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Genetics Unveil the Genealogical Ancestry and Physical Appearance of an Unknown Historical Figure: Lady Leonor of Castile (Spain) (1256–1275)

Sara Palomo-Díez [1,2,*], Cláudia Gomes [1,2], María Sonia Fondevila [3], Ángel Esparza-Arroyo [4], Ana María López-Parra [1,2], María Victoria Lareu [3], Eduardo Arroyo-Pardo [1,2] and Juan Francisco Pastor [5]

1 Health Legislation, Psychiatry, and Pathology Department, Medicine Faculty, The Complutense University of Madrid, 28040 Madrid, Spain
2 Forensic Sciences Group, Genetics and Toxicology, San Carlos Research Institute (IdISSC), Clínico San Carlos Hospital, 28040 Madrid, Spain
3 Medicine and Odontology Faculty, Santiago de Compostela University, 15705 Santiago de Compostela, Spain
4 GIR 'PrehUSAL', Department of Prehistory, Ancient History and Archaeology, University of Salamanca, 37007 Salamanca, Spain
5 Anatomy and Embriology Department, Medicine Faculty, Valladolid University, 47003 Valladolid, Spain
* Correspondence: spalomod@ucm.es; Tel.: +34-913-941-501

Abstract: Through this study, it has been possible to establish an accurate prediction of the physical characteristics, biogeographical origin, and genealogical ancestry of a previously obscured historical figure: The Princess Lady Leonor of Castile (1256–1275), one of the legitimate daughters of the Spanish King Alfonso X "The Wise". The genetic analysis of External Visible Characteristics in the mummified remains attributed to this Princess has allowed determining her origin by mitochondrial and nuclear DNA analysis, and her physical appearance for hair, eyes, and skin color by autosomal SNPs. The results show that the mummified remains correspond to a young European woman with black hair, green-hazel eyes, and white skin. Her physical appearance has not been possible to be compared with any pictorial source, but the biogeographical analysis results are consistent with the historiographic genealogical information.

Keywords: ancient DNA; biogeographical origin; mitochondrial DNA (mtDNA); Alfonso X of Castile "The Wise"; external visible characteristics (EVCs); History of Spanish royalty; genealogy

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1. Introduction

The present work will shed some light on the ancestry and physical appearance of Princess Leonor of Castile. To this commitment, we have applied ancient DNA techniques. We understand ancient DNA, which has a low quantity and quality of DNA molecules due to its significant age. Not always antiquity is the main cause of DNA degradation, but when we face up to any potential critical sample, like in this case, it must be implemented special analysis conditions and authenticity criteria (Gomes et al. 2019).

In a colloquium about kinship and legitimation, the Portuguese historian J. Mattoso (2011) referred to the importance of the Medieval research of words and language to understand the modalities and evolution of discourse, since those voices would be "a bit like mitochondrial DNA and chromosome Y, which is transmitted from generation to generation, and therefore, always keep some vestiges of previous generations". He was referring to the fact that these genetic lineage markers are transmitted unaltered from generation to generation to trace people's genealogy.

Beyond the fortunate metaphor of Mattoso, in the field of Medieval and Modern History, the application of Genetics has also changed, with the DNA analysis of individuals found in the necropolis, but also, for obvious reasons, of sets of remains from royal pantheons, for example from Aragon (Martínez Jarreta 2018) or Hungary (Olasz et al. 2019).

Works oriented to the identification of controversial characters are already frequent (Salerno et al. 2005; Haeusler et al. 2016) or to establish kinship among individuals buried together (Gomes et al. 2020; Gamba et al. 2011), without neglecting the detection of individuals related to skeletons with signs of serious diseases (Alves-Cardoso et al. 2022). Nowadays, it is even possible to determine the physical appearance of individuals by the genetic analysis of their skeletal remains (Gomes et al. 2017, 2020). Today it can be said, as Larmuseau and Bodner (2018) have pointed out, that genealogical genetic analyses have, above the merely anecdotal, significative biological, historical, social, and educational importance.

On this occasion, we present the study carried out on the remains of the Infanta Leonor, daughter of King Alfonso X the Wise, as a part of a project for the conservation, restoration, and dissemination of her remains and sepulcher.

Leonor of Castile, an Unknown Princess

The Spanish Princess Leonor of Castile (1256/1257–1275) was the fourth daughter of eleven legitimate sons and daughters of Alfonso X "The Wise" (Spain, 1221–1284) and the Queen Violante of Aragón (1236–1300) (de Salazar Acha 1990). On one hand, there is certain conflicting information about the life of Princess Leonor from different sources (González González and Iriarte 1993; Gutierrez Baños 2014). On the other hand, no registered graphical documents about her physical appearance have survived our days.

Concerning her biogeographical origin, the genealogy of her family is summarized in Figure 1. In summary, in this figure (Figure 1), it is possible to observe that, according to the historical sources, princess Leonor of Castile was fundamentally European and to some extent with Eastern Mediterranean ancestry.

However, it is interesting to analyze in more depth the maternal lineage of Lady Leonor due to the exclusively maternal lineage inheritance of one of the genetic markers studied in this work, mitochondrial DNA (mtDNA). Most of the maternal lineage of Lady Leonor is summarized in Figure 2.

The princess died when she was 19 years old of unknown causes (Martínez Santamarta and Robert 2010). She probably died in Montpellier (France); however, Alfonso X ordered that Leonor's remains be buried in the Royal Monastery of Saint Dominic of Caleruega (Burgos, Spain), where they were located up to the beginning of this research (Gutierrez Baños 2014).

The present study revolves around two objectives: on one hand, to determine the biogeographical origin of the remains to prove if the predicted biogeographical origin is consistent with the supposed European Princess' ancestry; and, on the other hand, to determine the most likely pigmentation phenotype (skin, eyes, and hair pigmentation) of Lady Leonor of Castile.

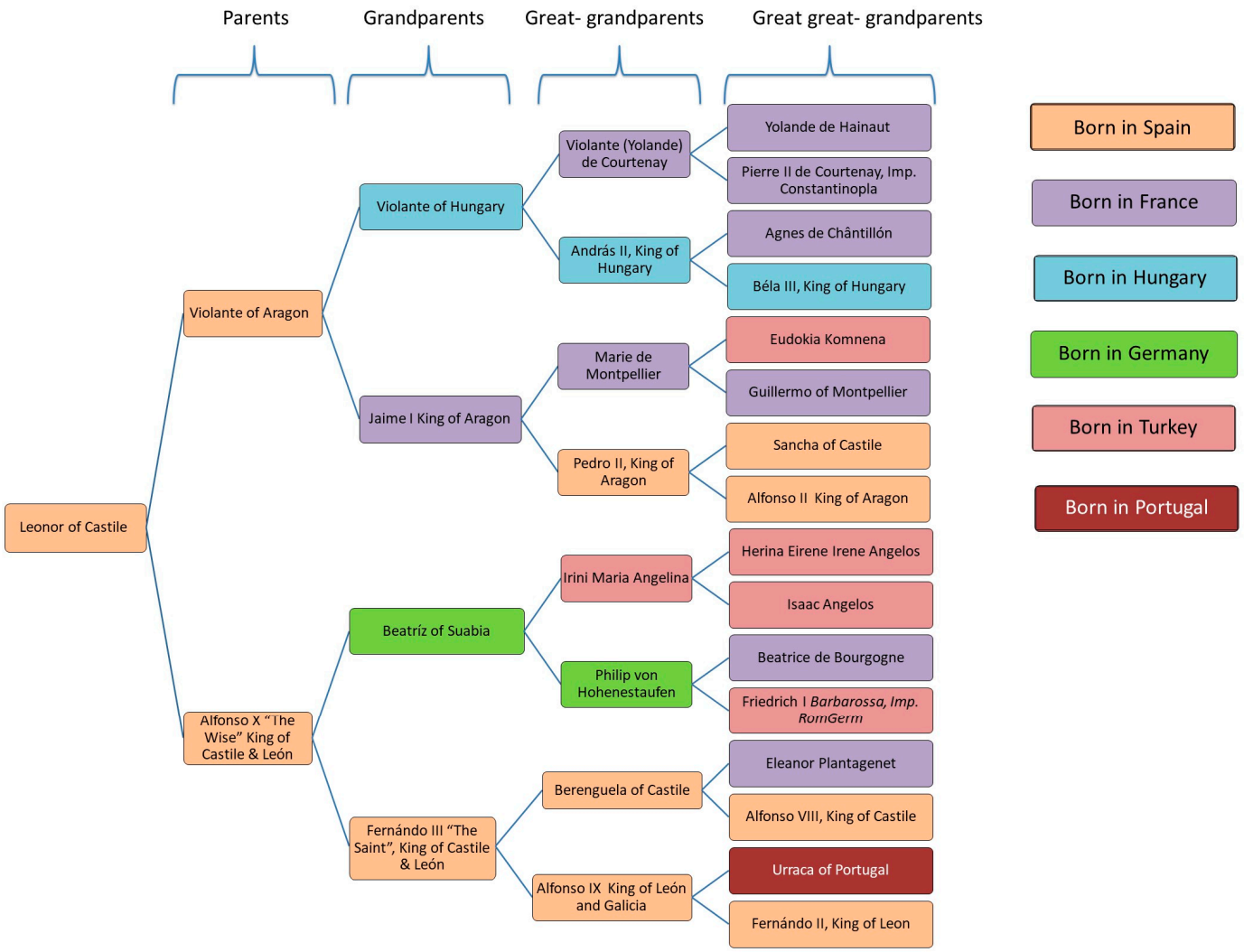

Figure 1. Genealogical tree of Lady Leonor of Castile.

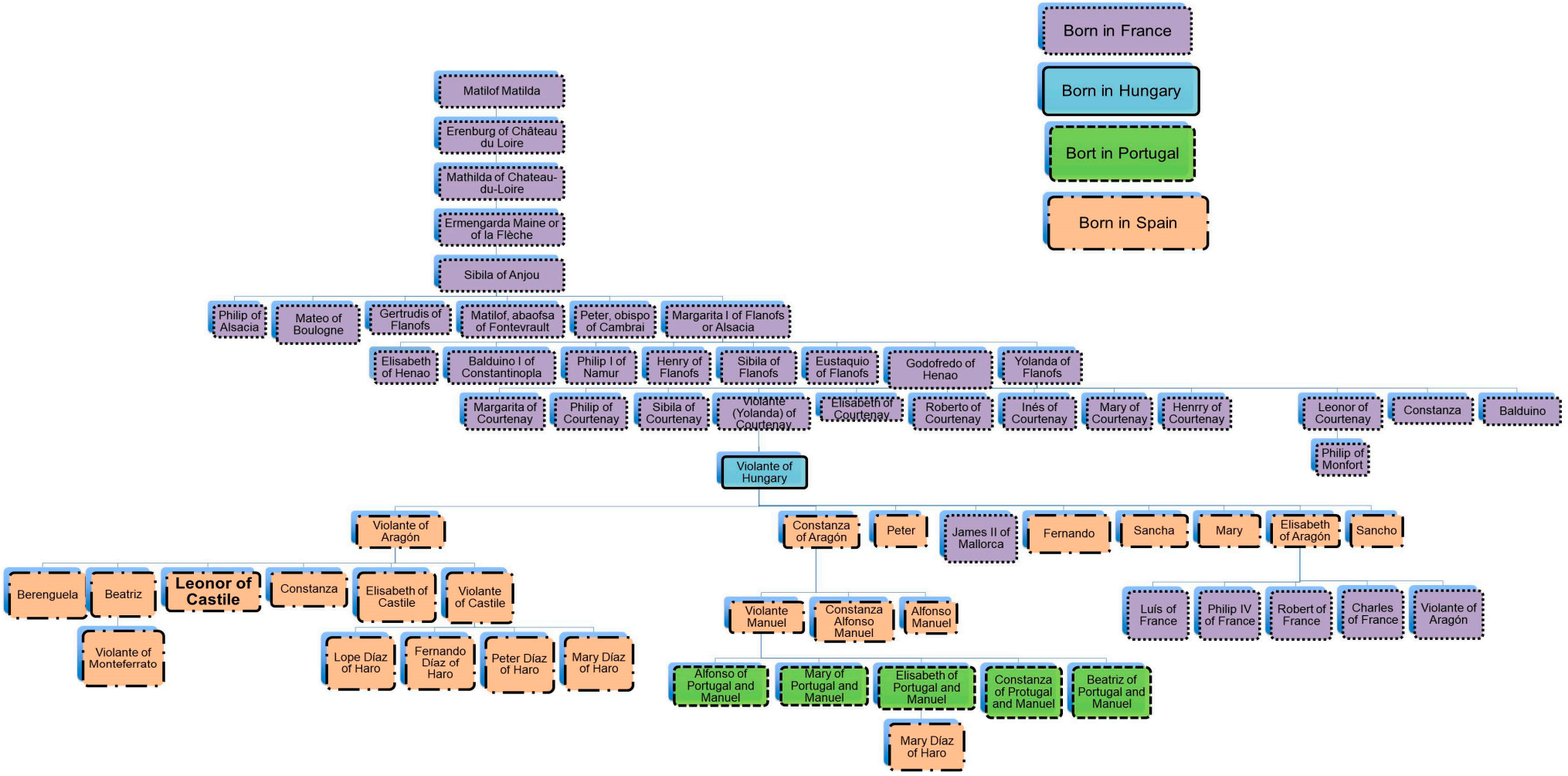

Figure 2. Maternal lineage Biological tree of Lady Eleanor of Castile. The scheme shows all the family members within Princess Eleanor's maternal lineage with whom they share the same mitochondrial DNA.

2. Materials and Methods

On June the 10th, 2014, the opening of the sarcophagus containing the mummified remains of Lady Leonor of Castile was carried out. Previously, the coffin had been subjected to a Helical Computed tomography and EDX analysis (Energy Dispersive X-Ray Analysis, an x-ray technique used to identify the elemental composition of materials) (Pastor et al. 2021). Subsequently, the coffin was opened and the remains found wrapped in a piece of white fabric. Through the first stage of the research, the clothing and other objects were removed to be analyzed and reconstructed.

To perform the genetic analysis, there were selected two well-preserved teeth: the lower left second premolar 1LDC1 and the lower left first molar 1LDC2 (Figure 3).

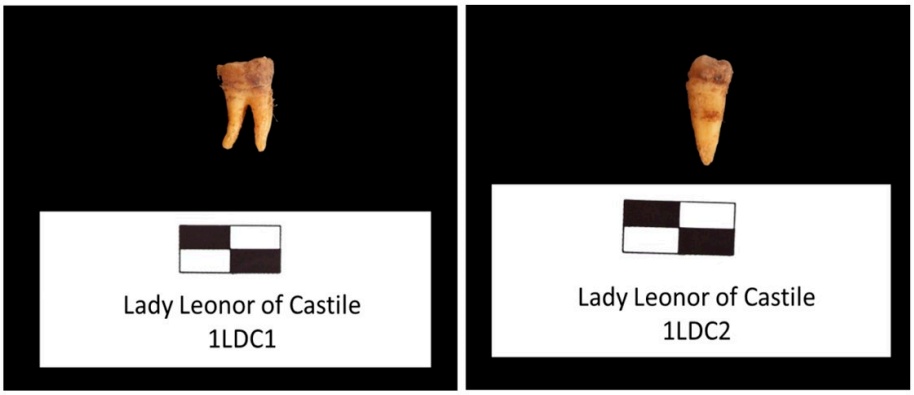

Figure 3. Selected samples to perform the genetic analysis.

Genetic analyses were performed in two specialized aDNA laboratories, hereinafter, Laboratory 1 (at Health Legislation, Psychiatry, and Pathology Department. Medicine Faculty. Complutense University of Madrid) and Laboratory 2 (at Medicine and Odontology Faculty, Santiago de Compostela University); both yearly tested and certified by GHEP-ISFG (Spanish and Portuguese speaking group of the International Society of Forensic Genetics).

DNA extraction was performed in Laboratory 1 following Gomes et al. (2015). Following this method, sample 1LDC1 and sample LDC2 were analyzed in separate processes. Sample 1LDC1 was pulverized, and the DNA from 1LDC2 was extracted without the physical destruction of the piece. And hereinafter, both sample DNA extracts were processed separately to replicate results.

(a) The amplification of the DNA regions of interest supplied us with two different kinds of information: biogeographical origin and externally visible characteristics (EVCs).

Biogeographical origin analysis: To study the individuals' most probable origin, it was taken into account two kinds of information: nuclear and mitochondrial DNA (mtDNA). To predict the most probable biogeographic origin with nuclear information, the SNPforID 34-Plex forensic ancestry test was used to analyze 34 SNPs (Phillips et al. 2007; Fondevila et al. 2013). The PCR conditions are collected in Table 1. After the analysis in an ABI 3500 Genetic Analyzer (ThermoFisher SCIENTIFIC), biogeographic SNPs allele information was analyzed using GeneMapper™ Software 5 (ThermoFisher SCIENTIFIC, Waltham, MA, USA).

Finally, statistical parameters and the most probable biogeographical region were obtained with the software The Snipper app. suite v.2.5 (Phillips et al. 2007), considering the dataset for three major populations: Europe, Africa, and East Asia. This analysis was performed in Laboratory 2.

To analyze the maternal origin by mtDNA and determine the most probable haplogroup(s), both HV1 and HV2 regions were analyzed in Laboratory 1.

Table 1. The SNPforID 34-Plex PCR conditions.

PCR Conditions			Cycling Protocol		
	Final Concentration	**Volume (mL)**			
Buffer	1 X	0.615			
BSA	3.2 mg/mL	0.615	95 °C	15 min	
Cl2Mg	6.3 mM	1.615	95 °C	30 s	
dNTPs	0.625 mM	0.4	60 °C	50 s	35 cycles
Primer	Variable	2	65 °C	40 s	
TaqGold	0.5 U	0.1	65 °C	6 min	
DNA	n/a	1–4 mL			

There were performed the amplification of 294 bp of the Hypervariable region I (HVRI) (positions 16,105–16,399) (Fernández 2005) and 345 bp of the Hypervariable region II (HVRII) (positions 55–400) (Martínez-Labarga and Rickards 1999). These regions were selected and incorporated into four overlapping PCRs using the Multiplex PCR kit of Qiagen® (Hilden, Germany). The analysis was performed in duplicate for each of the selected samples. The amplification results were tested by electrophoresis on agarose gels and positive amplification results were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) (Exo-SAP®). Sequencing products were visualized on an ABI3730 (Life Technologies: Carlsbad, CA, USA) automated capillary electrophoresis sequencer.

The capillary electrophoresis results were analyzed with the aid of the software Chromas® (http://technelysium.com.au/), and compared to the rCRS (Anderson et al. 1981; Andrews et al. 1999) applying Mutation Surveyor® V.4.0.6 software (Softgenetics, State College, PA, USA) (Mutation Surveyor® software n.d.). Genetics analyses. After haplotype determination, the most probable haplogroup(s) were determined by searching on the HaploGrep 2 online application (van Oven and Kayser 2009; van Oven 2015), on EmPOP mtDNA database v4/R11 (Huber et al. 2018; Parson and Dür 2007), on mtDNA manager (Lee et al. 2008), and Phylotree phylogenetic tree (van Oven and Kayser 2009; van Oven 2015). Finally, there was performed a search of close mtDNA haplogroups in the AmtDB database.

(b) For the external phenotypic prediction, 35 SNPs were analyzed in Lab 2 to calculate the most probable hair, skin, and eye color pigmentation, using the primers and PCR conditions of Ruiz et al. (2013) (Maroñas et al. 2014). The EVCs PCR conditions are collected in Table 2. For each externally visible characteristic (EVC), the hypotheses considered were (a) iris pigmentation (brown, intermediate, and blue); (b) hair pigmentation (fair versus dark, and for the color pigmentation (red, blond, brown, and black); and (c) skin pigmentation (white, intermediate, and black) (Maroñas et al. 2014). Statistical analysis was performed with the online Snipper app suite v2.5 software (Phillips et al. 2007).

Table 2. EVCs PCR conditions.

PCR Conditions			Cycling Protocol		
	Final Concentration	**Volume (mL)**			
buffer	1 X	0.625	95 °C	15 min	
BSA	3.2 mg/mL	0.625			
Cl2Mg	6.3 mM	1.625	95 °C	30 s	
dNTPs	0.625 mM	0.43	60 °C	50 s	35 cycles
primer	Variable	1.5	65 °C	40 s	
TaqGold	0.5 U	0.1			
DNA	n/a	1–4 uL	65 °C	6 min	

Along with the full analytical procedure, pre-PCR, PCR, and post-PCR procedures were performed in three physically separated and isolated areas. Ancient DNA laboratories were equipped with UV light lamps (254 nm) to ensure a sterile environment for the reliable analysis of ancient DNA samples. Additionally, all laboratory equipment was routinely irradiated with UV light and thoroughly cleaned with bleach (70%) before and after each experiment. All experimental analyses were conducted by a single researcher to reduce the chances of staff DNA contamination; additionally, the access to these laboratories was limited to two people during the whole time the analyses were carried out. All laboratory procedures (sample preparation, extraction, and PCR) were performed wearing disposable laboratory coveralls (masks, caps, glasses, shoe covers, and gloves). DNAse and RNase-free reagents and consumables were also employed. The possibility of modern DNA contamination was monitored with extraction blanks and at least three PCR-negative controls were included per DNA amplification round. So, authenticity criteria were followed, considering the laboratories' infrastructures, methodology, and interpretation of the results. Also, before the genetic analysis, two biochemical assays on macromolecular preservation were performed by the archaeological team, as recommended by Pääbo (Pääbo et al. 2004). To monitor exogenous mitochondrial DNA contamination of the samples, genetic profiles were recovered from all the people involved in sample manipulation (Table 3).

Table 3. Researchers' mitochondrial DNA profiles.

Laboratory Staff	Mitochondrial DNA Haplotype
RESEARCHER A	16362C 55G 150T 239C 263G 309.1C 309.2C 315.1C
RESEARCHER B	263G 315.1C
RESEARCHER C	16145A 16172C 16222T 16261T 16305T 73G 242T 263G 295T 309.1C 315.1C
RESEARCHER D	263G 315.1C
RESEARCHER E	16126C 16294T 16296T 16304C 73G 263G 315.1C
RESEARCHER F	73G 263G 315.1C

3. Results

At Laboratory 1, all the PCR reactions performed have provided quality mtDNA sequences without molecular damage or trace of contamination. Furthermore, all the obtained results were consistent among them. This fact provides further strength to the obtained results. Combining the different PCR amplifications has allowed us to determine the next consensus mtDNA haplotype: 16224C 16311C 73G 195C 263G 315.1C (range: 16,105–16,569; 1–390). This result was obtained at least twice from each sample and mtDNA fragment, always with the same result. Subsequently, the obtained results were analyzed to determine the mitochondrial haplogroup, and it was determined as K1a4a1a, with a private mutation on position number 195 of HRVII.

Concerning the amplification of 34 autosomal SNPs for biogeographical assignment carried out at Laboratory 2, results can be seen in Table 4. This table also shows the obtained LR results performed to discern if the individual belongs to a European, Sub-Saharan Africa, East of Asia, American or Oceanian population.

The autosomal SNPs analysis suggests a European origin in the same way as the mtDNA analysis.

Finally, the results of the EVC genetic analysis carried out in Laboratory 2 are summarized in Table 5. To determine each one of the physical characteristics studied, we performed the same methodology employed in the biogeographical origin analysis.

In light of these results, the most probably physical appearance of the Princes was black hair, green-hazel eyes, and white skin.

Table 4. Autosomal SNPs results for the Biogeographical origin analysis and Likelihood Ratio results of the statistical comparison of the alleged origins one against another. In columns, we can observe the SNP (single nucleotide polymorphisms) analyzed and the obtained results obtained for each one. At the bottom of the table, it is possible to view the Likelihood ratio (LR) results obtained to discern if the individual belongs to a European, Sub-Saharan Africa, East of Asia, American or Oceanian population, showing that the most probable biogeographical origin is European, attending to the different origin hypothesis.

SNP	**RESULT**	**SNP**	**RESULT**	**SNP**	**RESULT**
rs5997008	C	rs773658	C	rs2026721	G
rs2304925	GT	rs10141763	A	rs4540055	A
rs917118	G	rs182549	CT	rs1335873	A
rs1321333	T	rs1573020	A	rs16891982	C
rs2814778	C	rs896788	C	rs730570	T
rs1024116	A	rs2065160	A	rs1886510	NN
rs7897550	C	rs2572307	G	rs5030240	C
rs722098	AG	rs2303798	C	rs3827760	A
rs10843344	CT	rs2065982	A		
rs12913832	AG	rs3785181	C		
rs239031	NN	rs881929	NN		
rs2040411	AG	rs1498444	A		
rs1978806	T	rs1426654	T		
LIKELIHOOD RATIO RESULTS					
HYPOTHESIS				LR	
H_1: European origin vs. H_2: American origin				LR = 1.95 × 1016 vs. 1	
H_1: European origin vs. H_2: Asia origin				LR = 1.02 × 1018 vs. 1	
H_1: European origin vs. H_2: North-African origin				LR = 10382,18 vs. 1	
H_1: European origin vs. H_2: Sub-Saharian origin				LR = 2.18 × 1031 vs. 1	

Table 5. EVCs SNPs results and Likelihood Ratio results of the statistical comparison of the EVCs one against another. In columns, we can observe the SNP (single nucleotide polymorphisms) analyzed and the obtained results for each. At the bottom of the table, it is possible to view the Likelihood ratio (LR) results obtained to discern the different external visible characteristics (EVCs) studied: eyes, skin, and hair colors.

Iris Eye Color System		**Hair Color System**		**Skyn Color System**	
SNP	**RESULT**	**SNP**	**RESULT**	**SNP**	**RESULT**
rs12913832	AG	rs1129038	NN	rs10777129	NN
rs1129038	NN	rs11547464	NN	rs13289	C
rs11636232	CT	rs12913832	AG	rs1408799	G
rs12203592	C	rs12931267	C	rs1426654	A
rs12896399	A	rs1805006	C	rs1448484	A
rs1393350	G	rs1805007	NN	rs16891982	C
rs1667394	CT	rs1805008	G	rs2402130	A
rs16891982	C	rs1805009	G	rs3829241	C
rs1800407	C	rs28777	NN	rs6058017	NN
rs4778232	G	rs35264875	A	rs6119471	C
rs4778241	GT	rs4778138	CT		
rs7183877	A	rs7495174	CT		
rs8024968	G				
LIKELIHOOD RATIO RESULTS					
HYPOTHESIS	LR	HYPOTHESIS	LR	HYPOTHESIS	LR
H_1: Dark hair vs. H_2: Light hair	LR = 487.06 vs. 1	H_1: Green-hazel eyes vs. H_2: Brown eyes	LR = 26.71 vs. 1	H_1: White skin vs. H_2: Intermediate skin	LR = 7.54 × 10^5 vs. 1
H_1: Black hair vs. H_2: Brown hair	LR = 2.65 vs. 1	H_1: Green-hazel eyes vs. H_2: Blue eyes	LR = 2.64 × 10^6 vs. 1	H_1: White skin vs. H_2: Black skin	LR = 1.98 × 10^{10} vs. 1
H_1: Black hair vs. H_2: Red hair	LR = 215.63 vs. 1				

4. Discussion

We have determined that the human remains studied belong to mtDNA haplogroup K1a4a1a, a typical European haplogroup nowadays, this finding indicates a typical European or Middle East biogeographical ancestry; this is consistent with the European maternal lineage origin of the Princess. As regards ancient DNA, the earliest reported appearance of this haplogroup was dated around 8000 years B.P. in Tell Ramad (Siria) (Fernandez et al. 2014). Also, human remains belonging to haplogroup K have been discovered, which date around 7500–7300 B.P. One of the most popular findings was Ötzi (5000 B.P.), which precisely belongs to the K1 haplogroup (Rollo et al. 2006). Through searching on the AmtDB database of the K1a4a1a haplogroup, no individuals were found with the same haplogroup. Nevertheless, also in AmtDB, the closest haplogroup found in Medieval populations was located in Hungary (Vai et al. 2019). K1a clade (group of individuals who share the same lineage, K1a) was also present during Middle Ages in Germany (Veeramah et al. 2018). If we search the broader K1, it was also present during Middle Ages in Italy (Amorim et al. 2018; Vai et al. 2019), Spain (Olalde et al. 2019), and Poland (Stolarek et al. 2018); but the largest presence of K1 mtDNA haplogroup was located in Hungary. These results are consistent with the mtDNA family tree of Leonor Princess (Figure 2).

Moreover, autosomal SNPs analysis supports this European origin too. This second analysis furthermore provides a broader view of the Princess' origins, since the analyzed SNPs have been inherited through both the maternal and the paternal lineage and are consistent too with the historical genealogy of Lady Leonor´s family, with mainly European roots (Figures 1 and 2). The matching results for the prediction of biogeographical origin obtained through the two different methodologies employed, provide greater strength to the concluded ancestry.

It could be interesting to compare Lady Leonor´s mtDNA haplotype to that of any person belonging to this maternal genealogy tree. However, it has not been possible to find any previously generated genetic data from other individuals from this maternal lineage. However, the mtDNA and YSTRs profiles of her grandmother´s grandfather, the Hungarian king Bela III, is well known (Olasz et al. 2019); unfortunately, this information is not comparable to our results. Nevertheless, the newly obtained data can provide relevant information to complete the genetic familiar genealogy.

Moreover, the feature that genetic evidence supports as most likely white skin pigmentation seems also to support a possible European origin for the studied remains.

From the paleogenetic point of view, this work confirms the feasibility of genetics to establish the kinship and physiognomic features of individuals. Determining that she is most likely to have white skin, green-hazel eye color, and black hair. Thereby, the Princess obscured in the documentation acquires some visibility. In addition, its haplogroup, K1a4a1a, has been obtained, establishing an eventual link with other royal houses currently under investigation (Olasz et al. 2019).

Certainly, from a historical point of view, the knowledge of notable characters is not today one of the main objectives of investigations, but the realization of restoration and conservation works constitutes new potential sources, usable from different disciplines and prospects. In this sense, the present study has an experimental nature, of exploration possibilities, with which some bases are laid whose use will be greater when other similar ones that allow comparative approaches are published.

Working with these royalties sometimes allows for written descriptions of their physical appearance and sometimes even preserved remains—such as the nails of the blonde Norwegian princess Kristín Hákonardóttir, during some time Leonor's mother's rival, whose tomb was opened last century (Vargas Blanco 1968), and sometimes there are also some "portraits", such as the miniatures of the monarchs, queens or princess collected in the cardboards of the cathedrals from Oviedo and Compostela, which confront genetic inferences, but, as has been said, none of Lady Leonor seems to be known until now. In general, the possibilities of identification will be greater from the Late Middle Ages, with the progressive display of the pictorial portrait.

The viability of the comparisons also grows in the case of investigations such as those mentioned above of the royal pantheons of Hungary and Aragon. Although there were partial pantheons in San Isidoro (León, Spain) and Las Huelgas (Burgos, Spain), for complex historical reasons (Arias Guillén 2015) a single royal pantheon was not formed in the whole of the Crown of Castile that could have facilitated this type of genetic research, it is not impossible to carry it out, taking advantage—as in the present case—projects of rehabilitation of scattered graves. The location of the characters of the dynasties—for example, Leonor's maternal grandmother and transmitter of her mitochondrial DNA, Violante of Hungary, Aragonese Queen buried in the Royal Monastery of Santa María de Vallbona (Lérida, Spain)—may serve for the constitution of a genetic database. In this sense, the genetic analysis of Leonor of Castile comes to join those already available for some of his relatives, such as Bela III of Hungary and Agnes de Châtillon, grandparents precisely from the aforementioned maternal grandmother of Leonor (Olasz et al. 2019).

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