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Excavations at Sissi V

Preliminary Report on the 2017-2019 Campaigns

VOLUME 1&2

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3.3. Paleogenetic Analyses of Human Remains at Sissi

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1. Introduction

During the 2011 excavation campaign in Zones 1, 2 and 9 at Sissi, 14 human bone and tooth samples were selected from the remaining skeletal assemblage in an attempt to extract ancient DNA (**table 3.3.1**).

Zone - Room	Period	Container	Ind. N°	Sample N°	Nature	Identification	Excavator	DNA extract Label
1.18 S	MM IB / IIA	pithos	1	11-01-9521/OB 003	Tooth	Right alveolar bone with LRI2 to LRP1	Crevecoeur	**D1/D2**
1.18 S	MM IB / IIA	pithos	1	11-01-9509/OB 001	Tooth	ULM2 and ULM3	Crevecoeur	**K1/K2**
1.29	MM IIA	pithos	4	11-01-9574/SA 005	Tooth	Left alveolar bone with ULC to ULP2	Crevecoeur	**C1/C2**
9.2	MM IB / MM II		1	11-09-9827/SA005	Tooth	UC and UP1	Lambert	**H1/H2**
9.2	MM IB / MM II		2	11-09-9833/SA005	Bone	Right clavicle	Schmitt	**L1/L2**
9.2	MM IB / MM II		5	11-09-9857/OB 008	Bone	Right radius	Schmitt	**F1/F2**
9.2	MM IB / MM II		6	11-09-9866/OB 016	Bone	Left clavicle	Schmitt	**B1/B2**
9.2	MM IB / MM II	larnax	7	11-09-9682/OB 001	Tooth	LLC to LLP2	Lambert	**G1/G2**
9.2	MM IB / MM II		8	11-09-9888/SA 005	Tooth	Left alveolar bone with ULI1 to ULM3	Schmitt	**J1/J2**
9.2	MM IB / MM II			11-09-9854/OB 002	Tooth	ULC, ULP2 and ULdm2	Schmitt	**E1/E2**
9.2	MM IB / MM II			11-09-9890/OB 001	Bone	Right ulna	Schmitt	**A1/A2**
2.6	LM I	pyxis		09-02-1258/OB 018	Bone	Right femur	Crevecoeur	**N1/N2**
2.8	LM I	pyxis	1	10-02-2225/OB 007	Tooth	Ldc to Ldm1	Civetta	**M1/M2**
2.8	LM I	pyxis	1	10-02-2225/OB 014	Bone	Femur	Civetta	**I1/I2**

Tab. 3.3.1 Details of the Sissi samples submitted to genetic analyses and labels of the DNA extracts obtained. MM = Middle Minoan, LM = Late Minoan. Tooth identification as follow: *i.e.* LRI2 = Lower Right second Incisor, ULdm2 = Upper Left deciduous second molar, C/c = canine, P = premolar

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The sampling was done directly in the field for the human remains from Zones 1 and 9[5]. Their excavation for DNA extraction purposes followed the required protocol to limit contamination. The focus was on dental remains or the best-preserved long bone when teeth were unavailable. As soon as skeleton pieces related to the maxilla or the mandible appeared, the excavator cleaned the tools with alcohol and wore a mask, hygiene cap and two pairs of plastic gloves. When relevant material was identified, it was contained in a plastic bag which was put in another one containing the labels. The material was then transported in a cooler and stored in a freezer. Regarding Zone 2, the sampling of two immature individuals (two children) was done in the *apothiki* in 2011. The 14 samples were kept in the same freezer until the permit for exportation and DNA extraction was issued, two years later.

A total of €5000 (TTC) was provided for the genetic analysis of the 14 human remains originating from the site thanks to the funding support of the KULeuven. The main goals of the analyses were to document both population origin and potential kinship relations between individuals recovered in specific funerary structures. All analyses presented hereafter have been conducted in the Paleogenetic platform of the UMR 5199-PACEA. DNA results will be presented in chronological order (the analyses were conducted between 2015-2018) and DNA extraction labels will be used in the discussion for the sake of clarity.

2. 2015-2016 – Classical paleogenetic analyses

The genetic analyses first targeted uni-parental markers. We conducted the simultaneous genotyping of 18 SNPs (Single Nucleotide Polymorphisms) of the mitochondrial genome (mtDNA) coding region and 10 SNPs of the Y chromosome, permitting the characterization of main European maternal and paternal lineages. This analysis uses the iPlextechnique (Sequenom® technology), which is particularly sensitive and well adapted to the study of highly degraded DNA as was expected for the Sissi human remains. This genotyping enables the testing of DNA conservation in the samples and to determine maternal and paternal lineages (for men) simultaneously if DNA is sufficiently conserved.

The samples were decontaminated with diluted bleach and exposed to UV lights to destroy potential DNA molecules present on the samples' surfaces. A first set of DNA extracts was obtained for each sample (DNA extracts **A1** to **N1**) and was submitted to SNPs genotyping. It is important noting that extracts **D1**-**K1**, on the one hand, and **I1**-**M1**, on the other, were obtained from remains corresponding to the same individual. Results of this genotyping are presented in **table 3.3.2**. Since no Y chromosome SNPs could be genotyped, only mitochondrial genome SNPs are presented.

The majority of the mtDNA SNPs profiles obtained were fragmentary. This observation, combined with the impossibility of recovering any Y chromosome SNPs, demonstrated a severe degradation of the DNA molecules in the Sissi human remains. Moreover, several SNPs profiles were problematic since they consisted of a mix of different alleles for several mtDNA genome positions, corresponding to distinct maternal lineages. This type of 'mix' profile is usually indicative of highly fragmented/degraded DNA (leading to the presence of artefactual alleles) or of contamination by people with a maternal lineage distinct from that of the sample.

In order to test the presence of contamination of the Sissi human remains, we decided to characterize the maternal lineages of all the individuals (number 1 to 4) that manipulated the samples from the field to the lab and compare their haplogroups to those observed in the 'mix' profiles. The maternal lineage of manipulating individual n° 4 could not be determined since we could not obtain a sample, but this individual manipulated only two of the Sissi samples (the **M1** and **I1** DNA extracts) corresponding to the same individual found in Space 2.8. The haplogroups of the remaining manipulators was never detected in the 'mix' profiles, permitting us to exclude their implication in potential contamination. We could notably note that haplogroup H1 could be determined, despite partial SNPs profiles, on five different DNA extracts (extracts **B1**, **C1**, **D1**, **E1** and **N1**) and that this maternal lineage could not correspond to contamination by the different persons having handled them. Concerning the extract **M1**, a complete SNPs profile could be obtained and permitted to determine a lineage N1a or I. Unfortunately, in this specific case, we cannot exclude that this lineage corresponds to the manipulator n°

5 Permit 134536/80459/5208/1393 of 27/05/2014. The authors thank the Ministry of Culture and EFALAS as well as Dr. P. Iossif for their help.

4 since we could not genotype this person. Notably, this manipulator has also handled the sample for which we obtained the extract **I1** and for which the SNPs profile indicated a mixture of H1 and N1a/I haplogroups.

To go further in the maternal lineages' characterization, we analysed the first hypervariable region of the mitochondrial genome (HVR-I, nps 16,024 - 16,380) permitting to determine the haplotypes of the individuals. Four overlapping fragments (HVRIa / b / c / d) were amplified and sequenced following the procedures described in Rivollat *et al.* (2015). No reproducible result for HVR-I sequences could be obtained for all Sissi individuals. Unfortunately, all data in hands indicate major DNA degradation that impede to precise the Sissi individuals' maternal and paternal lineages.

Giving the difficulty to access the endogenous DNA of the Sissi human remains and given the potential contamination, we conducted a second process of DNA isolation on all samples (the **A2** to **N2** DNA extracts) including a short step of soaking in diluted bleach, followed by a short lysis step, before the usual isolation procedure (Boessenkool *et al.* 2017). The addition of these steps was reported as peculiarly efficient for eliminating contaminations (the bleach should destroy the contaminating DNA molecules attached to the surface of the mineral structure) and to recover endogenous DNA (attached more strongly to the mineral structure and consequently recovered during the second lysis step). Unfortunately, the SNPs genotyping conducted on this second set of DNA extracts gave no result which supports the hypothesis of very poor DNA concentration in the Sissi human remains that was apparently lost during these supplementary steps.

Thus, the first set of classical paleogenetic analyses conducted on the Sissi human remains demonstrated poor concentration of endogenous DNA in only a few samples. Very partial and non-conclusive results indicated the possible presence of H1 and N1a/I mitochondrial haplogroups in the Sissi community. It is worth noting that these maternal lineages have been encountered in notable frequencies among Minoan groups from the Lasithi plateau and dated between 4400 and 3700 BP (43.2 % haplogroup H, 8.1 % haplogroup I; Hughey *et al.* 2013). However, poor DNA conservation indications compelled us to use paleogenomic analyses more adapted to highly degraded DNA.

3. 2017-2018 – Paleogenomic analyses

DNA libraries were constructed from 12 different DNA extracts, with the QIAseq Ultra Low Input kit (QIAGEN). We targeted five DNA extracts obtained during the first process of DNA isolation (the **D1**-**F1**-**I1**-**M1**-**N1** extracts) and seven extracts obtained during the second process of DNA isolation (the **B2**-**C2**-**E2**-**F2**-**G2**-**I2**-**K2** extracts) in order to assess DNA conservation for samples that had given no SNPs results (second set of DNA extracts), problematic SNPs profiles (the **F1**-**I1** extracts) or complete/satisfying SNPs profiles (the **D1**-**M1**-**N1** extracts).

Of the 12 libraries constructed, three gave no signal during the Bio Analyzer quantification, indicating the total absence of DNA. These extracts derived from the second set of DNA extractions (the **C2**-**G2**-**K2** extracts). The remaining libraries (N=9) were submitted to shotgun sequencing on a Next Seq sequencer (75pb, paired-end). The results obtained for these nine libraries are compiled in **Table 3.3.3**.

The number of reads obtained for DNA libraries **B2**, **F2** and **N1** were too low to be exploitable (explaining the aberrant percentage of endogenous DNA content measured for them). Shotgun sequencing allowed us to obtain more than 200000 reads for the six remaining DNA libraries although several problems should be highlighted:

The presence of an important number of adapter dimers, which we did not manage to eliminate during selection size steps, due to very low DNA concentration in the extracts thus explaining the very low number of merged reads.

A low rate of reads mapping on the human genome reference (hg19), including an important rate of duplicates (*i.e.* reads present in several copies) indicating libraries with minimal complexity (low diversity of sequences). This low complexity causes a very important 'cluster factor'.

A low percentage of endogenous human DNA for all exploitable libraries. Only the libraries constructed from the **I1** and **M1** extracts yielded more than 1 % of endogenous DNA. The recovered human DNA showed typical ancient DNA characteristics (such as the presence of contaminations and short reads) but the limited complexity of these libraries excluded any further exploitation.

DNA extract	manipulator (mtDNAhaplogroup)	mitochondrial DNA SNPs																				mtDNAHaplogroup
		M		N					R													
		M*		N*	N1a	I	W	X	R*	HV*	H	H1	H3	V	J	T	U*	U4	U5	K	
		10400		10873	13780	10034	3505	6371	12705	14766	2706	3010	6776	4580	12612	1888	11467	11332	13617	10550	
		C/T		C/T	A/G	T/C	A/G	C/T	T/C	T/C	G/A	G/A	T/C	G/A	A/G	G/A	A/G	C/T	T/C	A/G	
A1	1 (I2a) - 3 (T2b)				A		G	CT	C	T					A		A	C	T		mix
		C		T	A		A	C	C	T	G	G	T		AG		A	C	T	A	
B1	1 (I2a) - 3 (T2b)	C			A		A	C	C	C	A	A	T			G	A	C	T	A	H1
		C			A		A	C	TC	C	A	A	T			G	A	C	T	A	
C1	2 (H11a) - 3 (T2b)	C					A		C			GA					A	C			H1
		C						CT	C		A	A	T							A	
D1	2 (H11a) - 3 (T2b)	C		T	A		A	C	C	C	A	A	T		A	G	A	C	T	A	H1
		C		T	A		A	C	C	C	A	GA	T		A	G	A	C	T	A	
E1	1 (I2a) - 3 (T2b)	C			A		A	C	C		A	GA	T		A	G	A		T	A	H1
		C			A		A			C		A	T						T		
F1	1 (I2a) - 3 (T2b)	C			A		A	C		TC	G	G	T				A	C	T	A	mix
		C			A		A	C	C		A	GA	T				A	C	T	A	
G1	1 (I2a) - 3 (T2b)	C		T	A			T	C	C	A				A		A	C	T	A	mix
		C					A		C	T			T			G	G			A	
H1	1 (I2a) - 3 (T2b)	C		T	A			CT		C	GA		T		A		A	C	T	A	mix
		C		T	A				C	C	G				A		A	C	T		
I1	4 (nd) - 3 (T2b)	C		T	AG		A	C	TC	T	GA	GA	T		A	G	A	C	T	A	mix
		C		T	G		A	C	T	TC	GA	GA	T		A	G	A	C	T	A	
J1	1 (I2a) - 3 (T2b)	C		T	A		C	C	C	C		A			A				T	A	mix
					A				TC	C	A	GA	T			GA	A		T		
K1	2 (H11a) - 3 (T2b)	C			A				C	C							A		T		mix
					A				C	TC	GA	A					A	C		A	
L1	1 (I2a) - 3 (T2b)	C			A		A		C	C		G	T				A		C		mix
					G		A		TC			G	T						T		
M1	4 (nd) - 3 (T2b)	C		T	G		A	C	T	T	G	G	T	G	A	G	A	C	T	A	N1a / I
		C		T	G		A	C	T	T	G	G	T	G	A	G	A	C	T	A	
N1	2 (H11a) - 3 (T2b)	C		T	A		A	C	C	C	A	A	T	G	A	G	A	C	T	A	H1
		C		T	A		A	C	C	C	A	A	T		A	G	A	C	T	A	

TAB. 3.3.2 RESULTS OF MTDNA SNPS GENOTYPING. EACH DNA EXTRACT WAS GENOTYPED TWICE TO CHECK FOR RESULTS REPRODUCIBILITY. CAPITAL LETTERS REPRESENT MATERNAL LINEAGES (HAPLOGROUPS), NUMBERS CORRESPOND TO THE ANALYSED GENOME POSITIONS, A/C/G/T CORRESPOND TO THE ALLELES IDENTIFIED FOR EACH SAMPLE AT THE GIVEN POSITION (ANCESTRAL ALLELES ARE IN BLACK WHEREAS DERIVED ALLELES ARE IN RED). THE MTDNA HAPLOGROUPS OF THE INDIVIDUALS THAT MANIPULATED THE SAMPLES ARE INDICATED

Sample Name	DNA extract	Total number of reads	Number of mergedreads	Number of reads mapped on Hg19	Number of reads mapped on Hg19 without duplicates	% duplicates	% endogenous DNA	Cluster Factor
A99-Sissi	B2	16	4	4	4	0.00	**28.57**	1.000
A102-Sissi	E2	201 154	91 482	2 196	1 076	51.00	**1.90**	2.041
A103-Sissi	F2	99	42	28	28	0.00	**28.57**	1.000
A106-Sissi	I2	283 675	138 911	1 389	435	68.68	**0.74**	3.193
T766-Sissi	F1	383 764	75 001	2 701	241	91.08	**0.93**	11.207
T767-Sissi	M1	280 596	35 778	15 090	3 385	77.57	**5.77**	4.458
T768-Sissi	N1	33	6	7	7	0.00	**41.18**	1.000
T773-Sissi	D1	535 073	75 836	4 079	281	93.11	**0.87**	14.516
T775-Sissi	I1	271 629	10 097	4 227	297	92.97	**8.82**	14.232

TAB. 3.3.3 RESULTS OF SHOTGUN SEQUENCING OF THE SISSI DNA LIBRARIES. BIOINFORMATIC ANALYSES WERE PERFORMED WITH EAGER

Overall, the genetic and genomic results obtained confirmed the very low rate or even the absence of endogenous human DNA in the Sissi human remains. For the rare samples that did provide satisfying rates of endogenous DNA, the low complexity of the libraries at hand (indicating a very low diversity of the human sequences recovered) excluded further genetic exploitation of these remains.

4. Conclusions

Although sampled and extracted with maximal precaution and analytical care, the 14 human remains from Sissi did not meet the required level of endogenous ancient DNA content needed for further investigation. As a consequence, the maternal lineages characterized for the Sissi group could not be fully authenticated. We may nevertheless note that the mtDNA haplogroups potentially identified were already described in the ancient Minoan population (Hughey *et al.* 2013) and may indicate genetic homogeneity for the Sissi community through time and space. Indeed, the main maternal lineage identified (haplogroup H1) was found in three zones of the site (Zone 1, 2 and 9), with the exception of one child from Zone 2. However, the maternal lineage of the latter one might be related to contamination.

Finally, we should highlight that, at the time of sampling, the potential of inner ear parts of the petrous bone as the best part of the body to retain ancient DNA was not yet known (Pinhasi *et al.* 2015). Given the state of preservation of the Sissi human remains, any further sampling should only focus on this anatomical part after its virtual preservation through micro-tomographic acquisitions.