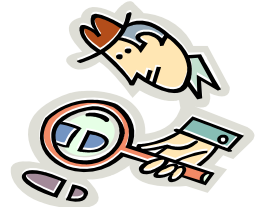
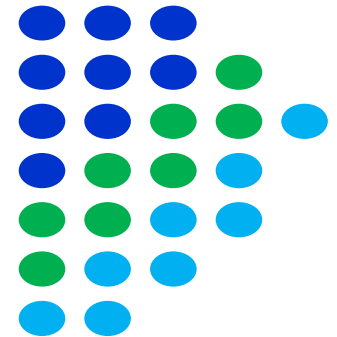




Mitchell M. Holland, Ph.D.  
Former Director, Forensic Science Program  
Professor, Biochemistry & MolBio  
Eberly College of Science  
Penn State University, University Park, PA  
Professor, University of Split (Honorary)



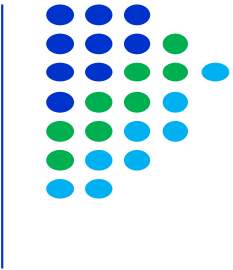
# ***A Forensic Genomics Approach for the Identification of Sister Marija Krucifiksa Kozulić of Rijeka***



26 June 2022



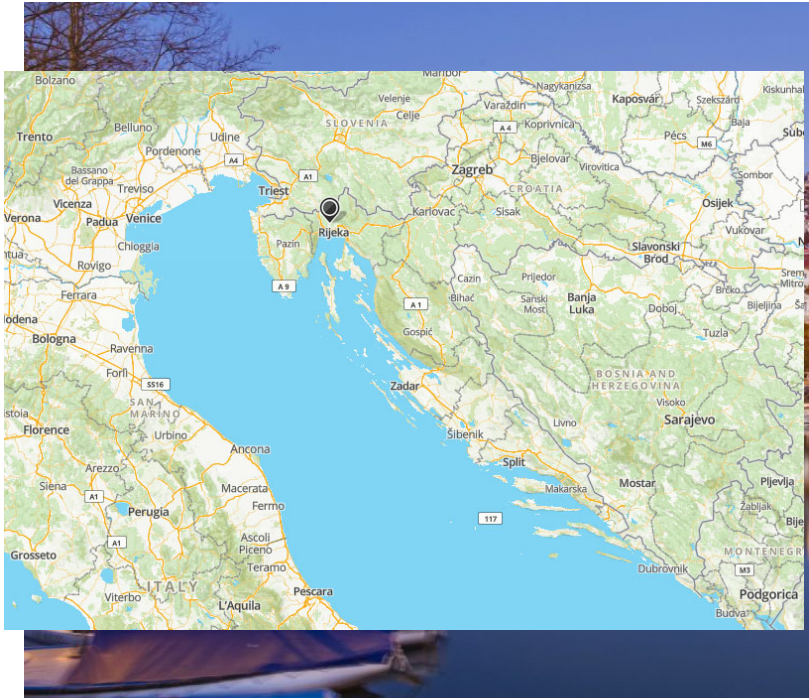
<http://forensics.psu.edu/research/dr.-mitchell-holland>



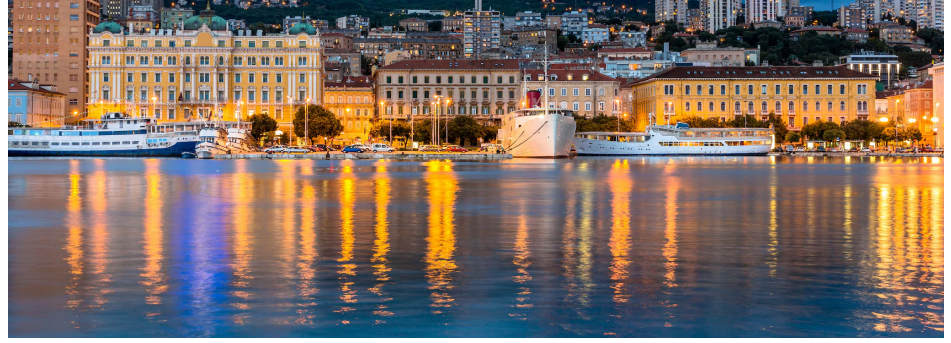
The story begins in the beautiful  
City of Rijeka







The story begins in the beautiful City of Rijeka







Age 15



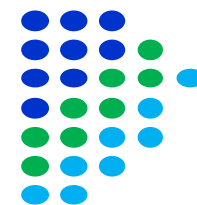
Sister Marija was born on  
the Island of Lošinj

September 20, 1852

Eldest of five children who survived  
from 11 siblings







Educated in Rijeka and Gorica in Italy

Poet, pastoral member, charitable-social worker, and teacher of music and foreign languages; she could speak Croatian, Italian, Hungarian, German, French, and Latin

Age 18



## The Kozulić shipyard at Pećine in Rijeka

Her family was quite wealthy – shipowners

However, two of her father's ships sank which left the family in hardship

This had a tremendous impact on Marija's outlook on the despair that families can face







She loved children

She spent much of her life  
educating disadvantaged,  
abandoned, orphaned children and  
providing them with a home

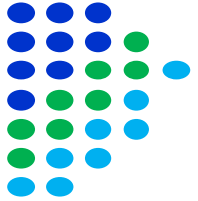




In **1895**, Sister Marija founded the Institute of the Sacred Heart of Jesus in Rijeka for poor children and youth

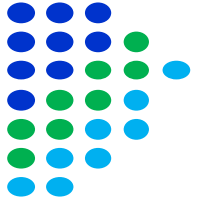






In **1899**, she founded the Order of Sisters of the Sacred Heart of Jesus in Rijeka, whose charter was the upbringing and education of children and youth with special needs

In **1904**, she took her vows and officially became a nun



Sister Marija was laid to rest on  
October 1, 1922, after rendering her  
soul to God on September 29<sup>th</sup>  
She was 70 yoa

***"I am gladly leaving the earth,  
because my mission here is done."***

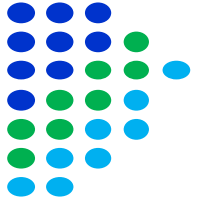
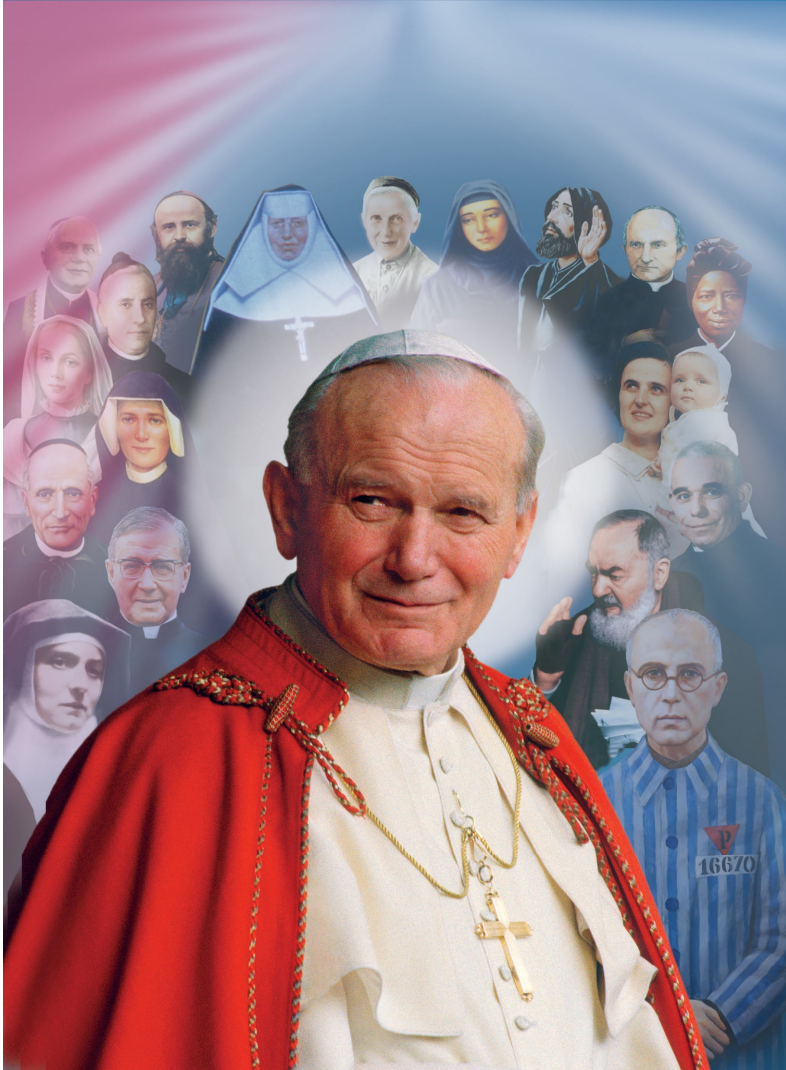
Citizens of Rijeka were heard crying:  
***"The Mother of Rijeka has died!"***  
***"A saint has died!"***





*"She was the true Mother of orphans, the poor and the abandoned whom she provided roof, shelter, food, clothing, upbringing and education. She blessed everyone, forever. With her devotion and with love of neighbors she dedicated her simple, pure, humble soul to the Glory of God. Her soul was brave, but without impudence; it was serious, but never strict. The sheltered orphans plead good people to pray for their beloved Mother to receive reward from the Heaven, for she had lived for the Heaven. May her soul rest in peace!"*





The beginning of the Canonization process of Sister Marija occurred in 2003 when Pope John Paul II visited Rijeka

Pope from 1978-2005

To the right is a collage of individuals he made a Saint during his reign as Pope

Pope John Paul II was beatified by Pope Benedict XVI in 2011 and made a Saint in 2013 by Pope Francis



# Canonization Process



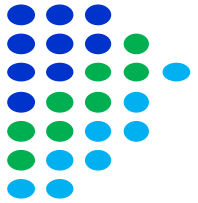
The process typically begins at least five years after the individual has been laid to rest to ensure that the case for sainthood can be properly evaluated

The local Bishop (diocese) can open an investigation into the life of an individual to determine whether they lived their life with sufficient holiness and virtue to be considered for sainthood – ***servant of God***





# Canonization Process



Show proof of a life of heroic virtue, and if the Pope agrees the individual is considered ***venerable***

The individual must have been responsible for at least two documented miracles after their death – after the first is documented they become beatified and are ***blessed***

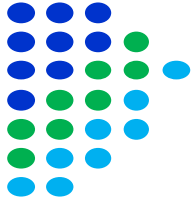


# Canonization Process



During the Canonization ceremony, the Pope conducts a special Mass, reading the individual's life history and then chanting a prayer in Latin that declares the person a Saint

The process leading up to Canonization is facilitated by a ***Postulator***

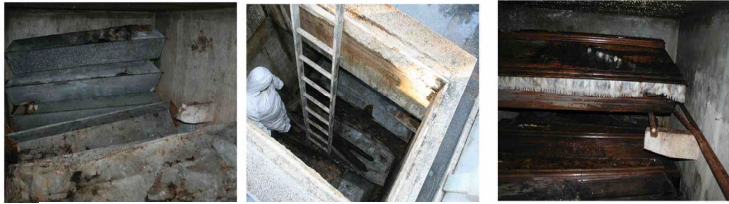
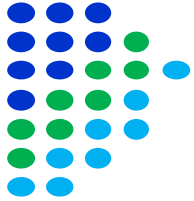


Sister Dobroslava Mlakić, *Postulator* in the  
Process of Beatification of Sister Marija

*Presents the case for Canonization*





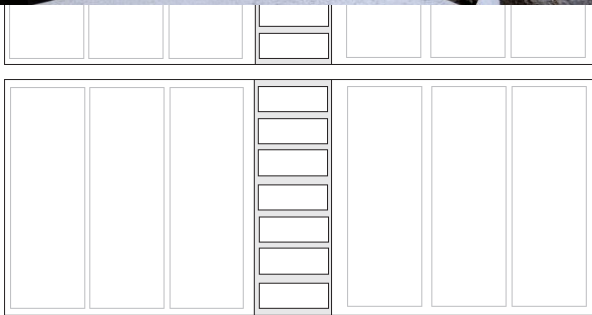


Dragan Primorac  
Željana Bašić  
Ivana Kružić  
Anja Petaros



Tin & wooden  
coffins along  
with bags of  
skeletal material

Transfer to plastic  
bags Circa 2006



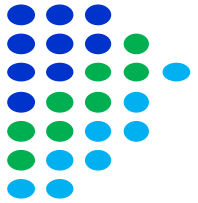
underneath the central part of the tomb two levels of wooden boxes (13 boxes)







# Commingled Remains



## Plastic Bag 2

Min of 3 People



## Plastic Bag 3

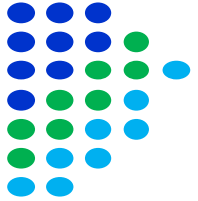
Min of 2 People







# Commingled Remains



## Plastic Bag 4

Min of 4 People



## Plastic Bag 5

Min of 3 People





# DNA Testing Approach



## Step 1: Find the sisters

Mitogenome sequencing of 12 femoral samples and 2 humeral samples

→ Identify samples with matching mtDNA haplotypes

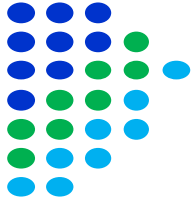
## Step 2: Confirm Kozulić pedigree

STR and SNP genotyping of samples with matching mtDNA haplotypes

→ Assess genetic relationship with Sister Marija's paternal niece



## Samples Tested for mitogenome Sequencing



Sample #	Bone	Side	Bag
42	Femur	R	2
44	Femur	L	2
45	Femur	Unknown	2
46	Femur	Unknown	2
65	Femur	L	3
60	Humerus	L	4
40	Femur	R	4
41	Femur	L	4
37	Femur	R	4
38	Femur	L	4
39	Femur	R	5
43	Femur	L	5
48	Femur	L	5
63	Humerus	R	5

Samples 45 & 46 either did not produce sequence information or the information could not be replicated

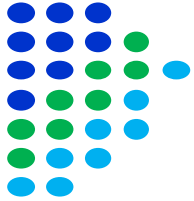
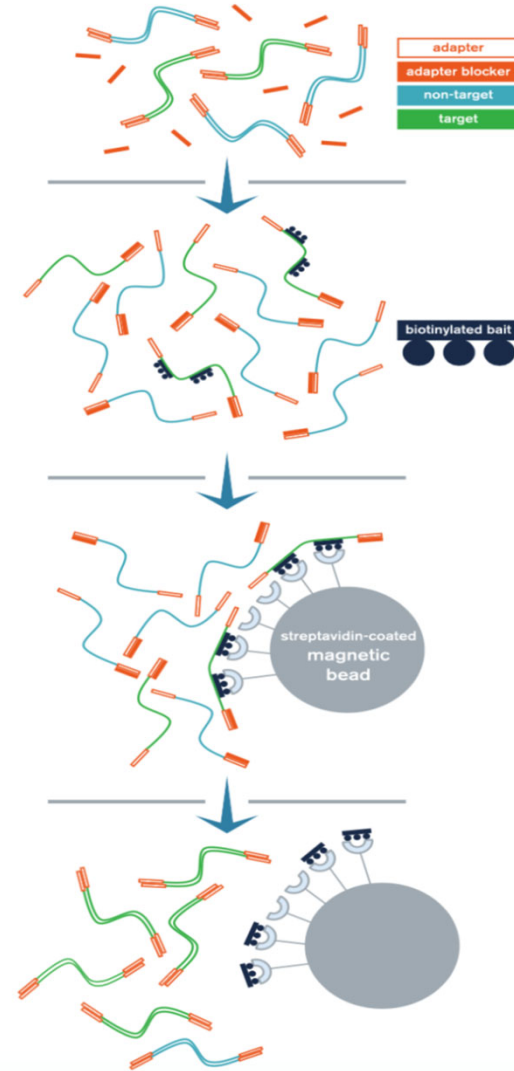




DNA from ~500 mg bone powder,  
demineralization, purification,  
NEBNext FFPE repair

Capture with a custom designed  
bait cocktail developed by AFDIL  
for the mitogenome

MPS on a MiSeq using 300-cycle  
paired end sequencing



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

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: [www.elsevier.com/locate/fsigen](http://www.elsevier.com/locate/fsigen)

Research paper

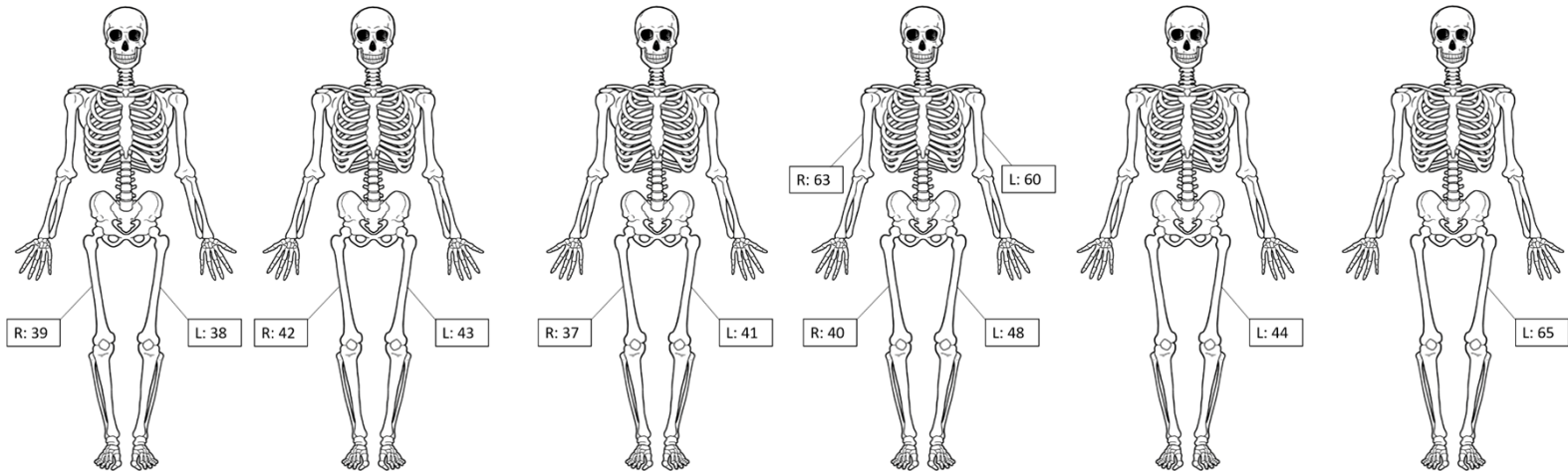
Performance evaluation of a mitogenome capture and Illumina sequencing protocol using non-probative, case-type skeletal samples: Implications for the use of a positive control in a next-generation sequencing procedure

Charla Marshall<sup>a,b,\*</sup>, Kimberly Sturk-Andreaggi<sup>a,b</sup>, Jennifer Daniels-Higginbotham<sup>a,b</sup>, Robert Sean Oliver<sup>a,b</sup>, Suzanne Barritt-Ross<sup>a,b</sup>, Timothy P. McMahon<sup>a</sup>

<sup>a</sup> 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  
<sup>b</sup> ARF Sciences, LLC, 9210 Corporate Blvd., Rockville, MD 20850, United States



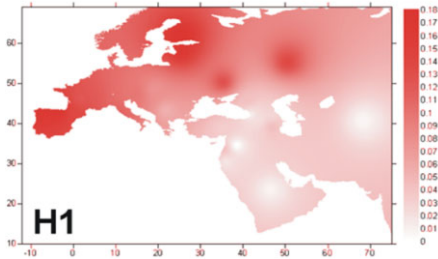
# Mitochondrial DNA Haplotypes



<b>Haplotype 1a</b> H1bu	<b>Haplotype 1b</b> H1bu	<b>Haplotype 2</b> H1a	<b>Haplotype 3</b> H1e1b	<b>Haplotype 4</b> V	<b>Haplotype 5</b> K1a5a
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38 in Bag 4  
39 in Bag 5

42 in Bag 2  
43 in Bag 5



b subclade is common in Eastern Europe

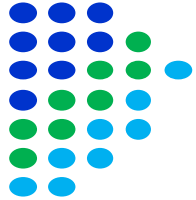
*genes* MDPI

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

Mitchell M. Holland <sup>1,\*</sup>, Kateryna D. Makova <sup>2</sup> and Jennifer A. McElhroe <sup>1</sup>

<sup>1</sup> Department of Biochemistry & Molecular Biology, Forensic Science Program, Eberly College of Science, Pennsylvania State University, University Park, PA 16802, USA; jam76@psu.edu  
<sup>2</sup> Department of Biology, Eberly College of Science, Pennsylvania State University, University Park, PA 16802, USA; kmakova@psu.edu  
 \* Correspondence: mmh20@psu.edu

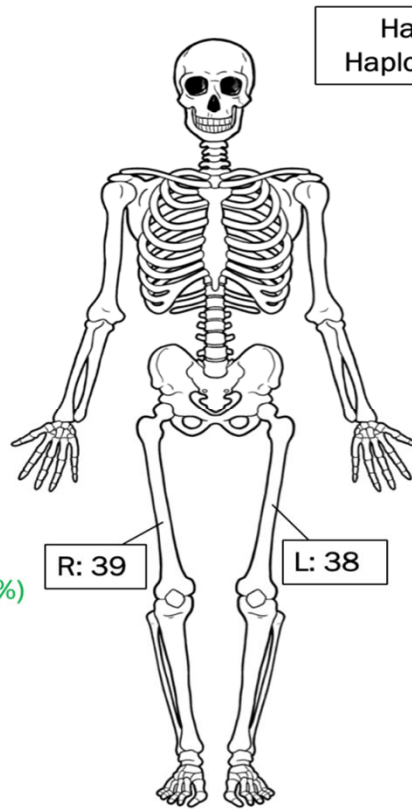
Received: 1 January 2018; Accepted: 20 February 2018; Published: 26 February 2018



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

Heteroplasmy

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

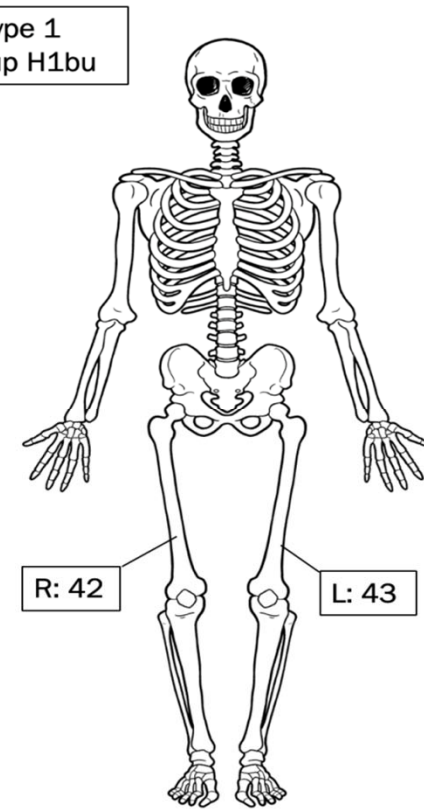


Haplotype 1  
 Haplogroup H1bu

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

Heteroplasmy

A13327A (12-13%)



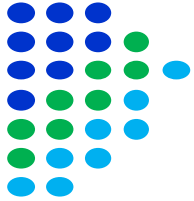
Control Region  
 Coding Region

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

No known disease state associated with the change

Reproduced by AFDIL

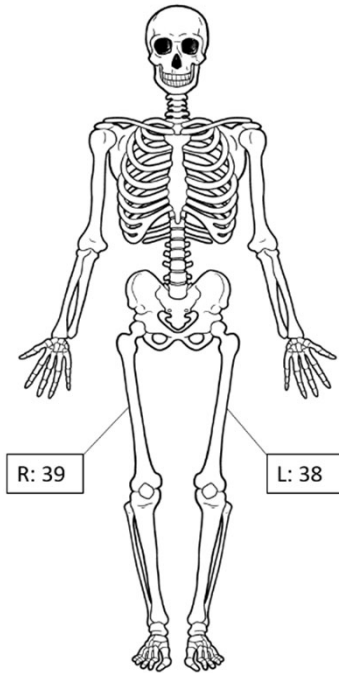




# Are these the Kozulić Sisters?

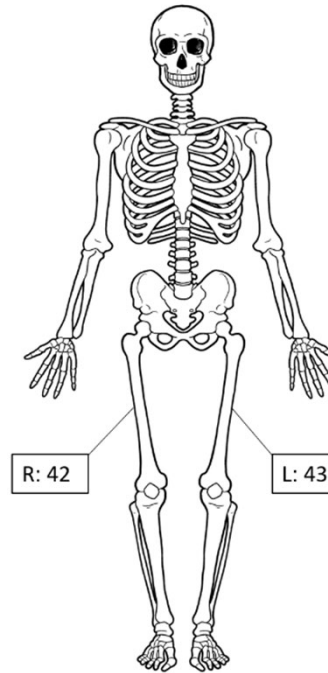


Marija



Haplotype 1a  
H1bu

263G  
315.1C  
750G  
1438G  
3010A  
4769G  
A5558G  
8860G  
12337insC  
13327R  
15326G  
16519C



Haplotype 1b  
H1bu

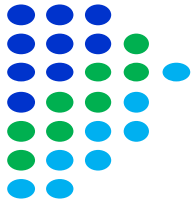
263G  
315.1C  
750G  
1438G  
3010A  
4769G  
5558G  
8860G  
13327R  
15326G  
16519C



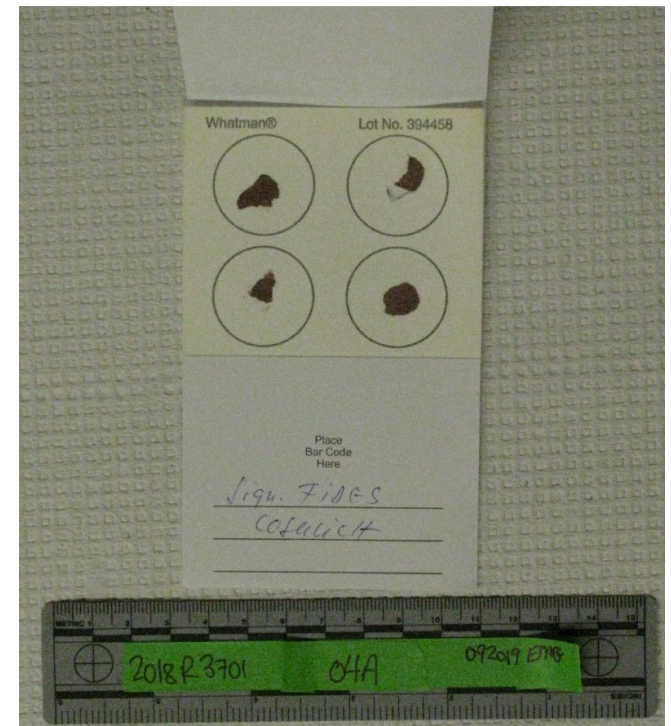
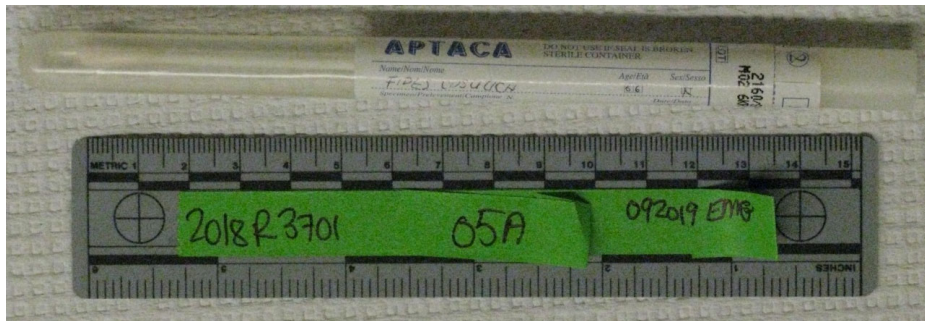
Tereza



# DNA Reference Samples from Paternal Niece



- Fides Kozulić
- Samples collected at age 66

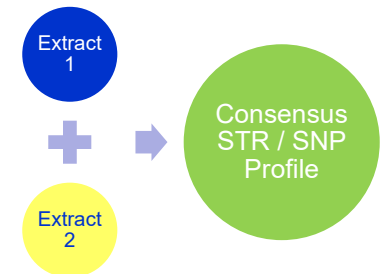






# STR/SNP Testing Methods



Target	Enrichment	Step 1	Step 2	Sequencing
<b>Autosomal STRs (n=29)</b>	Precision ID Globalfiler (all <300 bp; most <200 bp)	PCR	KAPA Hyper Library Preparation with 12-cycle PCR	MiSeq FGx 2x300 cycles V3-600 cartridge
<b>Autosomal Identity SNPs (n=90)</b>	Precision ID Identity (avg 132 bp)	PCR		NextSeq 550 75 cycles V2 high output cartridge



Y markers (2) included in the multiplex, but no results observed, as all samples were female

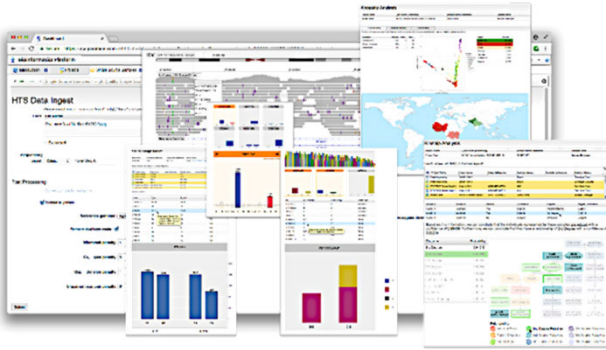
Article

**DNA Testing Reveals the Putative Identity of JB55, a 19th Century Vampire Buried in Griswold, Connecticut**

Jennifer Daniels-Higginbotham <sup>1,2</sup>, Erin M. Gorden <sup>1,2</sup>, Stephanie K. Farmer <sup>3</sup>, Brian Spatola <sup>4</sup>, Franklin Damann <sup>5</sup>, Nicholas Bellantoni <sup>6</sup>, Katie S. Gagnon <sup>7</sup>, Maria de la Puente <sup>8,9</sup>, Catarina Xavier <sup>9</sup>, Susan Walsh <sup>3</sup>, Walther Parson <sup>9,10</sup>, Timothy P. McMahon <sup>1</sup> and Charla Marshall <sup>1,2,10,\*</sup>



# STR and SNP Genotyping in Parabon Fx Software



	<b>Individual</b>	<b>STR Loci (max. 29)</b>	<b>SNP Loci (max. 90)</b>	<b>Total Loci (max. 119)</b>
Sample 39	H1bu – 1a	22 (76%)	67 (74%)	89 (75%)
Sample 43	H1bu – 1b	4 (14%)	38 (42%)	42 (35%)
Sample 40 (control)	H1e1b	29 (100%)	71 (79%)	100 (84%)
	Fides	27 (93%)	85 (94%)	112 (94%)



# Pairwise Kinship Analysis

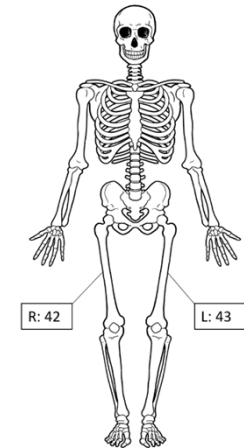
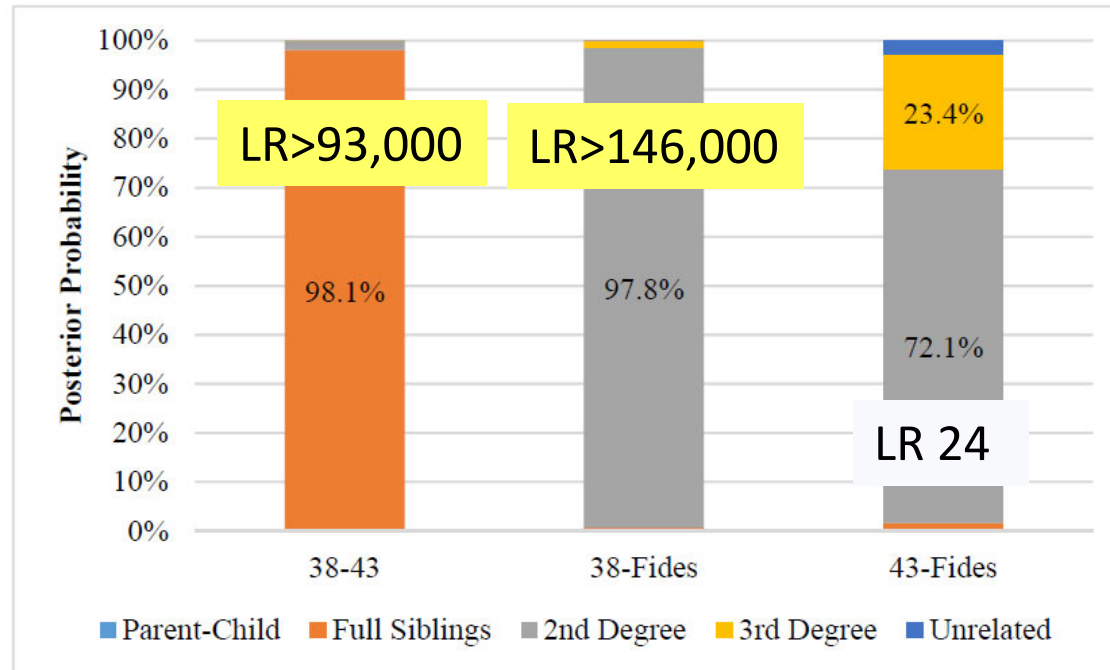
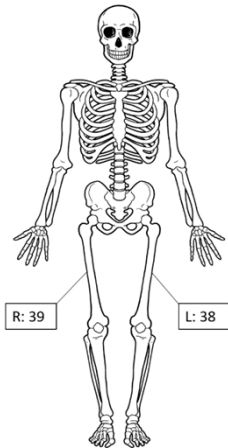


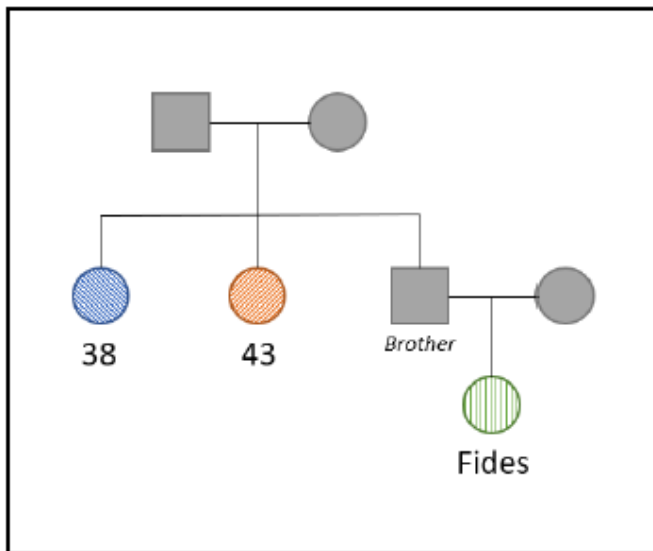
Figure 4. Posterior probability distributions of degrees of relatedness between pairwise comparisons of DNA profiles from samples 38, 43 and the buccal swab (Fides Kozulić). Probabilities greater than 5% are labeled. Sample 40 is not shown because all pairwise comparisons produced likelihood ratios less than one for all degrees of relatedness.



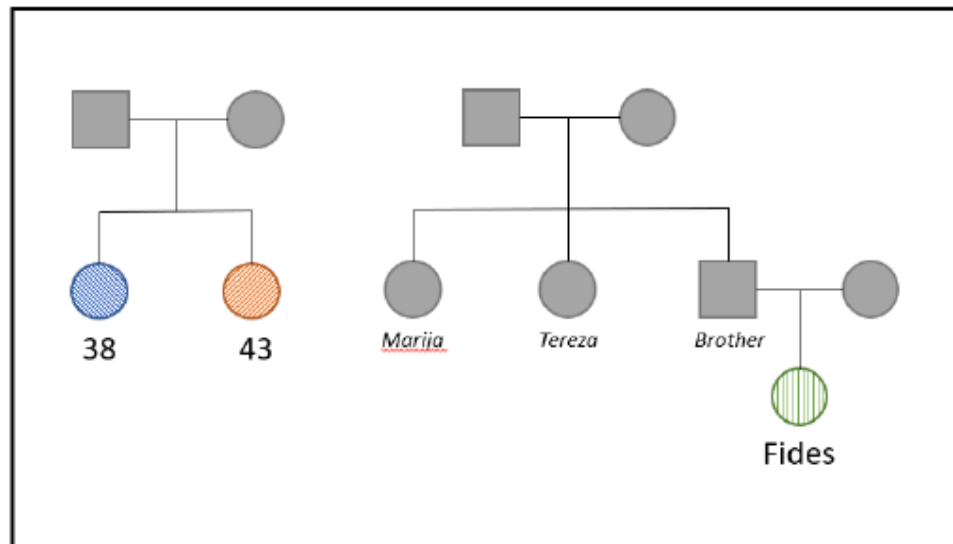
# Pedigree Kinship Analysis



Sisters from Kozulić family ( $H_1$ )



Sisters from another family ( $H_2$ )



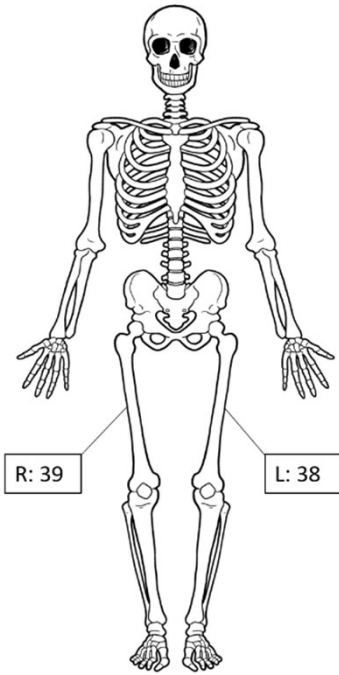
LR > 574,000





Marija

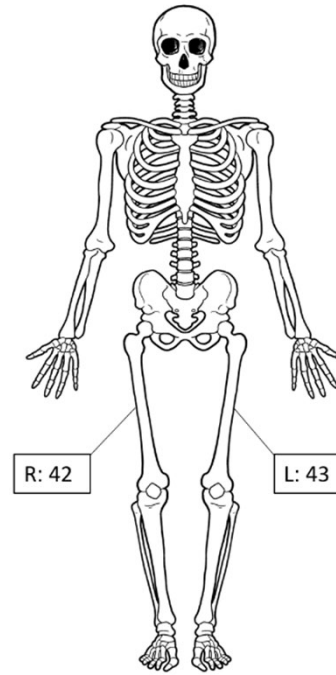
163±3.72 cm



Haplotype 1a  
H1bu

263G  
315.1C  
750G  
1438G  
3010A  
4769G  
A5558G  
8860G  
12337insC  
13327R  
15326G  
16519C

## Which One is Marija?



Haplotype 1b  
H1bu

263G  
315.1C  
750G  
1438G  
3010A  
4769G  
5558G  
8860G  
13327R  
15326G  
16519C

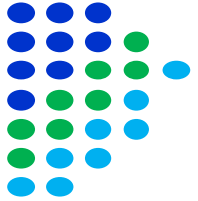


Tereza

165±3.72 cm

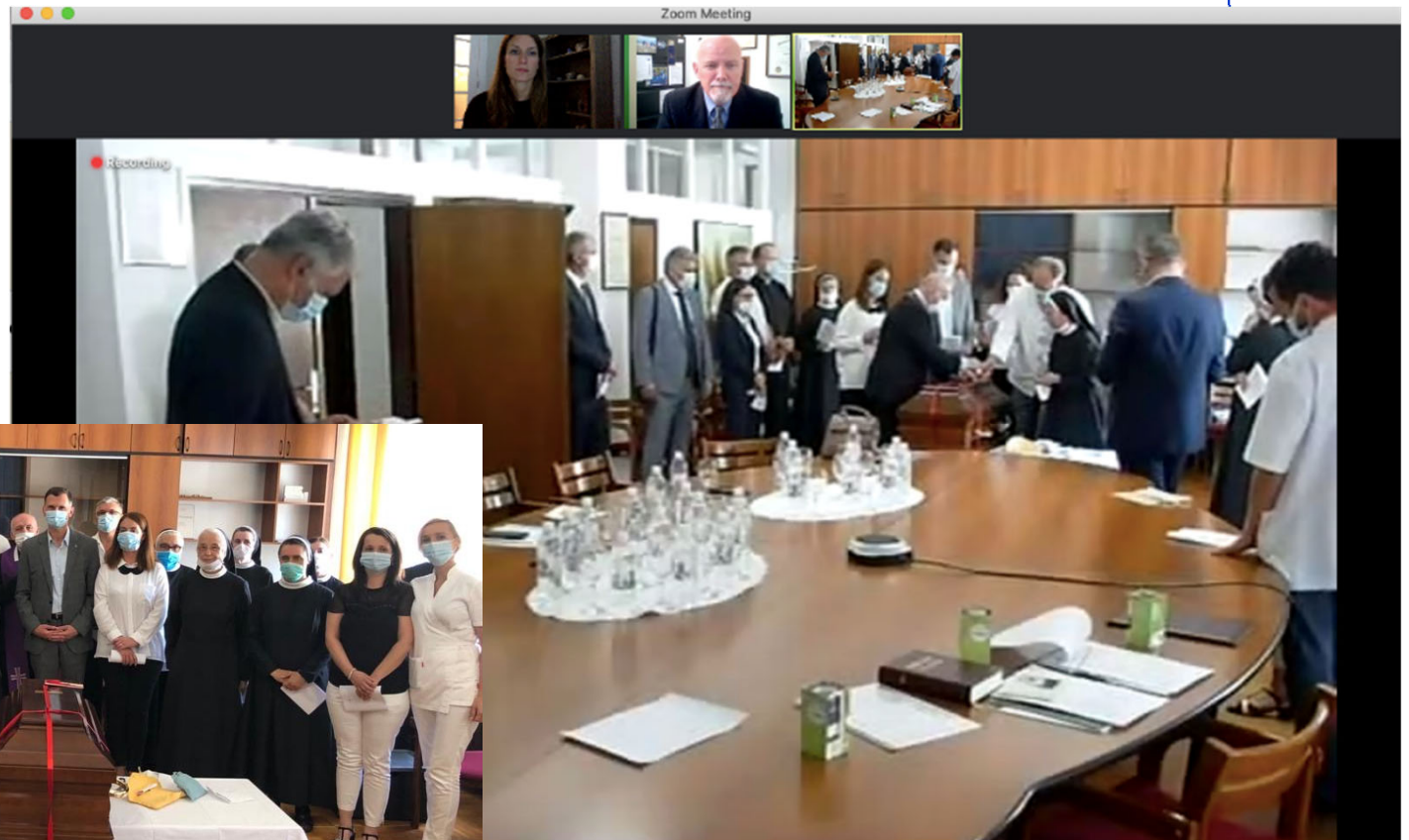


# Identification Ceremony



September 2, 2020

5:00-6:30 am EST





Dragan Primorac

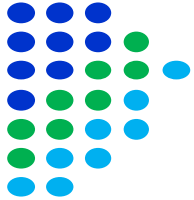
Sister Dobroslava Mlakić  
*Postulator of the Cause of Beatification*

Archbishop, Ivan Devčić



Sister Marija was finally laid to rest with her sister Tereza on September 2, 2020

You can visit her burial site and memorial at the House of Sister Marija



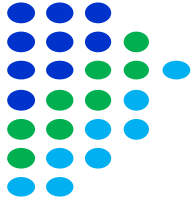
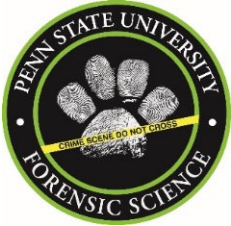




Church of St. Ana  
Rijeka, Croatia



*"Love has never impoverished anyone, rather a shining star of God's blessing shines over those who have love for their fellow human."*



mmh20@psu.edu



GCAT  
TACG  
GCAT

*genes*



Article

## A Forensic Genomics Approach for the Identification of Sister Marija Crucifiksa Kozulić

Charla Marshall <sup>1,2,3,\*</sup> , Kimberly Sturk-Andreaggi <sup>1,2</sup>, Erin M. Gorden <sup>1,2</sup>, Jennifer Daniels-Higginbotham <sup>1,2</sup>, Sidney Gaston Sanchez <sup>1,2</sup>, Željana Bašić <sup>4</sup>, Ivana Kružić <sup>4</sup>, Šimun Andelinović <sup>5,6</sup>, Alan Bosnar <sup>7</sup>, Miran Čoklo <sup>8</sup>, Anja Petaros <sup>9</sup>, Timothy P. McMahon <sup>1</sup>, Dragan Primorac <sup>3,5,10,11,12,13,14,15</sup>  and Mitchell M. Holland <sup>3,\*</sup>

*Genes* 2020, 11, 938; doi:10.3390/genes11080938



Sister Dobroslava

