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

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Oral Presentation
• 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

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

[Image: http://rosalind.info/media/microsatpcr2.gif]
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-SWG DAM 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

NEW!
• 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

- Primer Selection and Optimization
- Allele Sequencing and Ladder Design
- Validation Studies
- STR Genotyping
13 STR Multiplex

- Primer Selection and Optimization
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<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
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<tbody>
<tr>
<td>Dinucleotide</td>
<td>H09, ANUCS308</td>
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<tr>
<td>Trinucleotide</td>
<td>E07, D02, B05, B01, ANUCS301, ANUCS305, H06, ANUCS302, C11</td>
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<tr>
<td>Pentanucleotide</td>
<td>ANUCS501</td>
</tr>
<tr>
<td>Hexanucleotide</td>
<td>CS1</td>
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</table>
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)

FINAL 13-PLEX

6-FAM (Blue):
- D02
- C11
- H09
- B01

VIC (Green):
- E07
- 305
- 308
- B05
- H06

NED (Yellow):
- 501
- CS1

PET (Red):
- 302
- 301

LIZ (Orange):
- GS500-internal lane standard
13 STR Multiplex

Primer Selection and Optimization

Allele Sequencing and Ladder Design

Validation Studies

STR Genotyping
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
13 STR MULTIPLEX

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

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

- Extraction (N = 199) using Dneasy Plant Mini Kit
- DNA Quantitation (SYBR™ Green) using StepOne Real Time PCR Instrument
- PCR (Type-It® Microsatellite Kit) using Ependorf MasterCycler Gradient
- Electrophoresis using ABI 3500 Genetic Analyzer
- Genemapper® v 4.1 analysis
POSITIVE CONTROL

NEW!
• 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: ANUC S308, ANUC S302, ANUC S301, B01)
• UPGMA method with coefficient of ancestry $F_{st}$ 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
• 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
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 alleles detected in ANUCS308, ANUCS302, ANUCS301, B01

Primer-binding interactions

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

60°C

55°C
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
• 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
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Questions?

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