Ancient DNA study of six Portuguese's final Neolithic/Chalcolithic populations

Cristina Afonso[1]; Ana Maria Silva[1]; Mercedes Gonzalez-Ruiz[2]; Cristina Santos[2]; Assumpció Malgosa[2]

Introduction:

Ancient DNA techniques allows us to answer questions that osteological methods cannot always respond, like the sexual diagnosis in human remains, and helps on the resolution of population migration patterns.

Genetic analysis of ancient skeletal material is scarce in Portugal, with only one previous ancient DNA study preformed with Portuguese samples from this chronology [1].

The aim of this work is the comparison of the ancient populations samples with the study of contemporary and current reference populations, through the HVR-I region of the mtDNA and polymorphisms in the mtDNA coding region, in order to infer population distances; the determination of matrilineal kinships and the sexual identification through molecular methods.

Materials and Methods

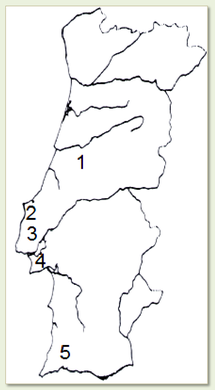

Figure 1: Map of mainland Portugal, with the excavation sites here studied (1 - DEA, 2 - PMI and PMII, 3 - CM, 4 - SP and 5 - MCI) (adapted from [3]).

Samples:

The sample was composed of 40 individuals dated from the late Neolithic/Chalcolithic (3637 to 1950 BC), from six Portuguese funerary monuments located in the centre/south of the mainland Portugal (Fig.1): Hypogeum of Monte Canelas I (MCI, 4460 ± 110 YBP), *tholos* of Paimogo I (PMI, 4250 ± 90 YPB), *tholos* of Paimogo II (PMII), natural cave of Cova da Moura (CM, 4715 ± 50 YBP), Hypogeum of São Paulo II (SP, 3969 ± 190 YBP) and Dolmen of Ansião (DEA, 4640 ± 90 YBP) [2-3].

DNA extraction:

The extraction of DNA was carried out through the recovery of powder from polpal cavity of dental pieces, following the method of phenol-chloroform, with the overnight addition of EDTA, recovering a final volume of 50 µl [4-5].

mtDNA analysis:

The analysis of the mtDNA was performed by sequencing 2 fragments of the HVR-I, between positions 16030-16420, and PCR-RFLP analysis of the coding region polymorphisms [6-7]. The samples in study were then compared with reference populations (Table 1), in order to perform a phylogenetic analysis.

Sexual determination:

The sexual determination was performed through the amplification of the amelogenin gene, on the X and Y chromosomes, and the amplification of the SRY gene from the Y chromosome [8-9].

Table 1: Reference populations used for phylogenetic analysis.

	Population	Code	N	Reference
Ancient Populations	Cova da Moura	CM	5	Present study
	Dólmen do Ansião	DEA	1	
	Paimogo I	PMI	10	
	Paimogo II	PMII	5	
	São Paulo	SP	6	
	Monte Canelas I	MCI	10	
	Paleolithic Spain/Italy	PaleoEI	7	[10-12]
	Mesolithic Europe	MesoEu	20	[13]
	Mesolithic Morocco	MesoMar	22	[14]
	Neolithic Syria	NeoSir	18	[11,15]
	Neolithic Scandinavia	NeoEs	21	[16-17]
	Neolithic Spain	NeoSp	11	[18]
	Calcolithic Spain	CalSp	10	[11,15]
	Pre-Roman Spain	PRSp	17	[19]
Modern Populations	Portugal (North)	NPt	100	[20]
	Portugal (Center)	CPt	82	
	Portugal (South)	SPt	59	
	Portugal	Pt	54	[21]
	Spain	Sp	71	
	Galicia	Glz	92	[22]
	Basque Country	PBsc	105	[21,23]
	Jordan	Jrd	6	[24]
	Egypt	Egt	67	[25]
	Nubia	Nub	80	
	Turkey	Trq	74	[26-27]
	Israel/Palestin	Plt	117	[28-29]
	Iraq	Irq	116	[28]
	Iran	In	12	
	Syria	Sir	69	
	Morocco (Berber)	MBer	62	[24,30]
	Morocco	Mar	39	
	Mauritania	Maur	35	

All results are preliminary, lacking cloning of the sequences obtained and replication of the results in an independent laboratory.

Results/Discussion

aDNA preservation:

Positive DNA amplification was possible in 92.5% of the samples. Amplification of the complete HVR-I of the mitochondrial genome was successfully performed in 72.5%.

Table 2: mtDNA results for each individual: HVR-I mutations and PCR-RFLP's. The samples identified with a * was only possible to sequence part of the HVR-I region.

Sample	HVR-I sequence (16030-16401)	Hg mtDNA (PCR-RFLP's)	Hg mtDNA (final)
CM 183*	CRS	H	**H**
CM 187	16189 16223 16278	X	**X2b**
CM 194	16111 16129	H	**H**
CM 202	16217 16270A	-	U5 or HV2
CM 208	16111T16129	-	?
DEA 50*	16356	-	H or U4
MCI 169.23*	CRS	-	H or HV* or R0
MCI 176.1	16294 16304	H	**H**
MCI 206.4*	CRS	H	**H**
MCI 282.3.1	16256	-	H or V
MCI 288.5	16256	H	**H**
MCI 337*	16356	H	**H**
MCI 384.11*	16311 16354	-	**H**
MCI 386.37	16256 16270 16274	-	**U5a1***
MCI 392/3*	16366	-	?
MCI 435.3	16093	H	**H**
PM 13297	CRS	-	H or HV*
PM 13514	16223 16278 16311	-	HV* or L3*/N*
PM 13622	CRS	U	**U**
PM 14063	16153 16156 16192	-	?
PM 14638	16192 16217 16270A	U	**U5b2***
PM 14714	CRS	H	**H**
PM 15079	16356	U	**U4**
PM 15986	16270	H	**H**
PM 17334	16147	-	**H**
PM 17480	CRS	-	H or HV* or R0
PMII 527	16183C 16189	H	**H**
PMII 542	16183C 16189	H	**H**
PMII 556	CRS	H	**H**
PMII 591	16292G	H	**H**
PMII 608	16093	-	?
SP 5730	CRS	H	**H**
SP 8001	CRS	H	**H**
SP 9222*	CRS	-	H or HV* or R0
SP 10280	16093	-	?
SP 18248	CRS	H	**H**
SP 18861	CRS	H	**H**

mtDNA variation:

It was possible to determine the mitochondrial haplogroups in 24 individuals (87.5 % by PCR-RFLP's and 12.5% through nucleotide positions of HVR-I) (Table 2):

- 79.17% of the individuals belong to haplogroup H;
- 16.67% to haplogroup U;
- 4.16% to haplogroup X.

This results suggests a Palaeolithic influence, since Neolithic haplogroups were not found.

Phylogeographic analysis:

A good separation of the samples belonging to the same haplogroup was achieved and the samples analysed in this study are correctly positioned in the phylogeny (Fig. 2).

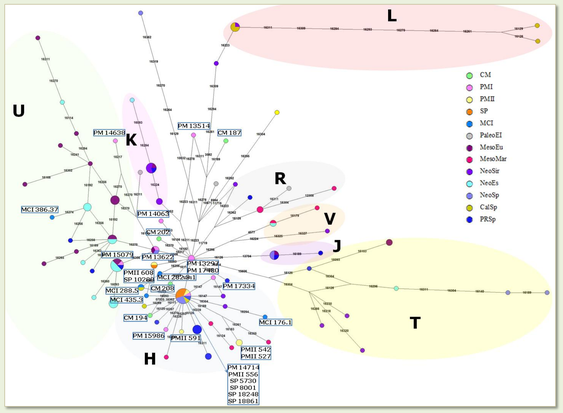

Figure 2: Reduced Median Joining Network of ancient reference samples used for comparison.

The majority of the sequences obtained aren't shared with other ancient populations. For these, additional haplotype comparisons were preformed with current populations from the Iberian Peninsula, Middle East and North of Africa. This showed us that, apart from 10 individuals, the haplotypes are shared with the Iberian Peninsula and Middle East populations, which indicates a genetic continuity and a lack of influence from Africa. The 10 non-shared haplotypes belong to European haplogroups, and differ from other European samples by one or more mutational steps in positions previously considered mutational hotspots [31].

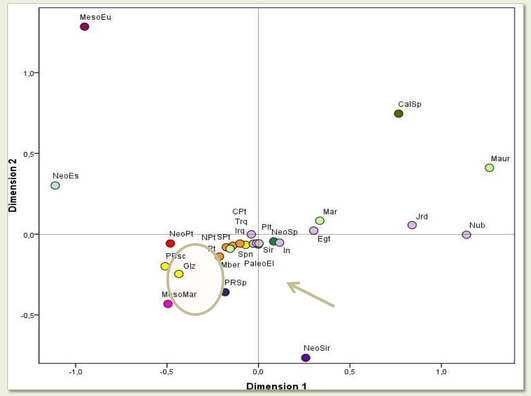

Figure 3: Multidimensional Scaling of the Reynolds genetic distance matrix.

The representation of the genetic distances between all the populations (ancient and modern) used for comparison (Fig. 3) shows that our population is closer to the populations of the Iberian Peninsula, especially those of Basque Country (PBsc) and Galicia (GLz), due to the high proportion of the H haplogroup, typical of those two populations. Regarding the ancient populations, the Portuguese Neolithic population (NeoPt) is closer to the one from the Paleolithic of Italy and Spain (PaleoEI), this may indicate a Paleolithic genetic origin of our samples.

Kinship and Sexual determination:

It was possible to determine probable matrilineal relationships, through similar uncommon haplotypes on three funerary monuments, two individuals from the natural cave of Cova da Moura (CM 194 and CM 208), two from the Hypogeum of Monte Canelas I (MCI 282.3.1 and MCI 288.5) and from *tholos* of Paimogo II (PMII 527 and PMII 542).

The sex determination through molecular methods was possible in only 16 individuals, of whom 62.5% were identified as female, and 37.5% as male (Table 3).

For six samples from MCI was possible to compare these results to those obtains through osteological methods, using the cranium and thalus [32-33], and there was an agreement from the two methods in two individuals (MCI 288.5 and MCI 384.11), and the remaining four samples had conflicting results, which can possibly be explained by erroneous results from the osteological methods.

Table 3: Sexual determination.

Sample	Sexual Determination	
	Molecular M.	Osteological M.
MCI 176.1	♀	♂
MCI 206.4	♀	♂
MCI 282.3.1	♂	-
MCI 288.5	♂	♂
MCI 384.11	♀	♀
MCI 386.37	♀	♂
MCI 392/3	♂	♀
MCI 435.3	♂	-
PM 13514	♂	-
PM 13622	♀	-
PM 14063	♀	-
PM 15986	♂	-
PM 17334	♀	-
PMII 527	♀	-
PMII 591	♀	-
SP 9222	♀	-

Conclusion:

- A good extraction efficiency was obtained (92.5%);
- The results of this study are more in line with the Cultural Diffusion Model, or at least with an explanation where the Neolithic genetic input was reduced, because they show a Palaeolithic origin of the samples;
- Prevalence of female individuals on the series used.

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[1] Centro de Investigação em Antropologia e Saúde, Life Science Department, University of Coimbra, 3000 – Coimbra, Portugal;
[2] Dep. Biologia Animal, Biologia Vegetal i Ecologia, Unitat d'Antropologia Biològica, Facultat Biociències, Universitat Autònoma de Barcelona, Spain

Merche.gruiz@gmail.com

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