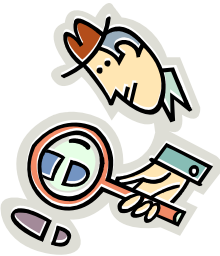
Mitchell M. Holland, Ph.D.
Professor, Biochem & MolBio
Former Director, Forensic Science Program
Eberly College of Science
Penn State University, University Park, PA



DCMPS of mtDNA Heteroplasmy: An Established Tool for Forensic Investigations

NJ State Police
Forensic Technology Center
8 Oct 2019

TECHTOUR 2019

http://forensics.psu.edu/research/dr.-mitchell-holland

Next Generation Sequencing (NGS) of mtDNA

… a Massively Parallel Sequencing (MPS) Approach to mtDNA Analysis

A recent search of the literature identified >29,000 published articles on MPS applications

>750 publications on MPS mtDNA
>85 publications on forensic MPS mtDNA

September 23rd | **September 24th** | September 25th | September 26th | September 27th

8:30 am - 5:00 pm

The Future is Now for MPS mtDNA Analysis

Location TBA

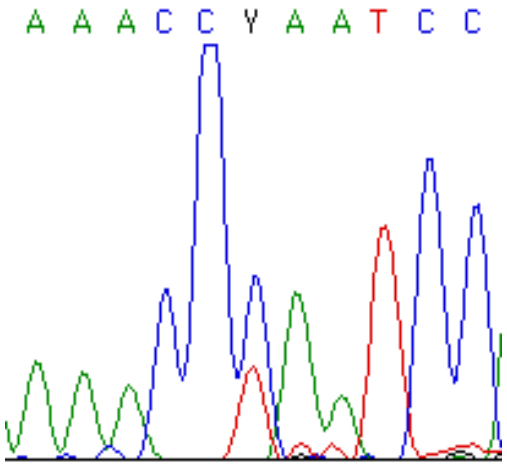
Michael Brandhagen *Co-Chair*
Forensic DNA Scientist, FBI Laboratory

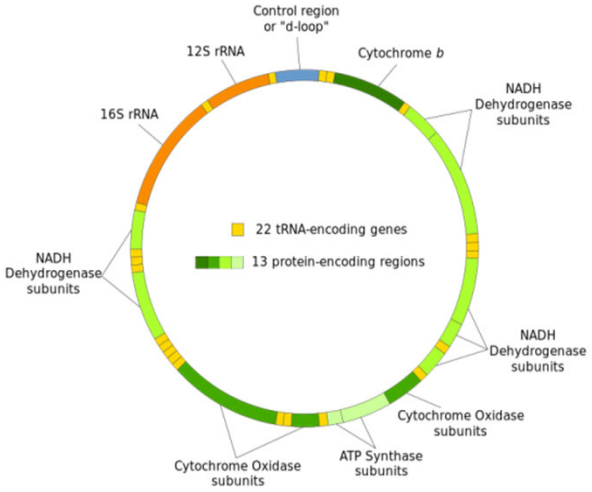
Mitch Holland *Co-Chair*
Associate Professor, Biochemistry and Molecular Biology, Eberly College of Science, Pennsylvania State University

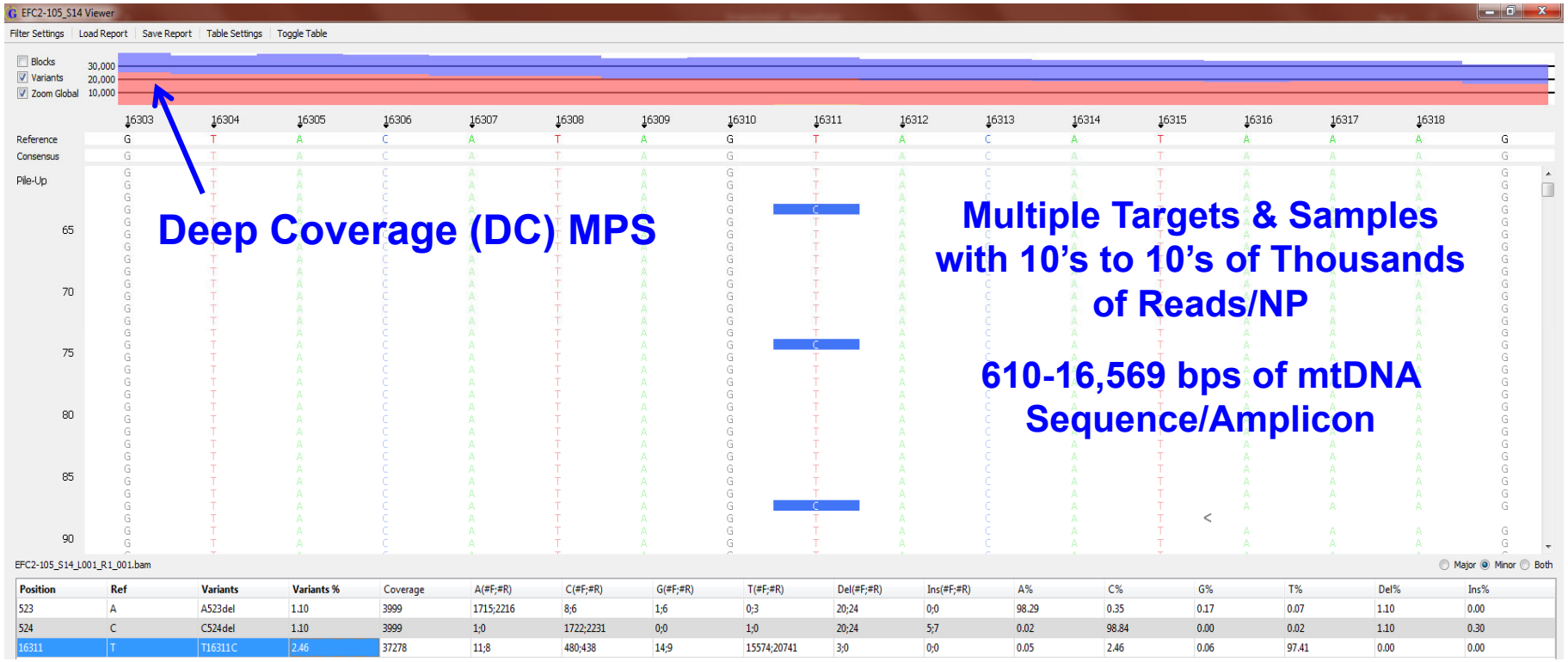
The purpose of this workshop is to educate the community on the availability of complete systems for MPS analysis of mtDNA; including enrichment approaches, library preparation methods, instrument choices, and analysis software solutions.

One Amplicon Target & Sample with One Read/NP

100-450 bps of mtDNA Sequence







Deep Coverage (DC) MPS

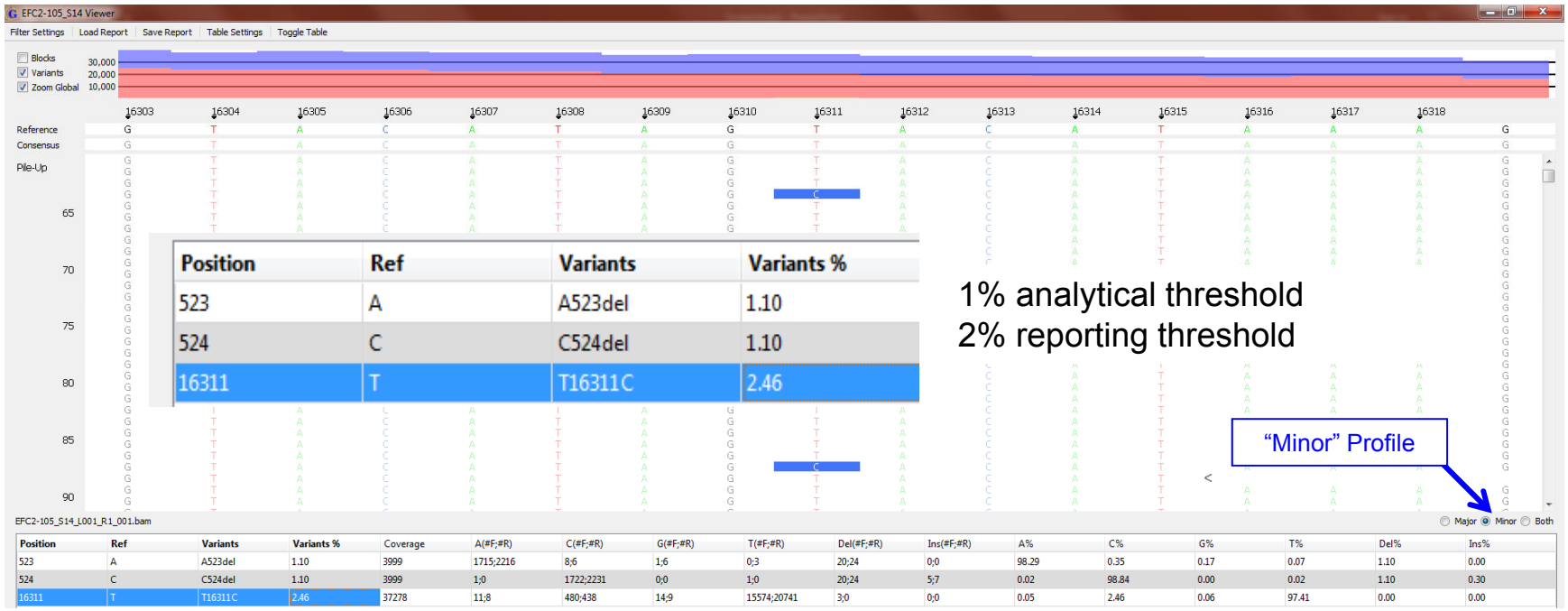
Multiple Targets & Samples with 10's to 10's of Thousands of Reads/NP

610-16,569 bps of mtDNA Sequence/Amplicon

Position	Ref	Variants	Variants %	Coverage	A(#F;#R)	C(#F;#R)	G(#F;#R)	T(#F;#R)	Del(#F;#R)	Ins(#F;#R)	A%	C%	G%	T%	Del%	Ins%
523	A	A523del	1.10	3999	1715;2216	8;6	1;6	0;3	20;24	0;0	98.29	0.35	0.17	0.07	1.10	0.00
524	C	C524del	1.10	3999	1;0	1722;2231	0;0	1;0	20;24	5;7	0.02	98.84	0.00	0.02	1.10	0.30
16311	T	T16311C	2.46	37278	11;8	480;438	14;9	15574;20741	3;0	0;0	0.05	2.46	0.06	97.41	0.00	0.00

MPS can resolve heteroplasmy

Threshold of 2%

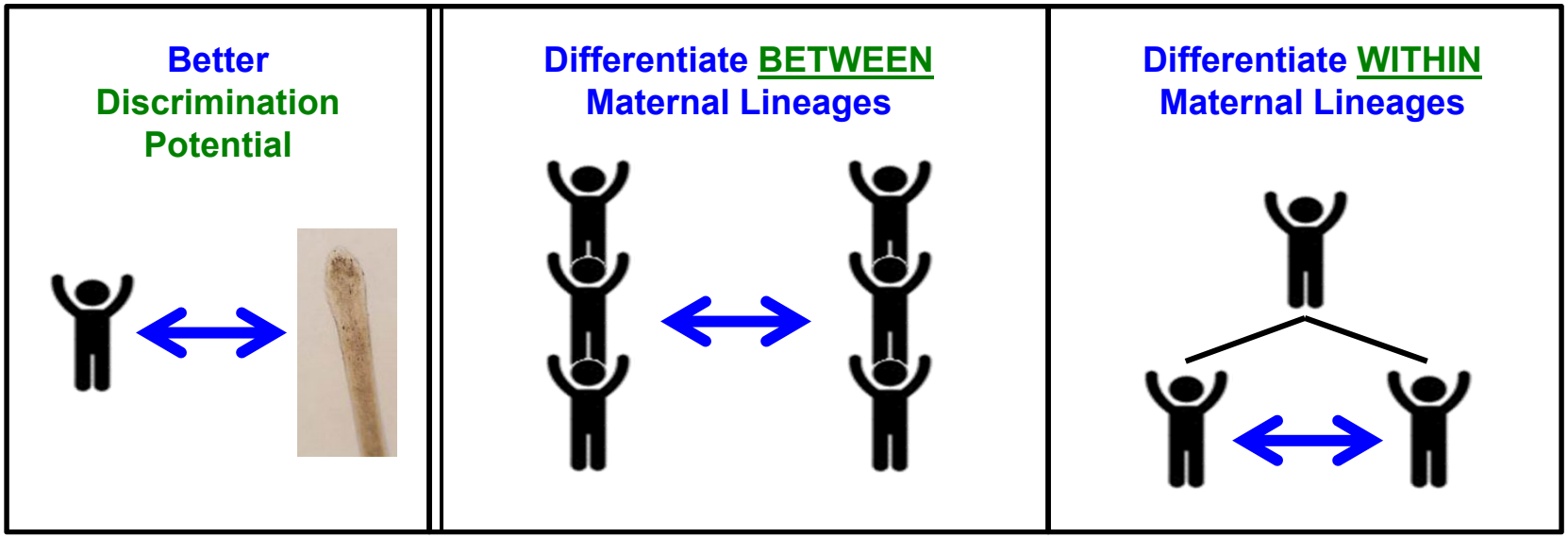


Position	Ref	Variants	Variants %	Coverage	A(#F;#R)	C(#F;#R)	G(#F;#R)	T(#F;#R)	Del(#F;#R)	Ins(#F;#R)	A%	C%	G%	T%	Del%	Ins%
523	A	A523del	1.10	3999	1715;2216	8;6	1;6	0;3	20;24	0;0	98.29	0.35	0.17	0.07	1.10	0.00
524	C	C524del	1.10	3999	1;0	1722;2231	0;0	1;0	20;24	5;7	0.02	98.84	0.00	0.02	1.10	0.30
16311	T	T16311C	2.46	37278	11;8	480;438	14;9	15574;20741	3;0	0;0	0.05	2.46	0.06	97.41	0.00	0.00

3/30 C variants = 10% Minor Variant Frequency (MVF)

2000 total read minimum/np
40 read minimum for minor variants
2% reporting threshold
(1% analytical threshold)
Balance ratio filters

Holland et al., FSIG 2017
GeneMarker™ HTS



DCMPS of mtDNA heteroplasmy allows us to accomplish these goals

44% of Mother-Child Pairs were Differentiated

genes

MDPI

Article

Deep-Coverage MPS Analysis of Heteroplasmic Variants within the mtGenome Allows for Frequent Differentiation of Maternal Relatives

Mitchell M. Holland [1,*], Kateryna D. Makova [2] and Jennifer A. McElhoe [1]

[1] Department of Biochemistry & Molecular Biology, Forensic Science Program, Eberly College of Science, Pennsylvania State University, University Park, PA 16802, USA; jam760@psu.edu
[2] Department of Biology, Eberly College of Science, Pennsylvania State University, University Park, PA 16802, USA; kmakova@bx.psu.edu
* Correspondence: mmh20@psu.edu

Received: 1 January 2018; Accepted: 20 February 2018; Published: 26 February 2018

Table 2. Differentiating heteroplasmy for 17 of the 39 mother-child pairs; buccal cell (Bu) and blood (Bl) samples. Frequency of heteroplasmy at each np in percentage (%), with the minor variant annotated as the letter after the np; i.e., T2746C is heteroplasmy at np 2746, with 20.11% of the C variant. Coverage and read distribution (forward reads, #For, compared to reverse reads, #Rev) are provided for each np of differentiating heteroplasmy, along with the gene annotation and whether sites in protein coding genes result in a synonymous change (Y) or not (N). The gene annotations include: CR = control region, 12S & 16S = 12S & 16S rRNAs, ATP6 = ATP synthase 6, ND = NADH dehydrogenase, $tRNA^{thr}$ = tRNA for threonine, and CO = cytochrome oxidase. Metadata for samples without the heteroplasmy are provided to illustrate that read percentages are clearly below reporting threshold and that coverage was adequate for this assessment.

Mother-Child Pair	Nucleotide Position	Sample Number	Major Allele	Coverage (≠For:≠Rev Reads)	Major Frequency (%)	Minor Allele	Coverage (≠For:≠Rev Reads)	Minor Frequency (%)	Gene Annotation	Synonymous (Y or N)	
T2746C		Mother - Bu (693)	T	2920:6014	79.67	C	655:1600	20.11	16S		
		Child - Bu (677)	T	4838:14038	99.64	C	1:9	0.053			
		Mother - Bl (M207)	T	14187:14328	80.3	C	3440:3528	19.62	16S		
		Child - Bl (M207-C)	T	24044:24176	99.88	C	6:12	0.037			
3	T9179C	Mother - Bu (1134)	T	3063:5076	85.02	C	538:892	14.93	ATP6	N (Val to Ala)	
		Child - Bu (1099)	T	6651:8730	99.82	C	8:7	0.097			
		Mother - Bl (M502G)	T	16583:20269	87.14	C	2468:2934	12.77	ATP6	N (Val to Ala)	
		Child - Bl (M501)	T	38769:44060	99.81	C	32:24	0.067			
4	G14040A	Mother - Bu (659)	G	5770:4227	92.01	A	474:381	7.86	ND5	Y (Gln)	
		Child - Bu (722)	G	20789:16141	99.86	A	8:12	0.054			
		Mother - Bl (M242)	G	13200:12992	94.07	A	831:811	5.89	ND5	Y (Gln)	
		Child - Bl (M242-C)	G	10355:10087	99.88	A	5:5	0.049			

… heteroplasmy must be observed in both tissues of one relative but not the other

Metadata: Differentiating

A total of **21 sites of heteroplasmy** were observed in the 17 mother-child pairs, with **no duplicates**, so some pairs had multiple sites of differentiating heteroplasmy

For two pairs, the **mother and child each** had a site of differentiating heteroplasmy

For a third pair, the **mother had one site and the child two sites** of differentiating heteroplasmy

Metadata: Differentiating

Of the 21 sites, 5 were observed in the control region, the most often targeted region of the mtgenome, and **16 sites were observed in the coding region**, further illustrating the value of sequencing the entire mtgenome

However, **5 of the 16 sites** in the coding region (~31%) resulted in **non-synonymous changes** to the protein sequence of the gene locus, so policy questions regarding potential connections to medical information will need to be answered

Table 1. Shared heteroplasmy for 12 of the 39 mother-child pairs. Frequency of heteroplasmy at each nucleotide position (np) in percentage (%), with the minor variant annotated as the letter after the np; i.e., A1656A (2.11%) is heteroplasmy at np 1656, with the first A as the reference sequence and the second A as the minor variant at 2.11% of the reads. Underlined letters following the np of heteroplasmy indicate a primary haplotype change between the mother and child. In two instances, heteroplasmy was not detected (ND) in a sample.

Mother-Child Pair	Sample Number	Shared Heteroplasmy
1	Mother-Bu (807)	A16183G (7.32%)
	Child-Bu (803)	A16183G (6.89%)
	Mother-Bl (M490)	A16183G (2.81%)
	Child-Bl (M490-C)	A16183G (2.46%)
2	Mother-Bu (618)	T16189C (7.74%)
	Child-Bu (606)	T16189C (11.07%)
	Mother-Bl (M249)	T16189C (2.81%)
	Child-Bl (M249-C)	T16189C (9.92%)
3	Mother-Bu (704)	T6152C (7.23%)
	Child-Bu (630)	T6152C (16.37%)
	Mother-Bl (M234)	T6152C (5.04%)
	Child-Bl (M234-C)	T6152C (16.48%)
4	Mother-Bu (762)	T10873C (2.53%)
	Child-Bu (702)	T10873C (6.66%)
	Mother-Bl (M210)	ND
	Child-Bl (M210-C)	T10873C (5.40%)

Overall, 22/39 pairs (~56%) had either shared (10), differentiating (5) or both (7) types of heteroplasmy

A total of **14 sites** of shared heteroplasmy (one family with three), with **no duplicates**, and only one site in common with the pairs exhibiting differentiating heteroplasmy (np 16093)

… are any of the shared or differentiating sites of heteroplasmy due to background noise/error in the MPS process, or to numts??

“Noise” in the System

“Noise” in the System

A.

	Metals-Power	Hair-Power	Blood/Buccal-Power	LowTemp-Power	LowTemp-Next	Buccal-Next
Total error	0.485±0.0049	0.325±0.1005	0.231±0.0663	0.297±0.0694	0.205±0.0021	0.182±0.0645
A error	0.118±0.0009	0.100±0.0417	0.052±0.0230	0.048±0.0035	0.070±0.0476	0.037±0.0121
C error	0.121±0.0006	0.085±0.0280	0.080±0.0191	0.069±0.0189	0.079±0.0297	0.060±0.0205
G error	0.131±0.0033	0.048±0.0227	0.044±0.0097	0.059±0.0013	0.061±0.0023	0.047±0.0204
T error	0.115±0.0038	0.091±0.0261	0.053±0.0145	0.049±0.0110	0.065±0.0243	0.039±0.0139

B.

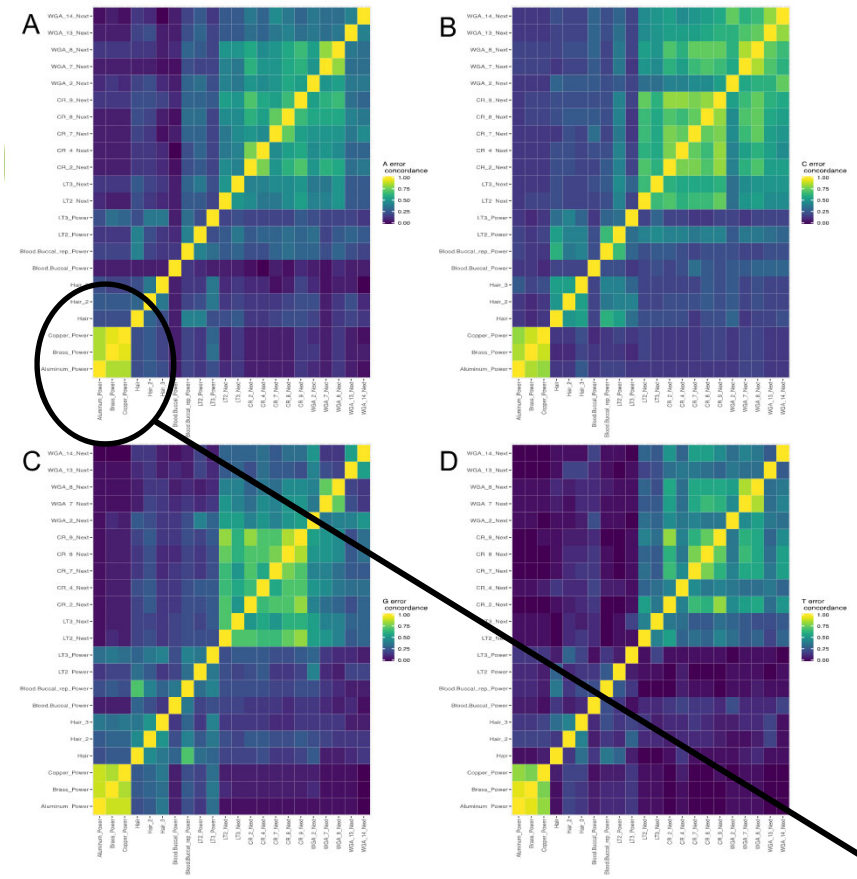
	Buccal-Next CR	Buccal-Next mtgenome
Total error	0.158±0.0720	0.166±0.0745
A error	0.032±0.0148	0.036±0.0164
C error	0.057±0.0273	0.063±0.0292
G error	0.036±0.0180	0.036±0.0171
T error	0.032±0.0126	0.030±0.0124

Background noise or error ranged from 0.030% to 0.131% across the four nucleotides.

Samples with increasing DNA damage saw increases in error.

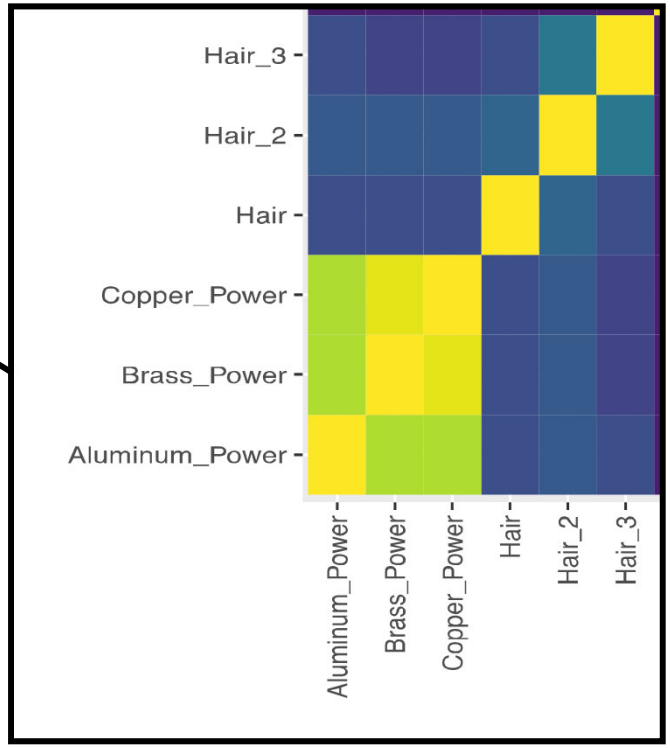
420+ million reads of data

Submitted for publication
Jennifer McElhoe



Comparison of sites with greatest error between MiSeq runs (i.e., datasets)

Dark Blue = zero concordance
Yellow = 100% concordance



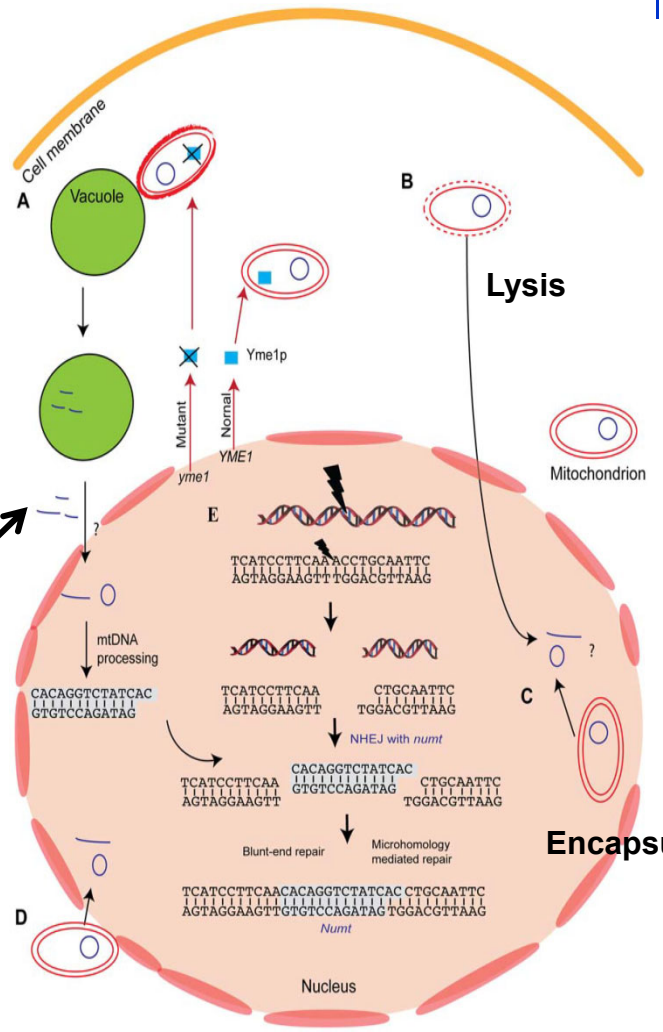
DNA recovered from the surface of ammunition components (Holland et al., FSIG 2019) gave the highest level of concordance

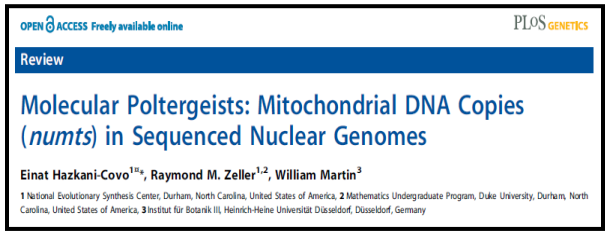
NUMTs

= nuclear mitochondrial DNA segments *
= nuclear mitochondrial sequences
= nuclear mitochondrial segments
= nuclear mitochondrial insertions

Estimate that ~750 NUMTs can be found in the nugenome, including some entirely intact mitogenomes, with ~4 unique NUMTs per individual

Most supported pathway is degradation of abnormal mitochondria




OPEN ACCESS Freely available online

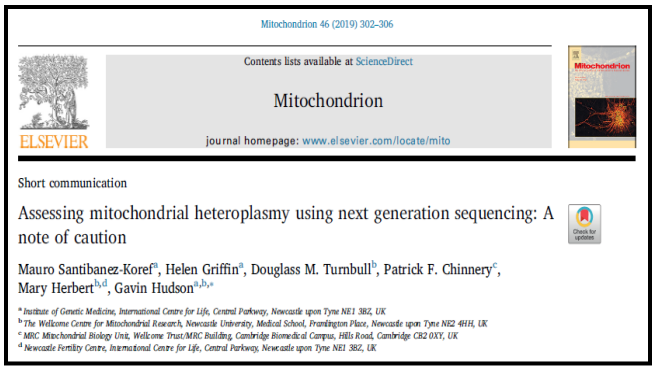
PLoS GENETICS

Review

Molecular Poltergeists: Mitochondrial DNA Copies (*numts*) in Sequenced Nuclear Genomes

Einat Hazkani-Covo[1¤*], Raymond M. Zeller[1,2], William Martin[3]

1 National Evolutionary Synthesis Center, Durham, North Carolina, United States of America, 2 Mathematics Undergraduate Program, Duke University, Durham, North Carolina, United States of America, 3 Institut für Botanik III, Heinrich-Heine Universität Düsseldorf, Düsseldorf, Germany



Mitochondrion 46 (2019) 302–306

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

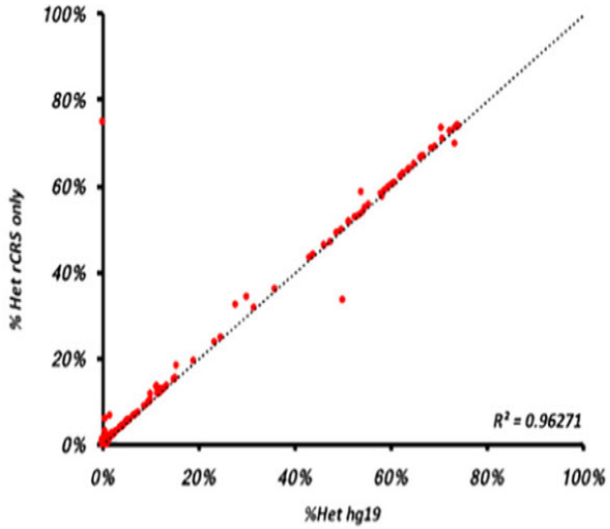
Short communication

Assessing mitochondrial heteroplasmy using next generation sequencing: A note of caution

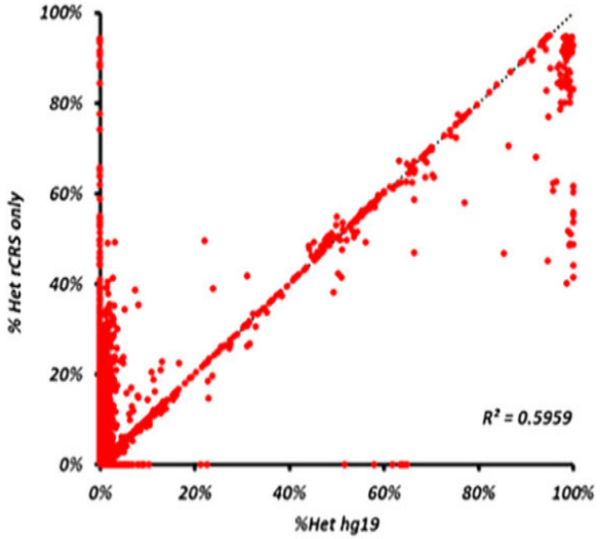
Mauro Santibanez-Koref[a], Helen Griffin[a], Douglass M. Turnbull[b], Patrick F. Chinnery[c], Mary Herbert[b,d], Gavin Hudson[a,b,*]

[a] Institute of Genetic Medicine, International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, UK
[b] The Wellcome Centre for Mitochondrial Research, Newcastle University, Medical School, Framlington Place, Newcastle upon Tyne NE2 4HH, UK
[c] MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0XY, UK
[d] Newcastle Fertility Centre, International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, UK

Heteroplasmy observed in whole mitogenome MPS data is dependent on enrichment method



2 amplicon approach



180 amplicon approach

As amplicon size decreases, the number of minor variants with discrepant frequencies can increase, with the majority of variant frequencies increasing when aligned to the rCRS only

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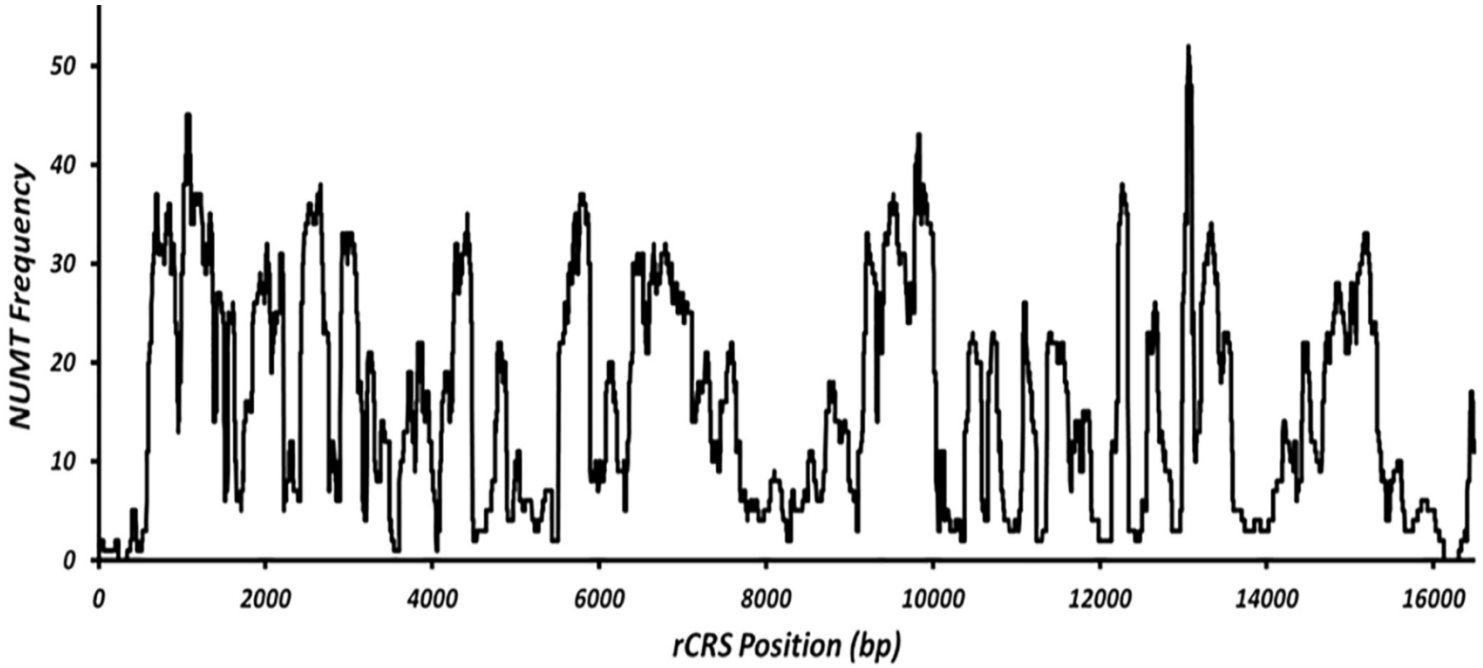
[c] MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0XY, UK
[d] Newcastle Fertility Centre, International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, UK

NUMT frequency is relatively low in the control region compared to sites across the coding region



Rates of DNA Duplication and Mitochondrial DNA Insertion in the Human Genome

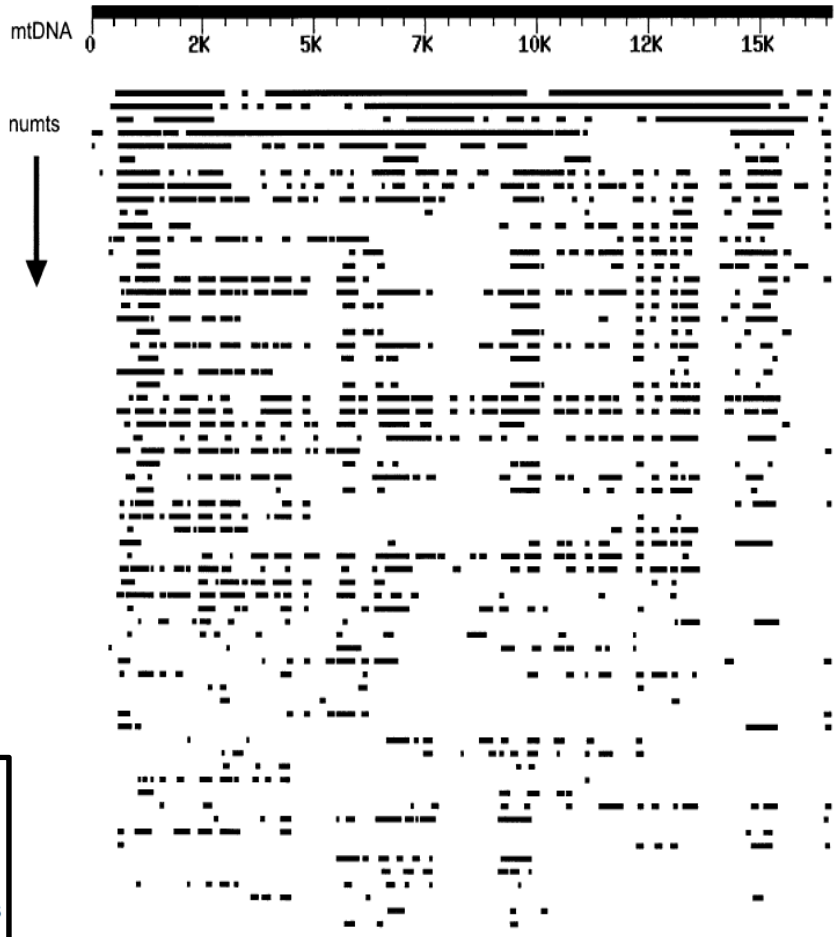
JOURNAL OF MOLECULAR EVOLUTION

Douda Bensasson, Marcus W. Feldman, Dmitri A. Petrov

School of Biological Sciences, Stanford University, 371 Serra Mall, Stanford, CA 94305, USA

NUMT copy number can be relatively high, but still far lower than actual mtDNA targets, so chances are low of detecting NUMT sequences

Bottom line: amplify larger targets and lower amounts of template DNA, and ultimately filter out phylogenetically inconsistent SNPs



Electrophoresis 2018, *39*, 2785–2797

Joseph David Ring[1,2]
Kimberly Sturk-Andreaggi[1,2]
Michelle Alyse Peck[1,2]*
Charla Marshall[1,2]

[1]Armed Forces Medical Examiner System's Armed Forces DNA Identification Laboratory (AFMES-AFDIL), DE, United States
[2]ARP Sciences, LLC, Rockville, MD, United States

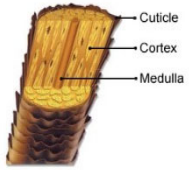
Research Article

Bioinformatic removal of NUMT-associated variants in mitotiling next-generation sequencing data from whole blood samples

mtDNA Workflow

MPS

DNA Extraction



DNA extraction is the same, but in many instances the amount of extract needed for amplification is reduced

- **Amplification of mtDNA**
- **Library Preparation**
- **Sequencing**
- **Data Analysis**

Identification of Marija Krucifiksa Kozulić

Sidney Gaston-Sanchez, Dragan Primorac, Šimun Anđelinović, Željana Bašić, Ivana Kružić, Alan Bosnar, Anja Petaros, Miran Čoklo, Jennifer Daniels-Higginbotham, Charla Marshall

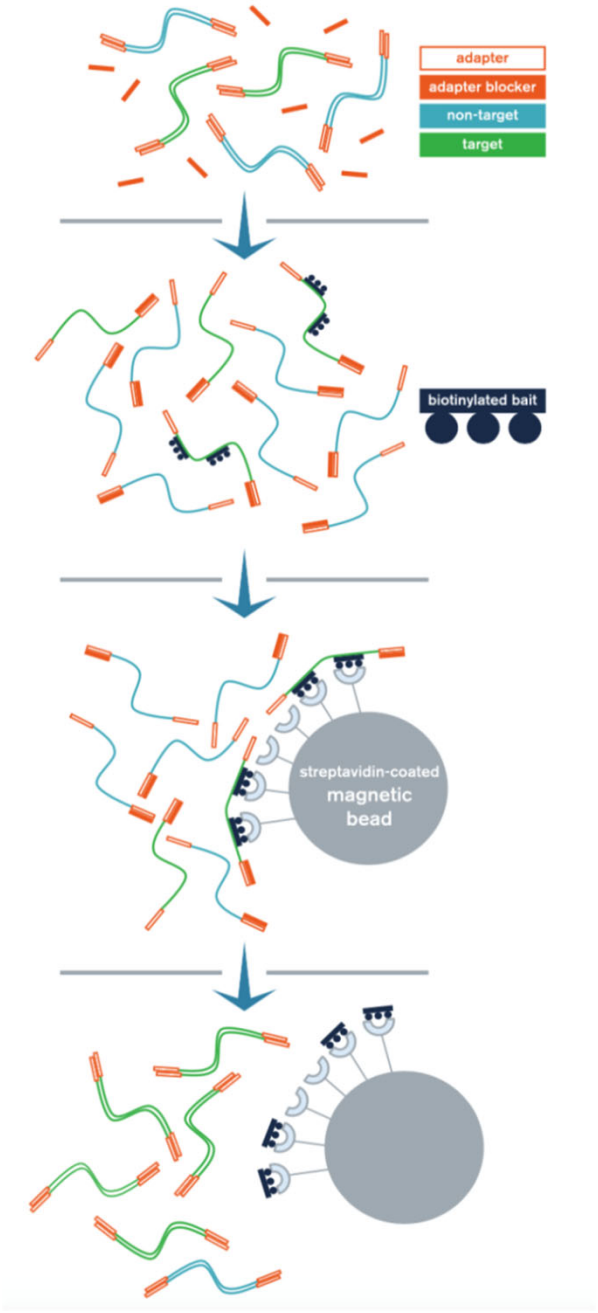
Archbishop Ivan Devčić, Roman Catholic Archdiocese of Rijeka and Sister Dobroslava Mlakić, Catholic Order of the Daughters of the Sacred Heart of Jesus, Rijeka

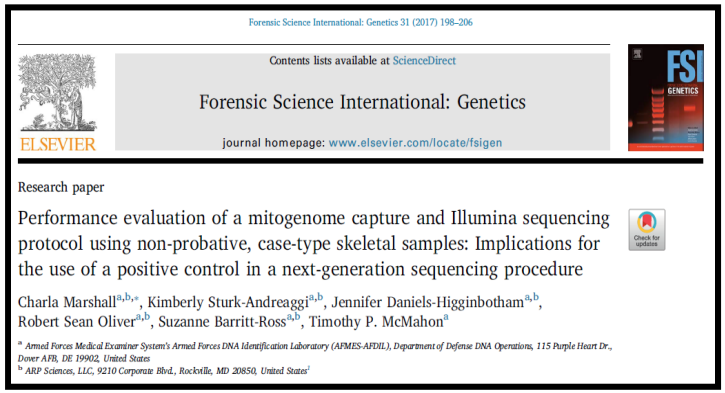
Sister **Marija Krucifiksa Kozulić** was a nun from Rijeka who dedicated her life to helping the poor and less fortunate. She is currently under consideration for beautification by the Vatican, which requires the identification of her remains.

Sister Kozulić died in 1922 and was buried in a tomb along with other nuns belonging to the Society of Sisters of the Sacred Heart of Jesus, including her biological sister, **Tereza Kozulić**.

Experts in varying disciplines such as forensic anthropology, pathology, and forensic molecular biology have contributed to the ongoing identification efforts.

Capture method using a custom designed bait cocktail developed by AFDIL for the entire mitogenome





Forensic Science International: Genetics 31 (2017) 198–206

Contents lists available at ScienceDirect

Forensic Science International: Genetics

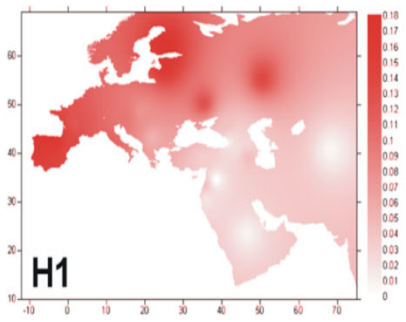
journal homepage: www.elsevier.com/locate/fsigen

Research paper

Performance evaluation of a mitogenome capture and Illumina sequencing protocol using non-probative, case-type skeletal samples: Implications for the use of a positive control in a next-generation sequencing procedure

Charla Marshall[a,b,*], Kimberly Sturk-Andreaggi[a,b], Jennifer Daniels-Higginbotham[a,b], Robert Sean Oliver[a,b], Suzanne Barritt-Ross[a,b], Timothy P. McMahon[a]

[a] Armed Forces Medical Examiner System's Armed Forces DNA Identification Laboratory (AFMES-AFDIL), Department of Defense DNA Operations, 115 Purple Heart Dr., Dover AFB, DE 19902, United States
[b] ARP Sciences, LLC, 9210 Corporate Blvd., Rockville, MD 20850, United States



b subclade is common in Eastern Europe

Haplotype 1
Haplogroup H1bu

Control Region
Coding Region

A263G
C309insC
C315insC
A750G
A1438G
G3010A
A4769G
A5558G
A8860G
A13327G
A15326G
T16519C

Heteroplasmy
A12337insC (32-34%)
A13327A (28-29%)

R: 39
L: 38

R: 42
L: 43

Heteroplasmy
A13327A (12-13%)

13327 is in the ND5 gene, with the A>G SNP causing a non-syn change from T>A

No known disease state associated with the change

Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II

Pavel L. Ivanov[1], Mark J. Wadhams[2], Rhonda K. Roby[2], Mitchell M. Holland[2], Victor W. Weedn[2] & Thomas J. Parsons[2]

nature genetics volume 12 april 1996

Likelihood Ratio

$$LR = \frac{p(E1/R) \times p(E2/R)}{p(E1/R') \times p(E2/R')}$$

p(E1/R) = the probability of the evidence (haplotype match between Marija and Tereza Kozulić) given the hypothesis that the remains are those of Marija Kozulić

E2 = the probability of co-occurrence of heteroplasmy

R' = given the hypothesis that the remains are unrelated

Modeling the approach from Ivanov et al, Nat Genet 1996

Maternal age effect and severe germ-line bottleneck in the inheritance of human mitochondrial DNA

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Likelihood Ratio

$$LR = \frac{p(E1/R) \times p(E2/R)}{p(E1/R') \times p(E2/R')}$$

Calculations are dependent on:

- **Haplotype frequencies (search, 1366 mitogenome profiles)**
- **Generational differences (known, 2)**
- **Mutation rates (empirical, 1/640)**
- **Heteroplasmy rates & sites (empirical, 5.6/10 & estimated 1/500)**
- **Generations to fixation (estimated, 1/20)**

EMPOP has 1366 mitogenome sequences in the database:
***Confidence Limit from Zero Proportion* = 0.00219 (1 in 457)**

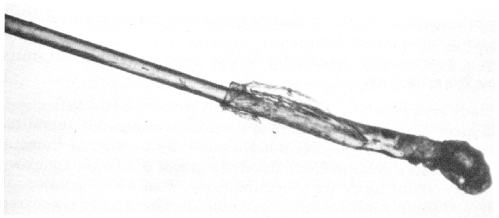
Likelihood Ratio

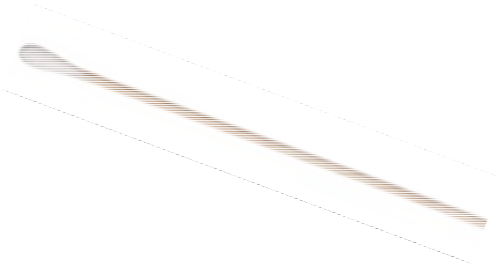
$$LR = \frac{p(E1/R) \times p(E2/R)}{p(E1/R') \times p(E2/R')}$$

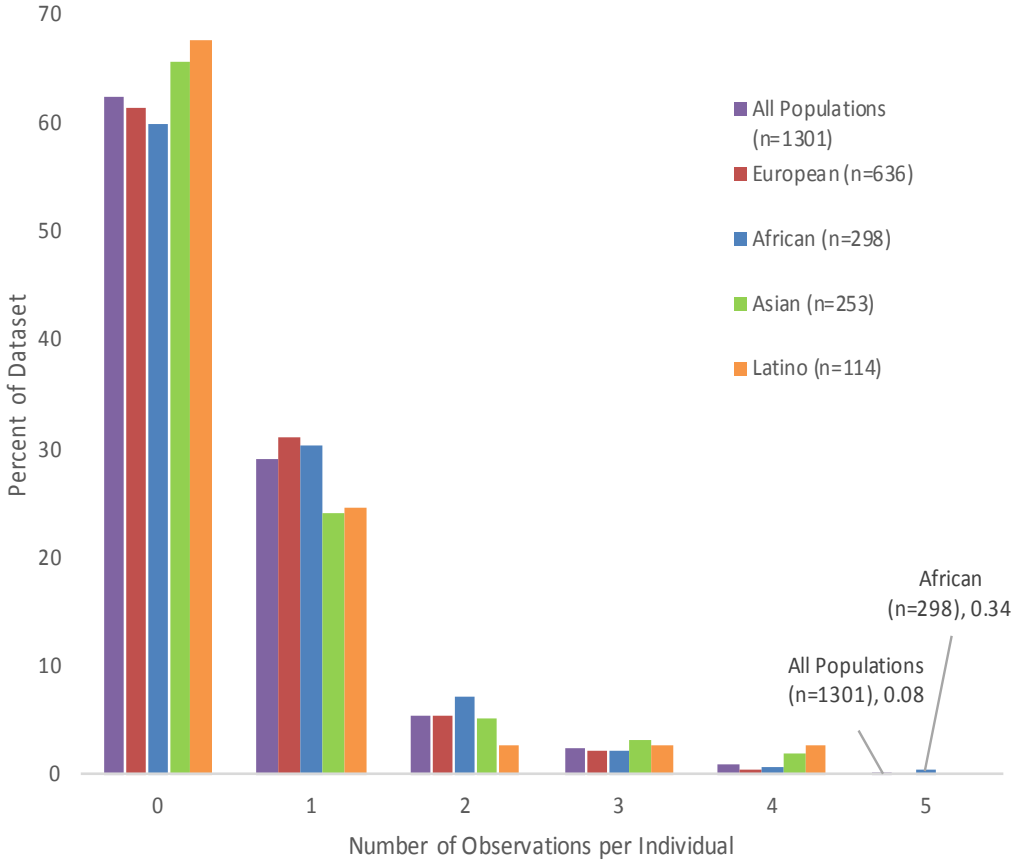
$= (455)(807) = \mathbf{367,185}$

Therefore, the DNA evidence is ~367,185 times more likely if one of the two set of remains are those of Marija Kozulić

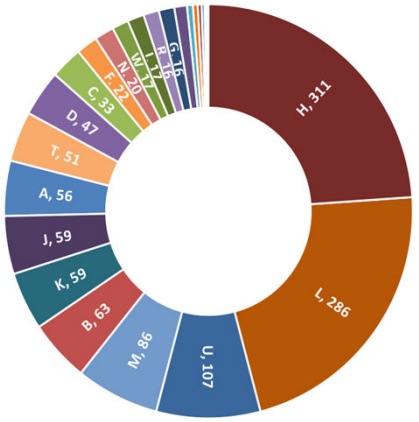
What if we just have an evidence to reference match?





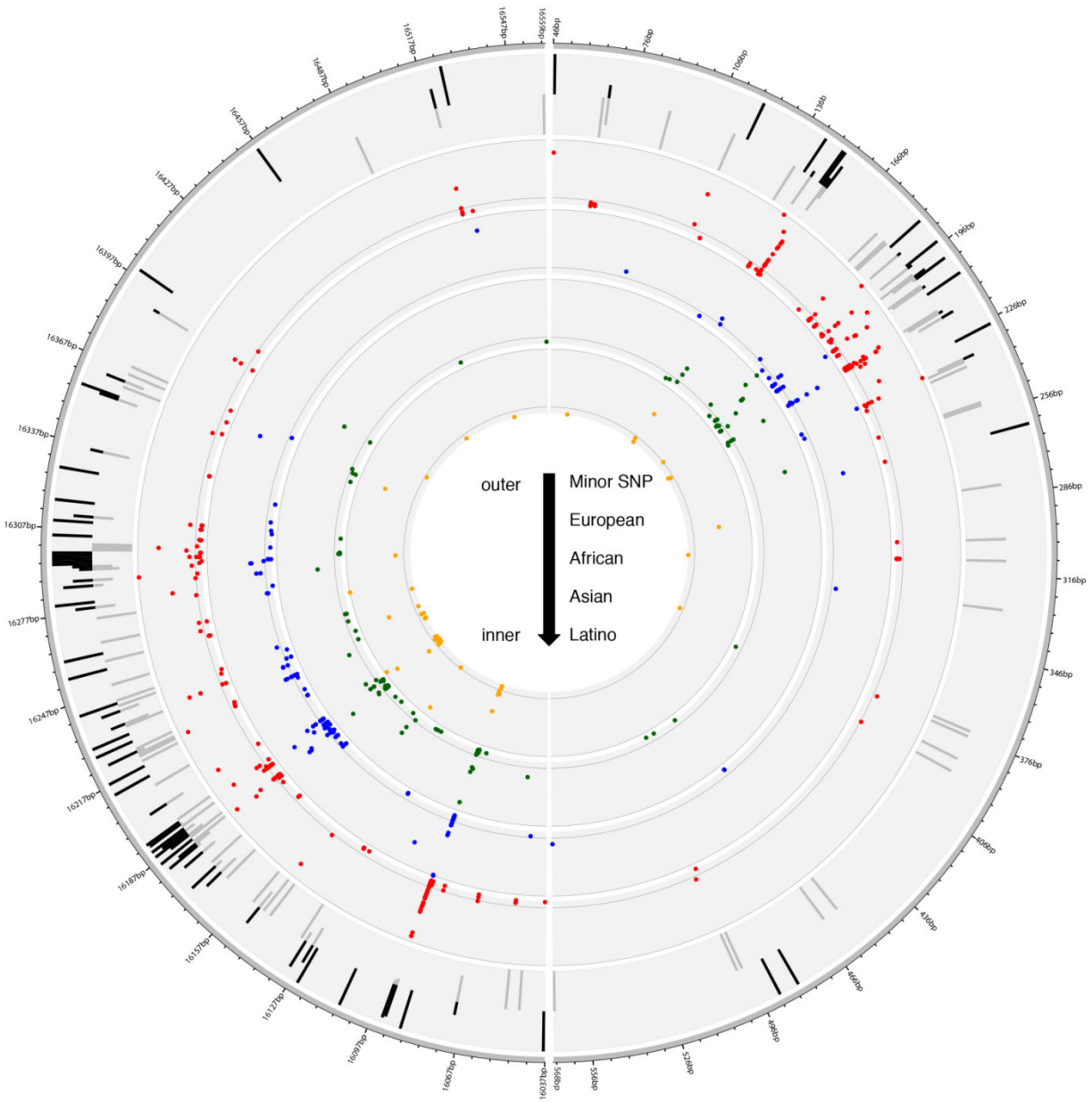


Rates of Heteroplasmy on a per Individual Basis



*Overall, 37.7% of the **combined** population of 1301 individuals of **European**, **African**, **Asian**, and **Latino** decent exhibited a site or sites of heteroplasmy. The rate of heteroplasmy is highly similar across the four population groups, representing 22 major haplogroups, the majority of which fall within H and L.*

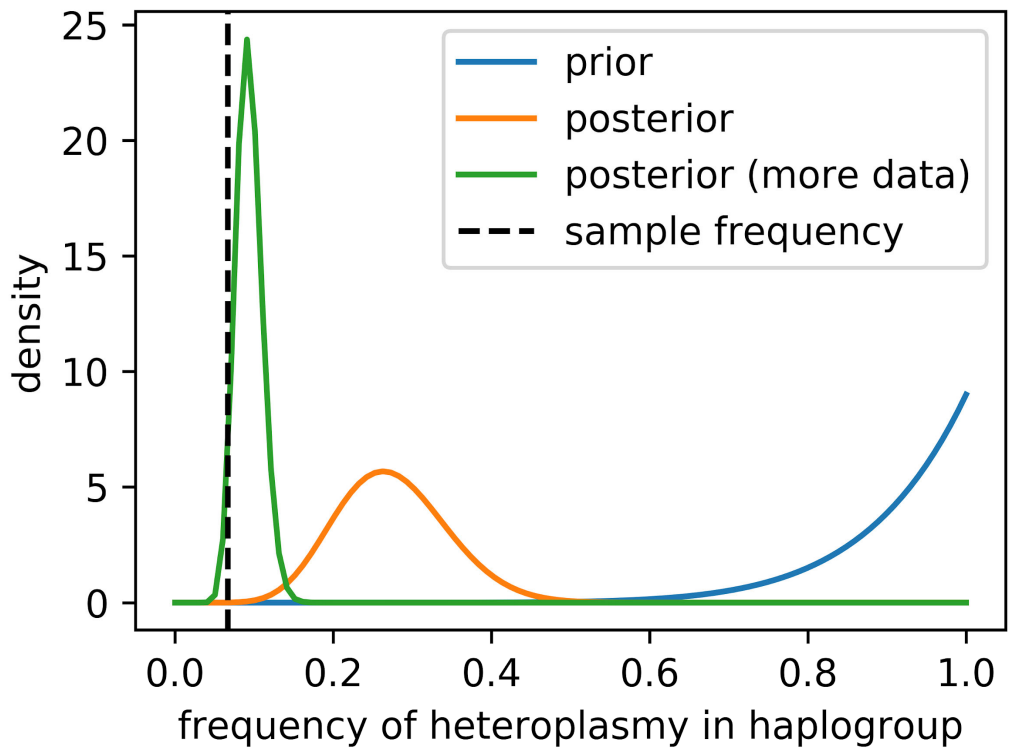
Therefore, heteroplasmy should be observed, on average, in more than one in three forensic cases.



*Sites of heteroplasmy across the CR, with numbering according to the rCRS. Sites are identified for **European** (n=636), **African** (n=298), **Asian** (n=253), and **Latino** (n=114) population groups.*

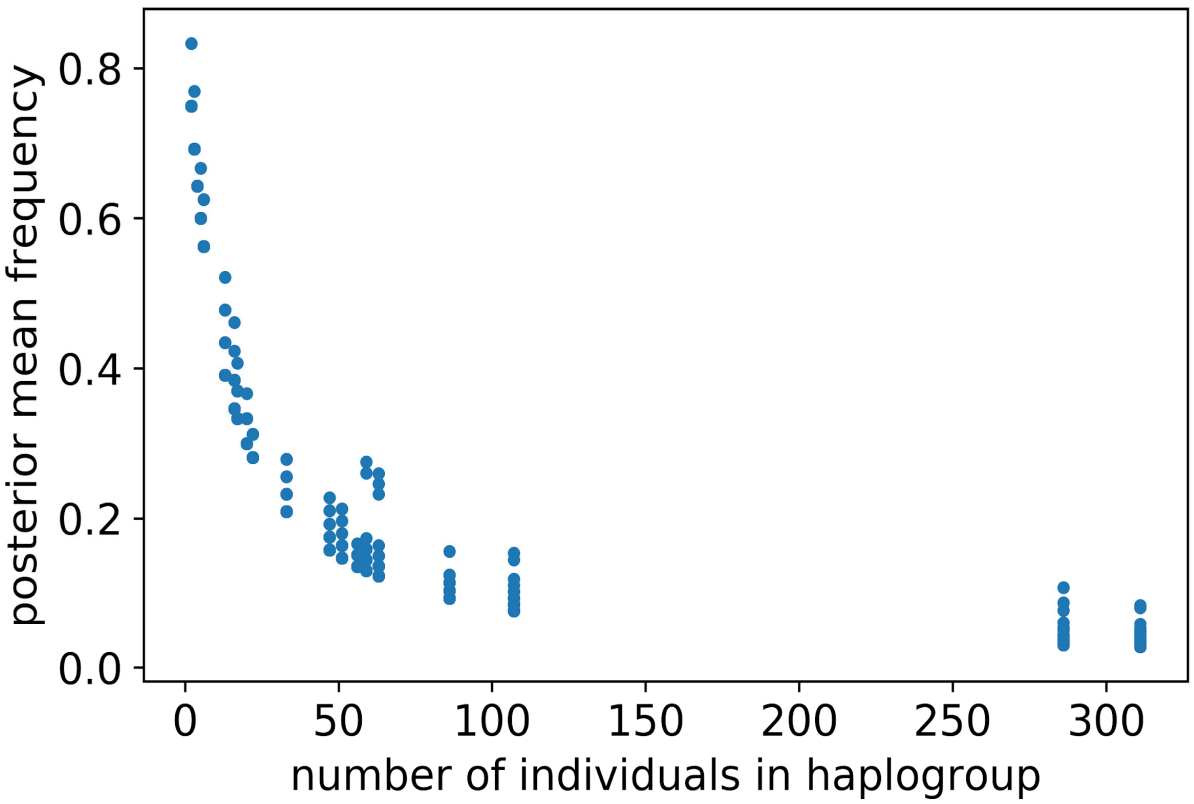
Inner doublet rings represent 2-10% heteroplasmy, with %'s above 10% moving outward for each group.

*The outer ring reflects the direction of the minor variant, with **Gray** bars the SNP and **Black** bars the reference sequence. The length of the bar represents the proportion of the minor variant when both scenarios are observed.*



*Schematic of Bayesian inference of heteroplasmy frequencies in a haplogroup. The prior distribution on the frequency of heteroplasmy in a haplogroup (**blue**) is skewed toward higher, or more conservative frequencies. The **orange** distribution shows the posterior distribution after observing two heteroplasmies in 30 observations, and the **green** distribution shows the posterior distribution after observing 20 heteroplasmies in 300 observations. The sample frequency of both samples is 6.7% (dashed vertical line).*

This model illustrates how the posterior distribution approaches the sample frequency as more data is collected.



Relationship between posterior mean heteroplasmy frequency and major haplogroup sample size. Each point represents the estimate of heteroplasmy occurrence rate in at a particular site in a particular major haplogroup. Estimates are shown only for site-haplogroup combinations at which heteroplasmy was observed at least once.

Frequencies decrease as the number of individuals in a population group increases.

Bottom Line

The majority of heteroplasmies are between 2-10%, strongly supporting MPS as the method of choice for mtDNA sequence analysis

Heteroplasmic sites are not evenly distributed, suggesting that selection plays a role in accumulation of heteroplasmies in the CR

The direction of heteroplasmy may be impacted by the location of the site, with the minor variant predominantly a SNP in void regions

Bottom Line

With the current dataset, co-occurrence of heteroplasmy will increase the LR by a factor of 10 or greater

With additional data, co-occurrence of heteroplasmy should increase the LR by a factor of 100-1000

Ultimately, the LR should be in the range of 100,000-1,000,000 for an mtDNA match when considering a haplotype match with the co-occurrence of heteroplasmy

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Daisy says hello!

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