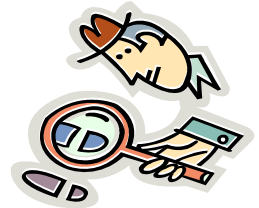
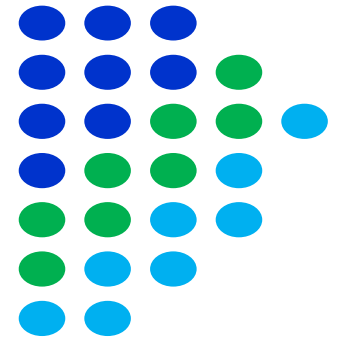




Mitchell M. Holland, Ph.D.
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Professor, Biochemistry & MolBio
Eberly College of Science
Penn State University, University Park, PA



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



**Green Mountain
DNA Conference**



27 July 2022

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



Article

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

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

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

^{1,2}**AFDIL (SNA)**

³Forensic Science Program, **Penn State University**

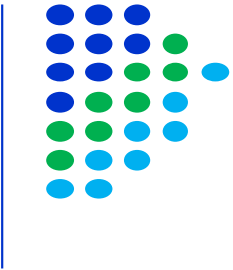
⁴Department of Forensic Sciences, **University of Split**

^{5,6}Medical School, University of Split (Clinical Hospital Center Split)

⁷Department of Forensic Medicine & Criminalistics, **University of Rijeka School of Medicine**

⁸Institute of Anthropological Research, **Center for Applied Bioanthropology**, Zagreb

⁹Department of Forensic Medicine, **National Board of Forensic Medicine**, Sweden



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

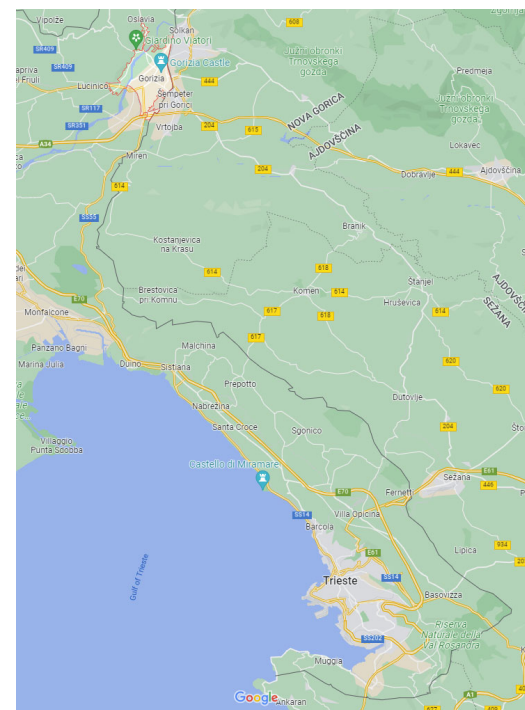


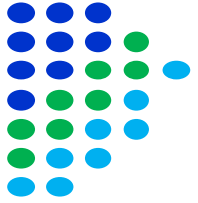


Educated in Rijeka and Gorizia, Italy

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

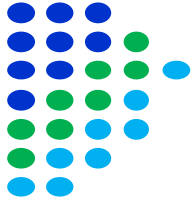
This had a tremendous impact on Marija's outlook on the despair that families can face





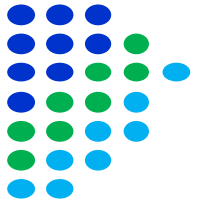
She loved children

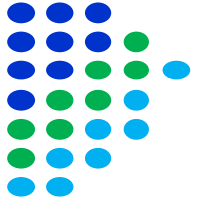
She spent much of her life
educating disadvantaged,
abandoned, orphaned children and
providing them with a home





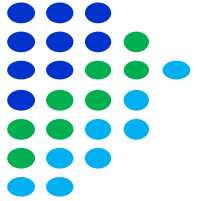
In **1895**, Sister Marija (43 yoa) founded the Institute of the Sacred Heart of Jesus in Rijeka for poor children and youth





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 (52 yoa)



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

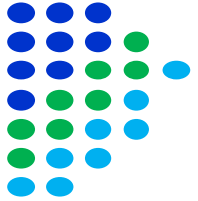
***"I am gladly leaving the earth,
because my mission here is done."***

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

John Paul II was Pope from 1978-2005

To the left 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

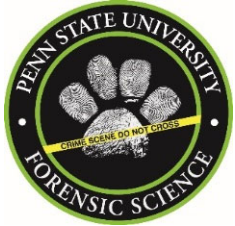


Canonization Process

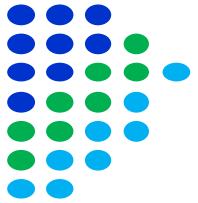


The process typically ***begins at least five years after*** the individual has been laid to rest to ensure that the case for sainthood can be properly evaluated

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***



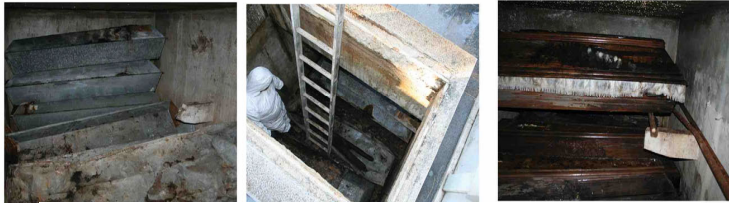
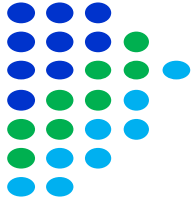
Sisters of the
Sacred Heart of
Jesus



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

Presents the case for Canonization



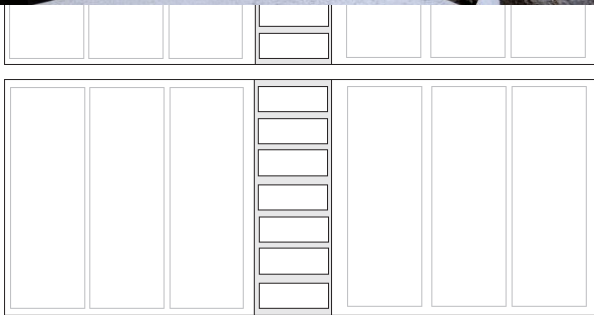


Dragan Primorac
Željana Bašić
Ivana Kružić
Anja Petaros



Tin & wooden
coffins along
with bags of
skeletal material

Transfer to plastic
bags Circa 2006



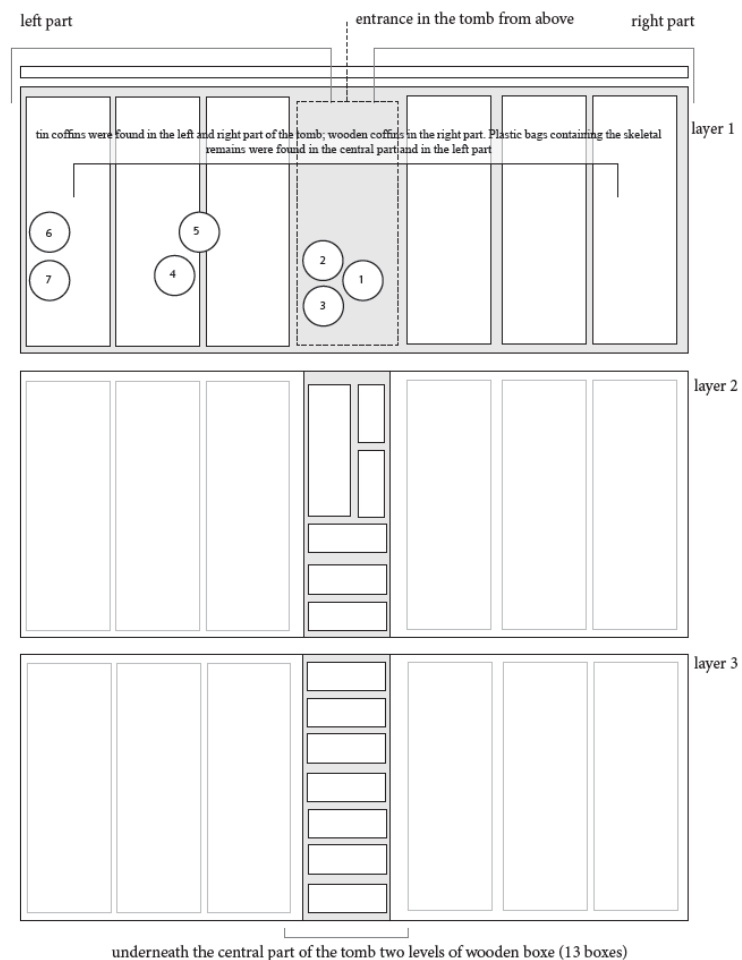
underneath the central part of the tomb two levels of wooden boxes (13 boxes)



Marija and her sister Tereza (Irena) were 2 of the 52 persons buried in the tomb belonging to the Society of Sisters of the Sacred Heart of Jesus

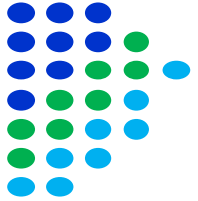
- | | |
|--|---|
| <ol style="list-style-type: none"> 1. Maria Geltrude Diracca 1876-1908 2. Maria Fabich 1873-1910 3. Maria Bradas 1873-1910 4. Gabriella Vittori 1887-1913 5. Gesualda de Zorzi 1857-1916 6. Francesca Piskar 1843-1918 7. Angelica Konigsmark 1885-1918 8. Michella Pillepich 1885-1920 9. Rosaria Vattovaz 1884-1923 10. Maria Margherita Rossoyich 1854-1938 11. Anna Domenica Gallovich 1868-1938 12. Alojzija Antić 1888-1964 13. Ignacija Kerum 1890-1971 14. Ksaverija Bakan 1900-1978 15. Mehtilda Brozović 1888-1982 16. Lucijana Čović 1947-1986 17. Margareta Nuić 1907-1988 18. Antonija Pavličević 1907-1988 19. Elizabeta Ivković 1908-1988 20. Renata Šafar 1916-1988 21. Terezija Szabo 1911-1993 22. Valerija Bendeković 1940-1993 23. Melanija Salopek 1942-1994 24. Pija Kerum 1933-1996 25. Ivana Kraljević 1920-1997 26. Silvina Mihaljević 1935-1998 27. Roberta Matešić 1937-1999 28. Agneza Činkole 1917-1999 29. Leticija Žderić 1947-2000 | <ol style="list-style-type: none"> 30. Stanislava Špoljarić 1911-2003 31. Eufemija Baf 1936-2003 32. Mirela Kamber 1952-2006 33. Kornelija Đapić 1933-2008 34. Skolastika Ivašković 1926-2008 35. Doroteja Galemanović 1937-2009 36. Kazimira Depikolozvane 1942-2010 37. Melanija Camenar 1898-1921 38. Tereza Kozulić 1861-1933 39. Marija Krucifiksa Kozulić 1852-1922 40. nepoznata časna sestra 1 41. nepoznata časna sestra 2 42. nepoznato djete 43. Francesca Buchwald 44. Herzegeh Antonia 45. Filomena Schlober 46. Antonia Rubessa 47. Caterina Fladnitzer 48. Gisella Nemeth 49. Maria Seliskar 50. Filomena Giovanello 51. Cecilia Micheli 52. Caterina Boldin |
|--|---|

Names in black font were identified through archaeological and anthropological analysis





Commingled Remains



Plastic Bag 2

Min of 3 People



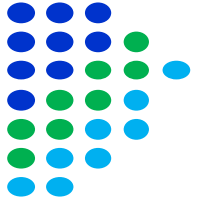
Plastic Bag 3

Min of 2 People





Commingled Remains



Plastic Bag 4

Min of 4 People



Plastic Bag 5

Min of 3 People





2-Phase DNA Testing Approach



Step 1: Find the sisters

Mitogenome sequencing of 12 femoral samples and 2 humeral samples

→ Identify samples with matching mtDNA haplotypes

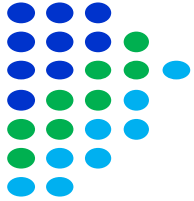
Step 2: Confirm Kozulić pedigree

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

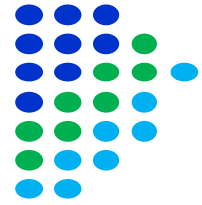
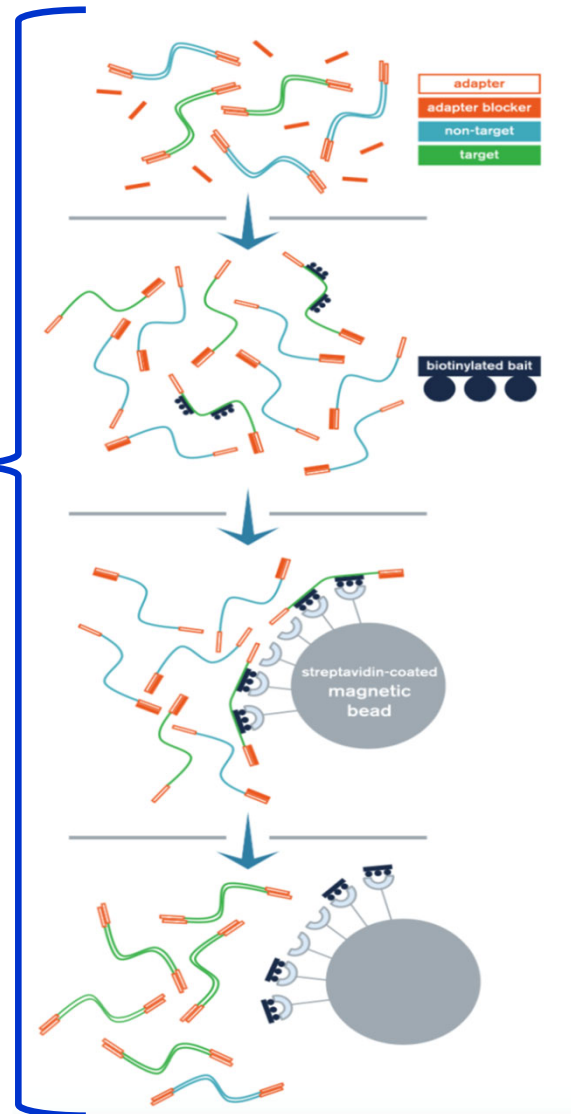
Samples 45 & 46 either did not produce sequence information or the information could not be replicated



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

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

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

Research paper

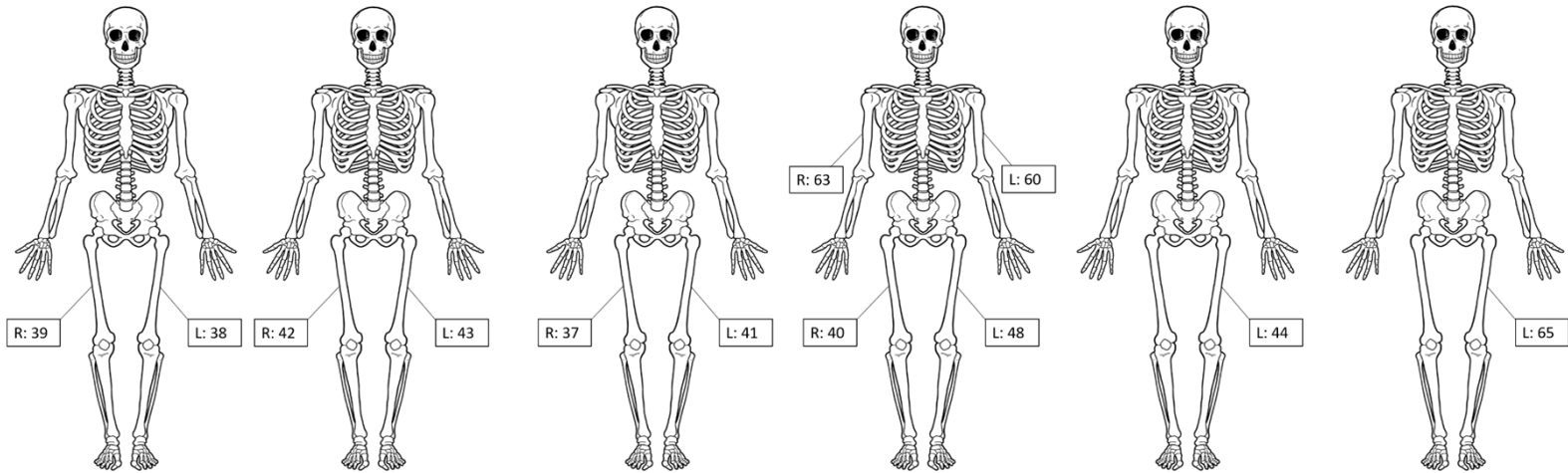
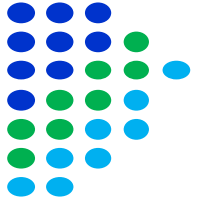
Performance evaluation of a mitogenome capture and Illumina sequencing protocol using non-probative, case-type skeletal samples: Implications for the use of a positive control in a next-generation sequencing procedure

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 ARF Sciences, LLC, 9210 Corporate Blvd., Rockville, MD 20850, United States



Mitochondrial DNA Haplotypes



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

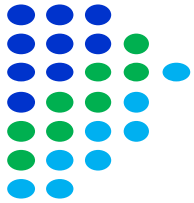
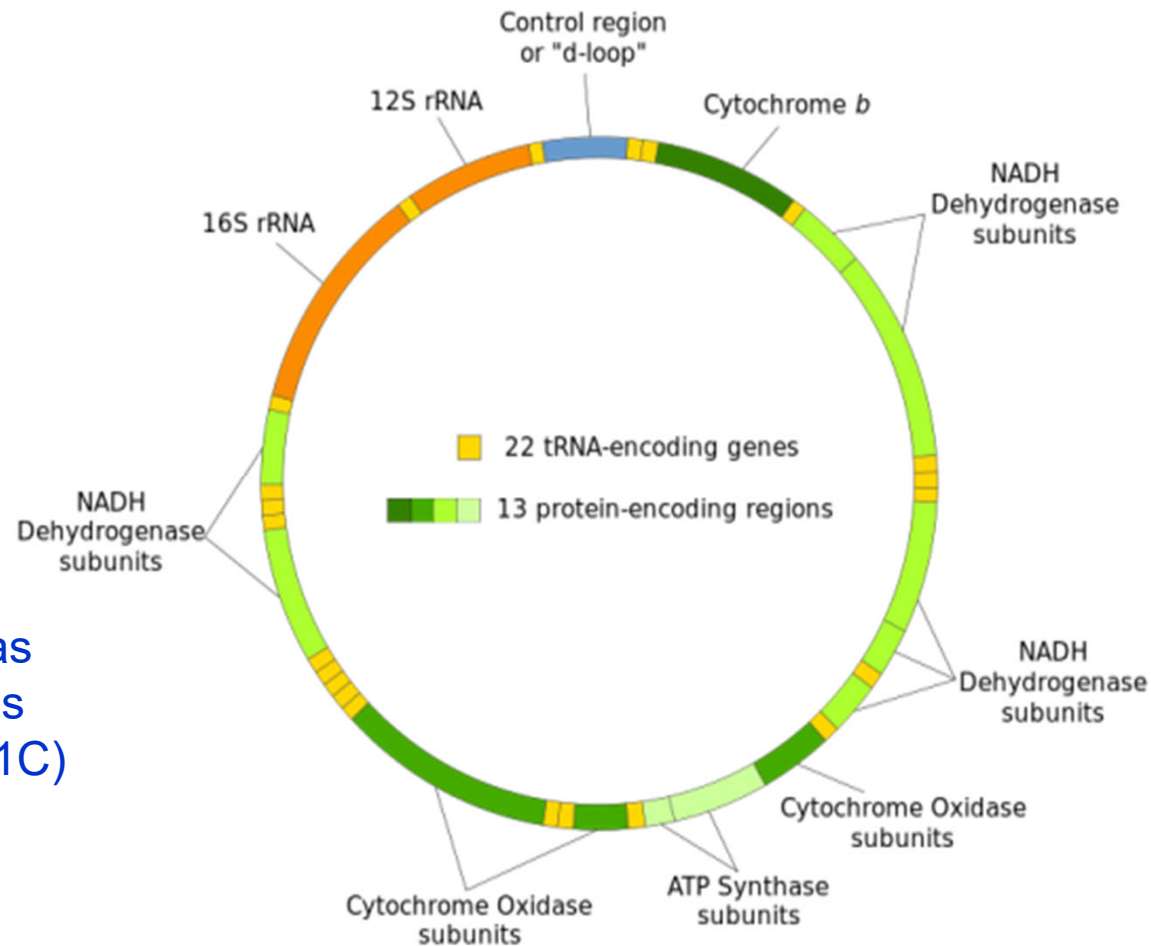
42 in Bag 2
43 in Bag 5

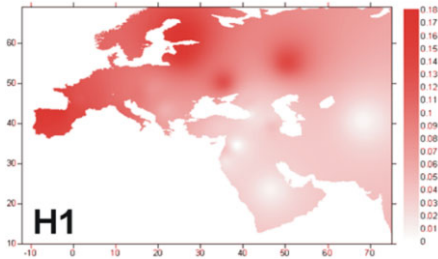


The mitogenome is
~16,569 bp in length

Numbering starts and
ends in the Control
Region

SNPs are annotated as
A8860G and insertions
as C309insC (or 309.1C)





b subclade is common in Eastern Europe

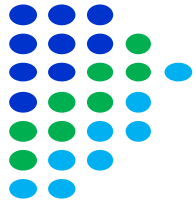
genes MDPI

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

Mitchell M. Holland ^{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; jam76@psu.edu
² Department of Biology, Eberly College of Science, Pennsylvania State University, University Park, PA 16802, USA; kmakova@psu.edu
 * Correspondence: mmh20@psu.edu

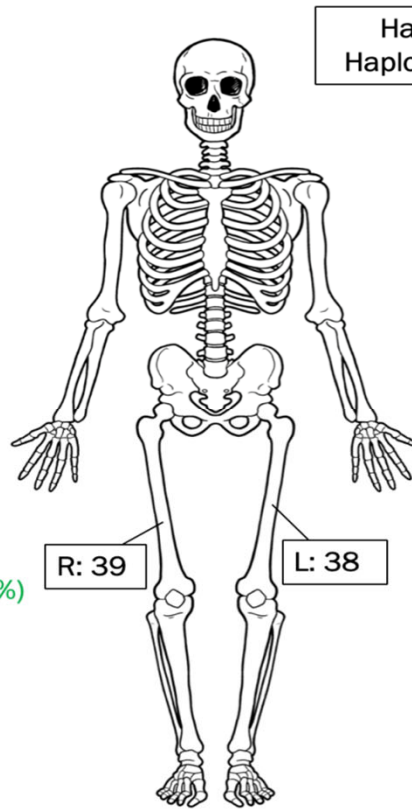
Received: 1 January 2018; Accepted: 20 February 2018; Published: 26 February 2018



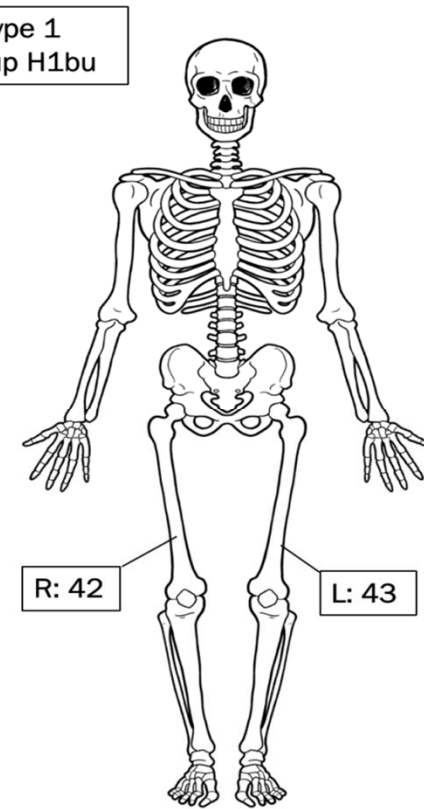
A263G
 C309insC
 C315insC
 A750G
 A1438G
 G3010A
 A4769G
 A5558G
 A8860G
 A13327G
 A15326G
 T16519C

Heteroplasmy

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



Haplotype 1
 Haplogroup H1bu



Control Region
 Coding Region

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

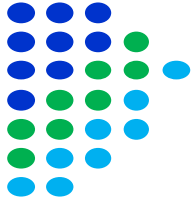
Heteroplasmy

A13327A (12-13%)

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

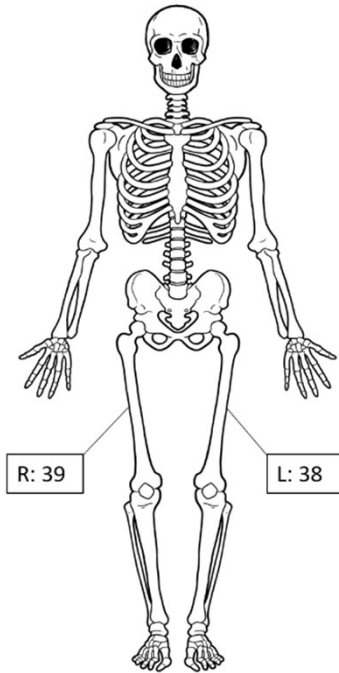
Reproduced by AFDIL



Are these the Kozulić Sisters?

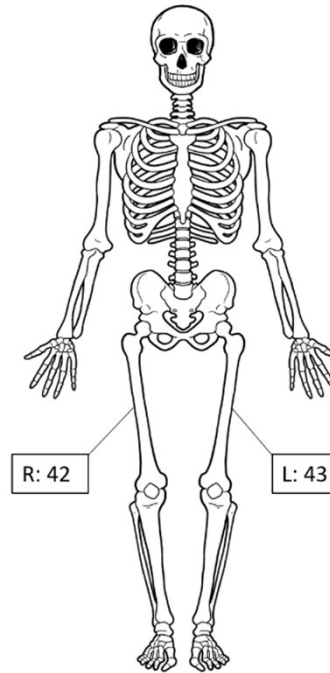


Marija



Haplotype 1a
H1bu

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



Haplotype 1b
H1bu

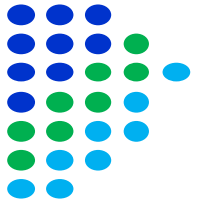
263G
315.1C
750G
1438G
3010A
4769G
5558G
8860G
13327R
15326G
16519C



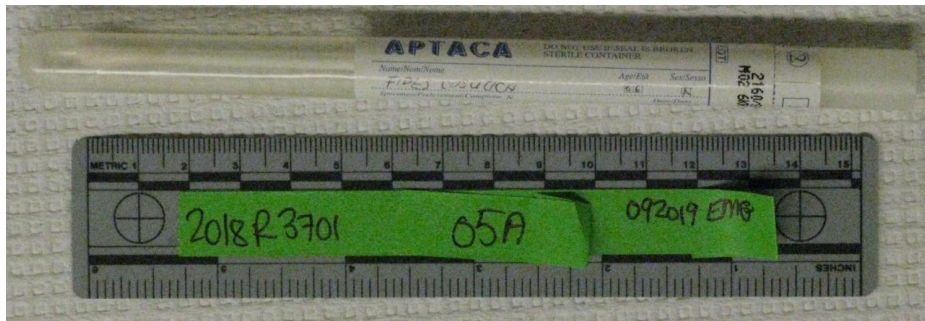
Tereza



DNA Reference Samples from Paternal Niece



- Fides Kozulić
- Samples collected at age 66

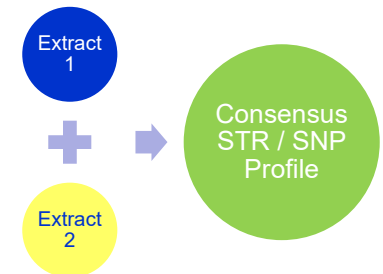






STR/SNP Testing Methods



Target	Enrichment	Step 1	Step 2	Sequencing
Autosomal STRs (n=29)	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
Autosomal Identity SNPs (n=90)	Precision ID Identity (avg 132 bp)	PCR		NextSeq 550 75 cycles V2 high output cartridge



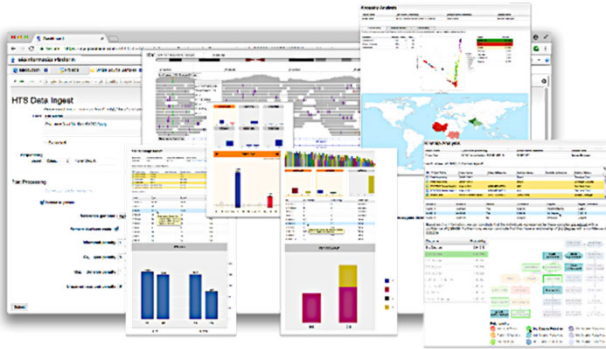
Y markers (2) included in the multiplex, but no results observed, as all samples were female

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

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

STR and SNP Genotyping in Parabon Fx Software



	Individual	STR Loci (max. 29)	SNP Loci (max. 90)	Total Loci (max. 119)
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

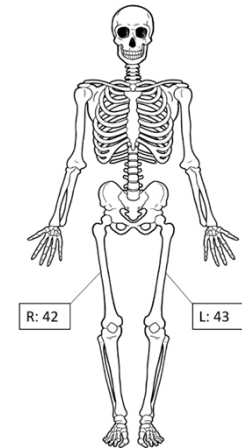
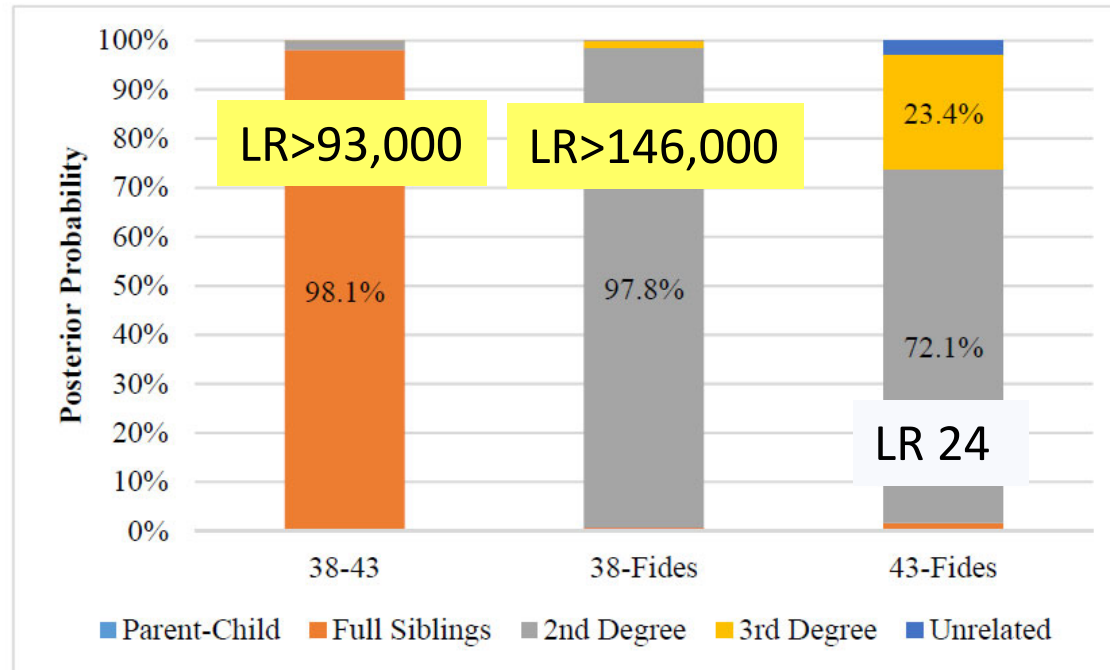
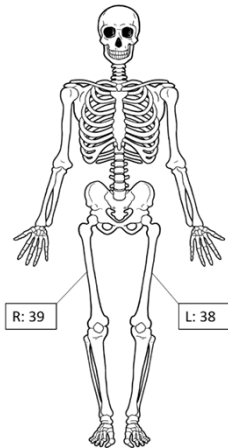
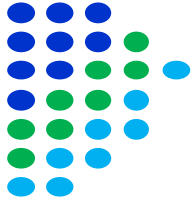
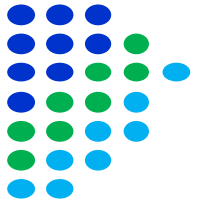


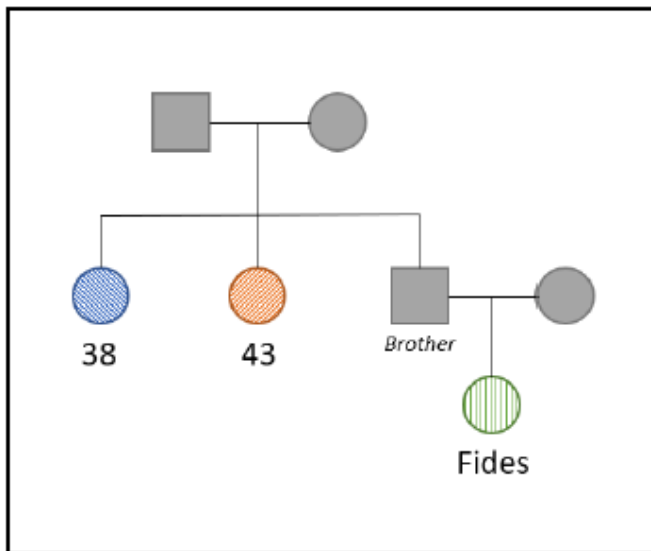
Figure 4. Posterior probability distributions of degrees of relatedness between pairwise comparisons of DNA profiles from samples 38, 43 and the buccal swab (Fides Kozulić). Probabilities greater than 5% are labeled. Sample 40 is not shown because all pairwise comparisons produced likelihood ratios less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



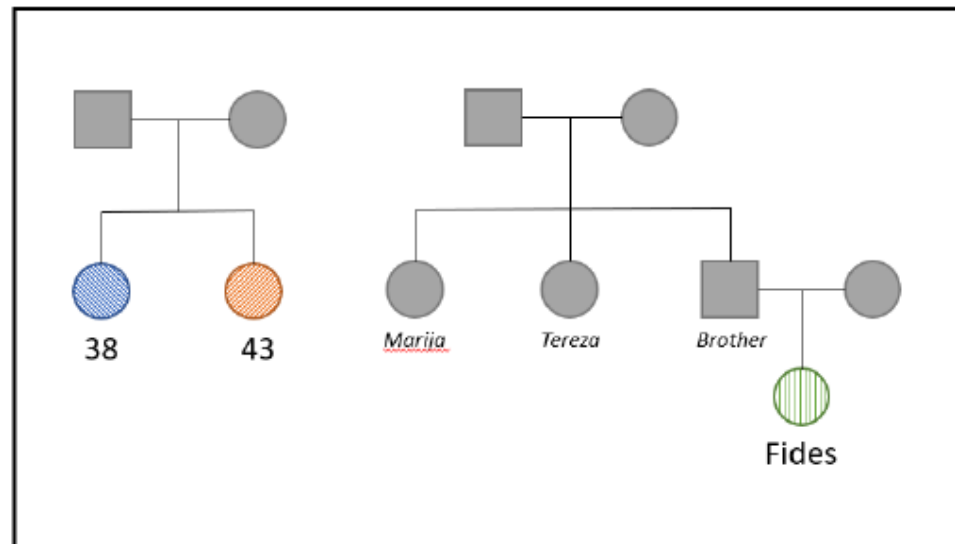
Pedigree Kinship Analysis



Sisters from Kozulić family (H_1)



Sisters from another family (H_2)

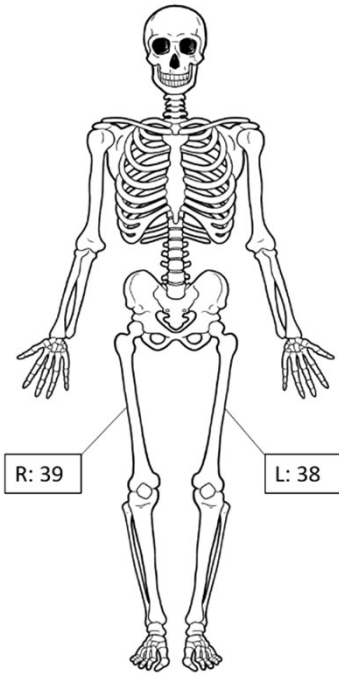


LR > 574,000



Marija

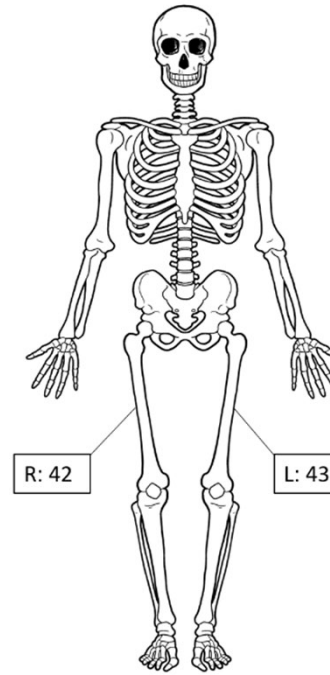
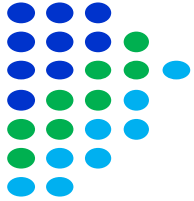
163±3.72 cm



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

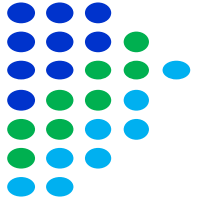


Tereza

165±3.72 cm

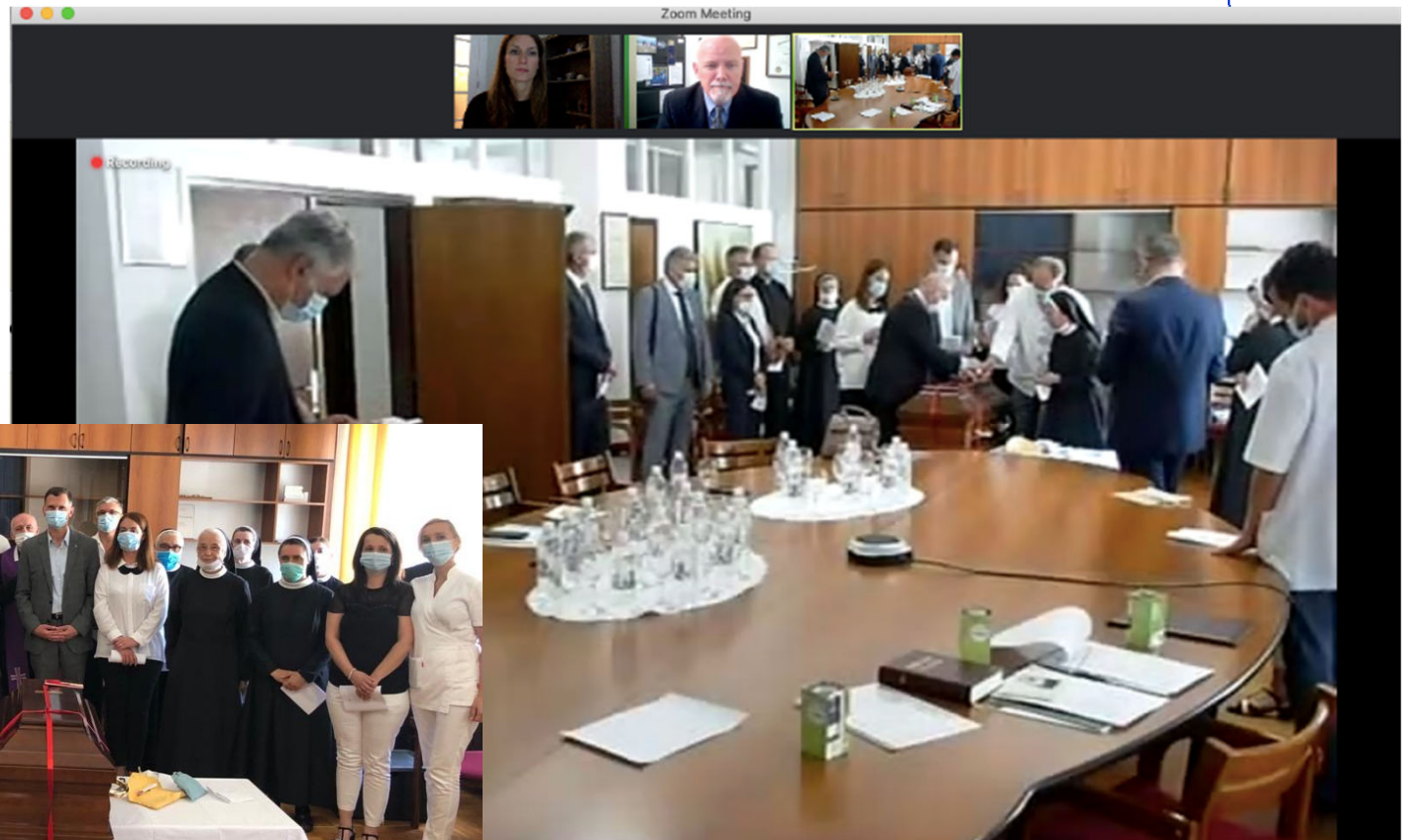


Identification Ceremony



September 2, 2020

5:00-6:30 am EST





Dragan Primorac

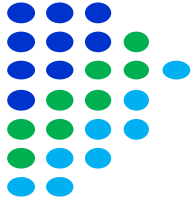
Sister Dobroslava Mlakić
Postulator of the Cause of Beatification

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





26 June 2022
ISABS Conference





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





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genes



Article

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

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

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



Sister Dobroslava

Green  ountain
DNA Conference