Y-STRs, mtDNA, and the Romanov Case

**NYC OCME
Dept of Forensic Biology**

New York City, NY
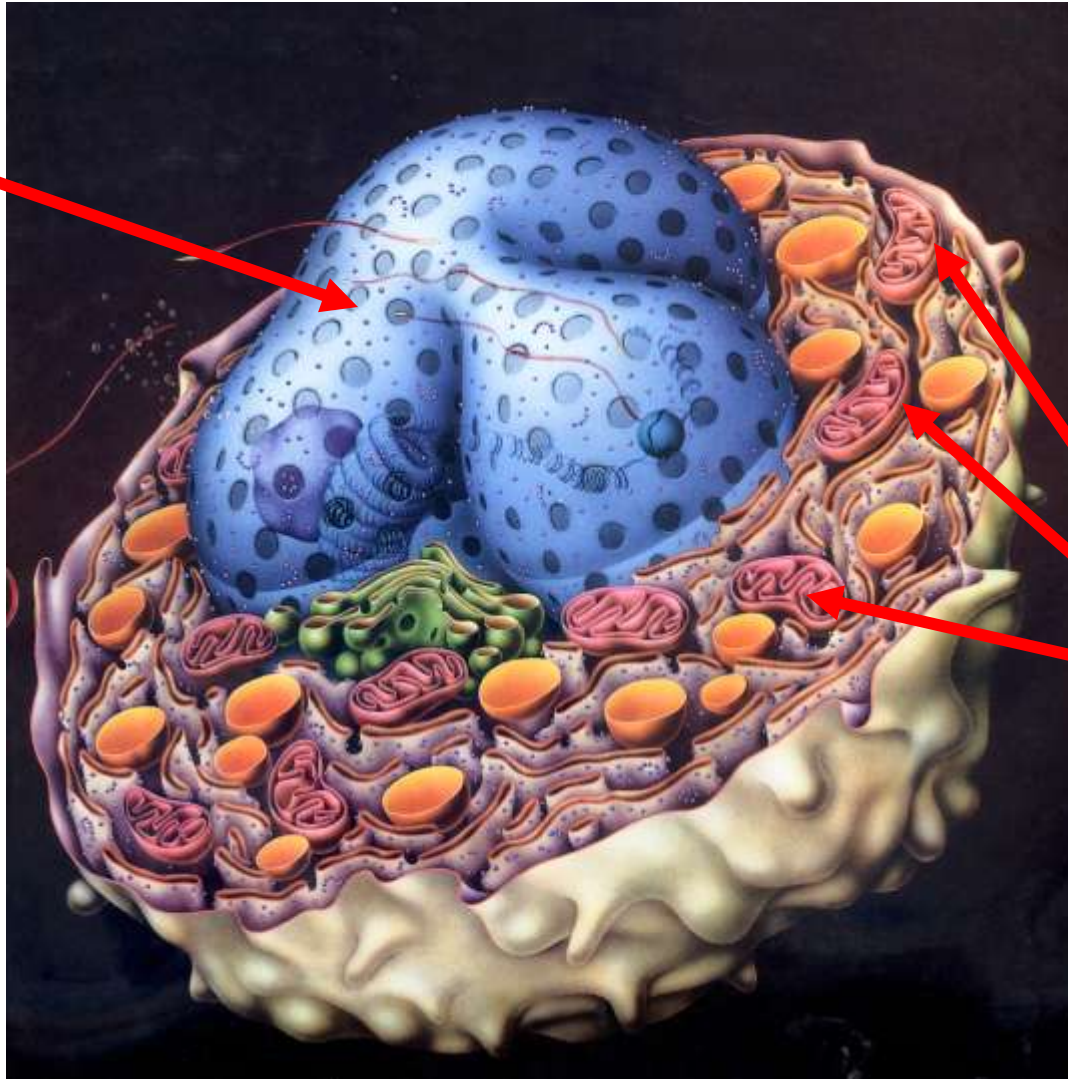**April 18, 2012**

Dr. Michael D. Coble
National Institute of Standards and Technology

michael.coble@nist.gov

The Human DNA Genome

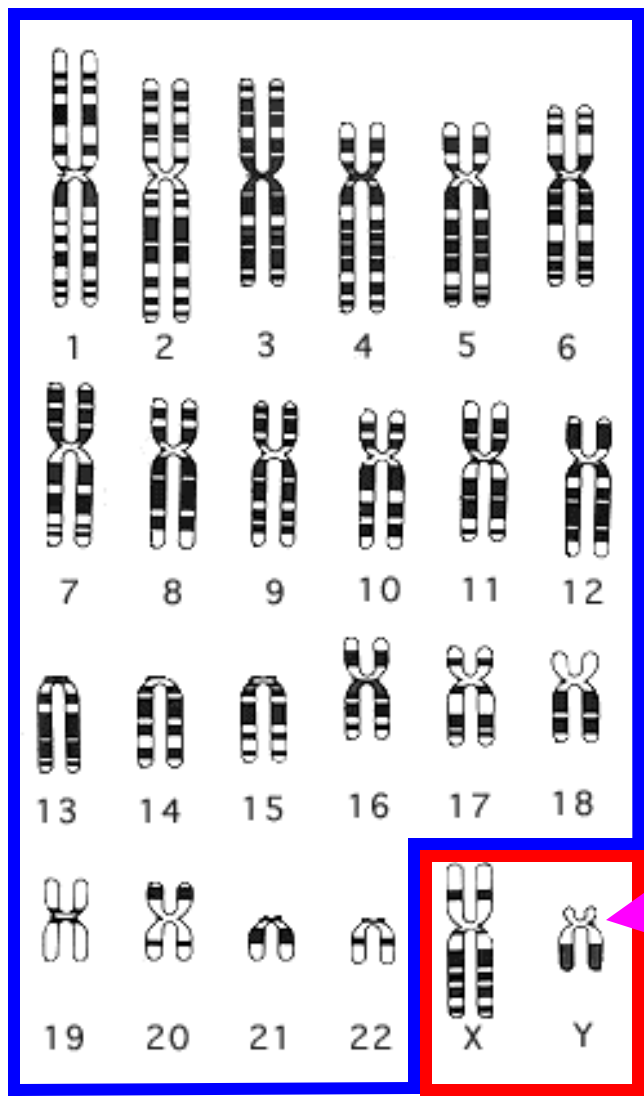

Nuclear DNA

~3.2 billion bp

High Power of Discrimination

Mitochondrial DNA

16.5 Kbp

High Copy #
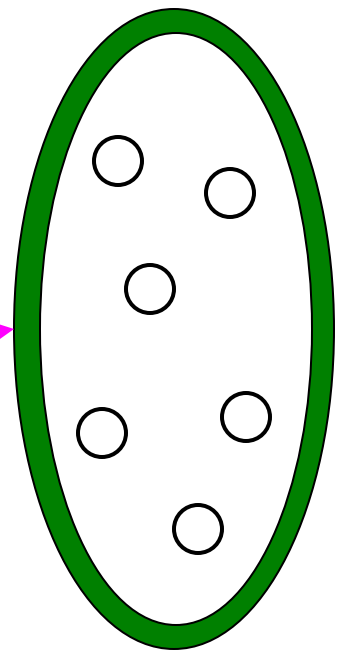
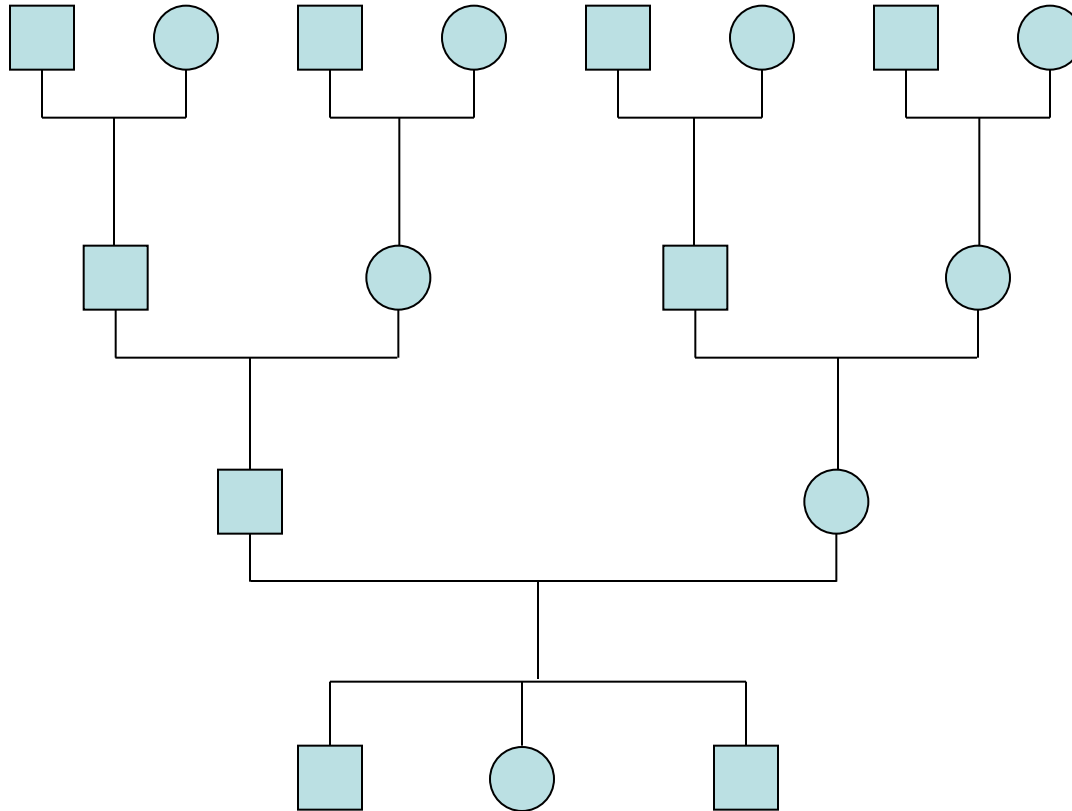# Cell Nucleus – 3.2 billion bp

Autosomes – 22 pairs – 2 copies per cell

Sex Chromosomes (XX or XY)

mitochondria – in cell cytoplasm
100s of mtDNA copies per cell

Lineage Markers
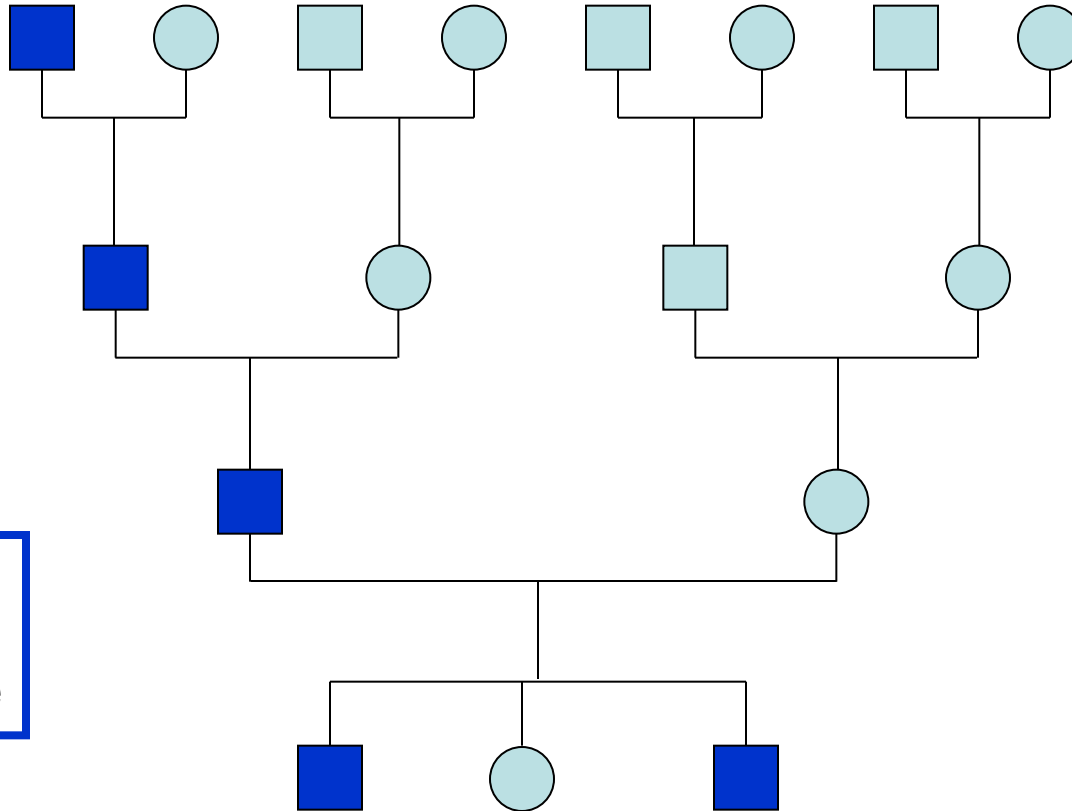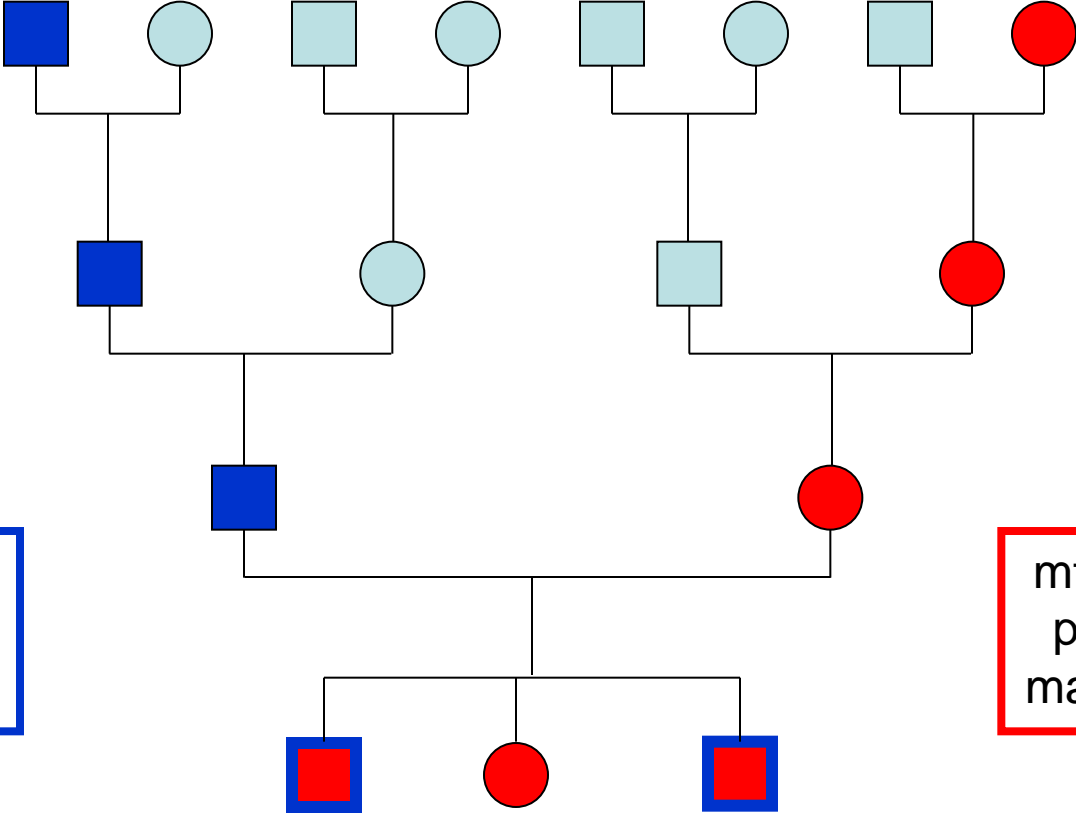

Autosomal DNA
1/8 from Great-grandparents

Lineage Markers

Y chromosome passed along paternal lineage

Autosomal DNA
1/8 from Great-grandparents
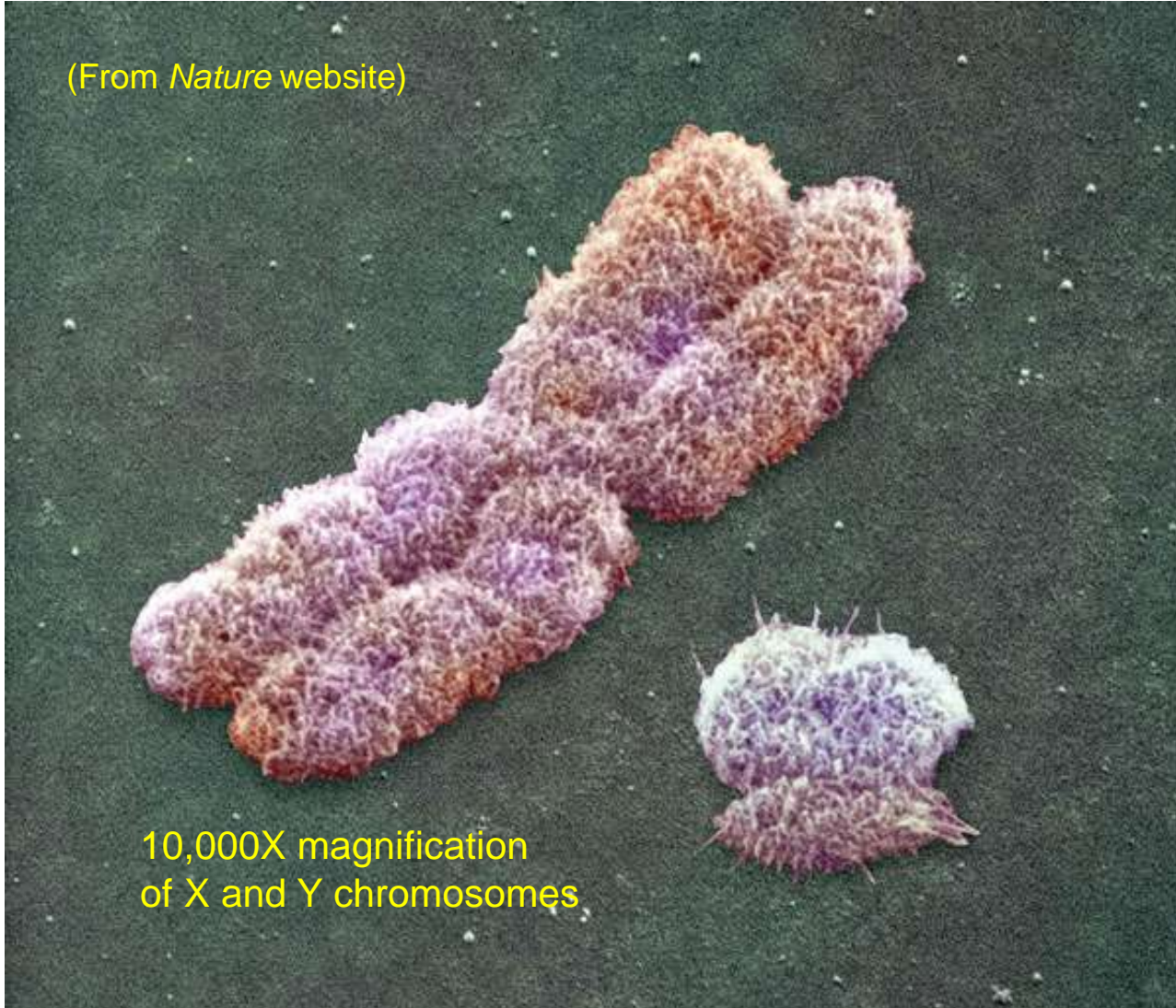
Lineage Markers

Y chromosome passed along paternal lineage

mtDNA genome passed along maternal lineage

Autosomal DNA
1/8 from Great-grandparents

The Y-chromosome

(From *Nature* website)

10,000X magnification
of X and Y chromosomes

Value of Y-Chromosome Markers

Application	Advantage
Forensic casework on sexual assault evidence	Male-specific amplification (can avoid differential extraction to separate sperm and epithelial cells)
Paternity Testing	Male children can be tied to fathers in motherless paternity cases
Missing Persons Investigations	Patrilineal male relatives may be used for reference samples
Human migration and evolutionary studies	Lack of recombination enables comparison of male individuals separated by large periods of time
Historical and Genealogical research	Surnames are usually retained by males; can make links where a paper trail is limited.

Disadvantages of the Y-Chromosome

- Loci are not independent of one another and therefore rare random match probabilities cannot be generated with the product rule; must use haplotypes (combination of alleles observed at all tested loci)

- **Paternal lineages possess the same Y-STR haplotype** (barring mutation) and thus fathers, sons, brothers, uncles, and paternal cousins cannot be distinguished from one another

- Not as informative as autosomal STR results
  - **More like addition (10 + 10 + 10 = 30) than multiplication (10 x 10 x 10 = 1,000)**

Various Types of Genetic Markers on the Human Y-Chromosome

Y-STRs
Short Tandem Repeats

GATAGATAGATAGATA

#Copies

12

13

14

15

16
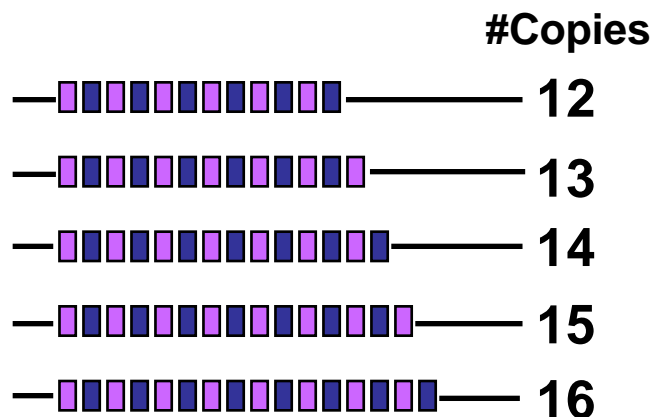
Multi-state characters
Quickly evolving (2×10^{-3}/gen)
High resolution haplotypes
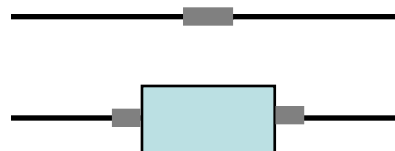
Y-SNPs
Single Nucleotide Polymorphisms

CG**A**TG

CG**G**TG

Insertion/deletions (indels)

Binary characters
Slowly evolving ($\sim 10^{-8}$/gen)
Low resolution haplogroups

52 Y STR Loci Mapped to Chromosomal Locations

>700 Y STRs now known

>300 Y SNPs characterized

-Butler (2003) Recent Developments in Y STR and Y SNP Analysis, *Forensic Sci. Rev.* 15:91-111
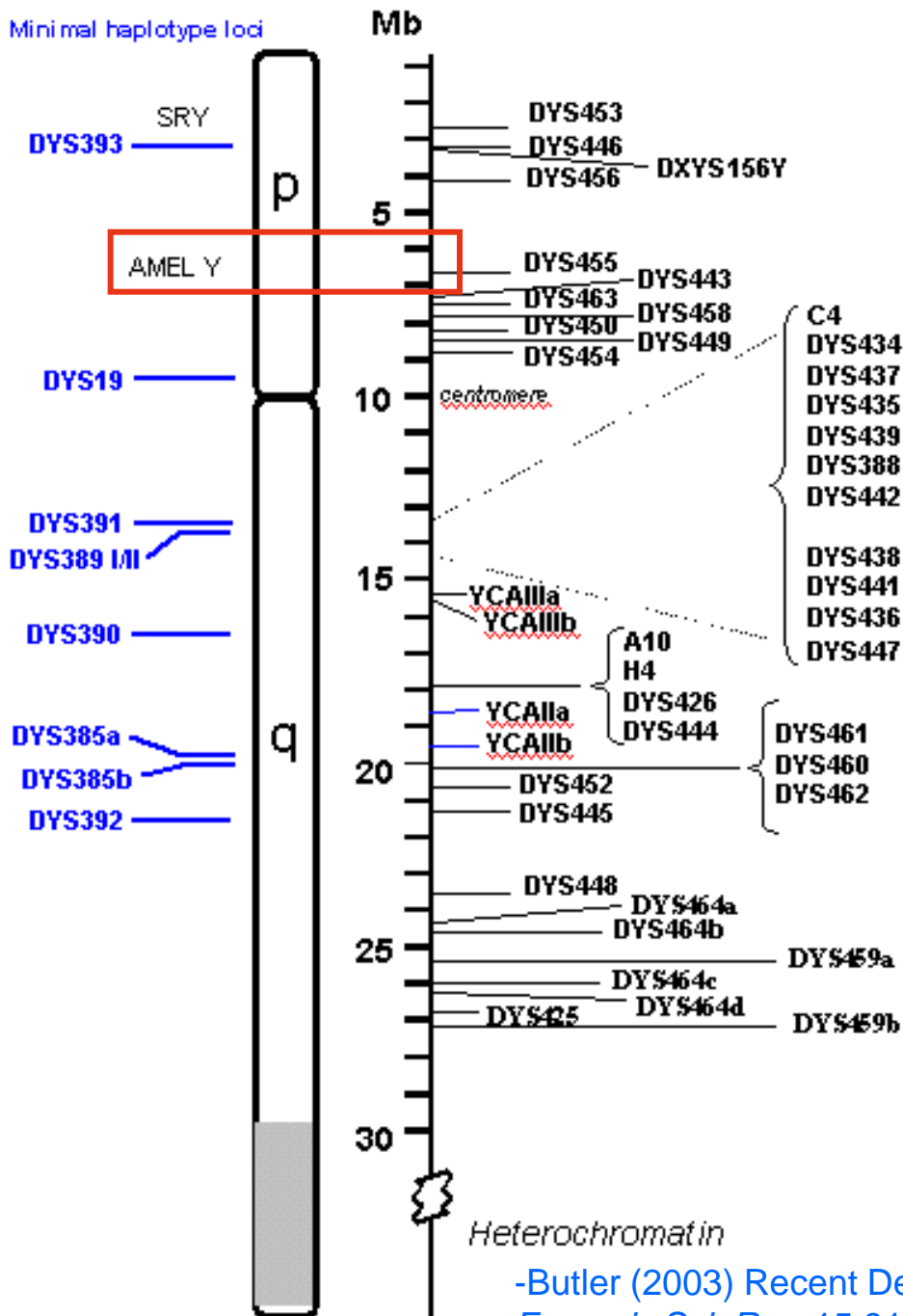
History of Y-STR Marker Discovery

1992 - DYS19 (Roewer et al.)

1994 - YCAI a/b, YCAII a/b, YCAIII a/b, DXYS156 (Mathias et al.)

1996 - DYS389I/II, DYS390, DYS391, DYS392, DYS393 (Roewer et al.)

1996 - DYF371, DYS425, DYS426 (Jobling et al.)

1997 - DYS288, DYS388 (Kayser et al.)

1998 - DYS385 a/b (Schneider et al.)

1999 - A7.1 (DYS460), A7.2 (DYS461), A10, C4, H4 (White et al.)

2000 - DYS434, DYS435, DYS436, DYS437, DYS438, DYS439 (Ayub et al.)

2000 - G09411 (DYS462), G10123 (de Knijff unpublished)

2001 - DYS441, DYS442 (Iida et al.)

2002 - DYS443, DYS444, DYS445 (Iida et al.); DYS446, DYS447, DYS448, DYS449, DYS450, DYS452, DYS453, DYS454, DYS455, DYS456, DYS458, DYS459 a/b, DYS463, DYS464 a/b/c/d (Redd et al.)

2002 – DYS468-DYS596 (**129 new Y STRs**; Manfred Kayser GDB entries)

2003 – DYS597-DYS645 (**50 new Y STRs**; Manfred Kayser GDB entries)

"Extended Haplotype"

"Minimal Haplotype"

U.S. Haplotype

From J.M. Butler (2003) Recent developments in Y-STR and Y-SNP analysis. *Forensic Sci. Rev.* 15:91-111

STR Markers with Low Stutter Products Benefit Forensic Analysis where Mixtures might be Present

YCAII a/b (dinucleotide repeat)

~50% stutter
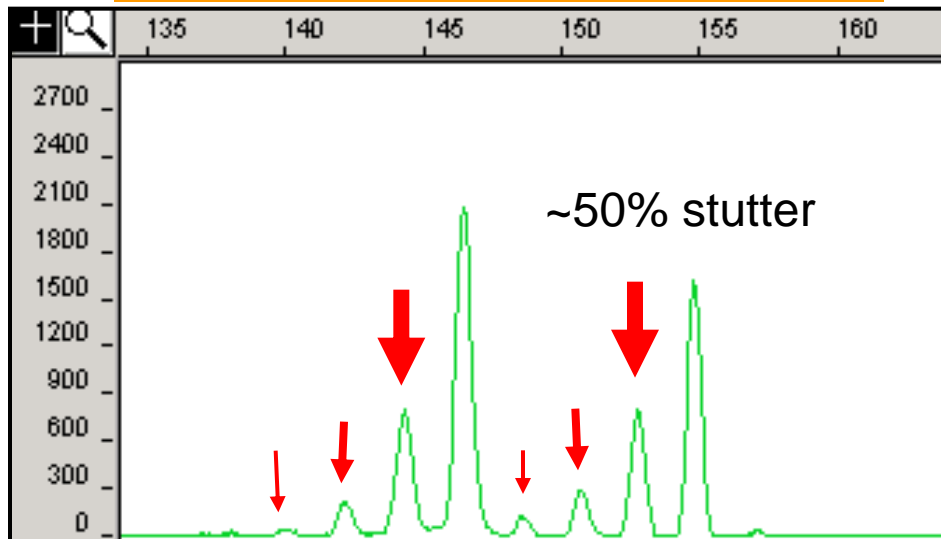
[CA]

11-24 repeats
YCC gene diversity 0.908
(Redd et al. 2002)

DYS448 (hexanucleotide repeat)

Almost no stutter observed

<2% stutter
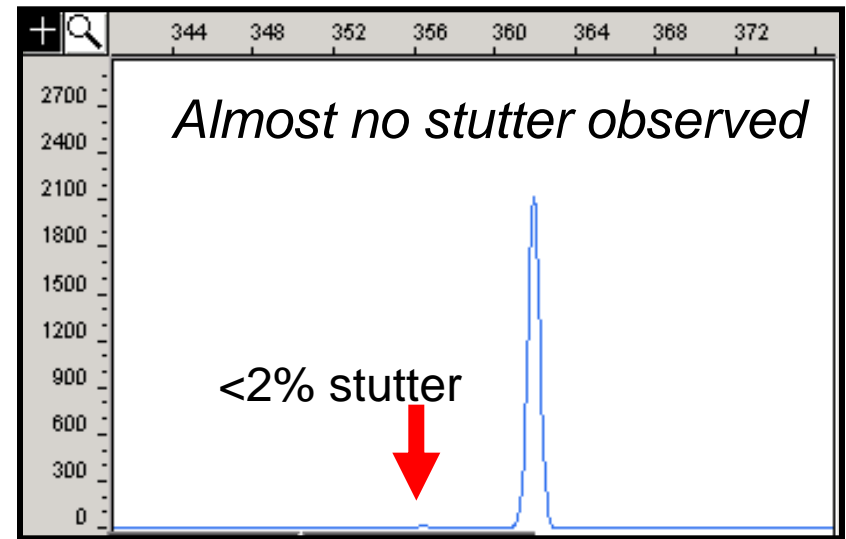
[AGAGAT]

20-26 repeats
YCC gene diversity 0.782
(Redd et al. 2002)

(A) DYS385 a/b

Multi-Copy (Duplicated) Marker

R primer

R primer

a

b
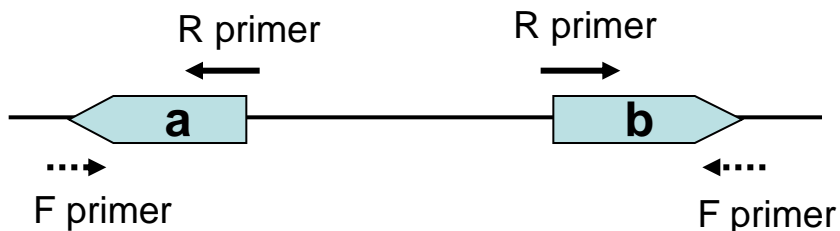
F primer

F primer

Duplicated regions are 40,775 bp apart and facing away from each other

a = b   a ≠ b
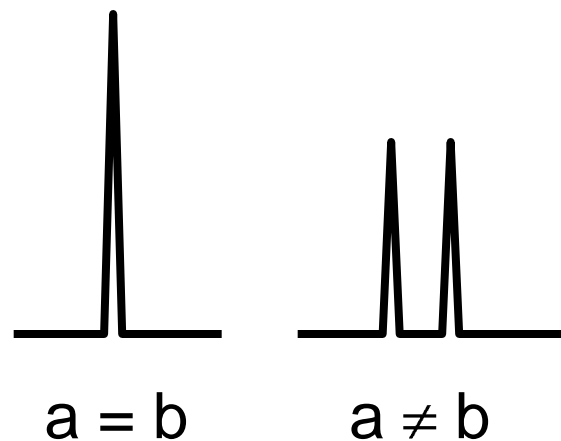
(B) DYS389 I/II

Single Region but Two PCR Products
(because forward primers bind twice)

I

II

F primer

F primer

R primer
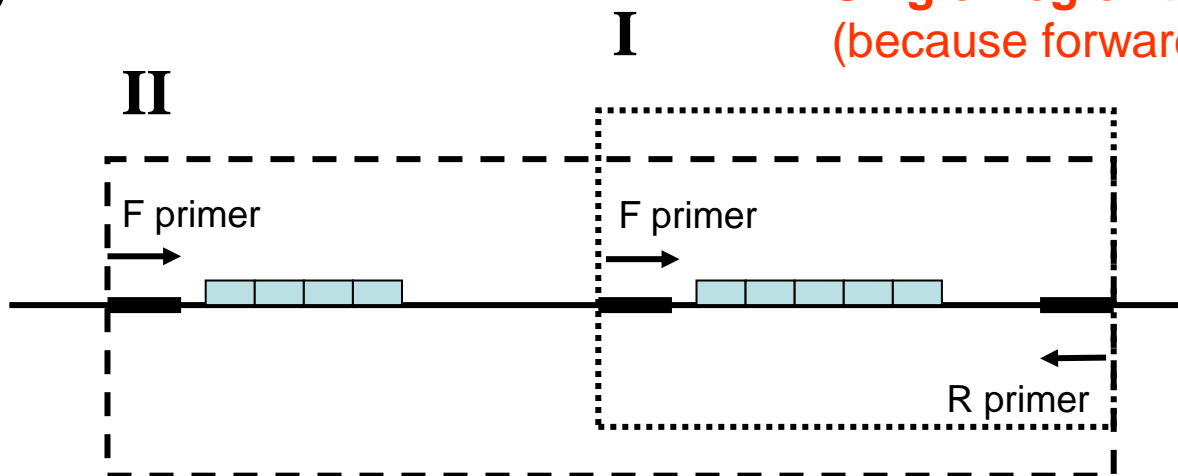
DYS389I   DYS389II

Primary Commercial Y-STR Kits

Allele size range and locus dye colors

PowerPlex® Y

Released by Promega Corporation in Sept 2003

	100 bp	200 bp	300 bp	400 bp
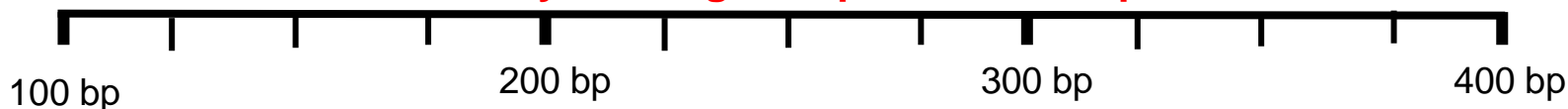
FL — DYS391, DYS389I, DYS439, DYS389II

JOE — DYS438, DYS437, DYS19, DYS392

TMR — DYS393, DYS390, DYS385a/b

3 dye colors
12-plex PCR

AmpFℓSTR® Yfiler™

Released by Applied Biosystems in Dec 2004

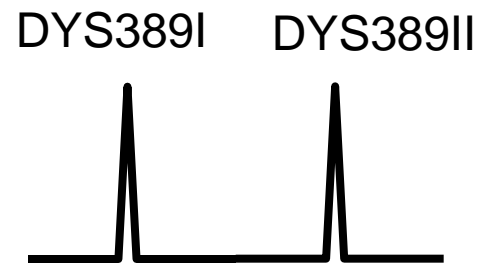|	100 bp	200 bp	300 bp	400 bp

6-FAM — DYS456, DYS389I, DYS390, DYS389II

VIC — DYS458, DYS19, DYS385a/b

NED — DYS393, DYS391, DYS439, DYS635, DYS392

PET — H4, DYS437, DYS438, DYS448

4 dye colors
17-plex PCR

Promega PowerPlex® Y Allelic Ladders
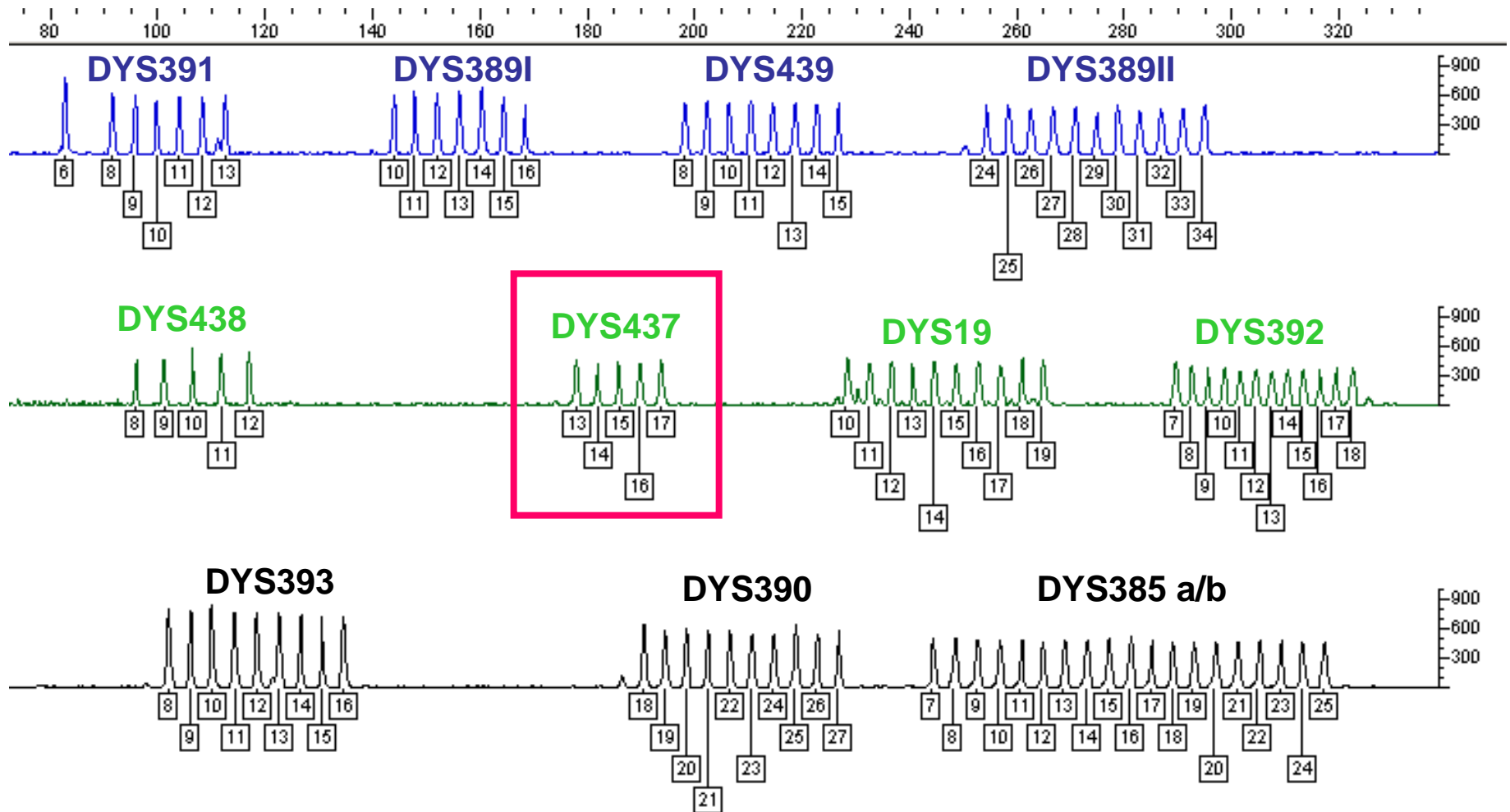
U.S. Core Loci + DYS437

Single amplification; ladders contain 103 alleles

Yfiler Allelic Ladders
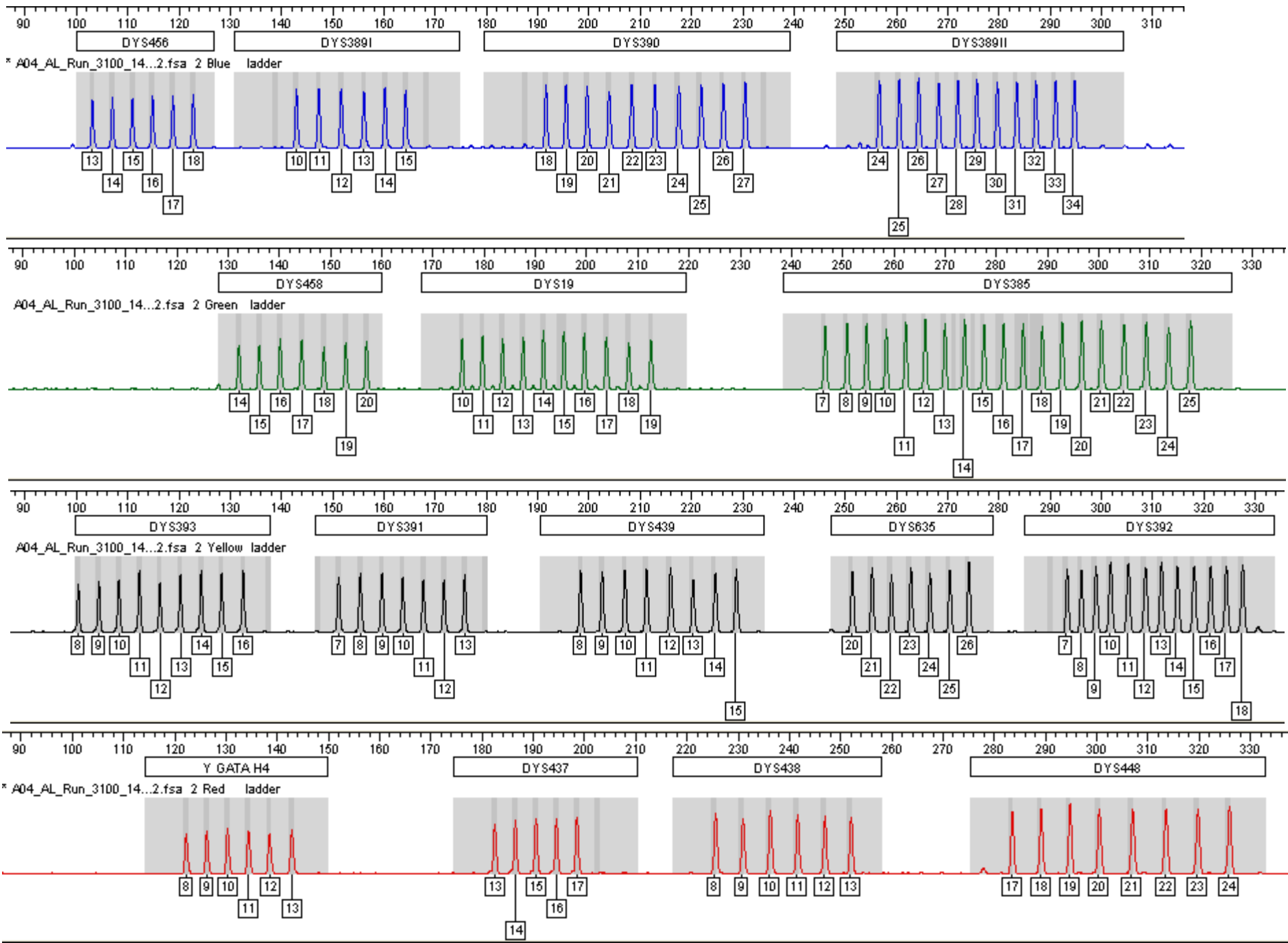
Coming Soon: PPY-23 from Promega

6 "New" Loci
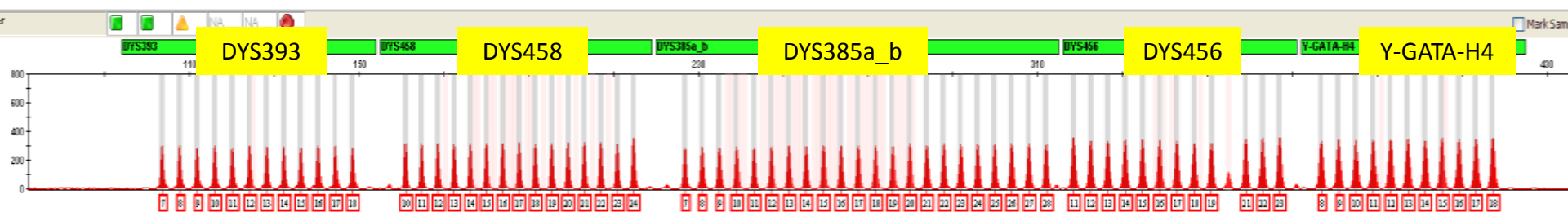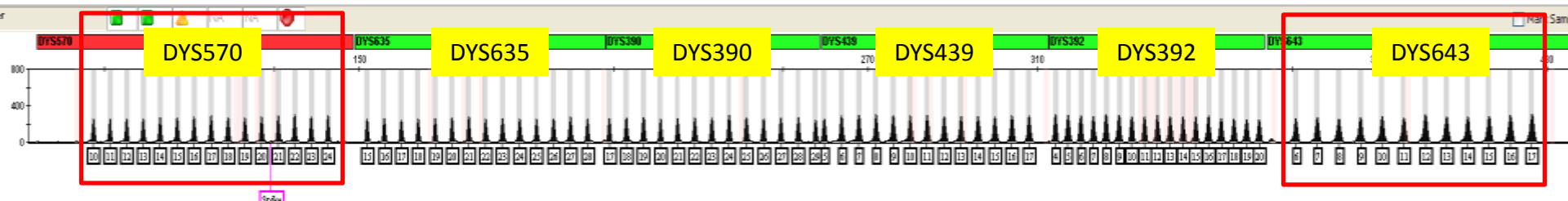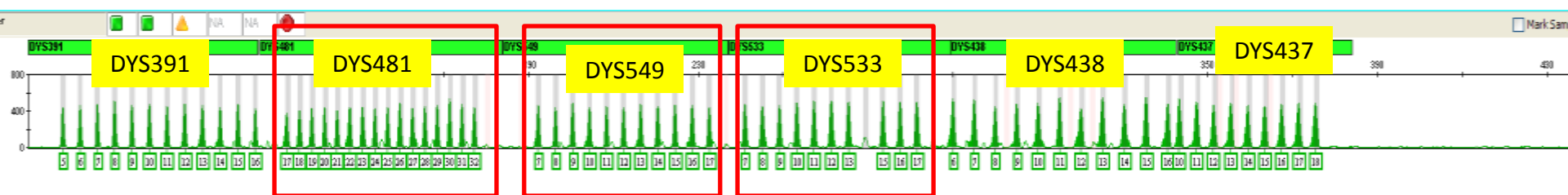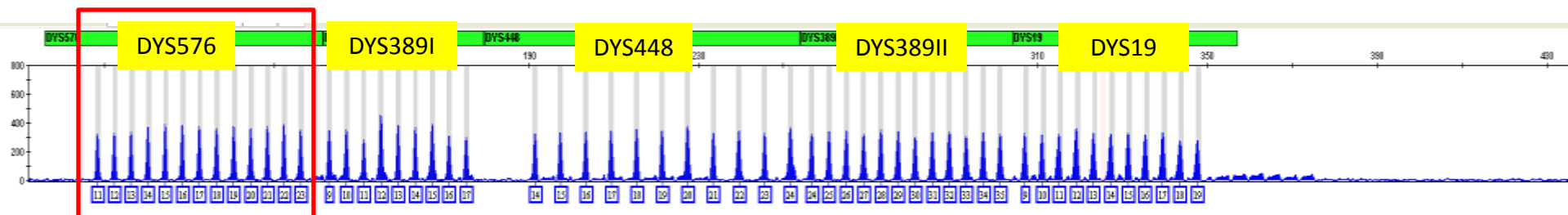
Mixture of 125pg Male + 400ng Female
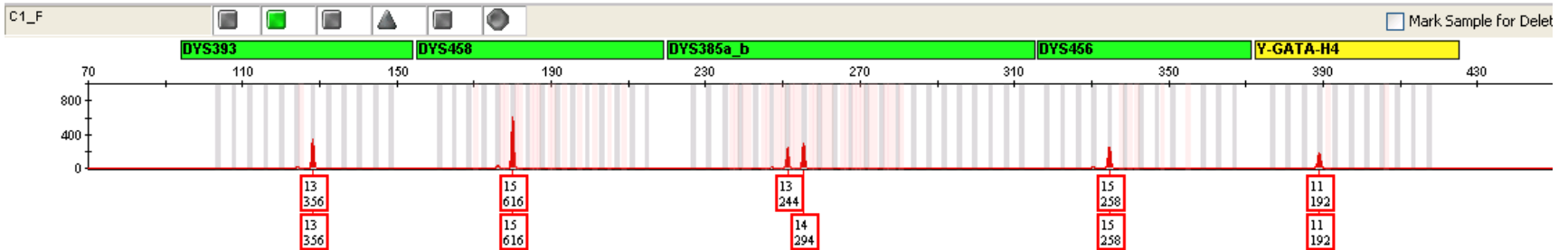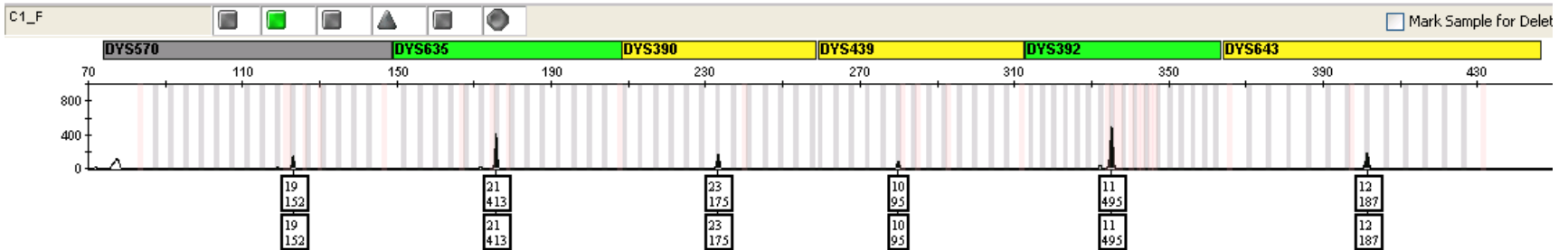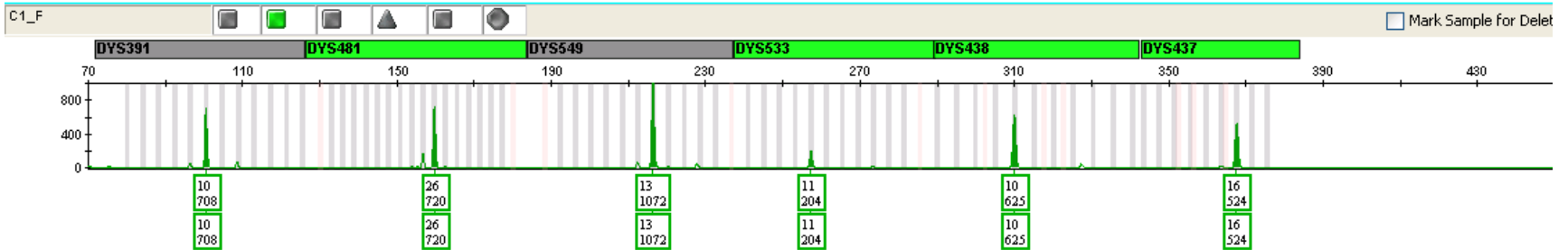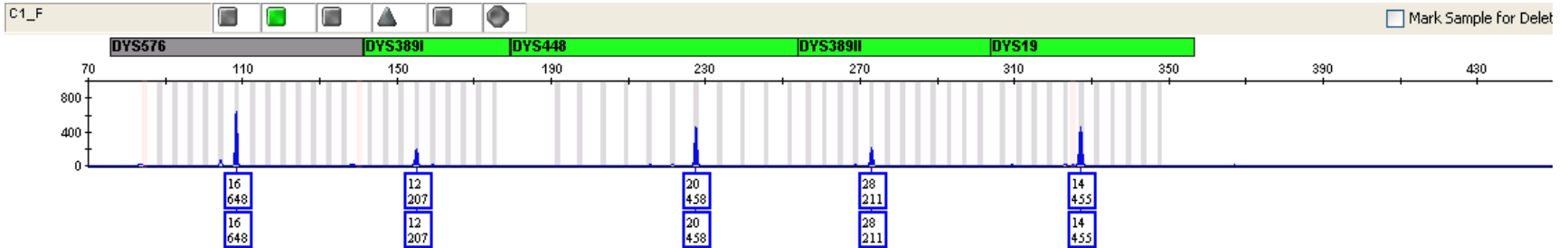
Mixture of 62.5pg Male + 400ng Female
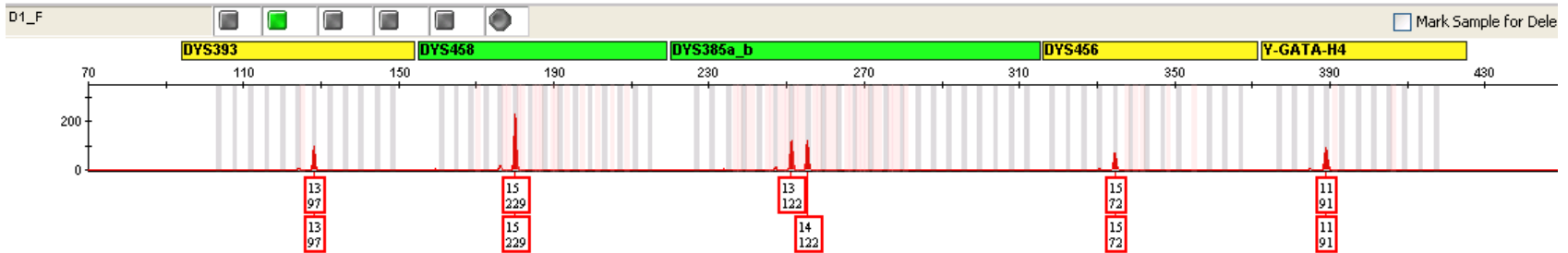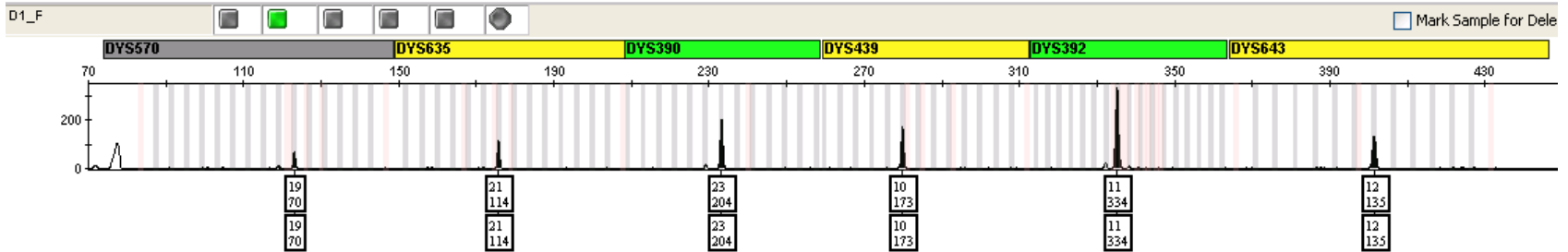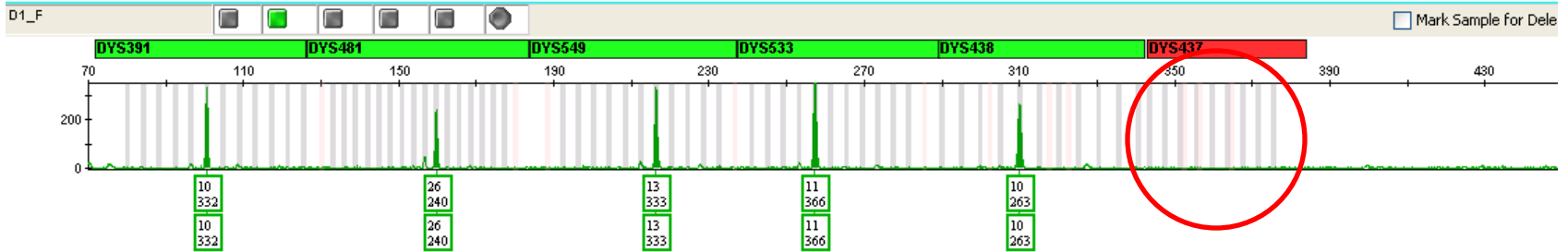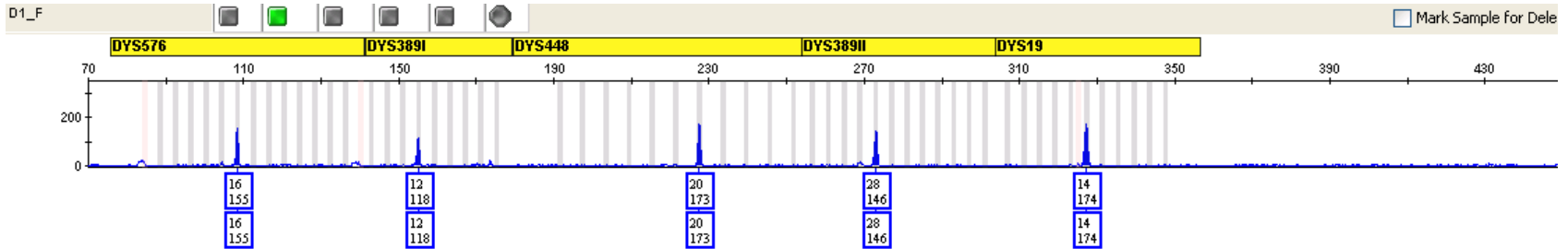
The value of Y-SNPs

Report

The Genetic Legacy of the Mongols

Tatiana Zerjal,[1] Yali Xue,[1,2] Giorgio Bertorelle,[3] R. Spencer Wells,[4] Weidong Bao,[1,5] Suling Zhu,[1,5] Raheel Qamar,[1,6] Qasim Ayub,[1,6] Aisha Mohyuddin,[1,6] Songbin Fu,[2] Pu Li,[2] Nadira Yuldasheva,[4,7] Ruslan Ruzibakiev,[7] Jiujin Xu,[5] Qunfang Shu,[5] Ruofu Du,[5] Huanming Yang,[5] Matthew E. Hurles,[8] Elizabeth Robinson,[1,*] Tudevdagva Gerelsaikhan,[1,+] Bumbein Dashnyam,[9] S. Qasim Mehdi,[5] and Chris Tyler-Smith[1]

Chris Tyler-Smith

Black Sea
Svan
Ossetian
Georgian
Caspian Sea
Lezgi
Armenian Azeri
KAZAKSTAN
RUSSIA
Aral Sea
UZBEKISTAN
TURKMENISTAN
Uzbek
Kurd
Turkmen
Tajik
Kazak
Kyrgyz
Uyghur
Xibe
Chinese Uyghur
Chinese Kazak
Han (Xinjiang)
MONGOLIA
Mongolian
Huizu
Han (Gansu)
Oroqen
Ewenki
Han (Inner Mongolia)
Inner Mongolian
Daur
Hezhe
Han (Heilongjiang)
Chinese Korean
Manchu
Korean
Japanese
Pacific Ocean

IRAN
AFGHANISTAN
Kalash
Balti
Hazara
Burusho
Brahul
Baloch
PAKISTAN
Makrani Baloch
Makrani Negroid
Parsi
NEPAL
BHUTAN
BANGLADESH
INDIA
Tibetan
Qiangzu
Han (Sichuan)
Buyi
Yaozu (Liannan)
Hani
Yaozu (Bama)
Lizu
Han (Guangdong)
CHINA
Shezu

n ≥ 60

n = 30

Star-Cluster Chromosomes

washingtonpost.com

Tom Robinson

DNA Shows Man a Descendant of Genghis Khan

By JILL LAWLESS
The Associated Press
Tuesday, June 6, 2006; 8:30 PM

LONDON -- Tom Robinson had long wondered about his family tree. He never suspected its roots might lie in the Mongolian steppe.

The Florida accountant knew that his great, great-grandfather had come to the United States from England _ but beyond that his research drew a blank. So he turned to the burgeoning field of "bioarchaeology," having his DNA tested to see what it revealed about his origins.

"I haven't done any conquering, per se."

The New York Times

In the Body of an Accounting Professor, a Little Bit of the Mongol Hordes

By NICHOLAS WADE
Published: June 6, 2006

Oxford Ancestors, the world's foremost and leading company in ancestral DNA analysis has uncovered the first American descendent of the great warlord **Genghis Khan**... **Tom Robinson**, Associate Professor of Accountancy and professional investment consultant, of Miami, Florida, USA.

It turns out that **Dr Robinson** is a direct descendent of **Genghis**, and he is the first American to find this out through a genetic test. His Y-Chromosome bears an astonishing seven out of nine possible genetic markers identical to **Genghis Khan's** (as DNA mutates over generations, two altering DNA markers is a remarkably low number for a period stretching over 700 years).

"It is a very precise match," Professor Sykes said.

Marker	Oxford Ancestors Robinson	Mongolian Benchmark
DYS19	16	16
DYS390	25	25
DYS391	10	10
DYS392	11	11
DYS393	13	13
DYS389I	13	13
DYS389II	31	29
DYS425	12	12
DYS426	12	11

Ghengis Khan – is thought to have belonged to the Asian YHg - C3

Additional testing by Family Tree DNA found that Tom Robinson belonged to Y-haplogroup R1a (W. European)

"A match at 10 fast-mutating sites is outvoted by a discrepancy at one slow-mutating site."

Dr. Chris Tyler-Smith
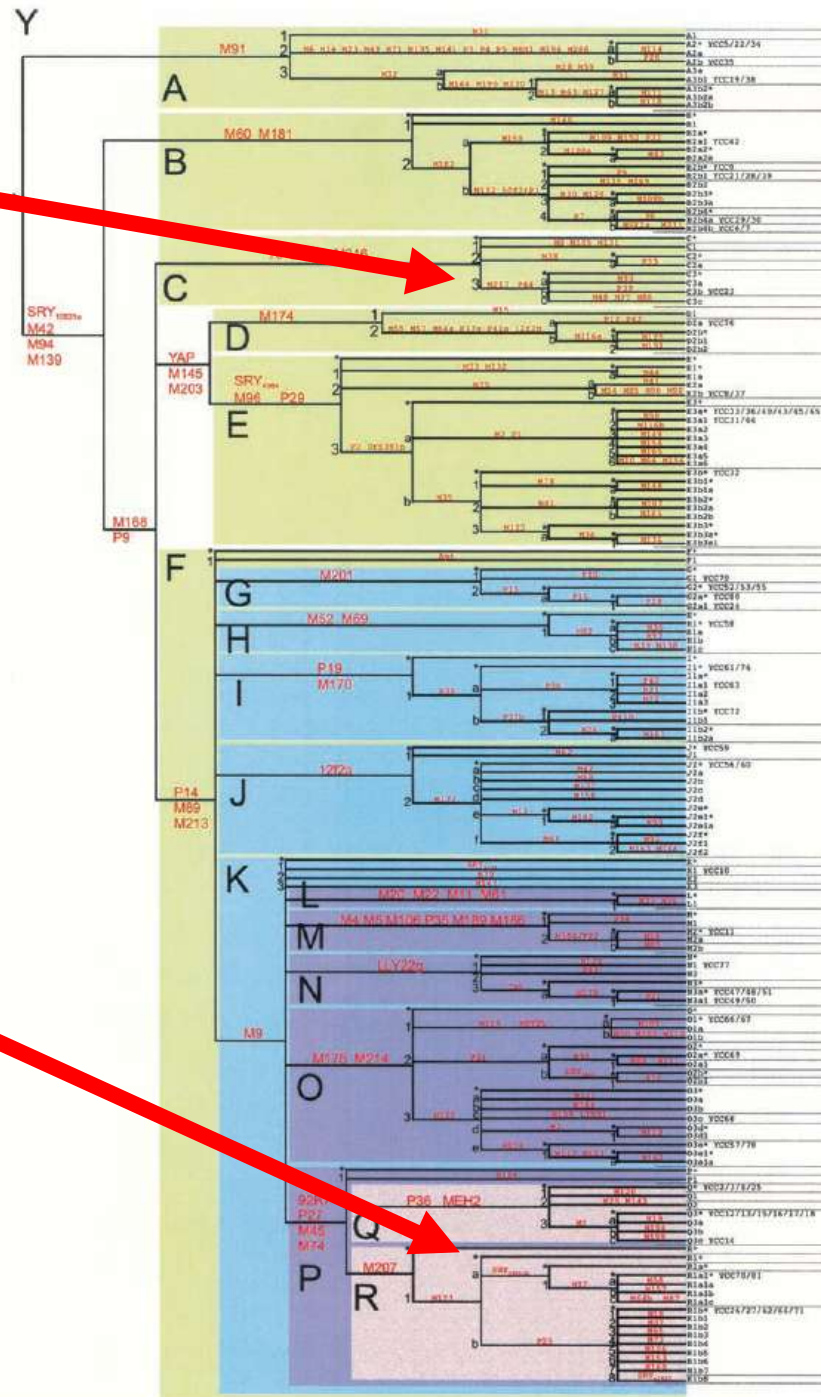
Recent Discussions with Y-STRs

STATS

Buckleton *et al.* (2011)

$$\tilde{p}_A \pm z_{(1-\alpha/2)} \sqrt{\frac{\tilde{p}_A(1 - \tilde{p}_A)}{n}} \text{ (two sided)}$$

and

$$\tilde{p}_A + z_{(1-\alpha)} \sqrt{\frac{\tilde{p}_A(1 - \tilde{p}_A)}{n}} \text{ (one sided)}$$

Described by
Holland and Parsons
(1999)

Normal Approximation of the 95% CI
for the Binomial Distribution

Buckleton *et al.*

- The problem – Y-STR haplotypes are not distributed as a normal approximation.

- For rare or limited types, the actual CI predicted by the normal approximation is at around 80-85% and not 95%.

- Clopper and Pearson determined the exact binomial distribution in the 1930s

THE USE OF CONFIDENCE OR FIDUCIAL LIMITS ILLUSTRATED IN THE CASE OF THE BINOMIAL.

By C. J. CLOPPER, B.Sc., and E. S. PEARSON, D.Sc.

CONFIDENCE BELTS FOR p (CONFIDENCE COEFFICIENT ·95)

SCALE OF p

SCALE OF $\frac{X}{n}$

FIG. 4.
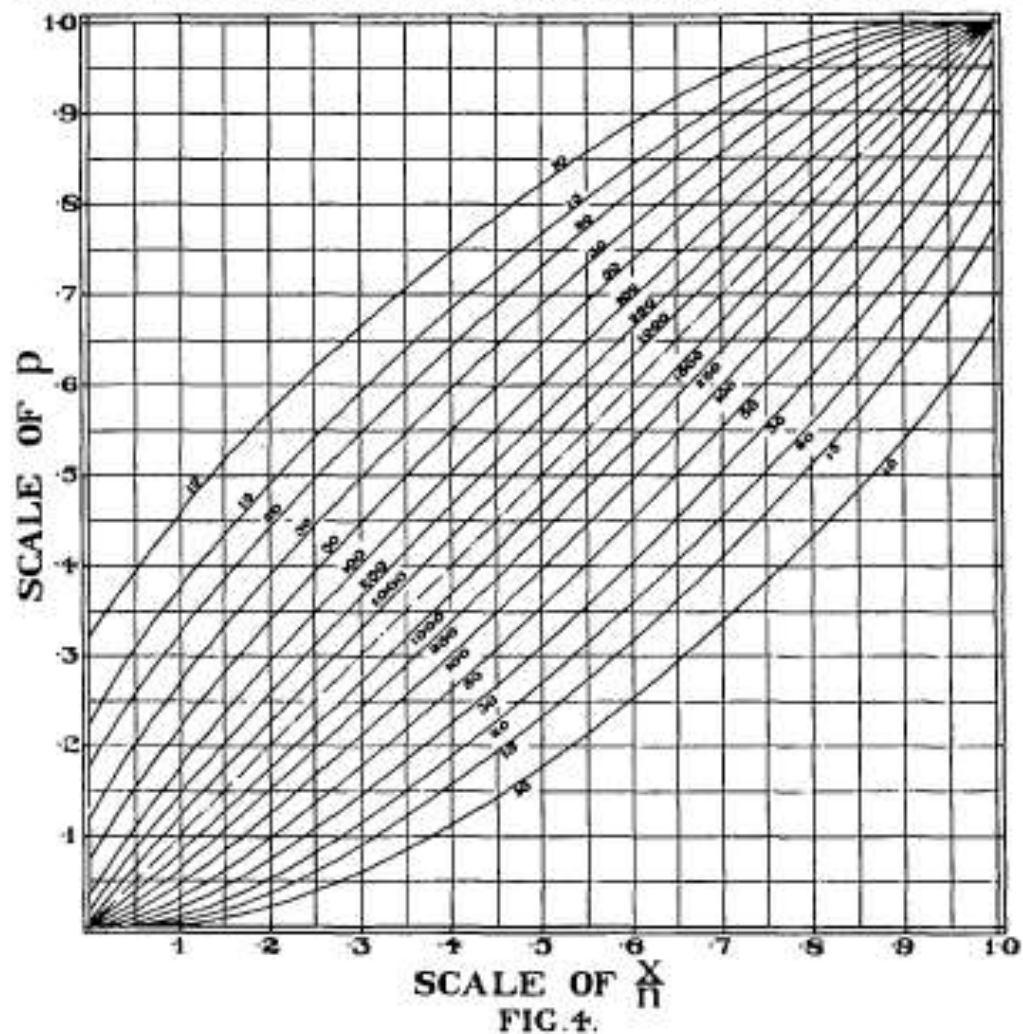
The Issue…

- Using the 95% Normal approximation is easy to calculate by hand…

- The Clopper Pearson – not so much.

$$\left\{\, \theta \,\middle|\, P\left[\mathrm{Bin}\left(n;\theta\right) \leq X\right] \geq \alpha/2 \right\} \bigcap \left\{\, \theta \,\middle|\, P\left[\mathrm{Bin}\left(n;\theta\right) \geq X\right] \geq \alpha/2 \right\}$$

Steven Myers (Cal DOJ) Worksheet

HaploCALc Haplotype Statistics Worksheet

	n	95778	
	x	22	

	p	1 in ...	
x/n	0.000229698	4400	
$1 - (\alpha)^{1/n}$			1-sided
1 - BetaInv	0.00032798	3000	1-sided
A&C	0.000325584	3100	1-sided
H&P	0.00031024	3200	1-sided ←
	0.00032567	3100	2-sided
C&P	0.00032798	3000	1-sided ←

Desired α	Desired Sig. Figs.
0.05	2

Cumulative Binomial Distribution

0.95

"Common Type" observed in the population – C&P ≈ 95% CI of H&P

HaploCALc Haplotype Statistics Worksheet

n	95778	
x	1	

	p	1 in ...	
x/n	1.04408E-05	96000	
$1 - (\alpha)^{1/n}$			1-sided
1 - BetaInv	4.95288E-05	20000	1-sided
A&C	4.6799E-05	21000	1-sided
H&P	2.76143E-05	36000	1-sided
	3.09043E-05	32000	2-sided
C&P	4.95288E-05	20000	1-sided

Desired α	Desired Sig. Figs.
0.05	2

Cumulative Binomial Distribution
0.95

HaploCALc Version 1.2

"Rare Type" observed once in the population –
C&P is more conservative than H&P

HaploCALc Haplotype Statistics Worksheet

n	95778	
x	0	

	p	1 in ...	
x/n	0		
$1 - (\alpha)^{1/n}$	3.12774E-05	32000	1-sided ⬅
$1 - \text{BetaInv}$	3.12774E-05	32000	1-sided
A&C	2.82473E-05	35000	1-sided
H&P	0		1-sided
	0		2-sided
C&P	3.12774E-05	32000	1-sided ⬅

Desired α	Desired Sig. Figs.
0.05	2

Cumulative Binomial Distribution

0.95

HaploCALc Version 1.2

"New Type" never observed in the population –
C&P is = Confidence Limit from Zero Proportion

Fundamental problem of forensic mathematics—The evidential value of a rare haplotype

Charles H. Brenner [a,b,*]

[a] School of Public Health, Forensic Science Group, U.C. Berkeley, Berkeley, CA United States
[b] DNA-VIEW, 6801 Thornhill Drive, Oakland, CA 94611-1336, United States

"The fundamental question to decide the evidentiary significance of a trait linking suspect to crime is not one of frequency but of probability: What is the probability for such a match to happen by coincidence when the suspect is innocent?"

mtDNA

Summary – mtDNA Characteristics

- High copy number of mtDNA.

- Maternal inheritance of mtDNA.

- Lack of recombination.

- High mutation rate compared to single copy nucDNA.
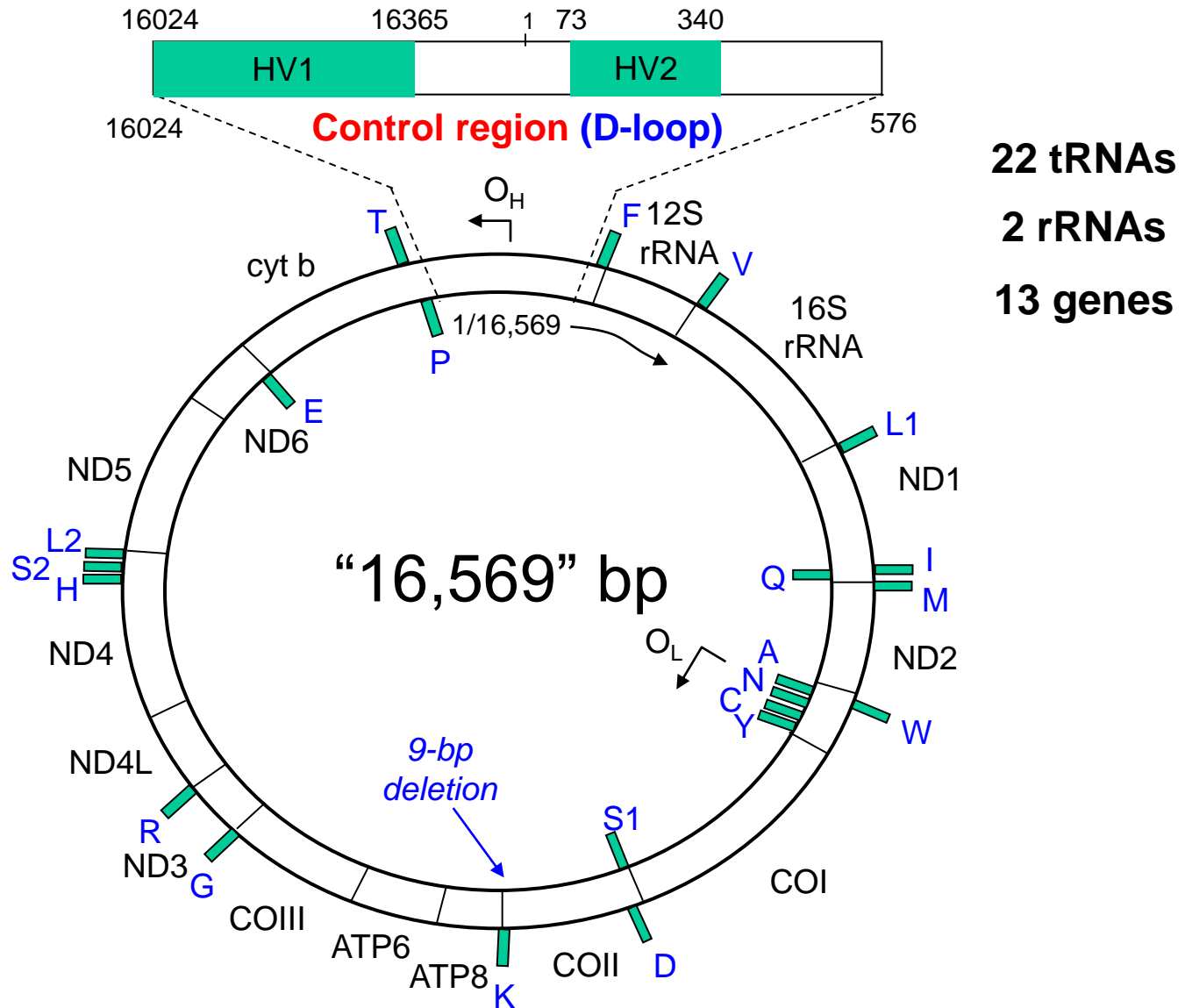
Figure 10.1, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Control Region (16024-576)

1,122 nucleotide positions

16024　　　　16365　　1　　73　　340

| HV1 | | HV2 | |

16024　　　　**Control region (D-loop)**　　　　576

(AC)$_3$
(AC)$_4$
(AC)$_5$
(AC)$_6$
(AC)$_7$
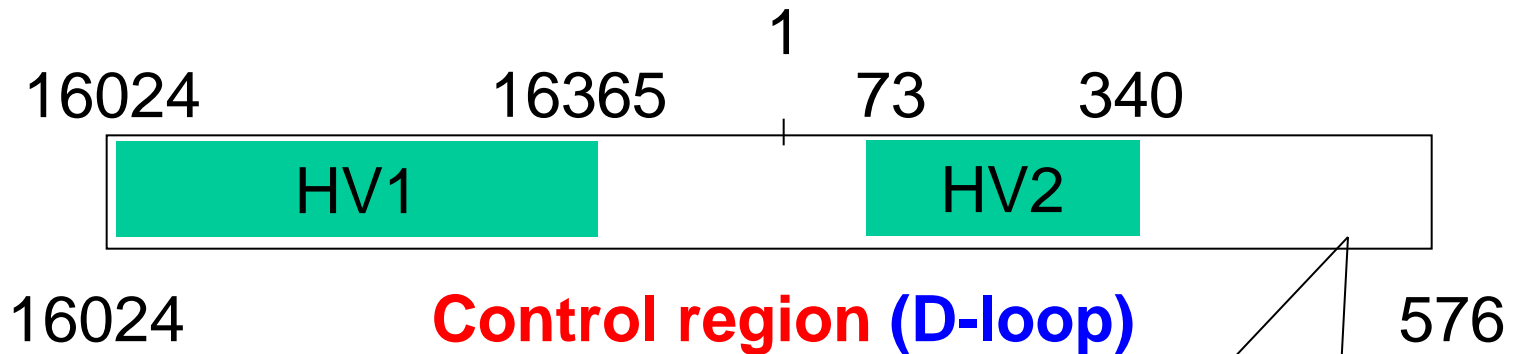
Forensic Focus

Typically only **610 bases examined**
  – (HVI: 16024-16365; HVII: 73-340)

Maternal Inheritance of mtDNA

- Fertilizing sperm contributes only nuclear DNA

- Cellular components including the mitochondria in the cytoplasm come from the mother's ovum

- Any sperm mitochondria that may enter a fertilized egg are selectively destroyed due to a ubiquitin tag added during spermatogenesis

- Barring mutation, a mother passes her mtDNA type on to her children

Candidates for mtDNA Testing

- Shed hairs lacking root bulb or attached tissue

- Fragments of hair shafts

- Aged bones or teeth that have been subjected to long periods of exposure

- Crime scene stains or swabs that were unsuccessful for nuclear DNA testing

- Tissues (muscle, organ, skin) that were unsuccessful for nuclear DNA testing

Terry Melton – International Symposium on the Application of DNA Technologies in Analytical Sciences

Process for Evaluation of mtDNA Samples

Extract mtDNA from evidence (Q) sample

Extract mtDNA from reference (K) sample

Performed separately and preferably after evidence is completed

PCR Amplify HV1 and HV2 Regions

PCR Amplify HV1 and HV2 Regions

Sequence HV1 and HV2 Amplicons (both strands)

Sequence HV1 and HV2 Amplicons (both strands)

Confirm sequence with forward and reverse strands

Confirm sequence with forward and reverse strands

Note differences from Anderson (reference) sequence

Note differences from Anderson (reference) sequence

Question Sample

Reference Sample

Compare Q and K sequences

Compare with database to determine haplotype frequency

Figure 10.4, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Interpretational Issues - Heteroplasmy

- Heteroplasmy – the presence of more than one mtDNA type in an individual

- Once thought to be rare, heteroplasmy exists (at some level) in all tissues

- Especially important in forensic mtDNA analysis of hair

HV2 Length Heteroplasmy

"Out of phase!"

Sequence 1 AAACCCCCCCCTCCCCCCGCTTC
Sequence 2 AAACCCCCCCCCTCCCCCCGCTTC
Sequence 3 AAACCCCCCCCCCTCCCCCCGCTTC

Point Heteroplasmy

"Y" Pyrimidine

"Hotspot" for heteroplasmy

16093
(C/T)

T A T G T A T Y T C G T A C A T

16086   16101

T A T G T A T T T C G T A C A T

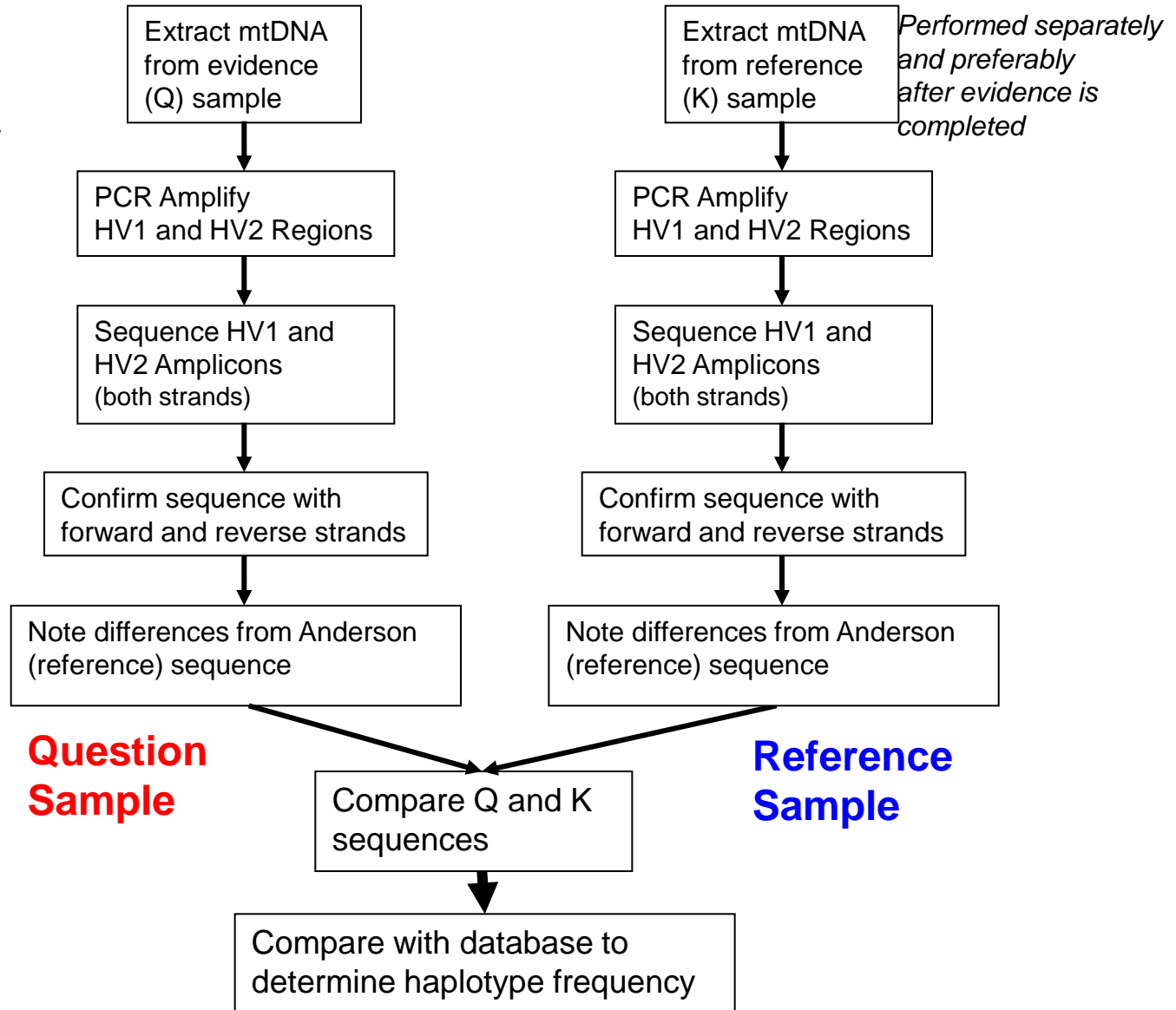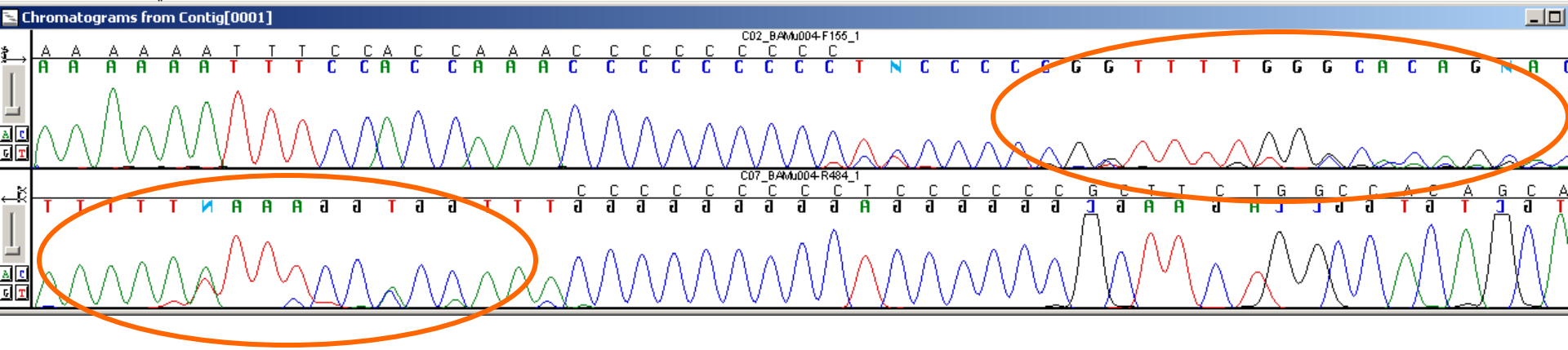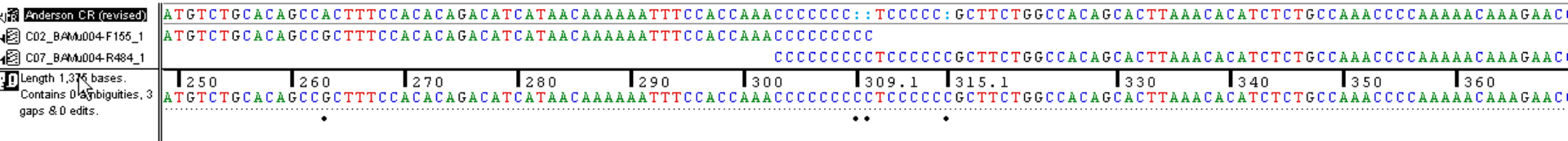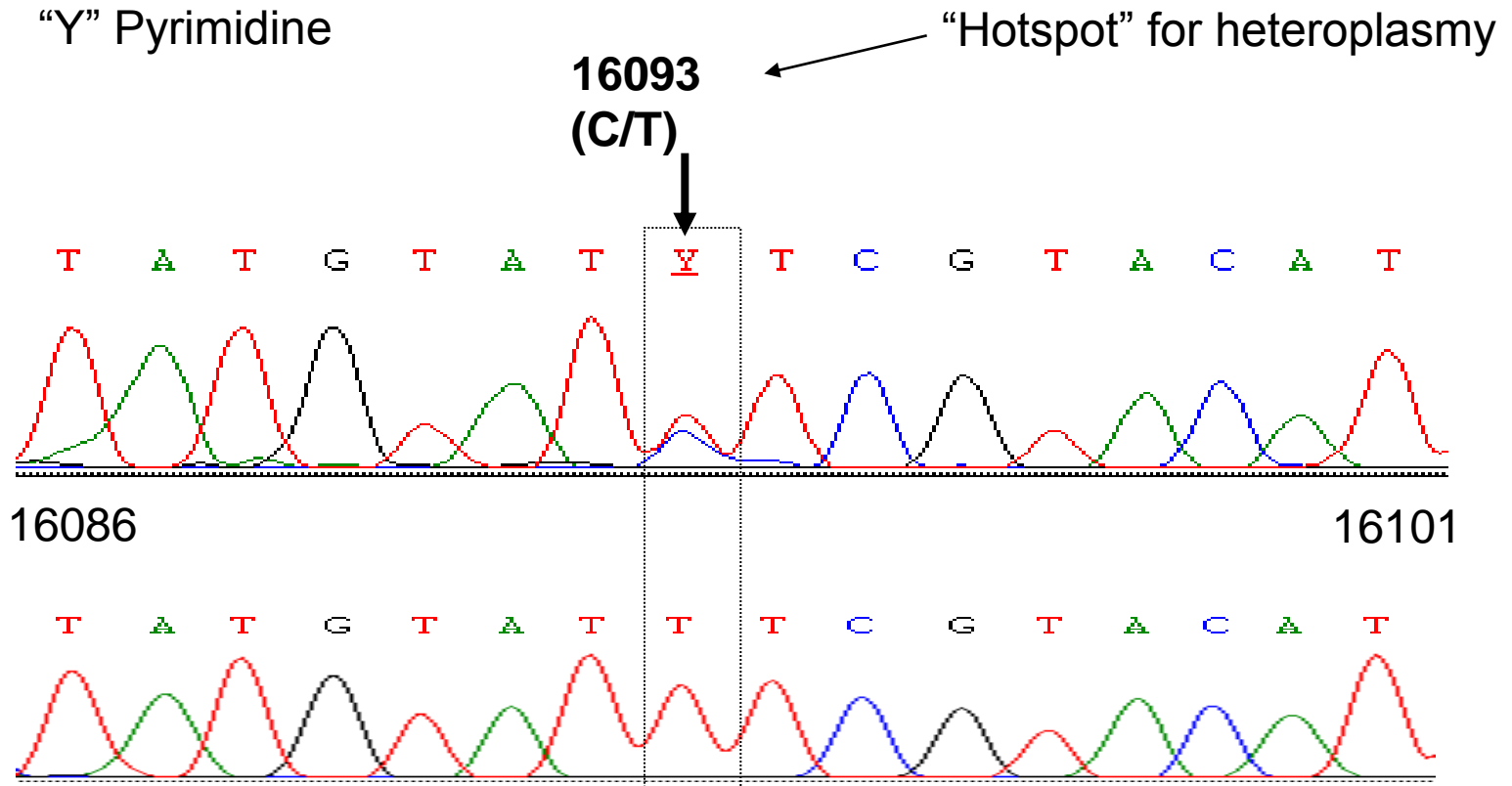Figure 10.9, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Origination of Heteroplasmy

Ovum – 100K mitochondria

Very little mito growth until implantation

Females – produce ~7 million ova during fetal development only a few hundred become mature oocytes

FIGURE 2. The mitochondrial genetic bottleneck
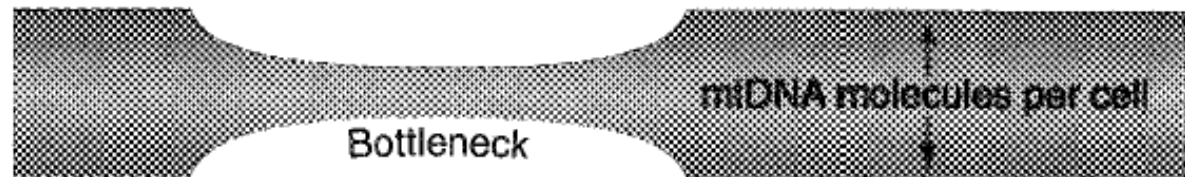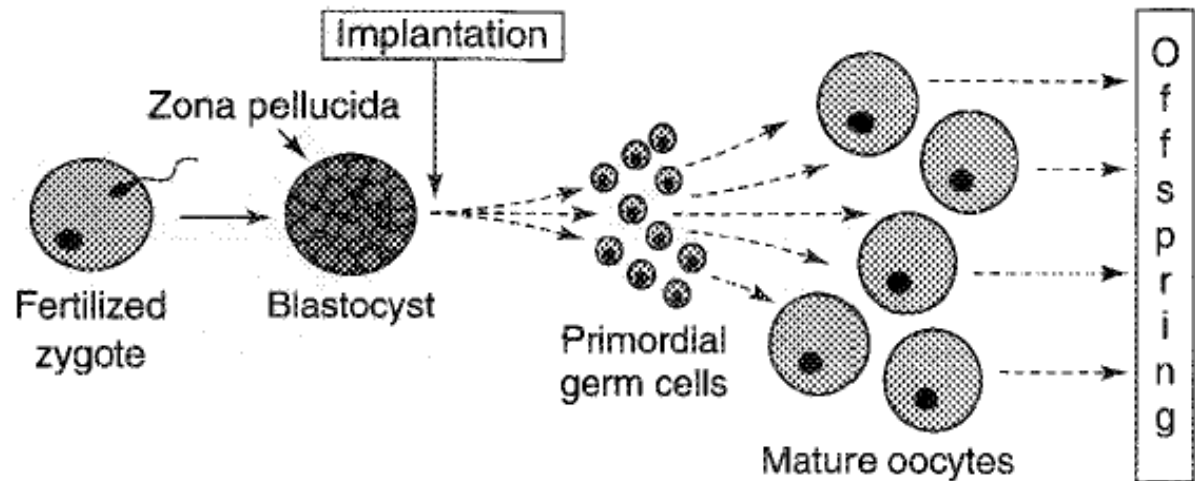
Chinnery *et al.* (2000) Trends in Genetics

ORIGINAL ARTICLE

Single lymphocytes from two healthy individuals with mitochondrial point heteroplasmy are mainly homoplasmic

Sabine Lutz-Bonengel · Timo Sänger · Walther Parson ·
Helena Müller · Joachim W. Ellwart · Marie Follo ·
Bernhard Bonengel · Harald Niederstätter ·
Marielle Heinrich · Ulrike Schmidt
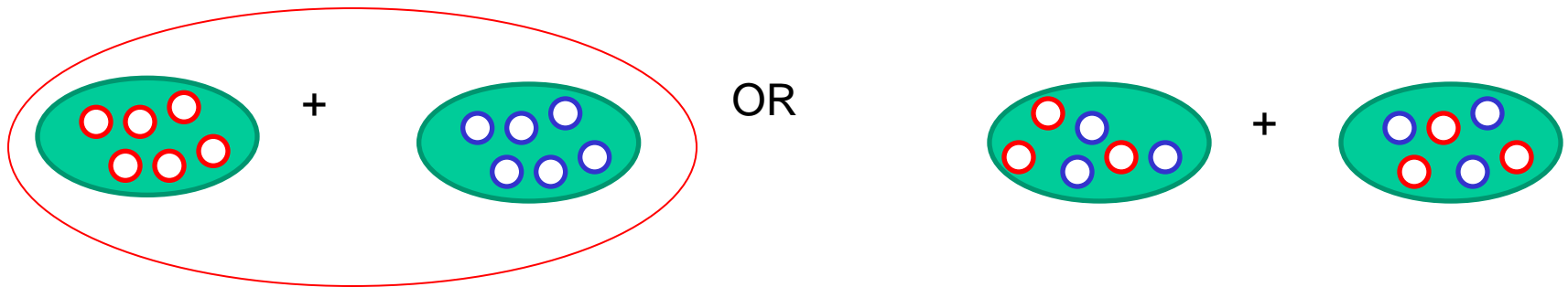
Detection of Heteroplasmic Mitochondrial DNA in Single Mitochondria

Joseph E. Reiner[1]*, Rani B. Kishore[1], Barbara C. Levin[2], Thomas Albanetti[3], Nicholas Boire[3], Ashley Knipe[3], Kristian Helmerson[1], Koren Holland Deckman[3]

1 Physical Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland, United States of America, 2 Material Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland, United States of America, 3 Department of Chemistry, Gettysburg College, Gettysburg, Pennsylvania, United States of America
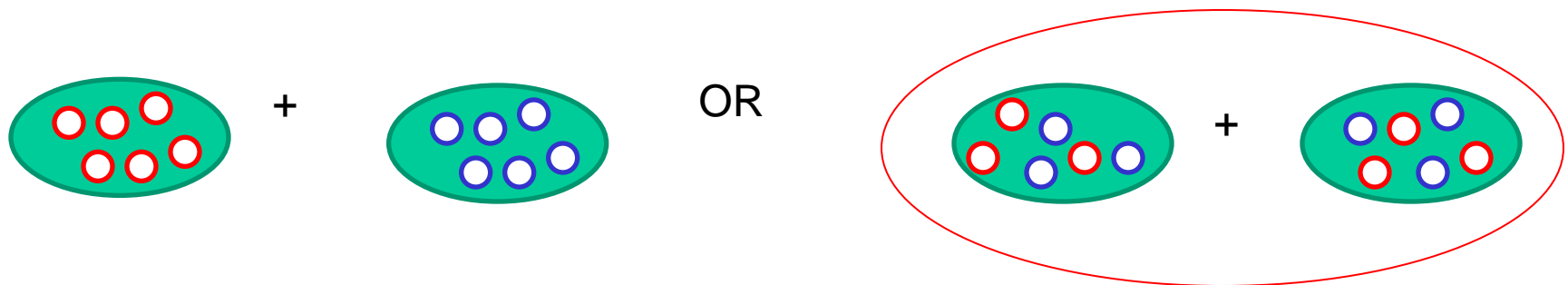
Improved extraction protocols for mtDNA testing

Current Extraction Protocols – Forensic mtDNA Labs
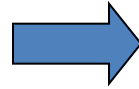
DNA Extraction
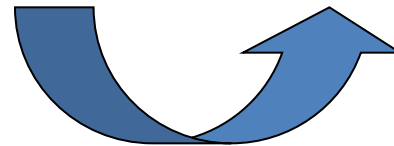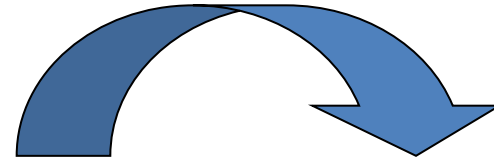
Demineralization protocol

- EDTA 0.5M, pH 8.5
- Detergent
- Proteinase K
- 1g powder
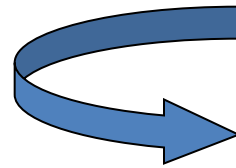
15ml extraction buffer

- Organic extraction (phenol-chloroform)
- Concentration and washes in filtration devices.

Casework SOP

10mM Tris, pH 8.0, 100mM NaCl, 50mM EDTA, pH 8.0, 0.5% SDS; ProK

Demineralization protocol

LCN-STR data-PP16

90-100% success

80-89.9% success

70-79.9% success

60-69.9% success

50-59.9% success

40-49.9% success

30-39.9% success

0-20% success
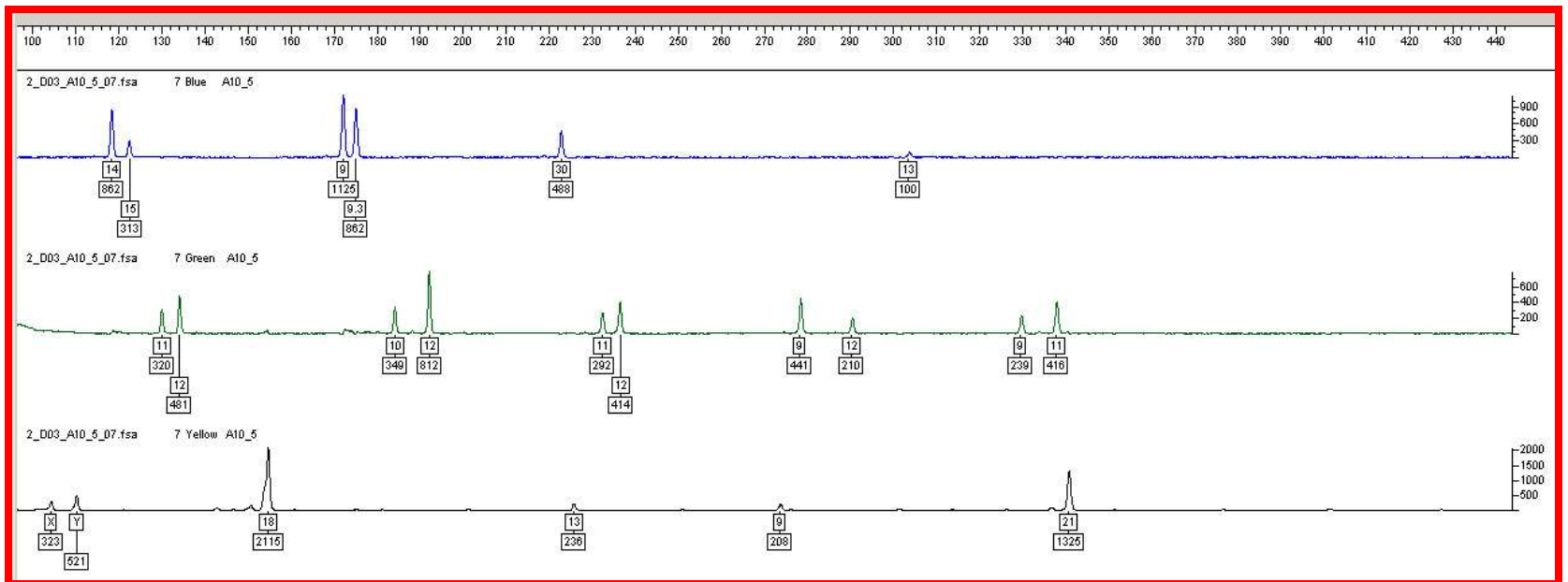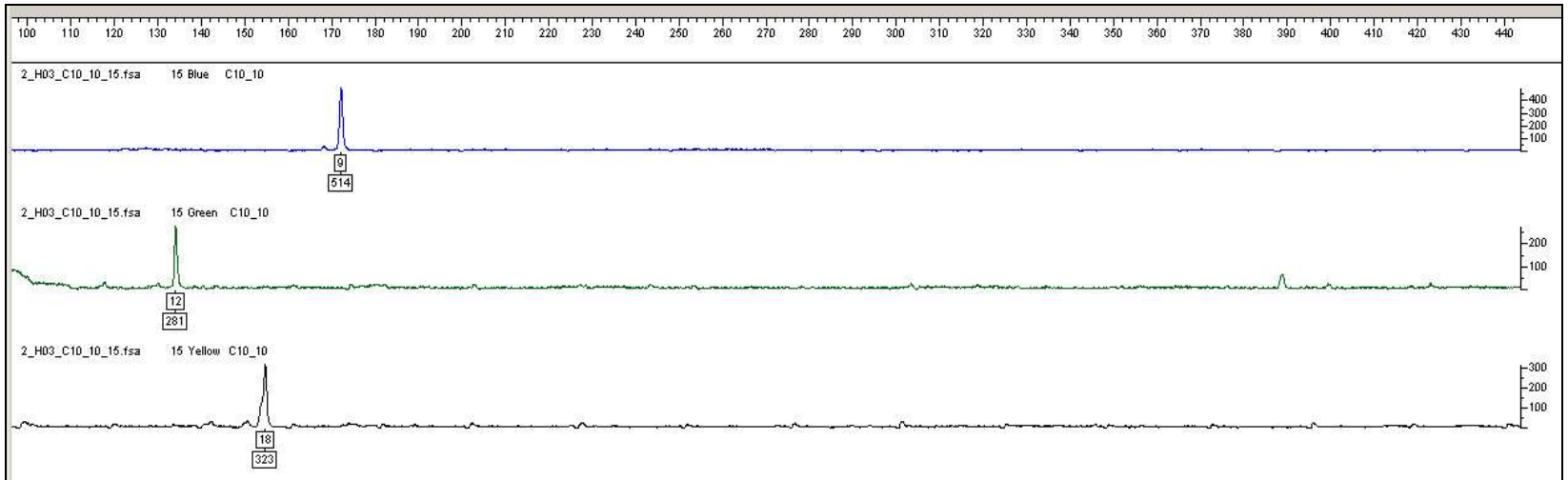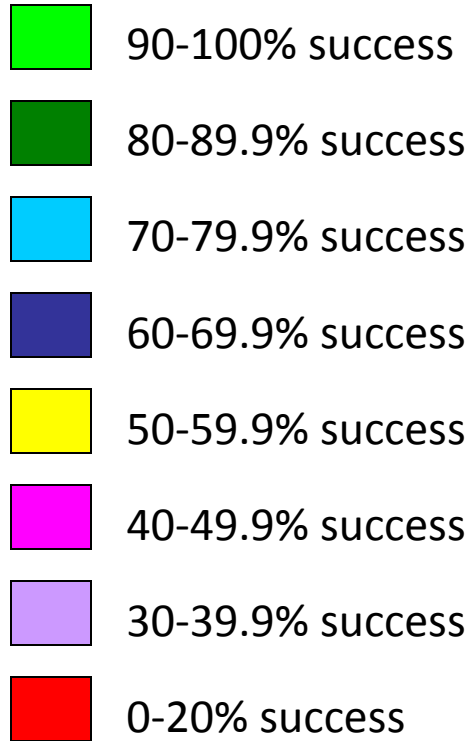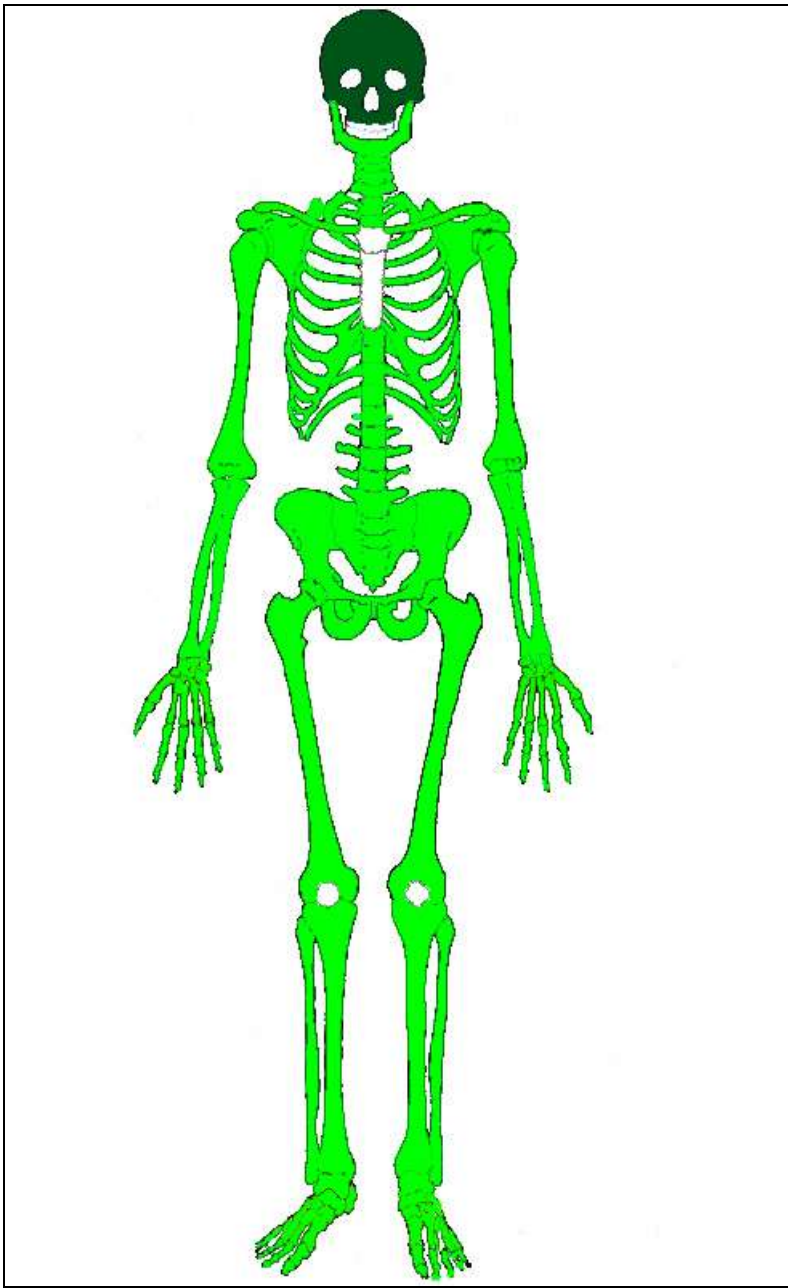
Demineralization success

736 samples processed

Courtesy of Suzie Barritt-Ross

Percent Success Rate by Fiscal Year
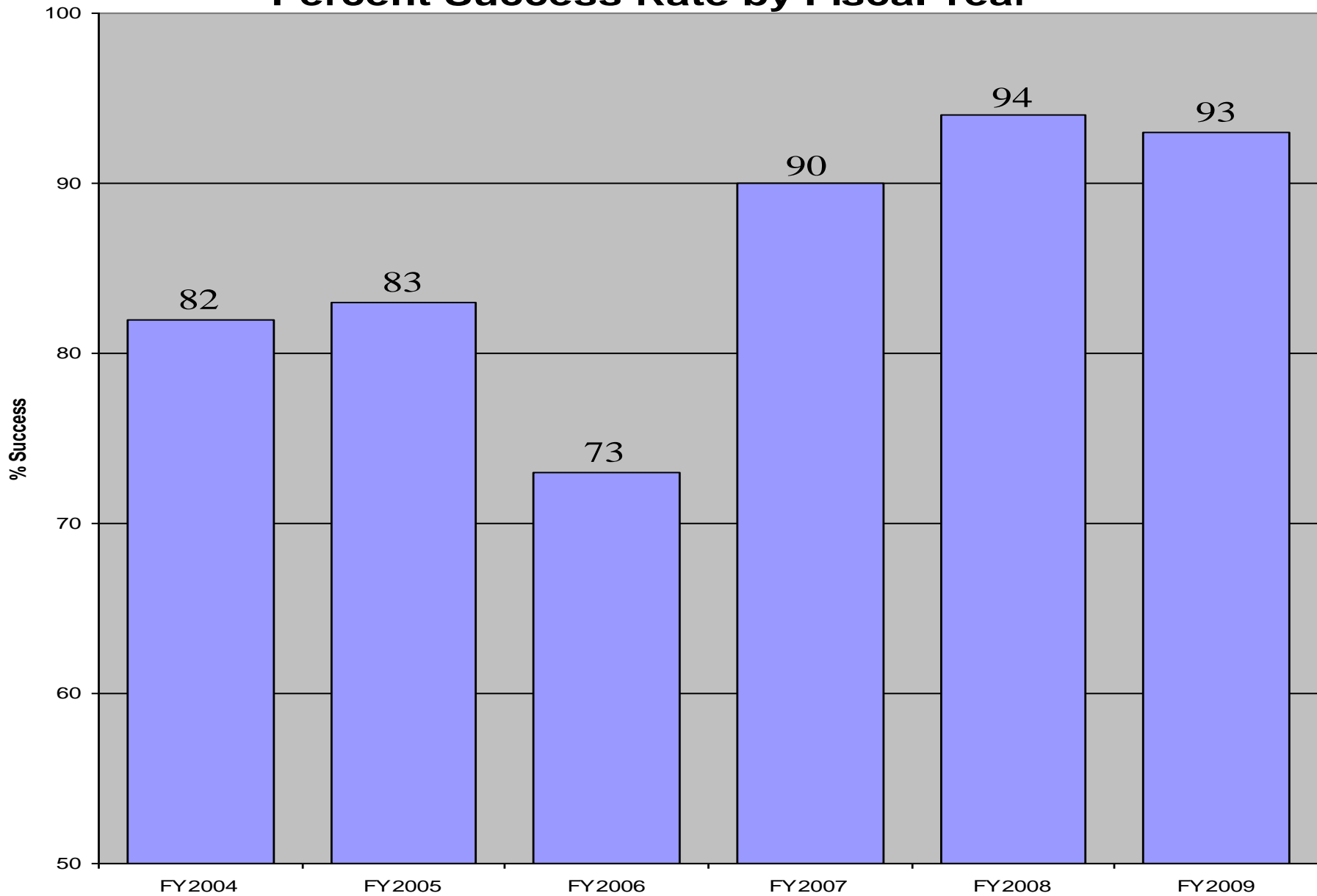
Courtesy of Suzie Barritt-Ross

Short communication

High efficiency DNA extraction from bone by total demineralization☆

Odile M. Loreille [*], Toni M. Diegoli, Jodi A. Irwin, Michael D. Coble, Thomas J. Parsons [1]

Armed Forces DNA Identification Laboratory, 1413 Research Blvd., Bldg. 101, Rockville, MD 20850, United States

Demineralization Protocol II

Demin. Buffer
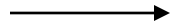
Ultra 4

Qiagen
Mini Elute

No Phenol   With Phenol

Post Qiagen Cleanup
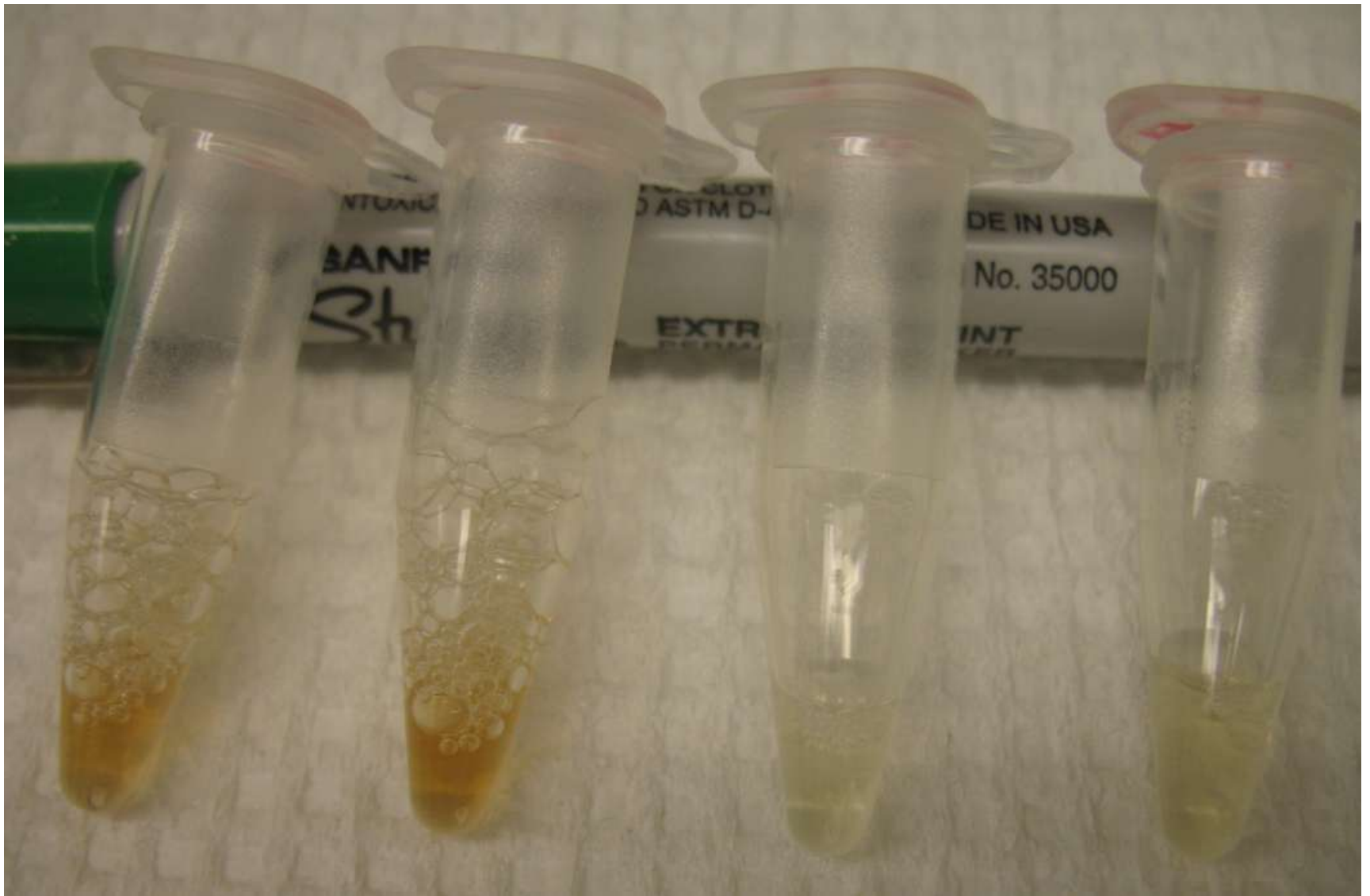
No Phenol   With Phenol

	CW-centricons 30	CW-ultra-4	RS + phenol	RS no phenol
Sample 01	8.866	7.722	10.344	7.472
Sample 02	0.425	0.834	1.257	1.092
Sample 03	0.05 (inhibited)	0.83	1.737	2.347
Sample 04	47	2.53*	59.11	50.76
Sample 05	1.959	1.785	3.464	3.394
Sample 06	9.189	7.83	12.494	10.632
Sample 07	5.692	12.599	11.128	8.373
Sample 08	2.127	0.935	3.418	2.964
Sample 09	10.93	2.27*	10.7	8.96
Sample 10	8.439	7.029	6.324	10.072

Recent Developments with mtDNA

Next Generation Sequencing

LETTERS

Heteroplasmic mitochondrial DNA mutations in normal and tumour cells

Yiping He[1], Jian Wu[1], Devin C. Dressman[1], Christine Iacobuzio-Donahue[2], Sanford D. Markowitz[3],
Victor E. Velculescu[1], Luis A. Diaz Jr[1], Kenneth W. Kinzler[1], Bert Vogelstein[1] & Nickolas Papadopoulos[1]

a.
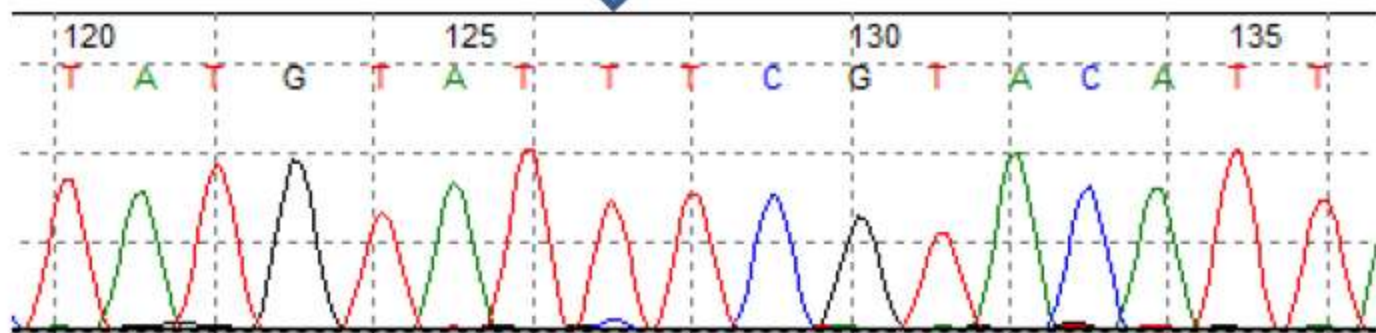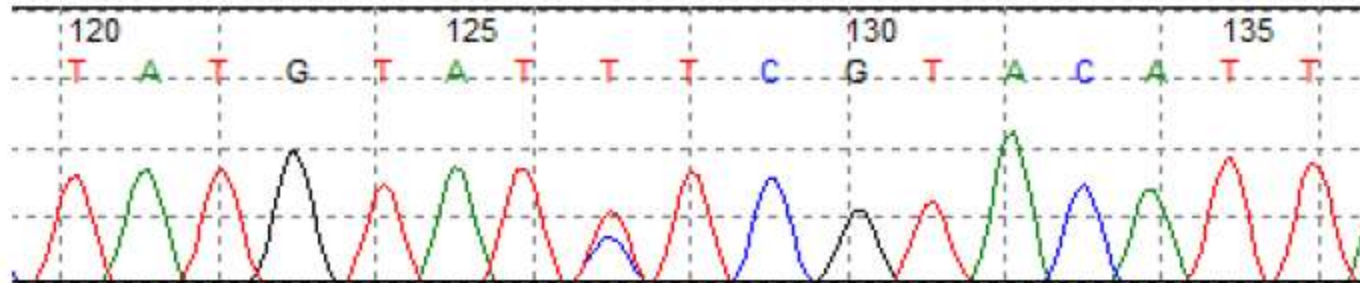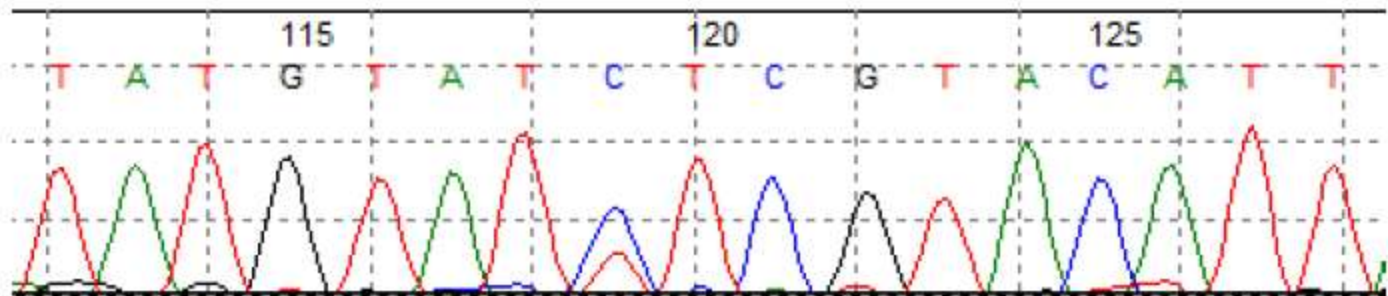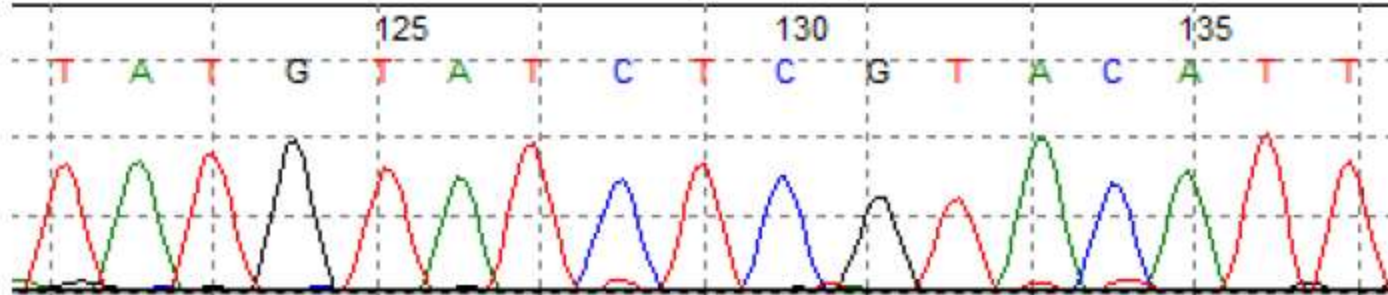
Because mtDNA template molecules are so numerous in comparison with nuclear DNA template molecules, they are also useful for forensic applications. Previous studies have shown variations in the length of mononucleotide tracts in mtDNA from hair roots compared with blood[29,30]. Our new results clearly show that heteroplasmies affect the entire mitochondrial genome, are common in normal individuals and vary markedly from tissue to tissue. Thus an individual, and perhaps even a single cell, does not have a single mtDNA genotype. Instead, tissues have a mixture of genotypes, a few of which may be maternally inherited and the remaining ones the result of somatic mutations. This suggests caution in excluding identity on the basis of a single or small number of mismatched alleles when the tissue in evidence (such as sperm) is not the same as the reference tissue of the suspect (such as blood or hair).

Table 2 | Heteroplasmic variants in different organs of the same individual (patient 11, 59 years old)

Position	Allele 1	Allele 2	Allele 1 frequency (%)										No. of tissues with variant	Minimum (%)	Maximum (%)
			Cerebrum	Heart	Skeletal muscle	Lung	Kidney	Spleen	Liver	Pancreas	Colon	Cerebellum			
60	C	T*	<0.35	<0.35	<0.35	<0.35	**2.16**	<0.35	**1.96**	<0.35	<0.35	<0.35	2	<0.35	**2.16**
64	A	C*	<0.35	<0.35	**1.73**	<0.35	0.36	<0.35	<0.35	<0.35	<0.35	<0.35	1	<0.35	**1.73**
72	C	T*	<0.35	<0.35	<0.35	<0.35	**18.4**	<0.35	**11.7**	<0.35	<0.35	<0.35	2	<0.35	**18.4**
73	G	A*	<0.35	<0.35	**2.27**	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	1	<0.35	**2.27**
74	G	T*	<0.35	<0.35	<0.35	<0.35	**2.64**	<0.35	<0.35	<0.35	<0.35	<0.35	1	<0.35	**2.64**
189	G	A*	1.06	0.92	**9.77**	0.37	<0.35	<0.35	0.91	0.39	<0.35	<0.35	1	<0.35	**9.77**
408	A	T*	<0.35	<0.35	**3.64**	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	1	<0.35	**3.64**
1983	C	T*	<0.35	<0.35	<0.35	<0.35	<0.35	**1.76**	<0.35	<0.35	<0.35	<0.35	1	<0.35	**1.76**
6078	C	A*	0.52	0.49	0.82	1.23	0.44	1.10	**2.99**	0.92	0.54	**1.60**	2	0.44	**2.99**
8021	G	A*	0.37	<0.35	<0.35	1.42	<0.35	**1.87**	<0.35	0.78	0.39	0.83	1	<0.35	**1.87**
11090	C	A*	0.88	0.67	**1.63**	1.12	1.04	**1.65**	1.46	**1.62**	1.02	1.24	3	0.67	**1.65**
14274	C	A*	0.54	**1.6**	0.41	<0.35	<0.35	<0.35	0.92	0.61	1.18	0.84	1	<0.35	**1.60**
16092	C	T*	0.60	0.53	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	0.54	**2.83**	1	<0.35	**2.83**
16093	C	T*	**67.4**	**43.6**	**7.44**	**73.0**	**90.9**	**81.6**	**70.9**	**63.8**	**62.2**	**60.1**	10	**7.44**	**90.9**
Total number of heteroplasmic variants			1	2	6	1	4	4	4	2	1	3	–	–	–

* The reference allele. Allele 1 frequencies of 1.6% or more are shown in bold.

Position	Allele 1	Allele 2	Skeletal muscle	Lung	
60	C	T*	<0.35	<0.35	
64	A	C*	**1.73**	<0.35	**N.D. by Sanger Sequencing**
72	C	T*	<0.35	<0.35	
73	G	A*	**2.27**	<0.35	**N.D. by Sanger Sequencing**
74	G	T*	<0.35	<0.35	
189	G	A*	**9.77**	0.37	
408	A	T*	**3.64**	<0.35	**N.D. by Sanger Sequencing**
1983	C	T*	<0.35	<0.35	
6078	C	A*	0.82	1.23	
8021	G	A*	<0.35	1.42	
11090	C	A*	**1.63**	1.12	**N.D. by Sanger Sequencing**
14274	C	A*	0.41	<0.35	
16092	C	T*	<0.35	<0.35	
16093	C	T*	**7.44**	**73.0**	
Total number of heteroplasmic variants			6	1	

Patient #	Age	Position	Allele 1	Allele 2
1	66	60	C	T*
1	66	72	C	T*
1	66	94	A	G*
2	77	60	C	T*
2	77	72	C	T*
2	77	94	A	G*
4	50	72	C	T*
5	35	72	C	T*
6	53	72	C	T*
6	53	94	A	G*
8	64	72	C	T*
9	42	60	C	T*
9	42	72	C	T*
9	42	94	A	G*
10	59	60	C	T*
10	59	72	C	T*

60, 72, 94
(Artifacts?)

Short communication

Current Next Generation Sequencing technology may not meet forensic standards

Hans-Jürgen Bandelt [a,b,*], Antonio Salas [a,b]

[a] *Department of Mathematics, University of Hamburg, 20146 Hamburg, Germany*
[b] *Unidade de Xenética, Instituto de Medicina Legal, Facultade de Medicina, and Departamento de Anatomía Patolóxica e Ciencias Forenses, Facultade de Medicina, Universidade de Santiago de Compostela, 15782 Galicia, Spain*

Patient 4

709 1888 (4917) (8697) (10463) 13368 14905 (15607) (15928) (16294)

T

4	72	T	C
4	73	A	G
4	200	A	G
4	263	A	G
4	709	G	A
4	750	A	G
4	1420	T	C
4	1438	A	G
4	1888	G	A
4	2141	T	C
4	4769	A	G
4	6249	G	A
4	6524	T	C

4	7028	C	T
4	8860	A	G
4	9117	T	C
4	11251	A	G
4	11719	G	A
4	11764	A	G
4	11812	A	G
4	12741	C	T
4	13368	G	A
4	14233	A	G
4	14687	A	G
4	14766	C	T
4	14905	G	A
4	15452	C	A
4	16126	T	C
4	16296	C	T
4	16324	T	C
4	16519	T	C
4	10726	G	A

Table 1
Deficiencies in the mtDNA sequences reported by He et al. [6].

Sample	HG[a]	Missed mutations (characterizing haplogroup)[b]
Patient 1	J1c3a1	15326 (H2a2), 2706 (H), 4216 (R2'JT), 3010[c] (J1), 13934 (J1c3)
Patient 2	J1b1a	15326 (H2a2), 2706 (H), 4216 (R2'JT), 3010[c] (J1), 16222 (J1b), 16261[c] (J1b), 5460[c] (J1b1)
Patient 3	J1c or J1c3a	15326 (H2a2), either 2706 (H) or 13934 (J1c3), 4216 (R2'JT), 3010[c] (J1)
Patient 4	T2a1b1	15326 (H2a2), 2706 (H), 4216 (R2'JT), 4917 (T), 8697 (T), 10463 (T), 15607 (T), 15928 (T), 16294[c] (T), 13965 (T2a), 13966 (T2a1b1)
Patient 5	N22	15326 (H2a2), 2706 (H), 16223[c] (R), 942 (N22), 16249[c] (N22)
Patient 6	U5a1	15326 (H2a2), 2706 (H), 13617 (U5), 16270 (U5), 16256[c] (U5a)
Patient 7	X2a2	15326 (H2a2), 2706 (H), 7028 (H), 16223[c] (R), 153 (X), 13966 (X), 16278[c] (X), 1719[c] (X2), 12397 (X2a'j), 8913 (X2a), 16213 (X2a)
Patient 8	J1c3a	15326 (H2a2), 2706 (H), 4216 (R2'JT), 3010[c] (J1), 13934 (J1c3)
Patient 9	J1c	15326 (H2a2), 2706 (H), 4216 (R2'JT), 3010[c] (J1), 185[c] (J1c)
Patient 10	H7c	15326 (H2a2), 4793 (H7)
CEPH 45♀	T2b3	15326 (H2a2), 1438 (H2), 14905 (T)
CEPH 45♂	H1	15326 (H2a2), 1438 (H2)
CEPH1377♀	T1	15326 (H2a2), 1438 (H2), 14905 (T)
CEPH1377♂	K1b1a	15326 (H2a2), 1438 (H2), 152[c] (K1b1a)

Short communication

Current Next Generation Sequencing technology may not meet forensic standards

Hans-Jürgen Bandelt [a,b,*], Antonio Salas [a,b]

[a] *Department of Mathematics, University of Hamburg, 20146 Hamburg, Germany*
[b] *Unidade de Xenética, Instituto de Medicina Legal, Facultade de Medicina, and Departamento de Anatomía Patolóxica e Ciencias Forenses, Facultade de Medicina, Universidade de Santiago de Compostela, 15782 Galicia, Spain*

"Before one can really set out to access to entire mtDNA genome data with relative ease for forensic purposes, one needs careful calibration studies under strict forensic conditions—or might have to wait for another generation."

The Identification of the two missing Romanov Children by DNA Testing

Michael D. Coble[1], Odile M. Loreille[1], Mark J. Wadhams[1], Suni M. Edson[1], Kerry Maynard[1], Carna E. Meyer[1], Harald Niederstätter[2], Cordula Berger[2], Burkhard Berger[2], Anthony B. Falsetti[3], Peter Gill[4,5], Walther Parson[2], Louis N. Finelli[1]

[1]Armed Forces DNA Identification Laboratory, Armed Forces Institute of Pathology, Rockville, Maryland, [2]Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria, [3]University of Florida, Gainesville, FL, [4]University of Strathclyde, Department of Pure and Applied Chemistry, Glasgow, United Kingdom, [5]Institute of Forensic Medicine, University of Oslo, Oslo, Norway.

ELSEVIER

Assessing ancient DNA studies

**M. Thomas P. Gilbert[1], Hans-Jürgen Bandelt[2], Michael Hofreiter[3] and Ian Barnes[4]**

[1]Ecology and Evolutionary Biology, The University of Arizona, 1041 E. Lowell St, Tucson, AZ 85721, USA
[2]Department of Mathematics, University of Hamburg, Bundesstr. 55, 20146 Hamburg, Germany
[3]Department of Evolutionary Genetics, Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, 04103 Leipzig, Germany
[4]The Center for Genetic Anthropology, Department of Biology, Darwin Building, University College London, Gower Street, London, UK, WC1E 6BT

- Isolation of work areas: to separate samples and extracted DNA from PCR amplified products.
- Negative control extractions and amplifications: to screen for contaminants entering the process at any stage.
- Appropriate molecular behaviour: owing to DNA degradation, the successful amplification of large DNA fragments in ancient DNA studies should be treated with caution.
- Reproducibility: multiple PCR and extractions should yield consistent results.
- Independent replication: the generation of consistent results by independent research groups.
- Associated remains: are associated remains equally well preserved, and do they show evidence of contamination?
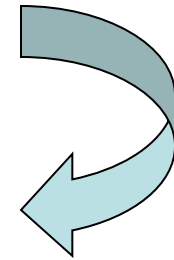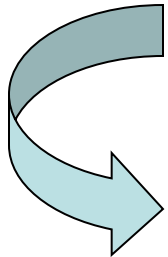
The Romanovs – Russia's Royal Family (1913)

Photo taken commemorating the 300th Anniversary of the Romanov Dynasty

Historical Background

- After spending several months in Tobolsk, the family is finally exiled to Siberia (Ekaterinburg).

The Romanovs in Tobolsk, Russia

The Romanov Family in captivity (left to right Tatiana, Tsarvitch Alexei, Maria (standing) Tsar Nickolas II, Anastasia, Olga)

Historical Background

- After spending several months in Tobolsk, the family is finally exiled to Siberia (Ekaterinburg).

- "I would go anywhere at all, only not to the Urals."   - Tsar Nicholas II

Dr. Eugene Botkin

Anna Demidova

Alexei Trupp

Ivan Kharitinov

Ipatiev House in Ekaterinburg

From the Sokolov collection at Harvard

The Romanov family was kept in Ekaterinburg under house arrest by the Bolsheviks from the end of April 1918 until their murder on July 17, 1918.

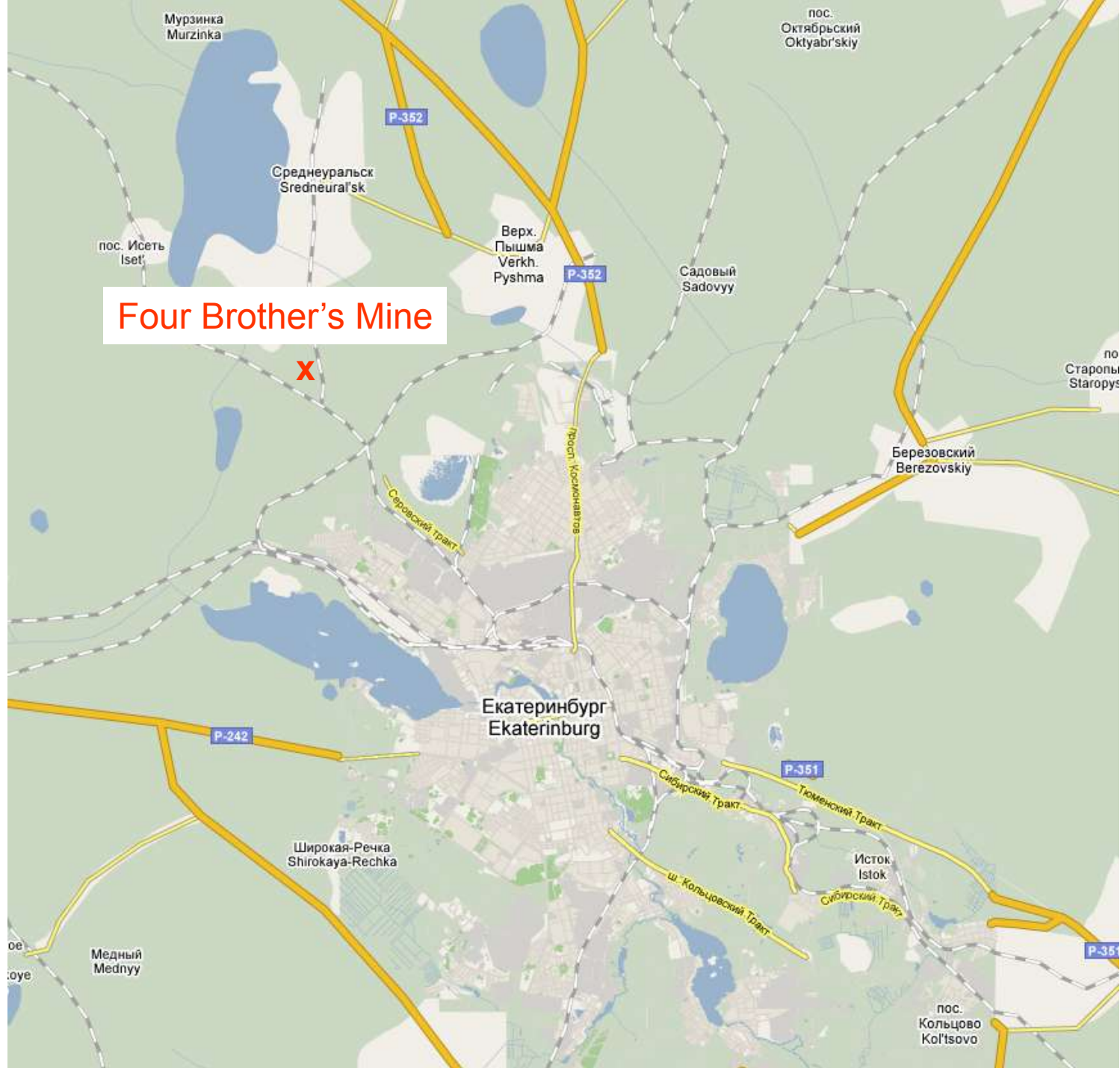From the Sokolov collection at Harvard

Courtesy of Peter Sarandinaki

Yakov Yurovsky
Chief Executioner

'Exécution du Tsar à Ekatérinenbourg le 17 juillet 1918'
by the french painter *Sarmat*
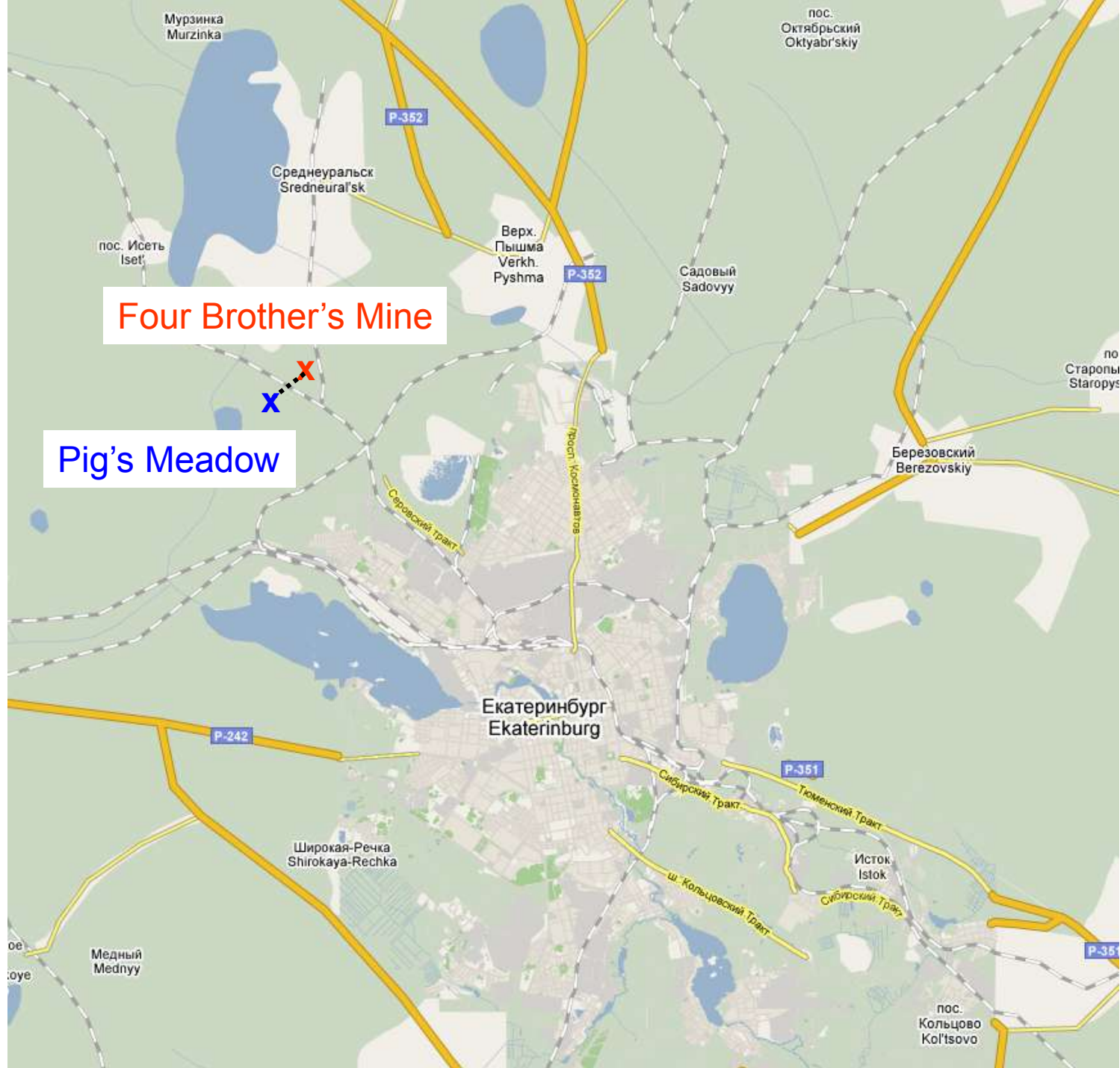
Four Brother's Mine

X

Koptiaki Road in July

Truck used to transport the remains

Excerpt from the Yurovsky Report

"Here (we) ignited a fire, and while the grave was being prepared, we cremated two corpses: Alexei and by mistake, instead of Alexandra Fedorovna, (we) cremated, apparently, Demidova. At the cremation site (we) dug a pit, laid down the bones, leveled it, again lit a large fire and with the ashes concealed any traces."

Excerpt from the Yurovsky Report

"Before laying down the other corpses, we doused sulfuric acid over them, filled the pit, sealed it with sleepers, the empty lorry drove over, (and) somewhat packed down the sleepers and (then we) finished. At 5-6 o'clock in the morning, (I) gathered every one and having declared to them the importance of the completed matter, having warned (them), that everyone must forget about what they saw and never talk about it with anybody."
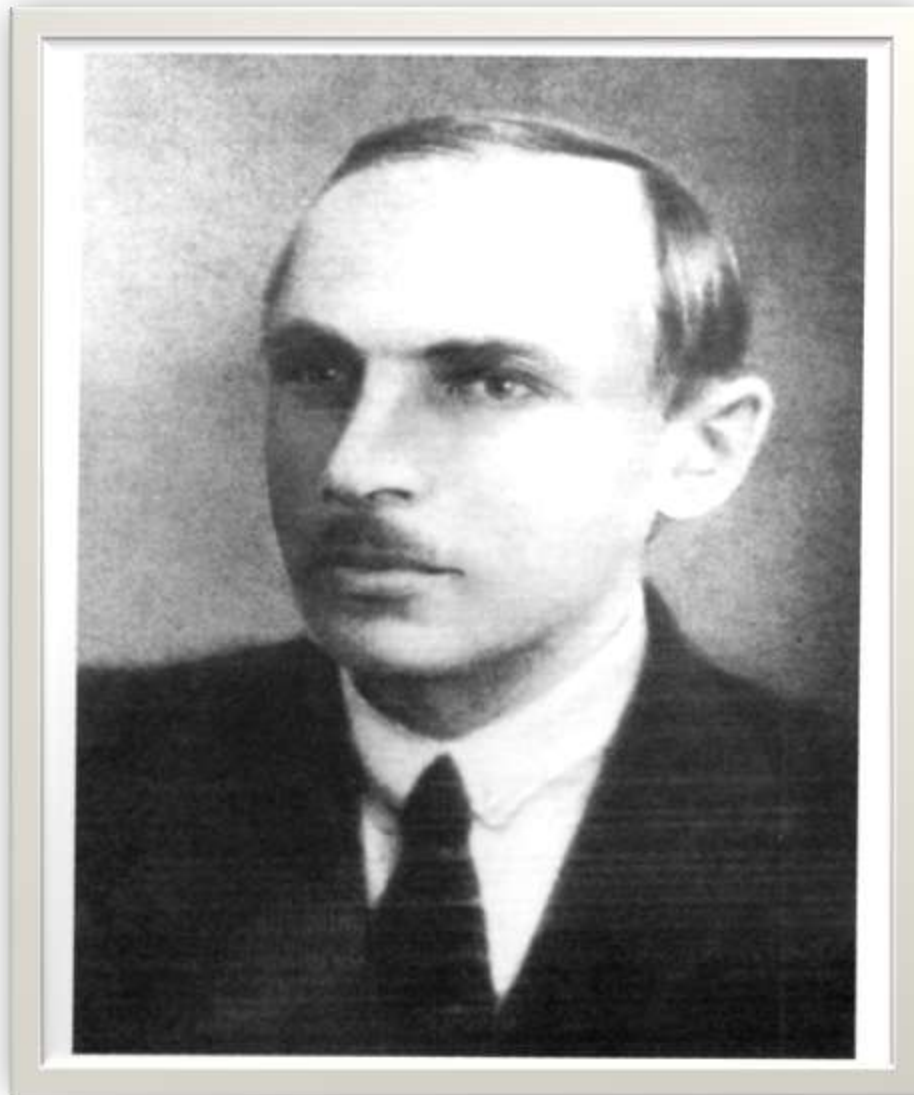
Investigator Nikolay Sokolov 1919

Photo from Dr. Alexander Avdonin

Courtesy of Peter Sarandinaki

Basement Room of the Ipatiev House where the Russian Imperial family was murdered on July 17, 1918 by members of the Ural Soviet

1919 Site Excavation at Four Brother's Mine Shaft

1919 photo taken by Sokolov of the small bridge at Pig's Meadow

Sokolov's photo of the Koptiaki Road standing on the wooden bridge

2007 dig site

From the Sokolov collection at Harvard

1920 photo of Peter Ermakov standing on the bridge

1978- Dr. Avdonin and his group discover the mass grave in Pig's Meadow

1979- Dr. Avdonin and his group planted bushes to hide their find

1991 – Official discovery of the mass grave and excavation of the remains.
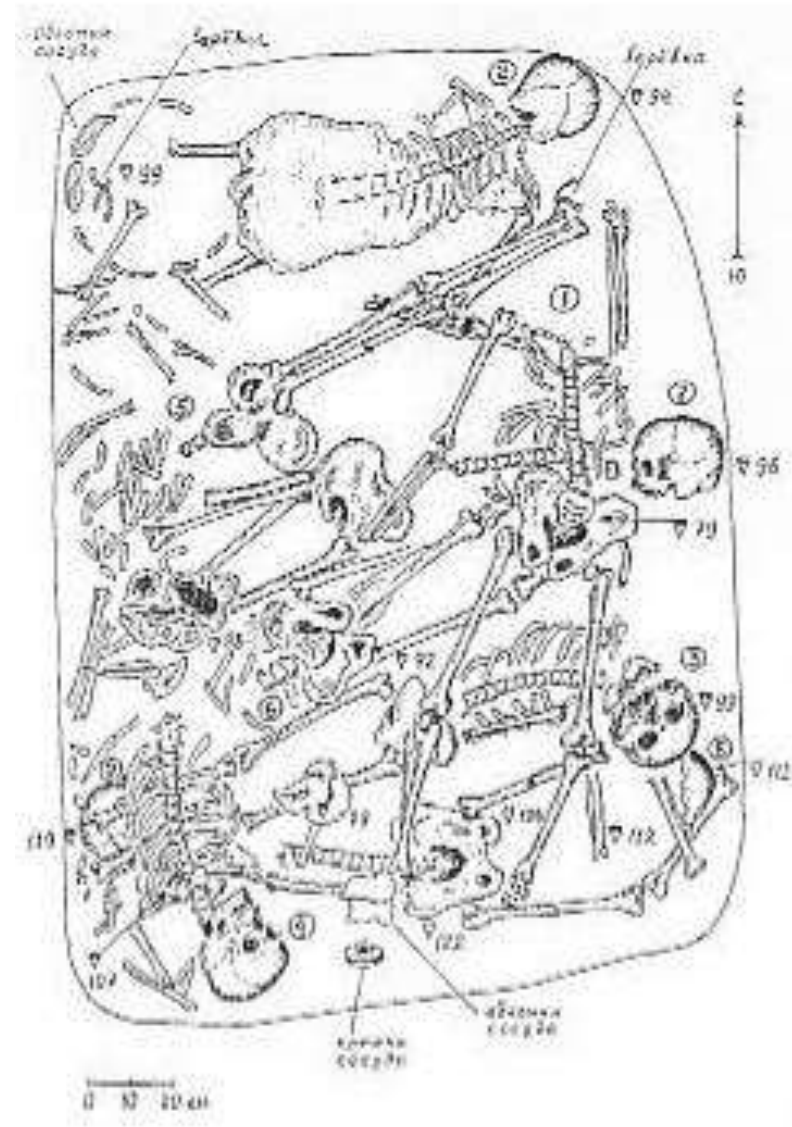
Identifications results comparison
of Russian (Abramov) and U.S. (Maples) teams

Skeleton #	Sex	Age	Size	Abramov conclusion	Maples conclusion
1	F	40-50	161-168	Demidova	
2	M	50-60	171-177	Botkin	
3	F	20-24	158-165	Olga	
4	M	45-55	165-170	Nikolai	
5	F	~20	166-171	Tatiana	Maria
6	F	~20	162-171	Anastasia	Tatiana
7	F	45-50	163-168	Aleksandra	
8	M?	40-50	?	Kharitonov	
9	M	+60	172-181	Trupp	
			Missing bodies ->	**Alexei and Maria**	Alexei and Anastasia

http://www.romanov-memorial.com/Final_Chapter.htm

Previous DNA Testing of the 1991 Remains

Identification of the remains of the Romanov family by DNA analysis

Peter Gill[1], Pavel L. Ivanov[2], Colin Kimpton[1], Romelle Piercy[1], Nicola Benson[1], Gillian Tully[1], Ian Evett[1], Erika Hagelberg[3] & Kevin Sullivan[1]

Table 1 STR genotypes[a] for the nine skeletons

Skeleton	HUMVWA/31	HUMTH01	HUMF13A1	HUMFES/FPS	HUMACTBP2
1 (servant)	14,20	9,10	6,16	10,11	ND
2 (doctor)	17,17	6,10	5,7	10,11	11,30
3 (child)	15,16	8,10	5,7	12,13	11,32
4 (Tsar)	15,16	7,10	7,7	12,12	11,32
5 (child)	15,16	7,8	5,7	12,13	11,36
6 (child)	15,16	8,10	3,7	12,13	32,36
7 (Tsarina)	15,16	8,8	3,5	12,13	32,36
8 (servant)	15,17	6,9	5,7	8,10	ND
9 (servant)	16,17	6,6	6,7	11,12	ND

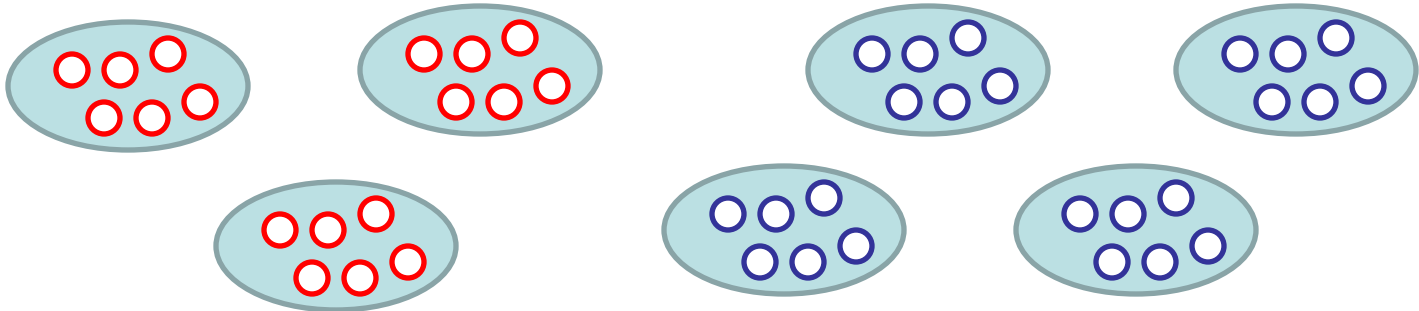[a]Allele designation for all loci except HUMACTBP2 is based on the number of repeat units (determined by sequencing of specific alleles — data not shown). The allele designation for HUMACTBP2 is based on an arbitrary scale identical to that of Kimpton et al.[2].

9 unique DNA profiles – Skeletons 4 (Tsar) and 7 (Tsarina) are consistent with being parents of skeletons 3, 5, and 6.

NOTE: STRs were used as a sorting tool here

Mitotype
16126C
16169T/C
16294T
16296T
73G
263G
315.1C

Louise of Hesse-Cassel

Tsar Nicholas II

Tsarina Alexandra

Prince Philip Duke of Edinburgh

Xenia Cheremeteff-Sfiri

Mitotype
16126C
16169T
16294T
16296T
73G
263G
315.1C

Mitotype
16111T
16357C
263G
315.1C

SOURCES: Gill et al. (1994) *Nature Genetics,* 6, 130-135.; Ivanov et al. (1996) *Nature Genetics,* 12, 417-420; Stone, R. (2004) *Science*, 303, 753.

D.N.A. Box 10.2, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Heteroplasmy

16169 T　　　　　**16169 C**

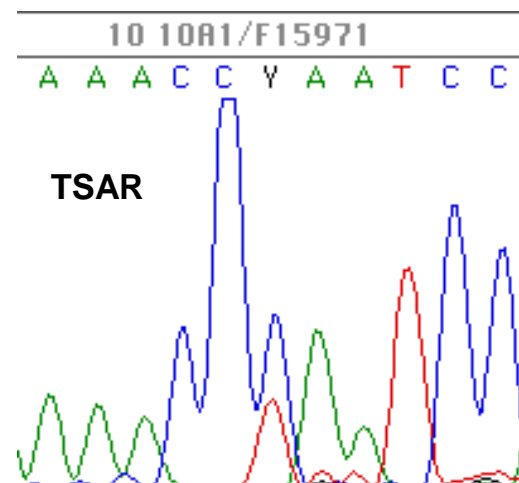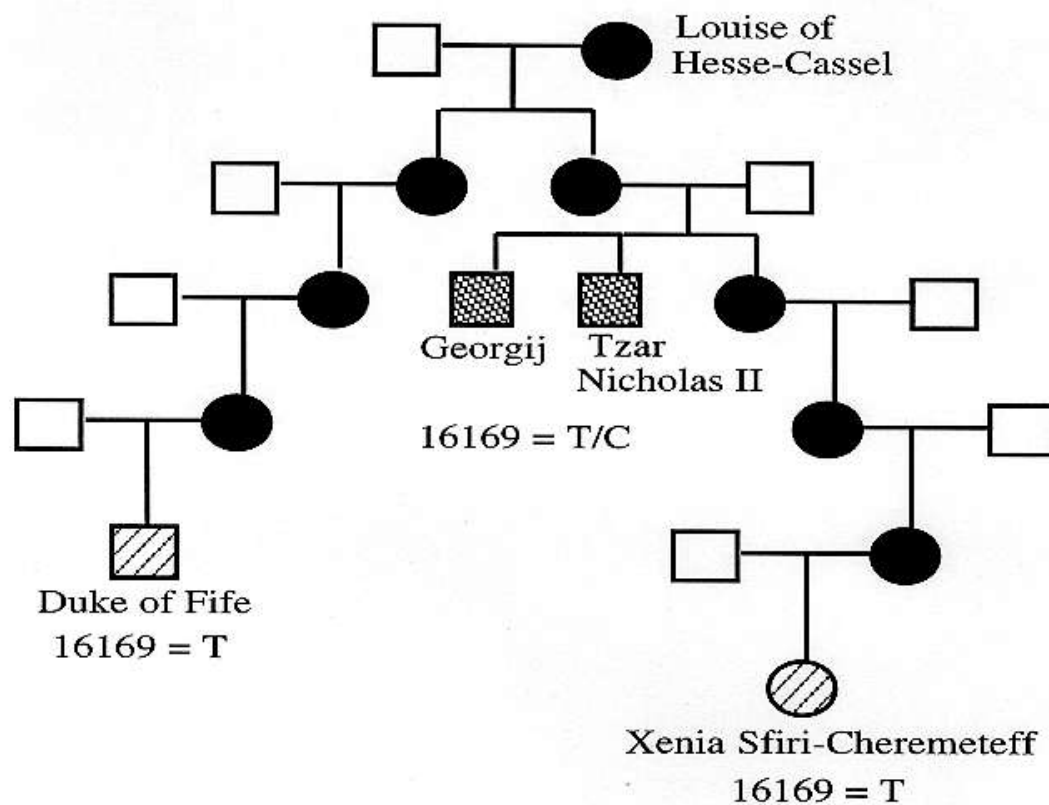10 10A1/F15971

A A A C C Y A A T C C
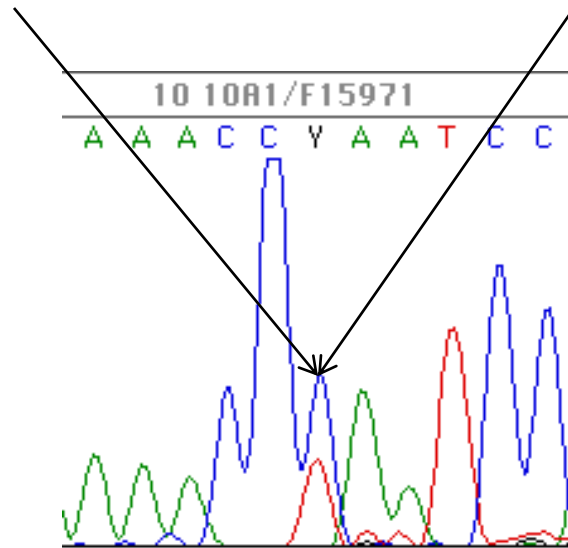
AFDIL – Confirmation of FSS

Dr. Tom Parsons

Concerns About the 1ˢᵗ DNA Testing

- Heteroplasmy – not well understood at the time. We now know that it is quite common.

- Relatively low statistical power – mtDNA database size of 200-300 individuals (LR = 70).

- STRs – in their infancy – only 5 markers were examined.

Despite the concerns – the evidence was overwhelming

Remains of Czar Nicholas II's Son May Have Been Found

FRIDAY, **AUGUST 24, 2007**

MOSCOW — The remains of the last czar's hemophiliac son and heir to the Russian throne, missing since the royal family was gunned down nine decades ago by Bolsheviks in a basement room, may have been found, an archaeologist said Thursday.
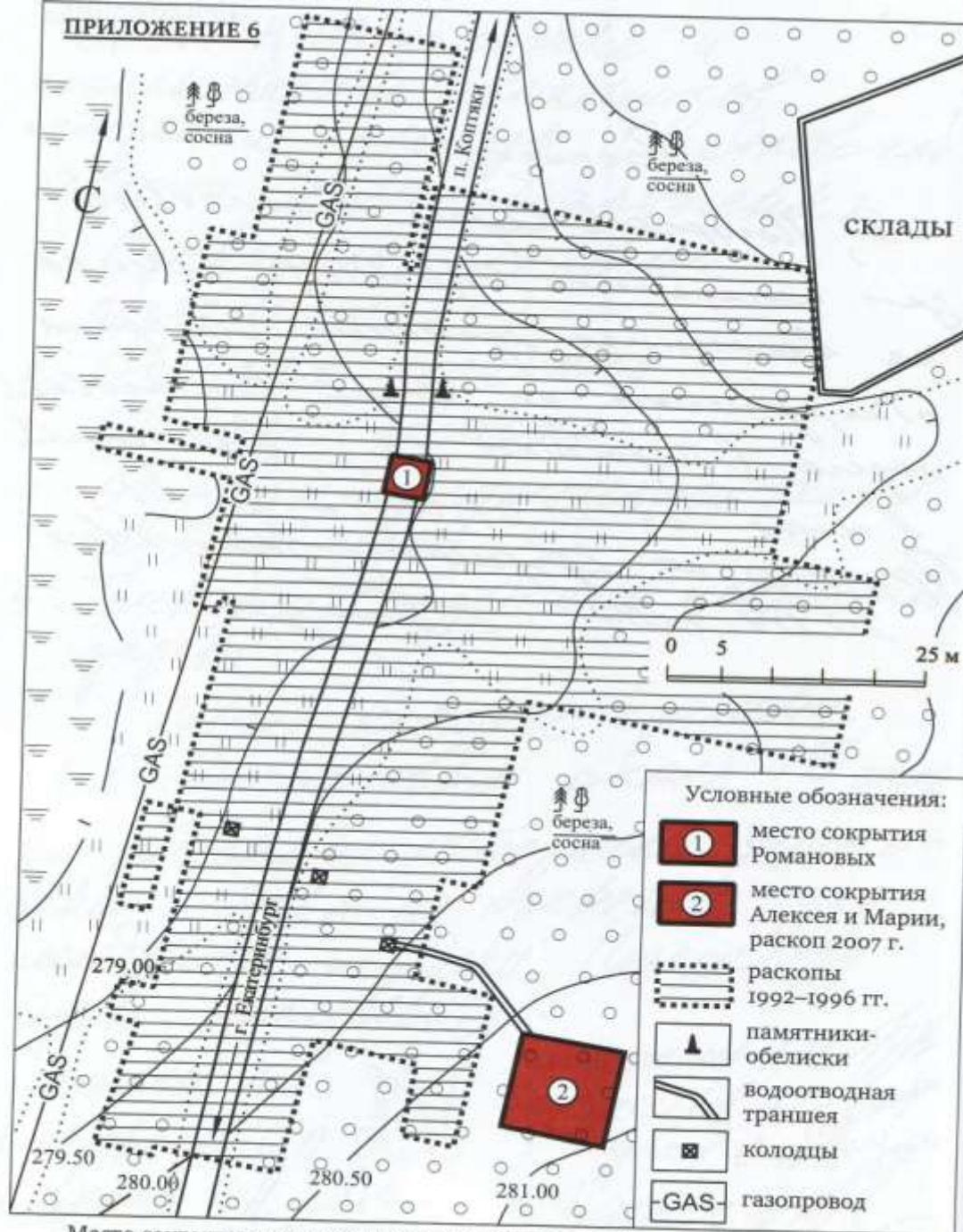
Prince Alexei, aged 8 or 9, in a detail from an official Russian royal-family photograph taken in 1913. His sister Grand Duchess Anastasia's hand drapes over his shoulder.

ПРИЛОЖЕНИЕ 6

С

береза,
сосна

береза,
сосна

п. Коптяки

склады

GAS

GAS

①

GAS

0 5 25 м

GAS

береза,
сосна

г. Екатеринбург

279.00

279.50

280.00

280.50

281.00

②

Условные обозначения:

① место сокрытия Романовых

② место сокрытия Алексея и Марии, раскоп 2007 г.

••••• раскопы 1992–1996 гг.

▲ памятники-обелиски

водоотводная траншея

⊠ колодцы

GAS газопровод

Место сокрытия останков членов Царской семьи. Топографический план.
Составил: Н. Г. Ерохин. Чертеж: Е. О. Святовой

№ 144.2 № 144.1 № 139

№ 104

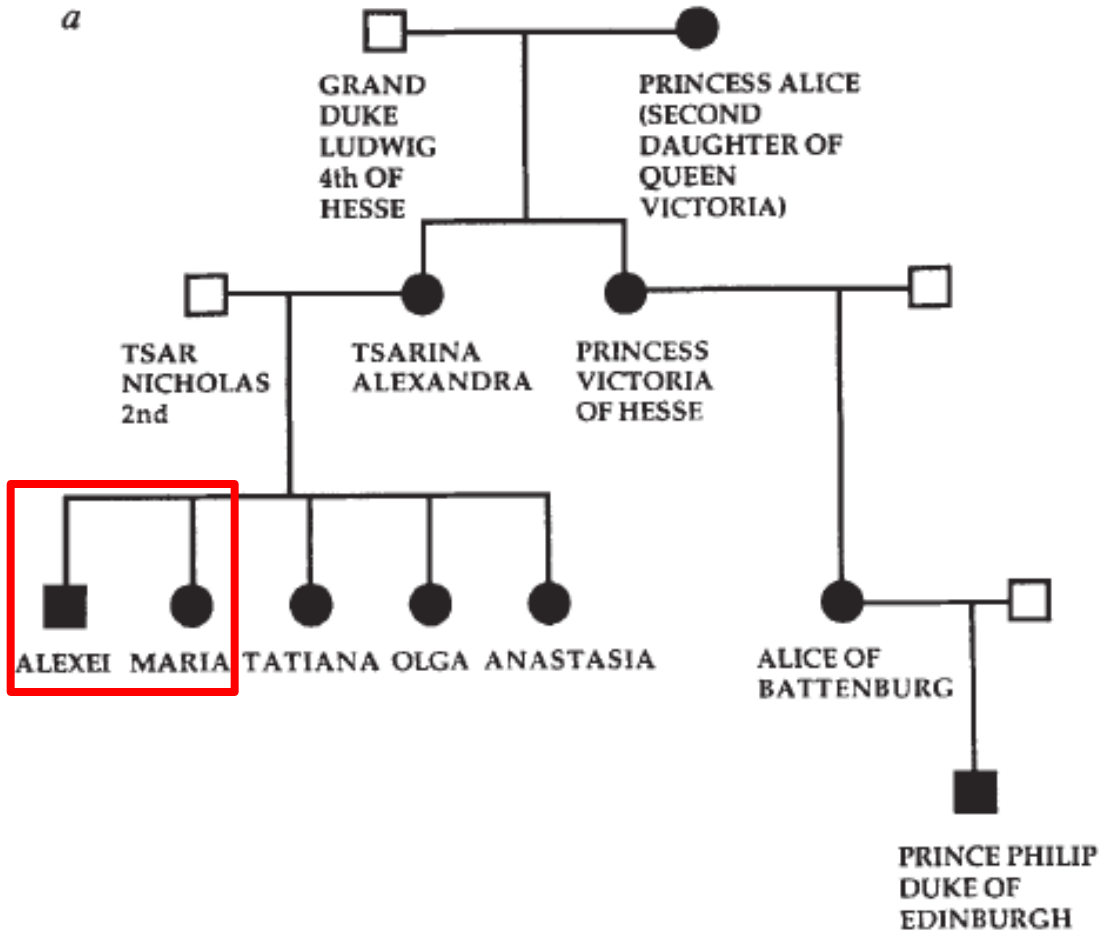№ 140

№ 141

№ 145

№ 142

№ 147
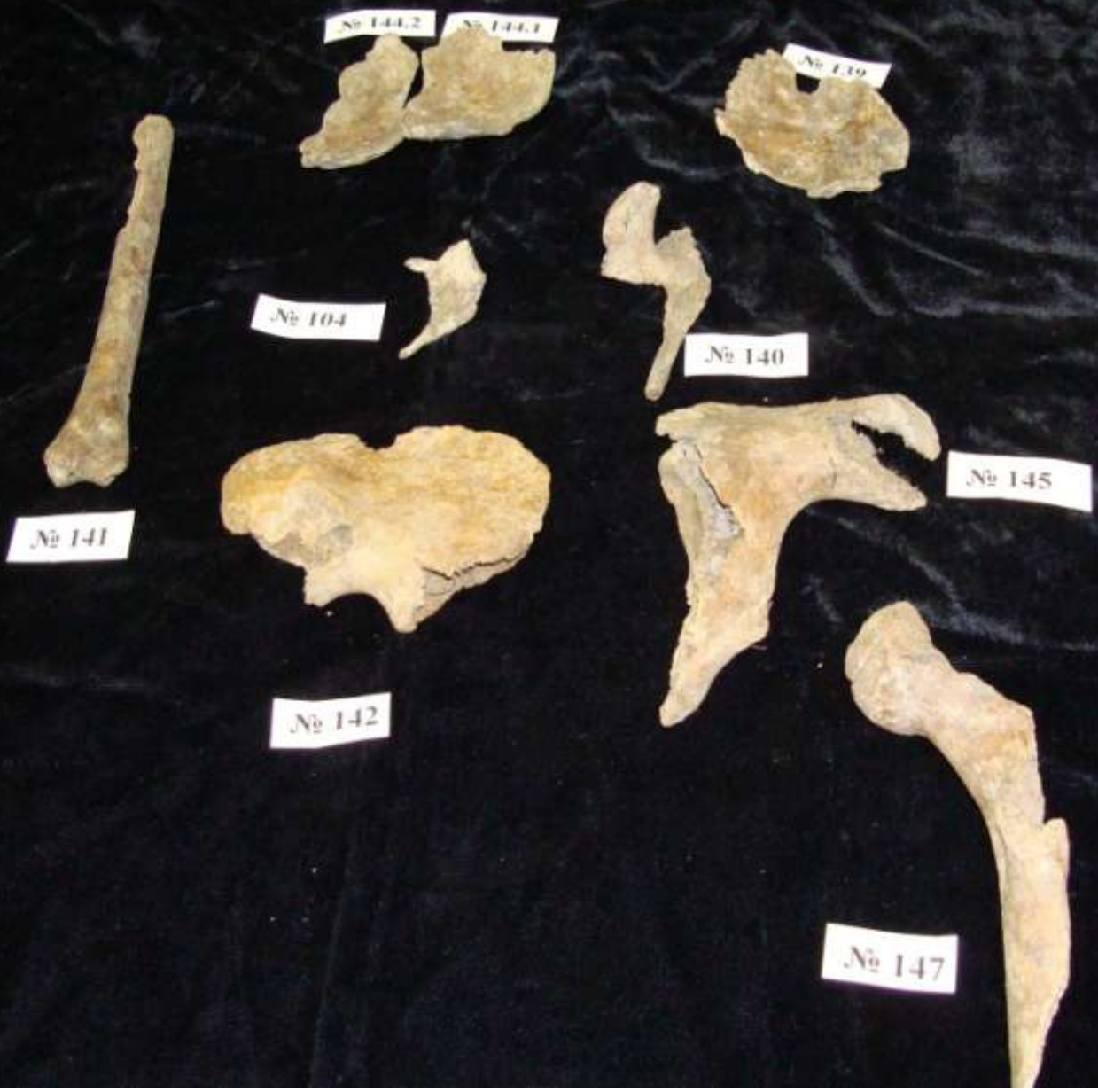
2007
2 sets of remains
Alexei and
Maria
???

~ 70 meters away

9 bodies
Tsar Nicolas II
Tsarina Alexandra
Olga
Tatiana
Anastasia
Anna Demidova
Alexei Trupp
Dr. Botkin
Ivan Kharitinov

a

GRAND DUKE LUDWIG 4th OF HESSE — PRINCESS ALICE (SECOND DAUGHTER OF QUEEN VICTORIA)

TSAR NICHOLAS 2nd — TSARINA ALEXANDRA — PRINCESS VICTORIA OF HESSE

ALEXEI MARIA TATIANA OLGA ANASTASIA

ALICE OF BATTENBURG

PRINCE PHILIP DUKE OF EDINBURGH

mtDNA Analysis

16111 C-T
16357 T-C
263 A-G
315.1 C

Gill *et al.* (1994)
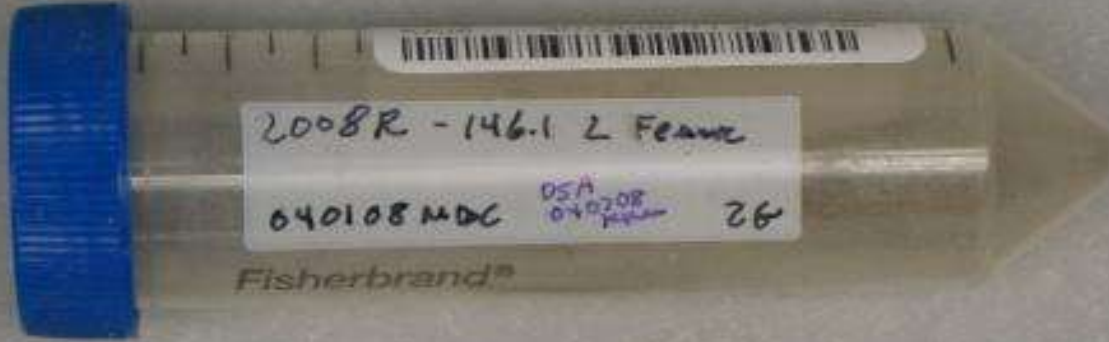
16111 C-T

16357 T-C

Sample 147
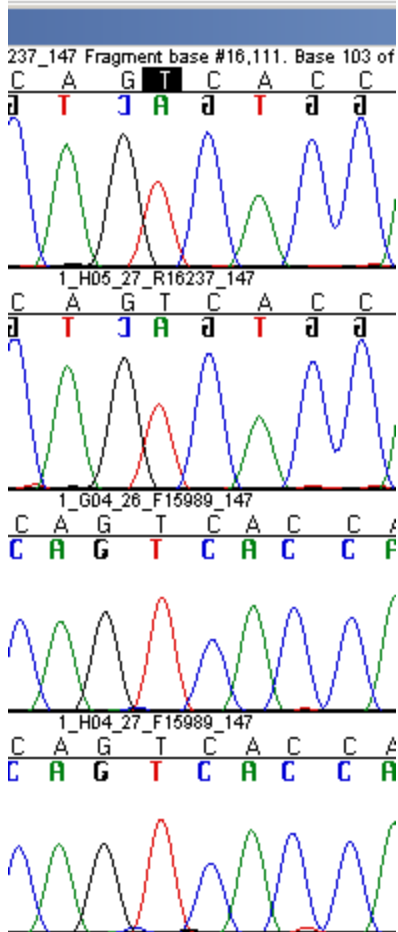
Table 1. Sequences of the samples recovered from "Grave #2" in August 2007 and tested in this study.

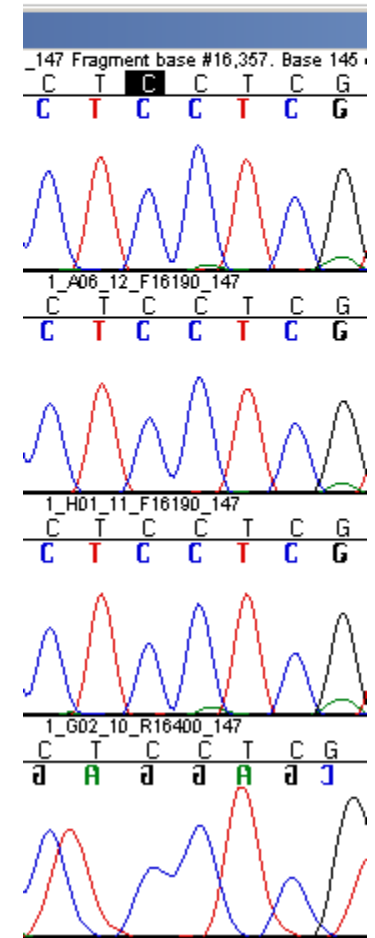| Bone	Russian #	Region Sequenced	Sequence
Right humerus	141	16024-16391 and 35-369	16111T, 16357C, 263G, 315.1C
Occipital fragment	139	no results	–
Occipital fragment	144.1	16024-576	16111T, 16357C, 16519C, 263G, 315.1C, 524.1A; 524.2C
Right os coxae-♀	145	16024-16391 and 35-369	16111T, 16357C, 263G, 315.1C
Left femur	146.1*	16024-576	16111T, 16357C, 16519C, 263G, 315.1C, 524.1A; 524.2C
Right femur - ♀	147*	16024-576	16111T, 16357C, 16519C, 263G, 315.1C, 524.1A; 524.2C
Right scapula	140	16024-16391 and 35-369	16111T, 16357C, 263G, 315.1C
Cranial fragment	143	16024-16391 and 35-369	16111T, 16357C, 263G, 315.1C
Left ilium	142	no results	–

Samples marked with an asterisk (*) were tested by AFDIL and GMI.
doi:10.1371/journal.pone.0004838.t001

Coble *et al.* (2009)

The "Tsarina" mtDNA Sequence

- Has not been observed in a database of **21,546** individuals (4,839 individuals in the US FBI mtDNA database and 16,707 individuals from an internal AFDIL Research Section database).

- mtDNA results agree with previous sequence data from Gill *et al.* 1994

nuclear DNA (STR) Testing

Sample 146.1
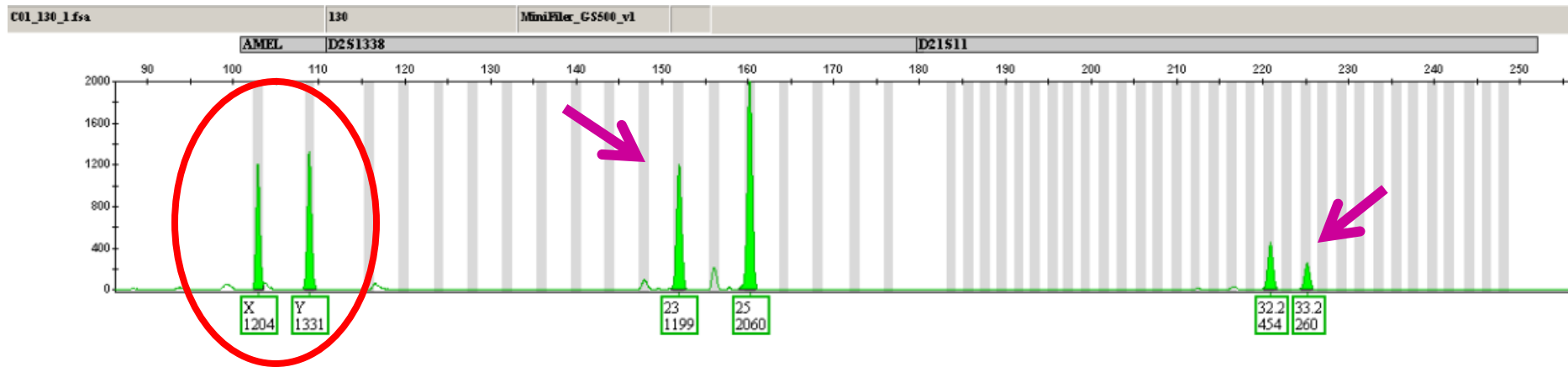
Sample 147
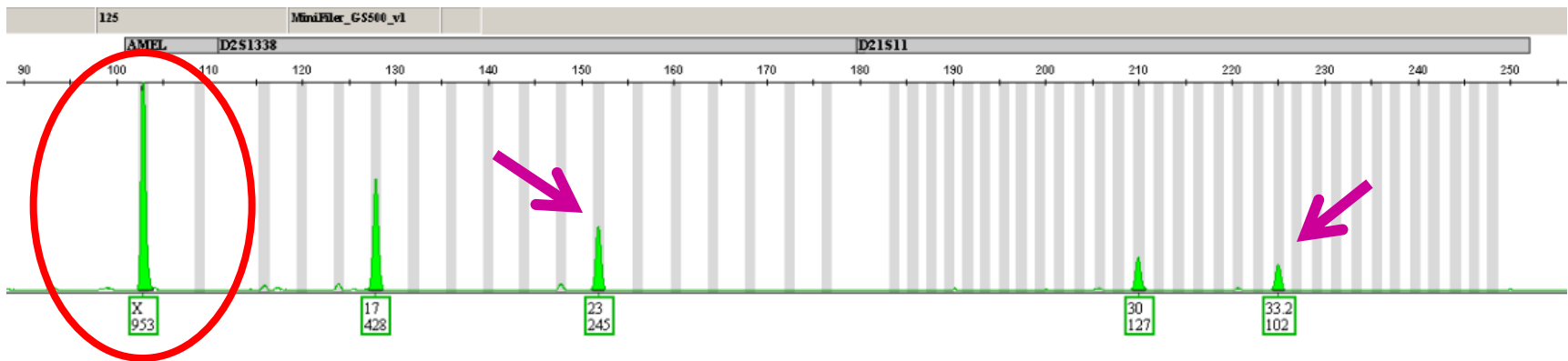
Scenario: Samples 146.1 and 147 as Sibs

$$LR = \frac{Pr\ (E \mid H_1)}{Pr\ (E \mid H_2)}$$

(The samples are siblings)

(The samples are NOT siblings)

Cumulative LR = **5.63 Million**

Can These Remains be Children of Tsar Nicholas II and Tsarina Alexandra?

Table 2. Samples recovered from "Grave #1" in the early 1990s and tested in this study.

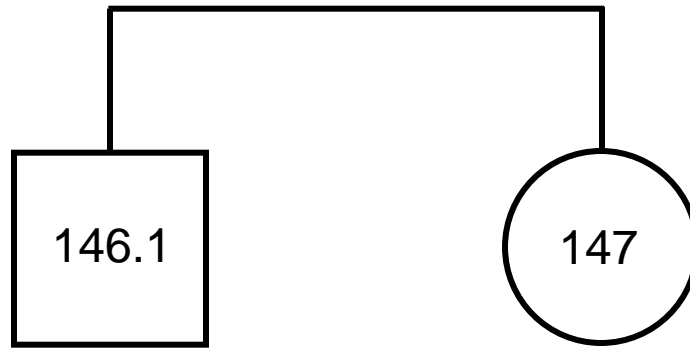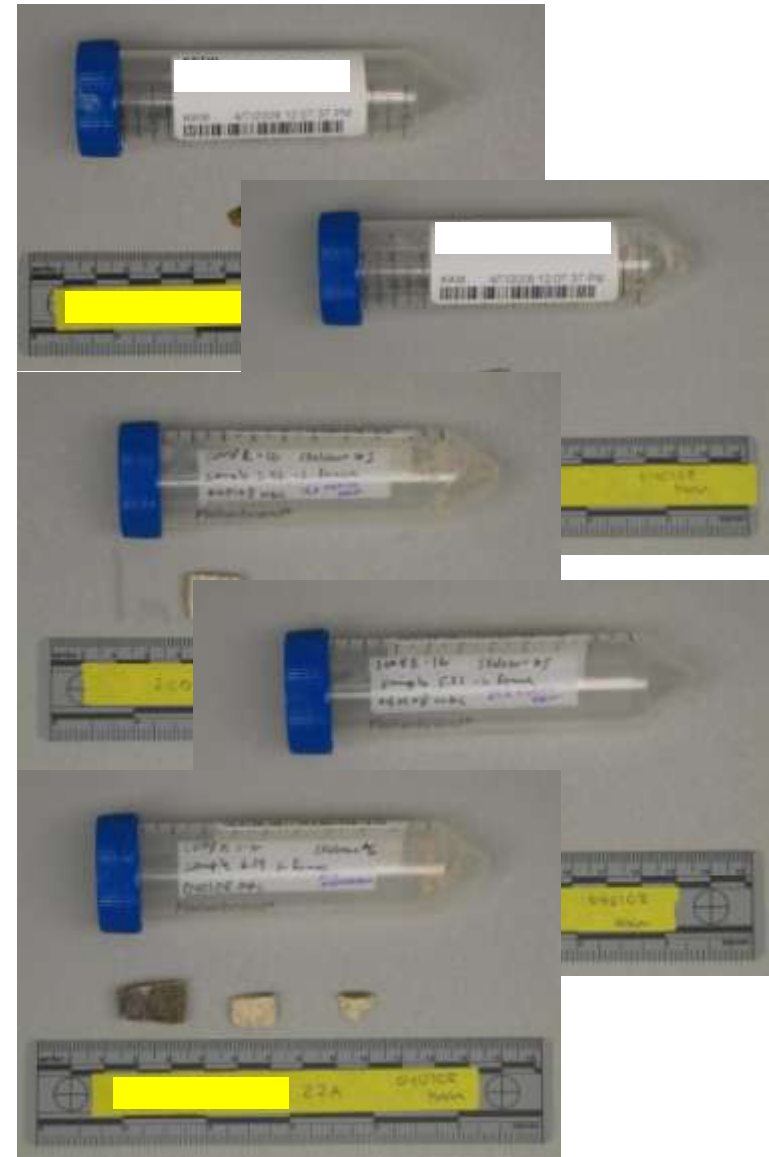| Skeleton	Attribution	Samples	Bone/Teeth
# 3	Olga	3.46*	Fragment of a left femur
		3.4	Partial tooth
#4	Nicholas	4.29	Fragment of a rib
		4.51*	Fragment of a calcaneus
		4a*	Partial tooth
		4.44	*Fragment of a pelvis*
#5	Tatiana	5.21*	Fragment of a left femur
		5.29	Fragment calcaneus
#6	Anastasia	6.14*	Fragment of the diaphyse of a left femur
		6.16*	Fragment of the diaphyse of a left tibia
#7	Alexandra	7.48	Fragment of a pelvis
		7.49*	Fragment of the diaphyse of a left tibia
		7a	Partial tooth
		7.40	*Fragment of the diaphyse of a left femur*

Coble *et al.* (2009)
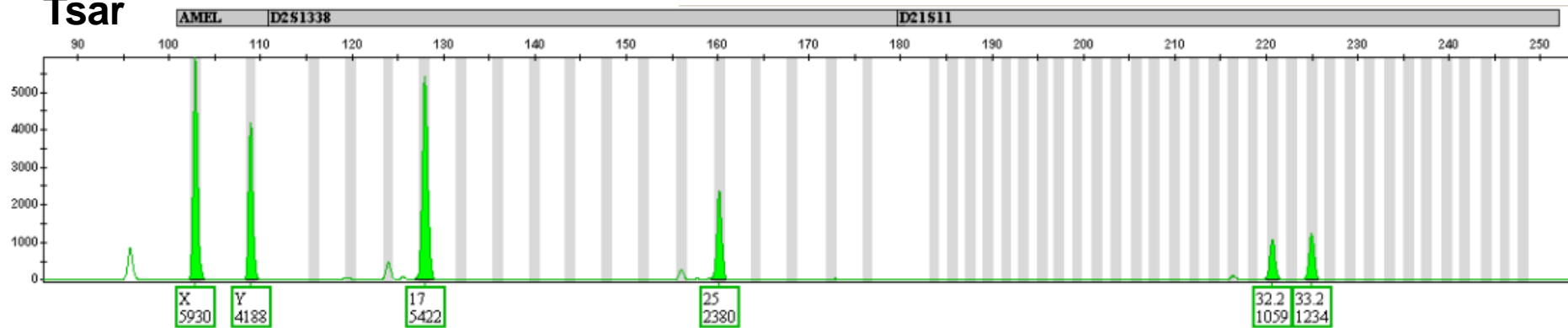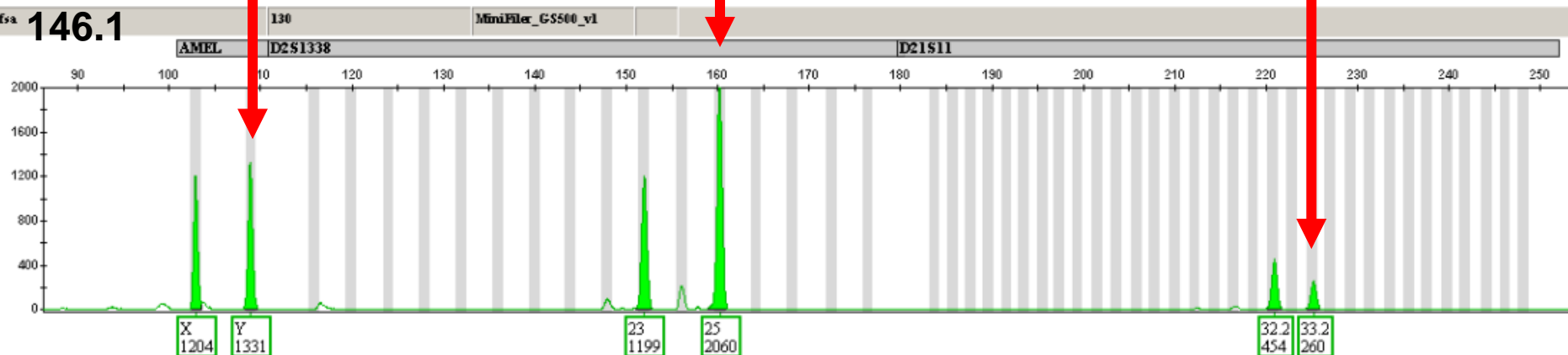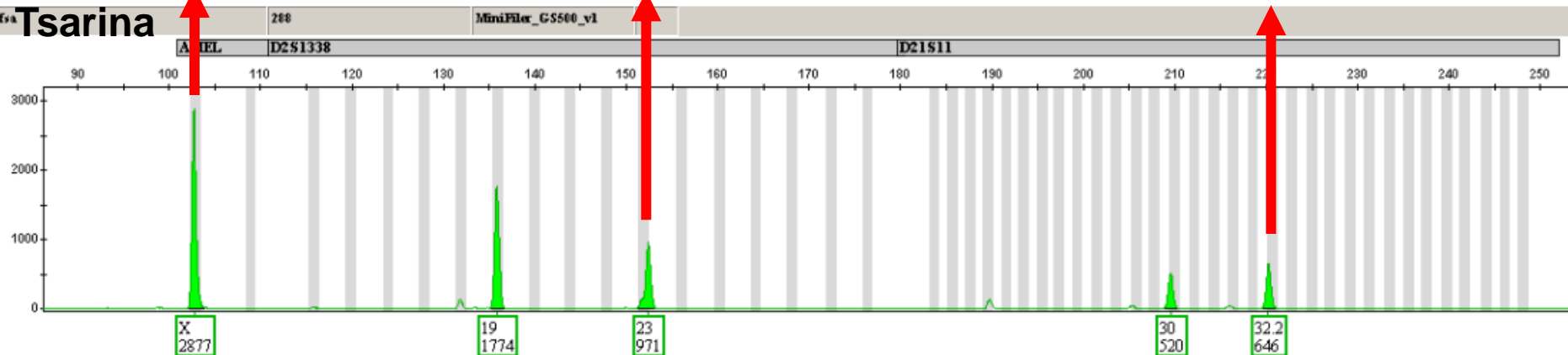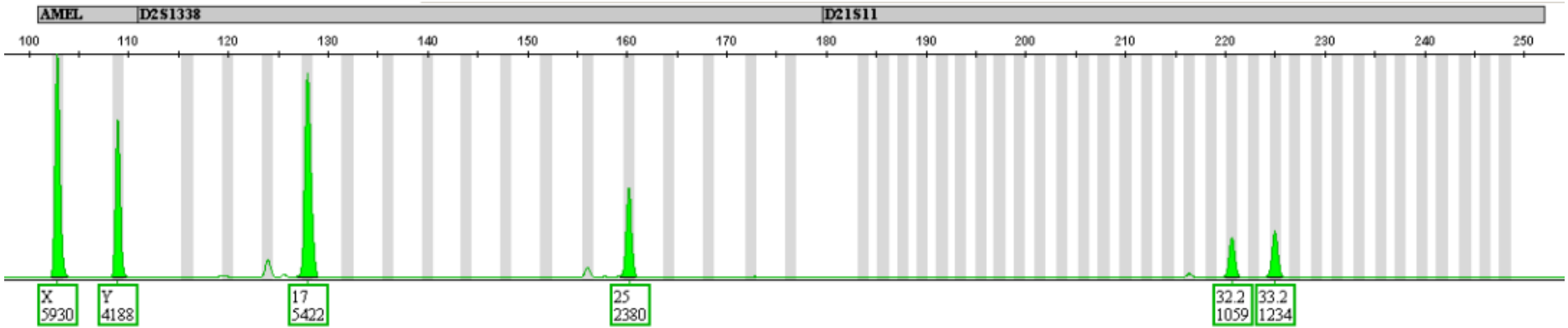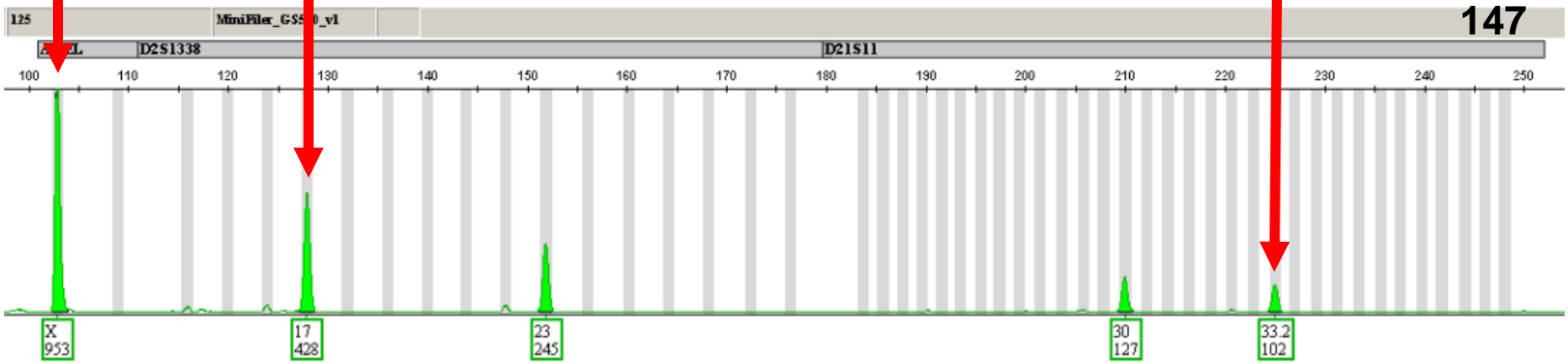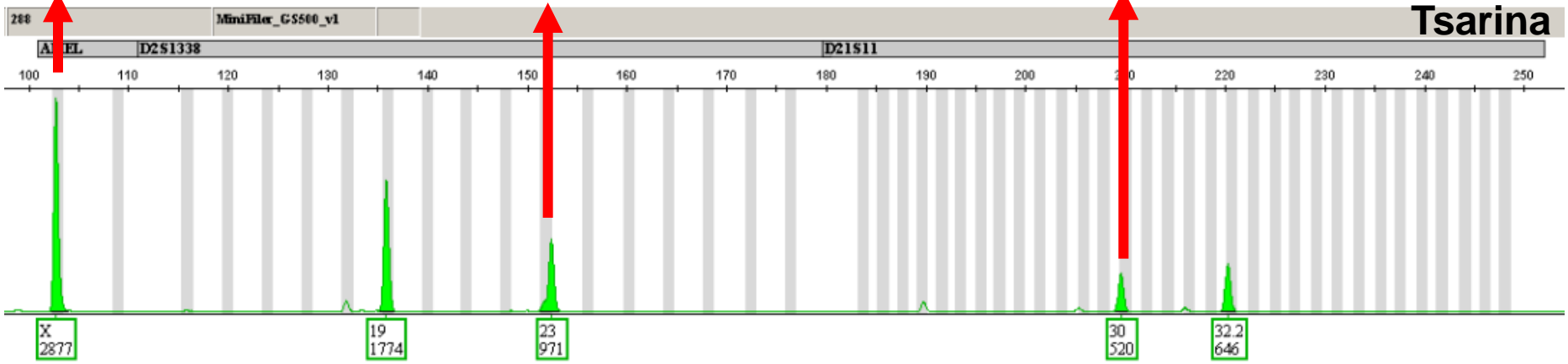
	AMEL	D2S1338						D21S11								
100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250

X 5930 | Y 4188 | 17 5422 | 25 2380 | 32.2 1059 | 33.2 1234

147

125 MiniFiler_GS500_v1

	AMEL	D2S1338						D21S11								
100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250

X 953 | 17 428 | 23 245 | 30 127 | 33.2 102

Tsarina

288 MiniFiler_GS500_v1

	AMEL	D2S1338						D21S11								
100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250

X 2877 | 19 1774 | 23 971 | 30 520 | 32.2 646

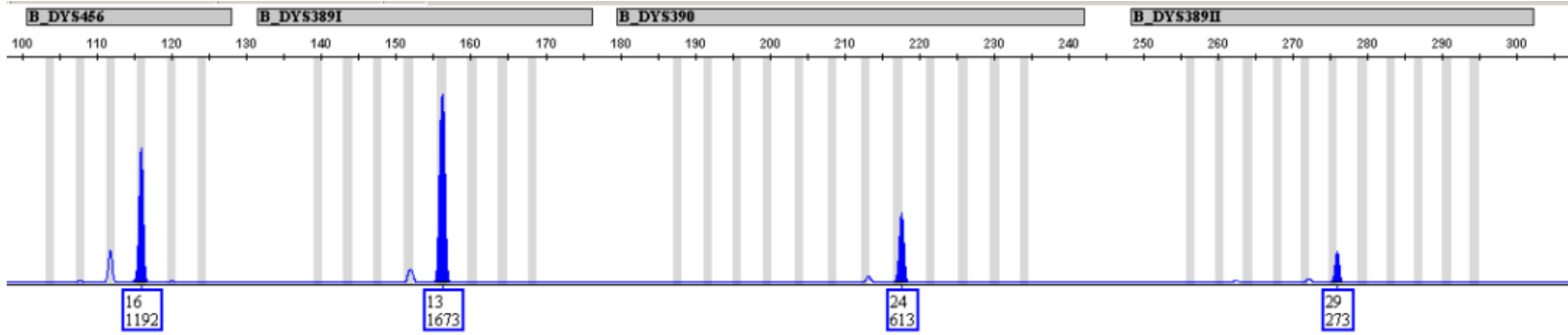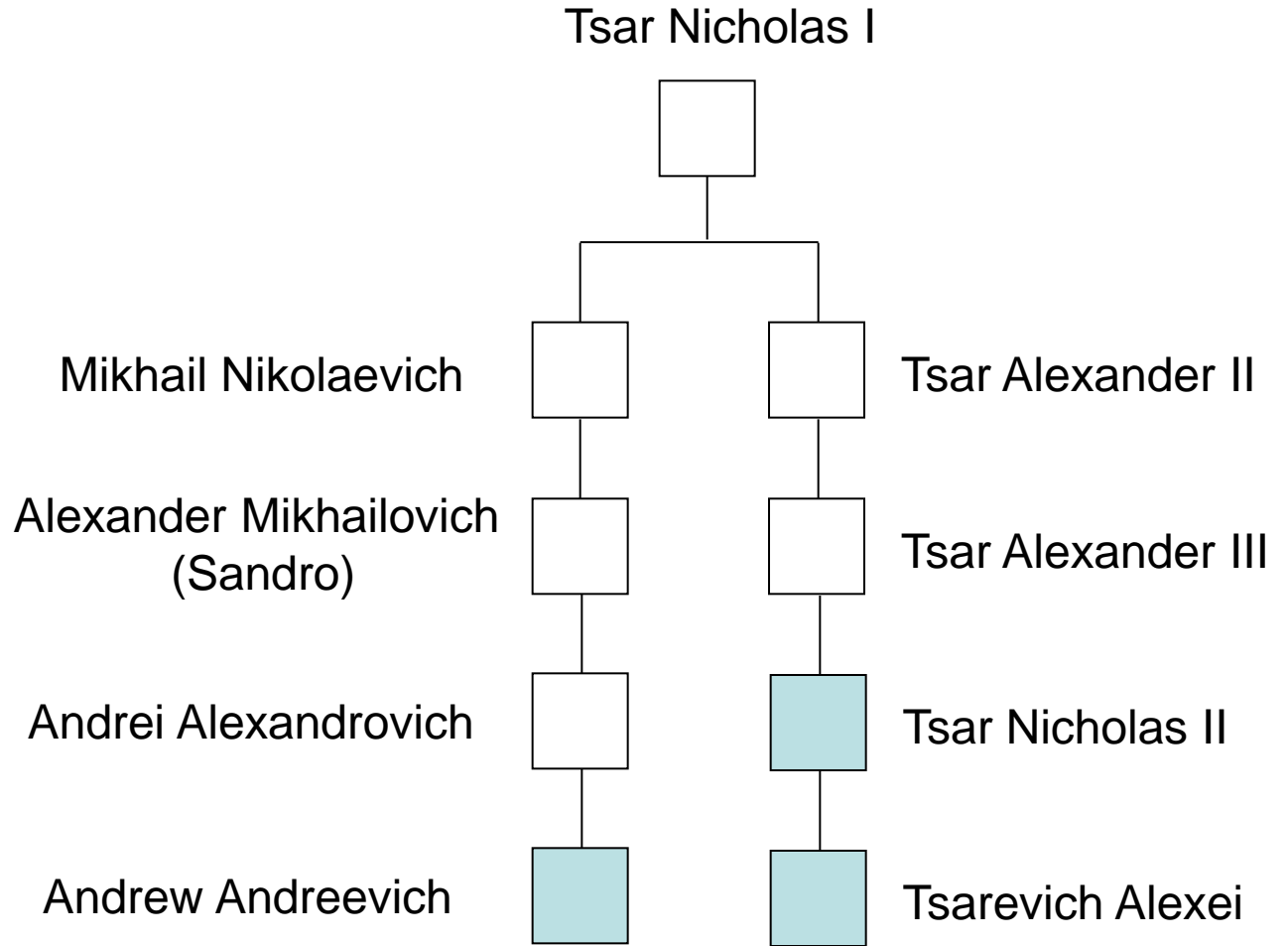| Marker	Sample 4.3 Tsar Nicolas II	Sample 7.4 Tsarina Alexandra	Sample 3.46 Olga	Sample 5.21 Tatiana	Sample 6.14 Anastasia	Sample 147 Maria	Sample 146.1 Alexei
Amelog	X, Y	X, X	X, X	X, X	X, X	X, X	X, Y
D3S1358	14, 17	16, 18	17, 18	17, 18	16, 17	17, 18	14, 18
TH01	7, 9.3	8, 8	8, 9.3	7, 8	8, 9.3	7, 8	8, 9.3
D21S11	32.2, 33.2	30, 32.2	30, 33.2	32.2, 33.2	30, 33.2	30, 33.2	32.2, 33.2
D18S51	12, 17	12, 13	12, 12	12, 12	13, 17	12, 17	12, 17
D5S818	12, 12	12, 12	12, 12	12, 12	12, 12	12, 12	12, 12
D13S317	11, 12	11, 11	11, 11	11, 11	11, 11	11, 11	11, 12
D7S820	12, 12	10, 12	12, 12	10, 12	12, 12	10, 12	12, 12
D16S539	11, 14	9, 11	11, 11	11, 11	11, 14	9, 11	11, 14
CSF1PO	10, 12	11, 12	11, 12	11, 12	10, 11	10, 12	10, 12
D2S1338	17, 25	19, 23	17, 19	23, 25	17, 19	17, 23	23, 25
vWA	15, 16	15, 16	15, 16	15, 16	15, 16	15, 16	15, 16
D8S1179	13, 15	16, 16	13, 16	15, 16	13, 16	15, 16	15, 16
TPOX	8, 8	8, 8	8, 8	8, 8	8, 8	8, 8	8, 8
FGA	20, 22	20, 20	20, 22	20, 20	20, 22	20, 22	20, 22
D19S433	13, 13.2	13, 16.2	13.2, 16.2	13.2, 16.2	13, 16.2	13, 13	13, 13.2

Coble *et al.* (2009)
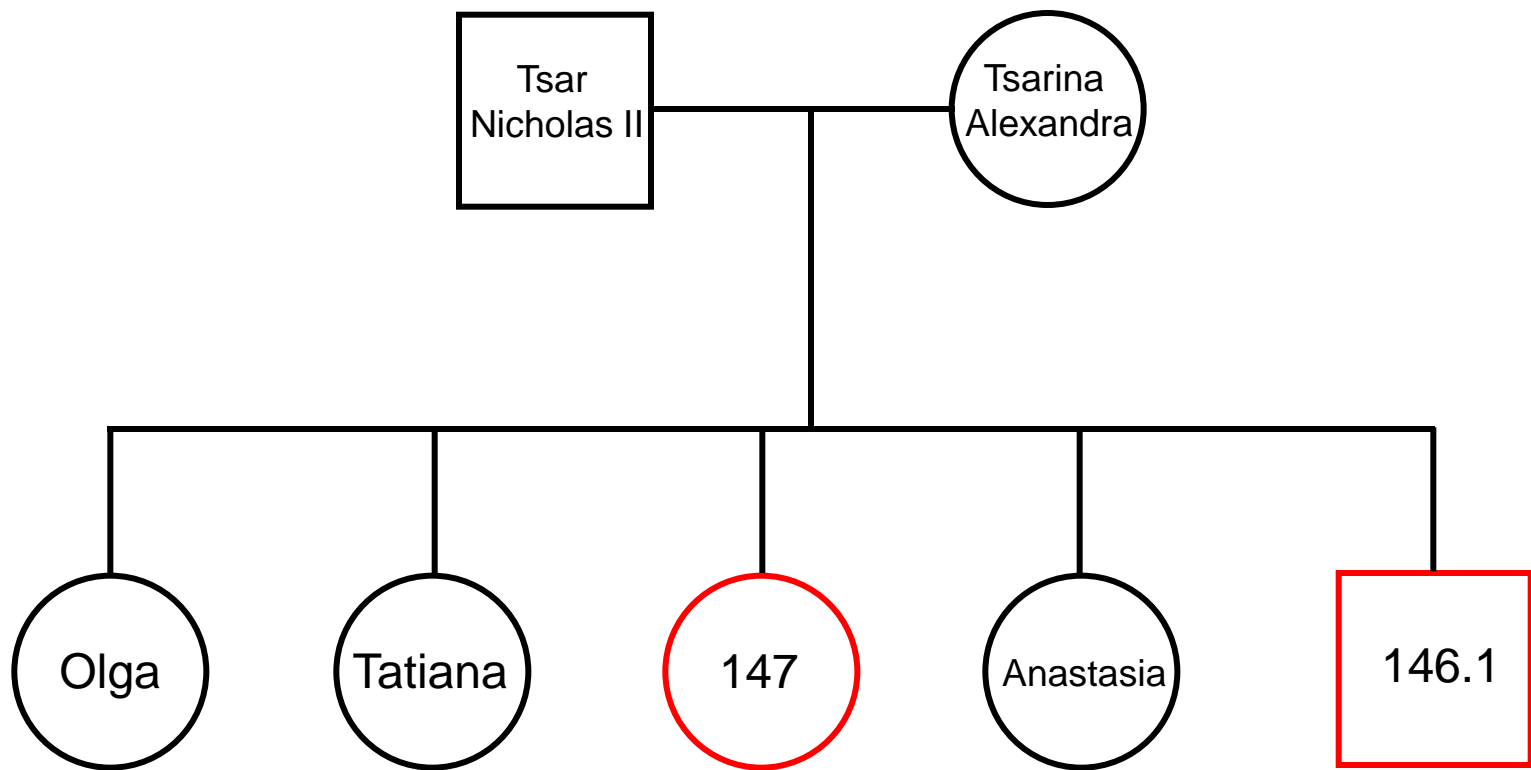
Scenario: Samples 146 and 147 are members of the Romanov Family

Cumulative LR = **4.36 Trillion to 80 Trillion**
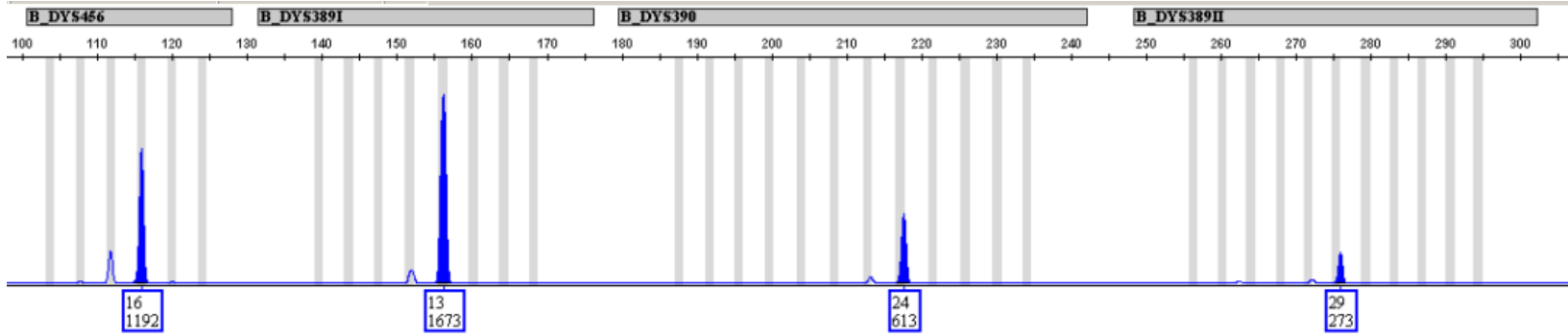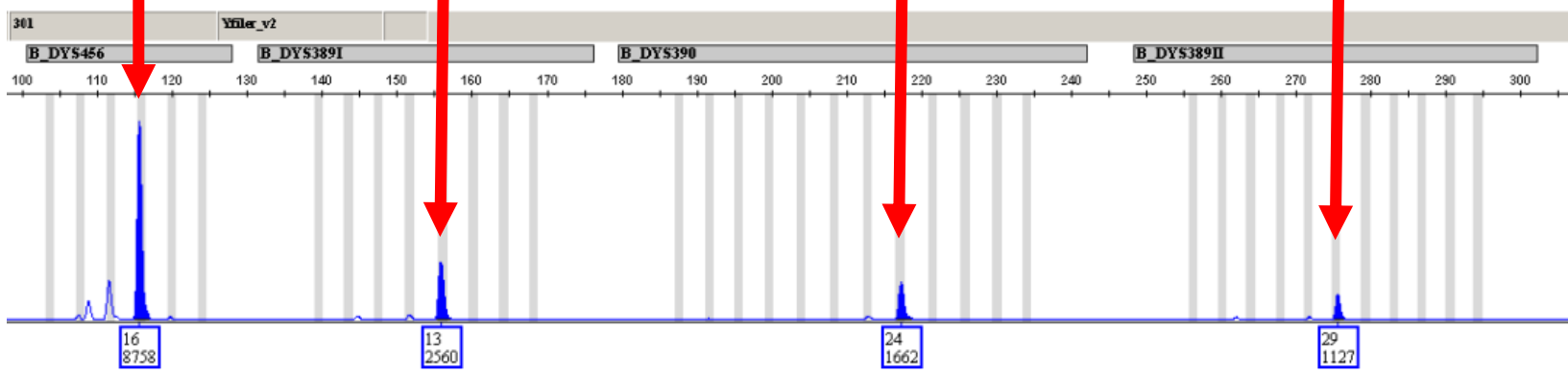
Y-chromosome DNA (Y-STR) Testing

Alexei

Tsar Nicholas I

Mikhail Nikolaevich

Alexander Mikhailovich
(Sandro)

Andrei Alexandrovich

Andrew Andreevich

Tsar Alexander II

Tsar Alexander III

Tsar Nicholas II

Tsarevich Alexei

The "Romanov" Y-STR Type

- Has not been observed in a database of **20,000+** individuals.

- (http://www.yhrd.org)

Table 4. Y-STR haplotype for Nicholas, Alexei and Andrew Romanov.

DYS19	DYS389I	DYS389II	DYS390	DYS391	DYS392	DYS393	DYS385a/b
14	13	29	24	10	13	13	11, 14

DYS438	DYS439	DYS437	DYS448	DYS456	DYS458	DYS635	YGATAH4
12	11	15	19	16	17	24	12

doi:10.1371/journal.pone.0004838.t004

Coble *et al.* (2009)

The Imposters

Anna Anderson Manahan

Establishing the identity of Anna Anderson Manahan

Peter Gill, Colin Kimpton, Rosemary Aliston-Greiner, Kevin Sullivan, Mark Stoneking, Terry Melton, Julian Nott, Suzanne Barrit, Rhonda Roby, Mitchell Holland & LTC Victor Weedn

FSS
Penn State University
AFDIL

Table 1 Short tandem repeat STR results

	VWA	TH01	F13A1	FES/FPS	ACTBP2	AMELOGENIN
Tsar (Skeleton 4)[a]	15,16	7,9,3[b]	7,7	12,12	11,32	X,Y
Tsarina (Skeleton 7)[a]	15,16	8,8	3.2[c],5	12,13	32,36	X,X
Anna Anderson (intestine sample)	14,16	7,9.3	3.2,7	11,12	15,18	X,X

Table 2 Mitochondrial DNA sequences

Origin of sample	DNA source	Length sequenced (bp)	16111	16126	16266	16294	16304	16357
Reference sequence	–	–	C	T	C	C	T	T
Duke of Edinburgh (Great nephew of Tsarina)	Blood sample	403	T	.	.	.	.	C
Anna Anderson	Intestine sample	403	.	C	T	T	C	.
Anna Anderson	Hair sample	344–362 (3 hairs)	.	C	T	T	C	.
C. Maucher (Great nephew of Schanzkowska	Blood sample	380	.	C	T	T	C	.

., Sequence unchanged from reference sequence.

Total number of "Russian imperial children" claimants since 1918:

Granny Alina

Olga: 28 claimants

Tatiana: 33 claimants

Maria: 53 claimants

Anastasia: 33 claimants

Alexei: 81 claimants

Vassili Filatov

Magdalen Veres

Eugenia Smith

Joseph Veres

Heino Tammet

Alexei Poutziato

All of the Romanovs were executed in the early morning of July 17, 1918

For more information…

PLoS one

Mystery Solved: The Identification of the Two Missing Romanov Children Using DNA Analysis

Michael D. Coble[1][9]*, Odile M. Loreille[1][9], Mark J. Wadhams[1], Suni M. Edson[1], Kerry Maynard[1], Carna E. Meyer[1], Harald Niederstätter[2], Cordula Berger[2], Burkhard Berger[2], Anthony B. Falsetti[3], Peter Gill[4,5], Walther Parson[2], Louis N. Finelli[1]

1 Armed Forces DNA Identification Laboratory, Armed Forces Institute of Pathology, Rockville, Maryland, United States of America, 2 Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria, 3 University of Florida, Gainesville, Florida, United States of America, 4 Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, United Kingdom, 5 Institute of Forensic Medicine, University of Oslo, Oslo, Norway

http://www.plosone.org/article/info:doi/10.1371/journal.pone.0004838