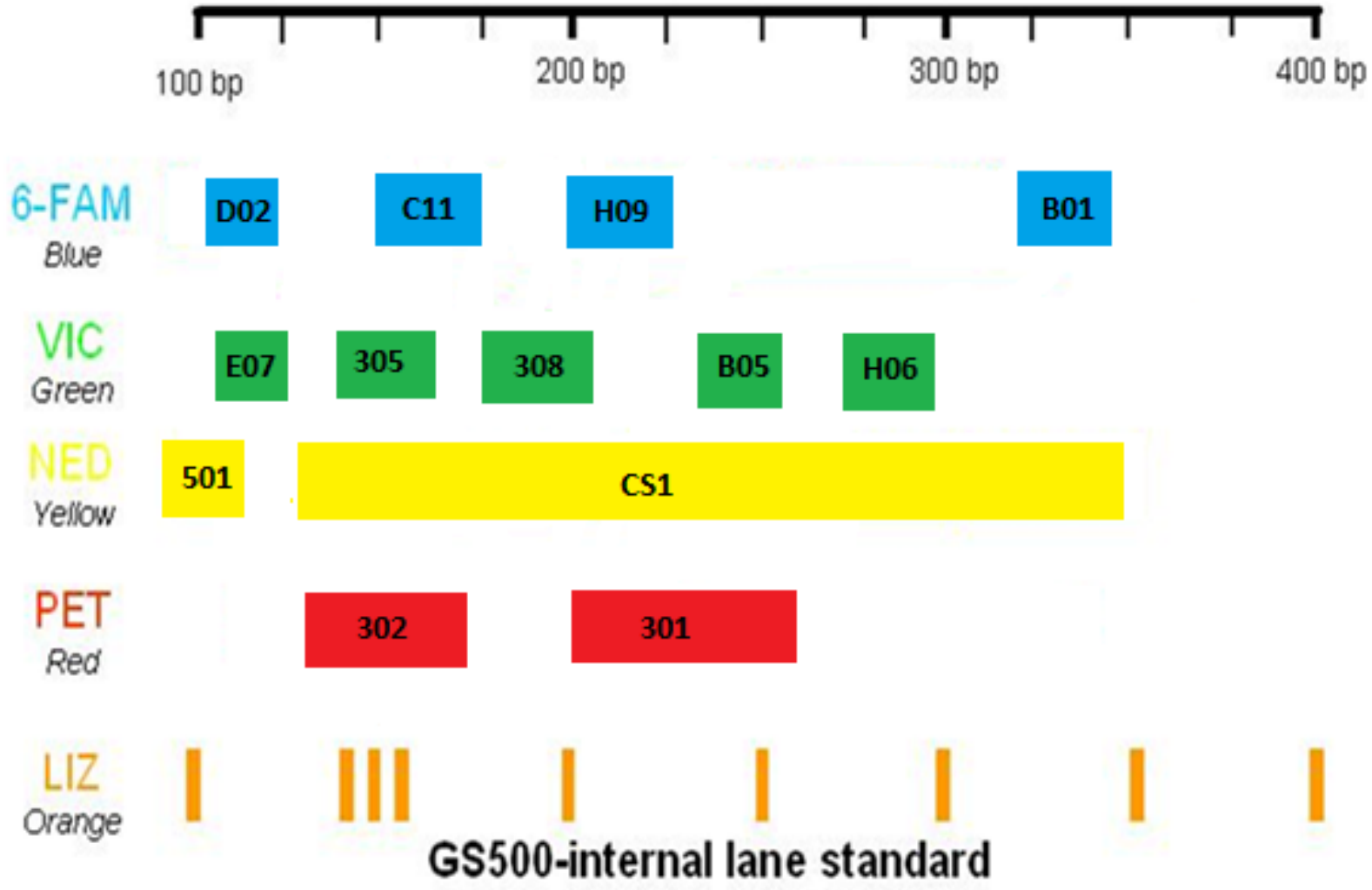
Type	Name
Dinucleotide	H09, ANUCS308
Trinucleotide	E07, D02, B05, B01, ANUCS301, ANUCS305, H06, ANUCS302, C11
Pentanucleotide	ANUCS501
Hexanucleotide	CS1

MULTIPLEX OPTIMIZATION

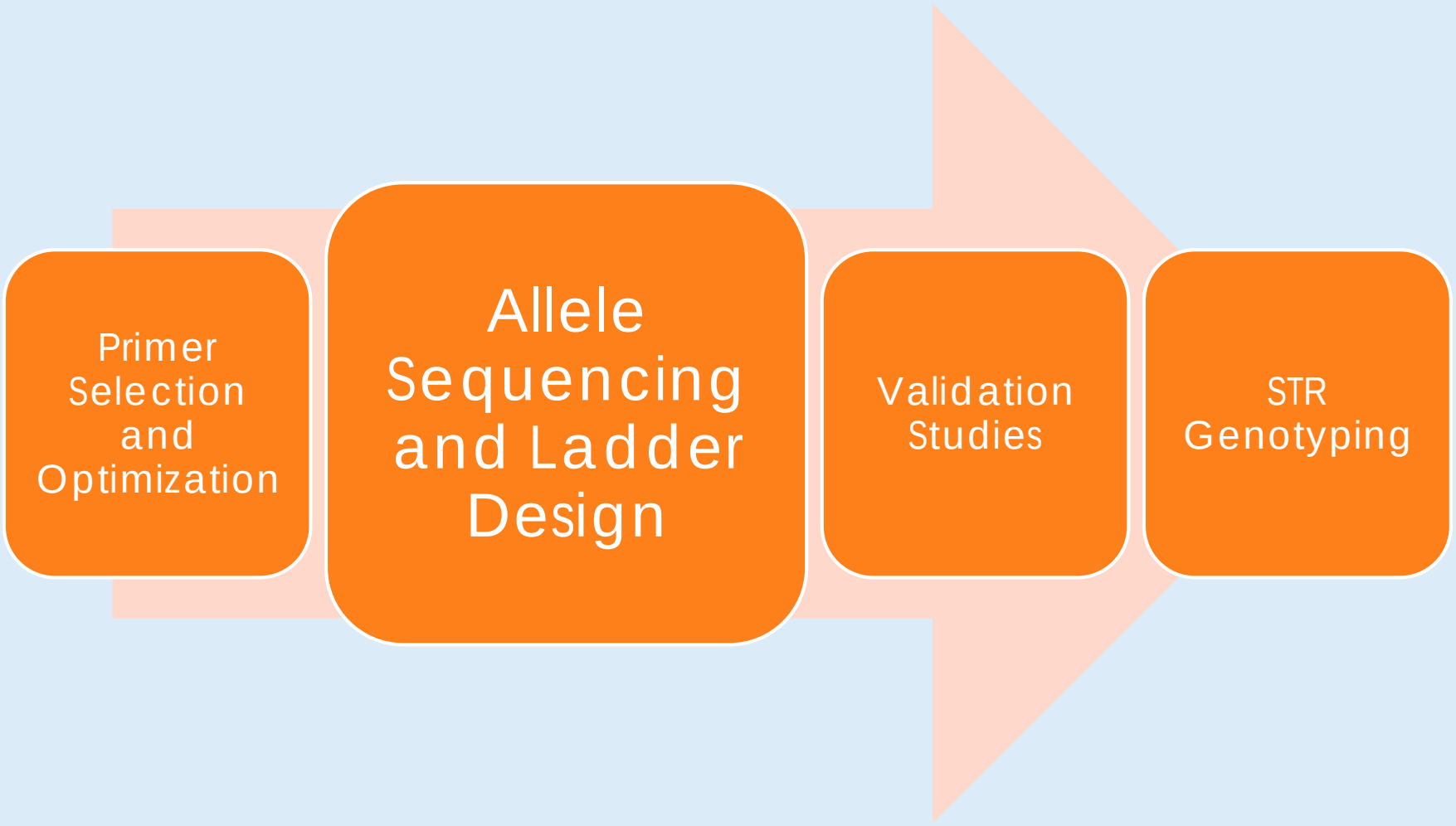
- Multiplex manager software, using published forward and reverse primer sequences, to detect potential primer-primer interactions
- Primer titration for multiplex
  - Type-IT® Microsatellite PCR Kit (QIAGEN)

http://www.selectscience.net/images/products/QIAGEN_ACTIVE_71.jpg.ashx?width=270&height=268

FINAL 13-PLEX



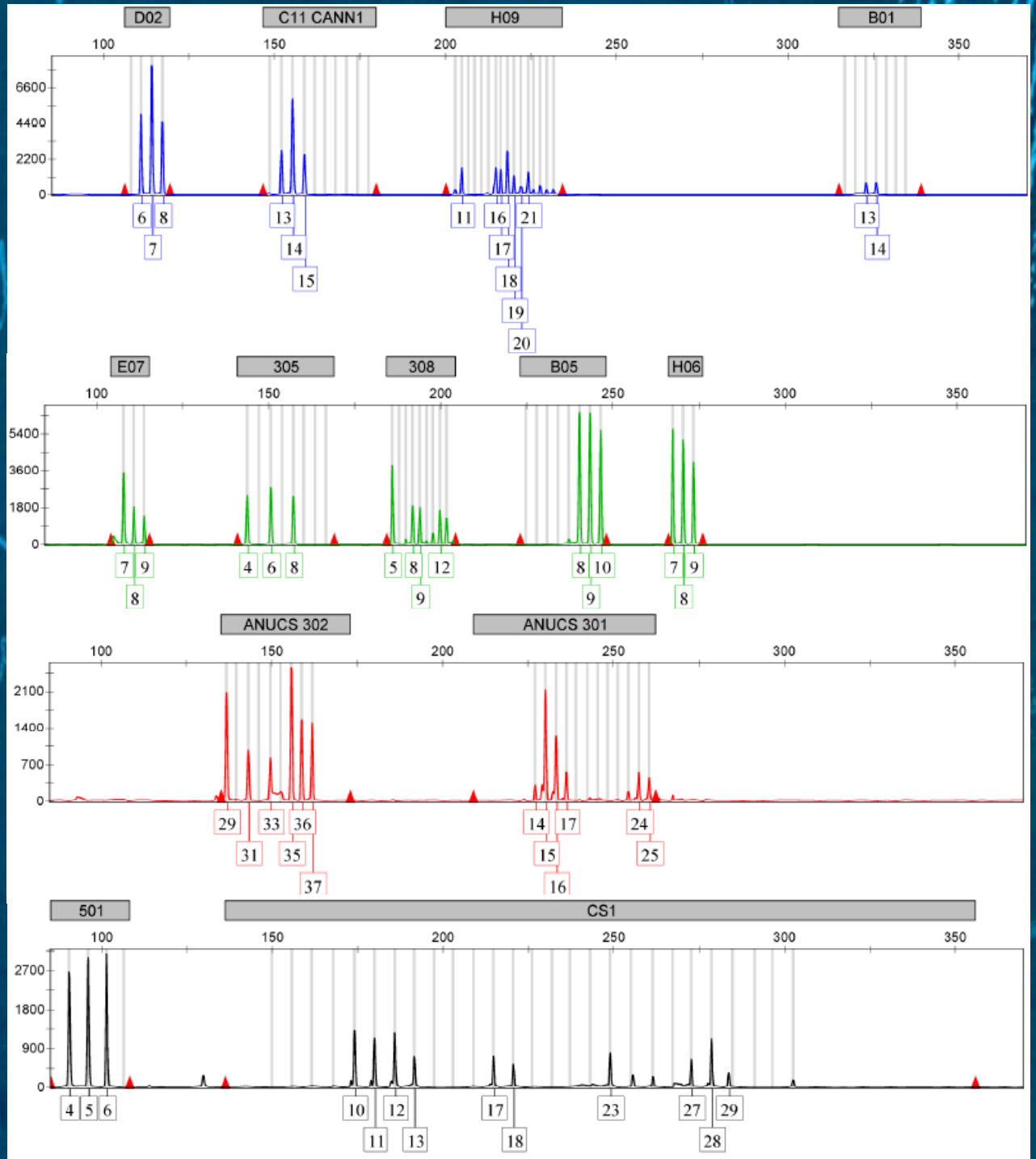
13 STR MULTIPLEX



SEQUENCING/ALLELIC LADDER

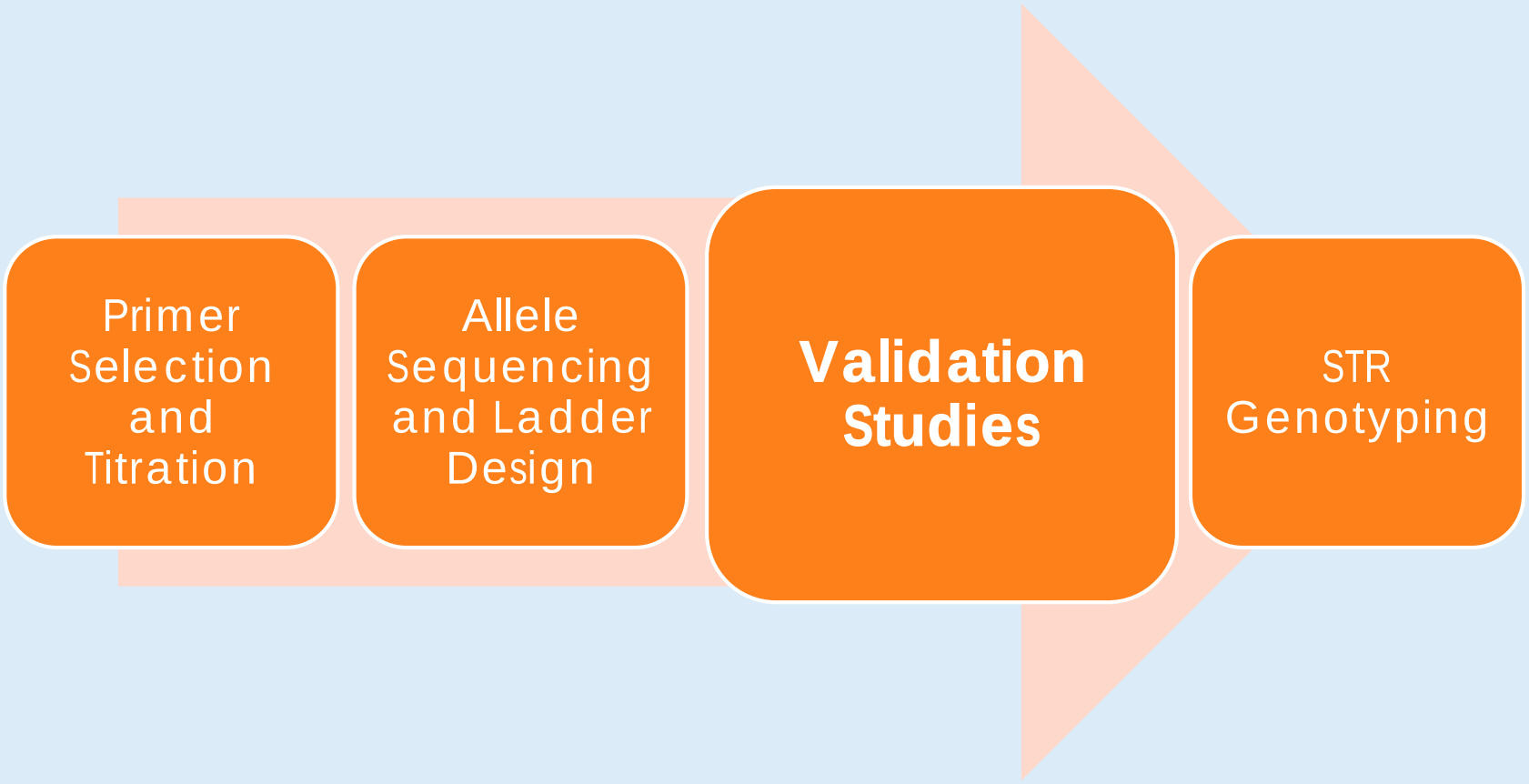
- Two to eight alleles per marker
- Big Dye Direct® Cycle Sequencing Kit
- Centri-Sep™ purification columns
- Geneious Pro Software
- Allelic Ladder Design

ALLELIC LADDER



NEW!

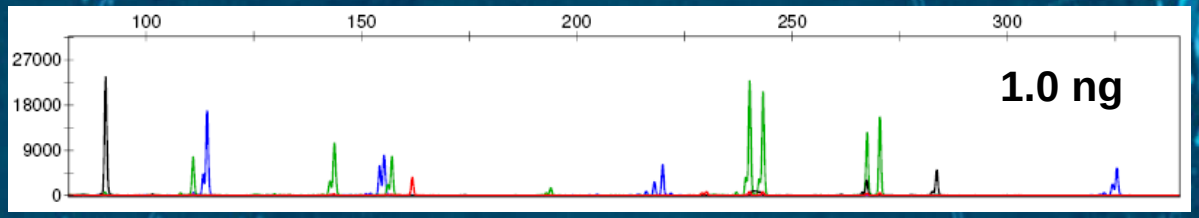
13 STR MULTIPLEX

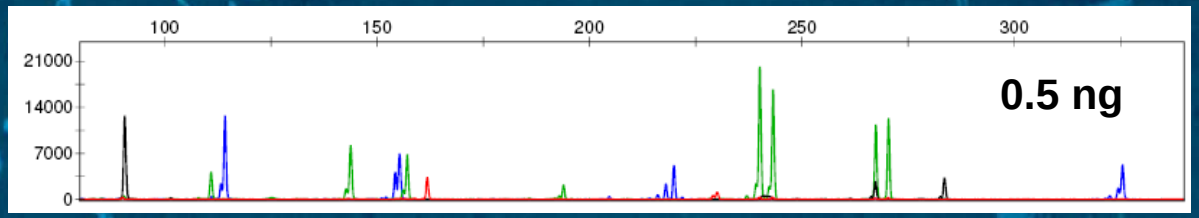


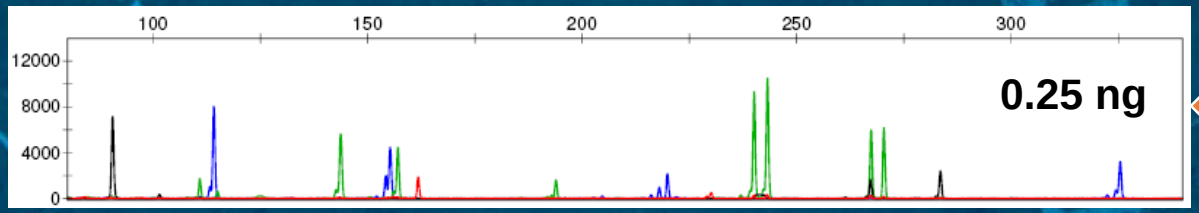
VALIDATION STUDIES

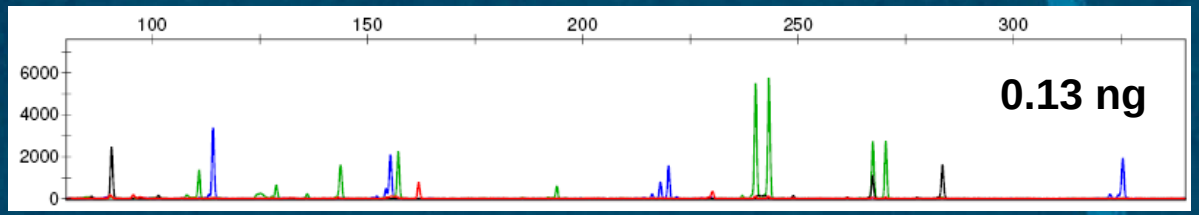
- The optimized developed system was validated according to SWGDAM guidelines:
  - Sensitivity
    - Dynamic range of assay (1.0 ng – 31.2 pg)
  - Species specificity
    - Plants (*Humulus lupulus* and *Nicotiana tabacum*)
    - Animals
    - Human

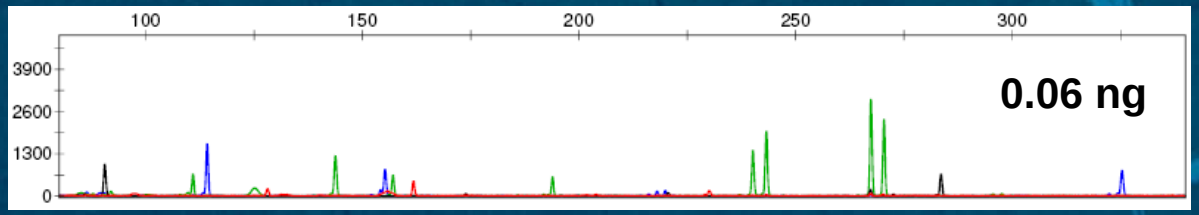
SENSITIVITY

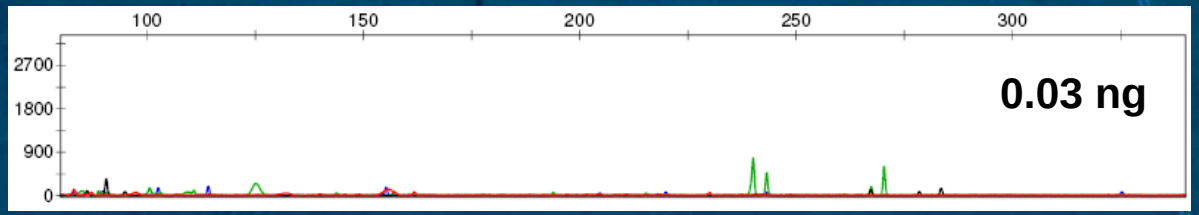




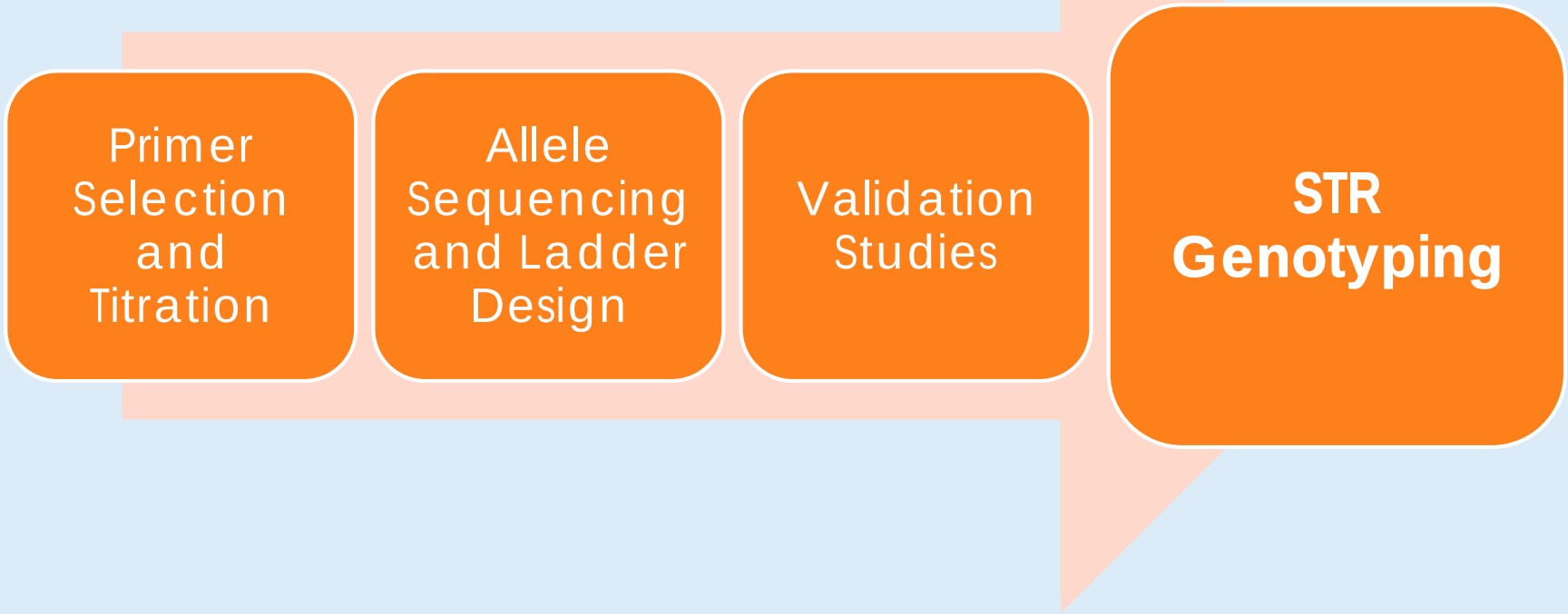








13 STR MULTIPLEX



STR GENOTYPING

Extraction (N =199)
Dneasy Plant Mini Kit

↓

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

↓

PCR (Type-It® Microsatellite Kit)
Ependorf MasterCycler Gradient

↓

Electrophoresis
ABI 3500 Genetic Analyzer

↓

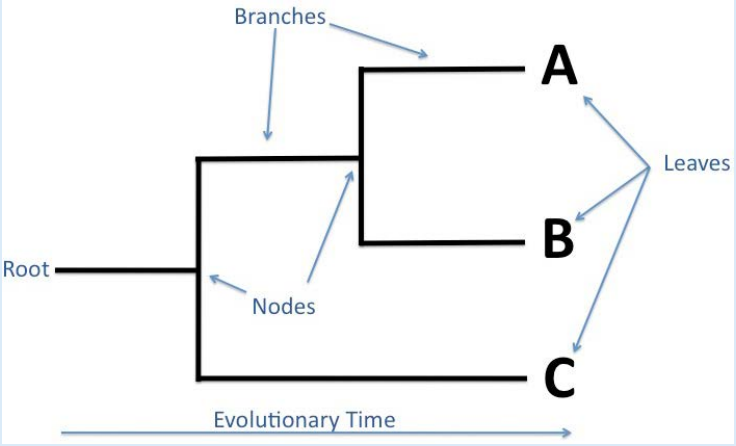
Genemapper® v 4.1 analysis

STR RESULTS

- Determine number of multi-locus genotypes and genotype sharing among samples
  - Only 4 duplicate genotypes within seizures were found
- 127 samples generated full profiles (64%)
  - 36% partial profiles (Locus dropout: ANUCS308, ANUCS302, ANUCS301, B01)

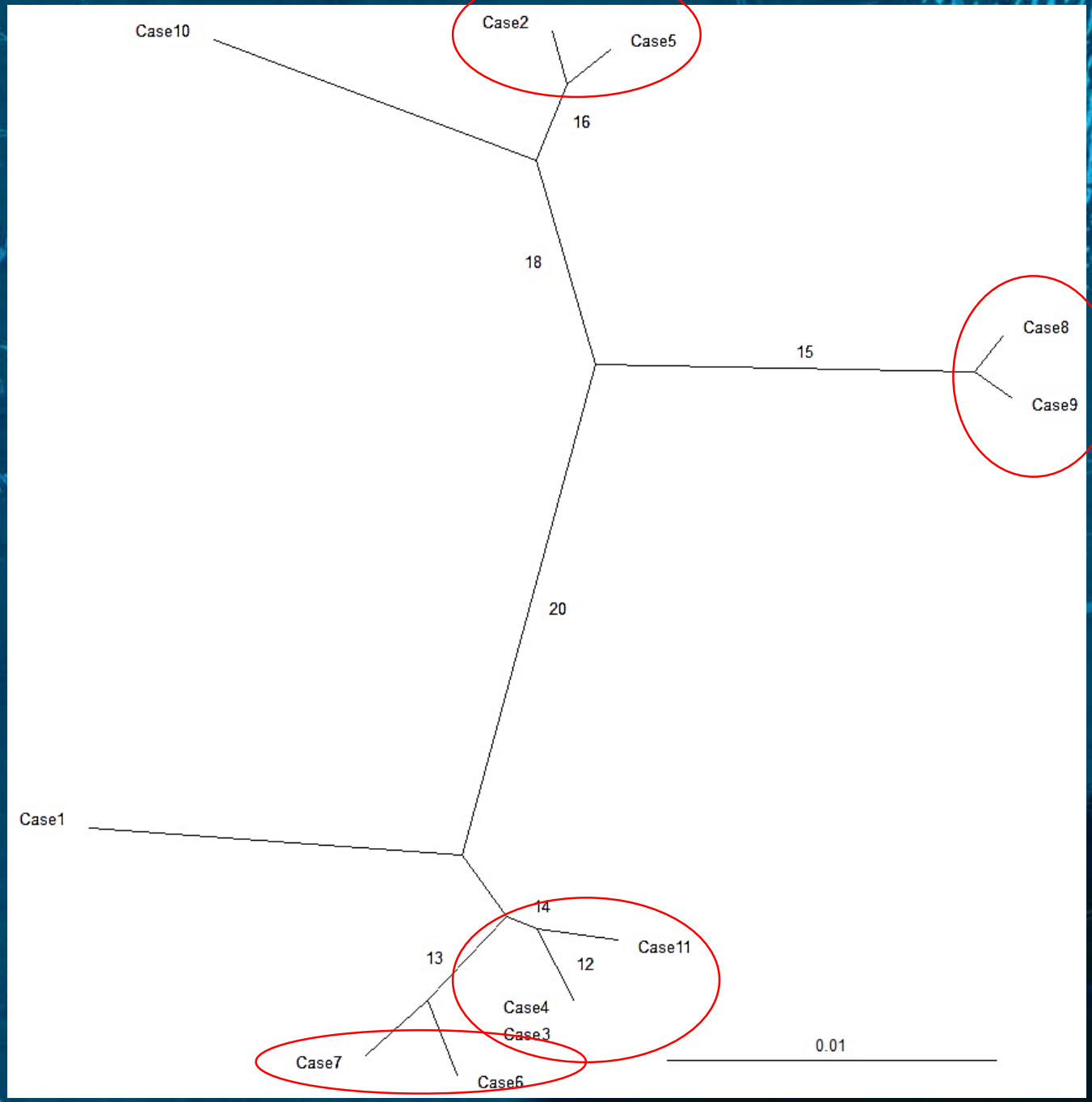
PHYLOGENETIC

- UPGMA method with coefficient of ancestry *Fst* as genetic distance (GDA software)
- Genetic association confirmed with Arlequin software
- Screen for the presence of homogeneous sub-populations among samples



https://arthropoda.files.wordpress.com/2010/01/phylogenies1.jpg

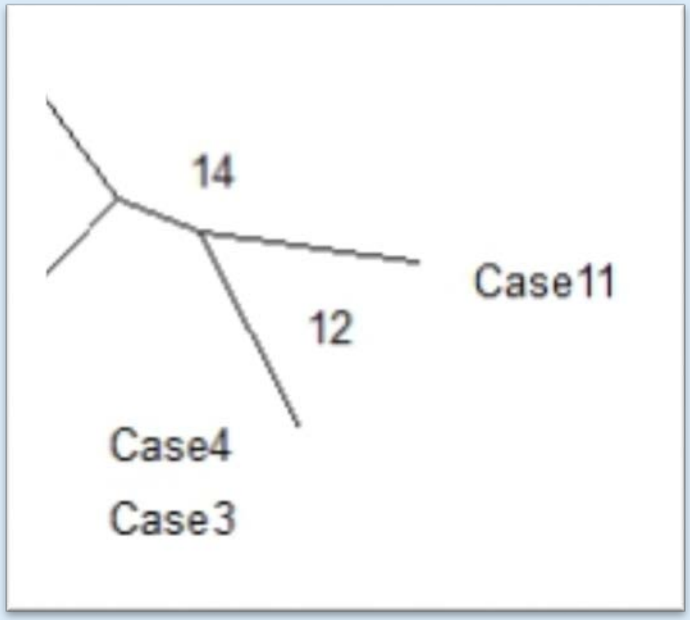
UPGMA Method, GDA



NEW!

HOMOGENOUS SUB-POPULATION

- Genetic association of three pairs of cases
  - Subset of samples (N=97): homogeneous subpopulation (low *Fst*) in Hardy – Weinberg equilibrium and linkage equilibrium (GDA).



GDA: 95% confidence interval bootstrapping; *Fst* = 0

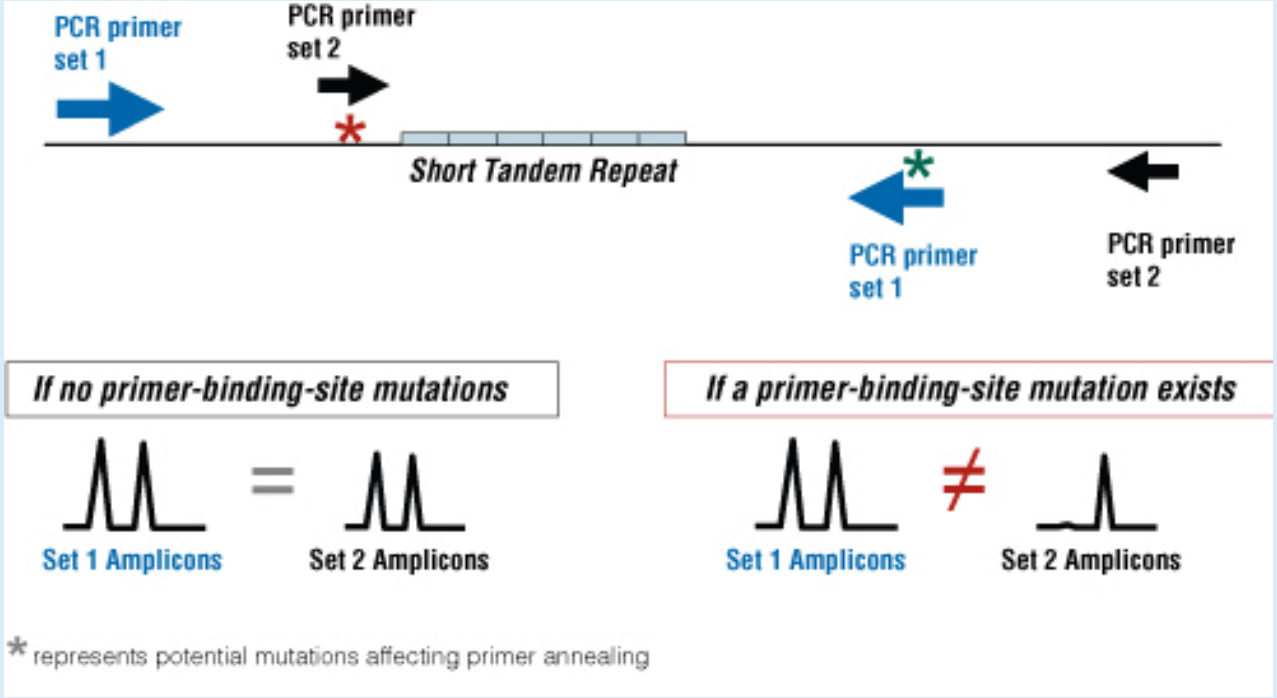
REFERENCE POPULATION DATABASE

- Population genetic statistics (PowerStats v1.2) and parameters of forensic interest:
  - Allele Frequencies
  - Hardy-Weinberg Equilibrium
  - Screen for null alleles (GENEPOP)
  - Random match probability
  - Power of Discrimination

The combined power of discrimination of this multi-locus system was 1 out of 70 million.

NULL ALLELE ANALYSIS, GENEPOP

- Null alleles detected in ANUCS308, ANUCS302, ANUCS301, B01
- Primer-binding interactions

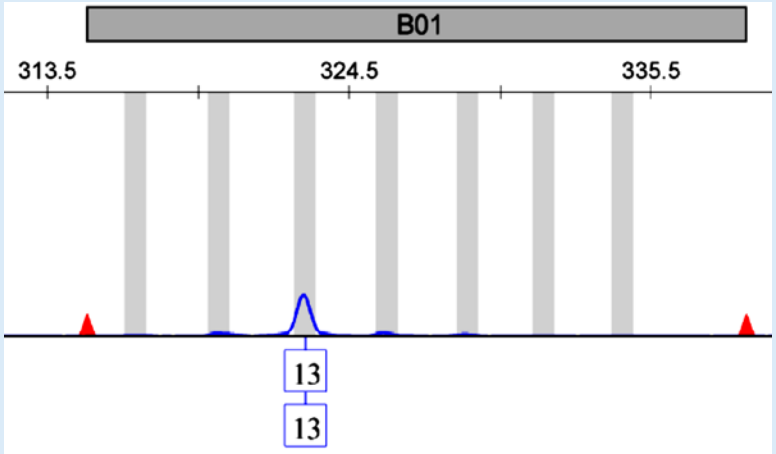


http://www.promega.com/~/media/images/resources/figures/profiles%20in%20dna/1301/hillfig01.jpg?la=en

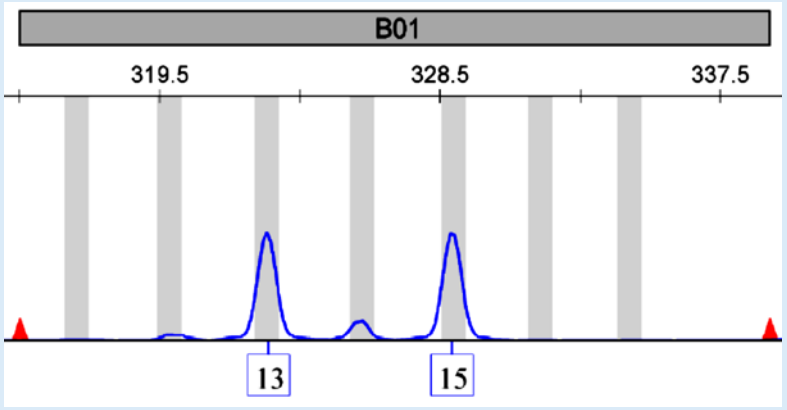
ADDITIONAL STUDIES WITH NULL ALLELES

- True annealing temperatures determined
  - All less than 60ºC
- Recovery of heterozygote peaks at true T_m
  - Less stringent binding

60ºC



55ºC



CONCLUSIONS

1. Development of a real-time PCR quantitation system (calibration curve showing $r^2 = 0.99$ + melting curve showing primer specificity)
2. Evaluation of 13-loci STR multiplex + ISFG recommendations
3. Detection of null alleles
4. Phylogenetic tree
5. Reference population database

POTENTIAL IMPACT

- Provide forensic community 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

ACKNOWLEDGEMENTS

- Sam Houston State University:
  - Dr. David Gangitano
  - Dr. Sheree Hughes – Stamm
  - Lori Powe
- Travel funded by NIJ GRF (2015-R2-CX-0030)

Sam Houston State University

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.

FSF Emerging Forensic Scientist Award
Oral Presentation

Questions?

Rachel Houston
rmh034@shsu.edu

Sam Houston State University