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Maternal and paternal lineages in King Tutankhamun's family

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Abstract

In this study, analysis of the mitochondrial and Y-chromosomal haplogroups was used to provide information about the phylogenetic groups of Tutankhamun's family members, and their presence among the reported contemporary Egyptian population data. The mitochondrial and Y-chromosomal DNA analysis of Tutankhamun's family confirms our previous data of the royal family pedigree, with multiple controls authenticating all results. The proposed sibling relationship between Tutankhamun's parents, KV55 (Akhenaten) and KV35YL, is further supported. The royal lineage is composed of the Y-chromosome haplogroup R1b and the mitochondrial haplogroup K. Population genetics point to a common origin at ca. 14 000-28 000 years before present locating to the Near East.

Introduction

The analysis of mitochondrial DNA (mtDNA) has been an approach of interest to study the history of populations' migration and phylogeny (Macaulay *et al*. 1999, 232–249; Cavalli-Sforza & Feldman 2003, 266–275; González *et al*. 2006, 124; Torroni *et al*. 2006, 339–345; Baca & Molak 2008, 39–61). Compared to nuclear DNA, the hypervariable regions of mtDNA accumulate mutations rapidly. Being non-recombining and maternally inherited, mtDNA has become the molecular marker of choice to investigate genetic flow, to study human evolution, and to construct distinct phylogenetic groups (Giles *et al*. 1980, 6715–6719; Richards *et al*. 1996, 185–203; Macaulay *et al*. 1999, 232–249; Cavalli-Sforza & Feldman 2003, 266–275; Pakendorf & Stoneking 2005, 165–183; González *et al*. 2006, 124; Torroni *et al*. 2006, 339–345).

However, mtDNA identifies only the female perspective of a population and is liable to genetic selection (Pakendorf & Stoneking 2005, 165–183). Therefore, it has been integrated and supported with the study of paternally inherited Y-chromosomal DNA. This latter has been used to establish a global Y-chromosome phylogenetic tree analogous to that of the mitochondrial one (Jobling & Tyler-Smith 2003, 598–612; Baca & Molak 2008, 39–61).

One of the most renowned royal families in ancient Egypt was that of the late 18th Dynasty (ca. 1550–1295 B.C.); including the pharaoh Akhenaten and his successor, Tutankhamun. The identity of the mummies, the relationship between them, and the description of inherited genetic diseases and pathological hallmarks in this dynasty have not been fully elucidated. Also, it is still not known to what extent endogamy has contributed to the early demise of the young king Tutankhamun and the consequent ending of the 18th Dynasty. Genetic fingerprinting together with statistical analysis was used in our previous study, to reveal the paternal and maternal autosomal lineages of Tutankhamun's family (Hawass *et al.* 2010, 638–647). The inherited autosomal half-allele, the partial Y-chromosomal data, and the possible trio (mother, father and child) figuring of Tutankhamun's family warranted the assembly of the most possible pedigree of the king's direct lineages. Yuya and Thuya were confirmed as the great-grandparents of Tutankhamun (generation I). Amenhotep III and KV35EL were identified as his grandparents (generation II), and the KV55 male and the younger lady in KV35 (KV35YL) were found to be his sibling parents (generation III). Consolidating these findings with the historical records, KV55 could be identified as Akhenaten and KV35EL as Queen Tiye (Hawass *et al.* 2010, 638–647; Habicht *et al.* 2016, S216–S231).

To expand the knowledge of the royal pedigree, another study was conducted to investigate the family maternal and paternal lineages. The current study employed analysis of the mitochondrial and Y-chromosomal haplogroups to test our previous results, (Hawass *et al.* 2010, 638–647) and to learn more about the genealogy of Tutankhamun's family.

Materials and Methods

DNA extraction, authentication and contamination monitoring:

Strict adherence to requirements of ancient DNA authentication, including laboratory design and practice standards, was adopted for proof of reliability and quality of the results (Richards *et al.* 1995, 291–299; Roberts & Ingham 2008, 600–613; Hawass *et al.* 2010, 638–647; Hawass *et al.* 2012, e8268; Keller *et al.* 2012, 698; Khairat *et al.* 2013, 309–325). DNA sampling and extraction were done following the published protocols for ancient DNA (Scholz & Pusch 1997, 61–64; Hawass *et al.* 2010, 638–647; Hawass *et al.* 2012, e8268). Sampling, DNA extraction, amplification, and sequencing were carried out in distinct working areas. Surfaces and equipment were continuously decontaminated with DNase and bleach and exposed to UV irradiation. Procedural components were strictly controlled and tested for the presence of contaminants. Negative controls were processed alongside each procedure to detect possible contamination with exogenous DNA sources (Tully *et*

al. 2001, 83–91; Pusch & Bachmann 2004, 957–964; Gilbert *et al.* 2005, 541–544; Lamers *et al.* 2009, 40–55; Haak *et al.* 2010, e1000536; Hawass *et al.* 2010, 638–647; Fulton 2012, 1–11; Khairat *et al.* 2013, 309–325). DNA fingerprinting for the lab members was carried out to exclude the presence of staff DNA contamination. Reproducible results were verified through bacterial cloning of amplicons (Pääbo *et al.* 2004, 645–679; Fernández *et al.* 2014, e1004401) and through the analyses of different biopsies per mummy, where 3–6 bone samples were taken from various body areas of each studied mummy (Hawass *et al.* 2010, 638–647). Recruitment of a dedicated laboratory separated from the original one, run by an independent research team, was used for replication purposes (Gilbert *et al.* 2005, 541–544; Lamers *et al.* 2009, 40–55; Hawass *et al.* 2010, 638–647; Fulton 2012, 1–11; Hawass *et al.* 2012, e8268).

Mummies and subjects:

Ten mummies were investigated in this study (Table1), including Tutankhamun, his identified parents, grandparents, and great grandparents. In addition to the analyzed 10 mummies, the published data of a father-son pair [Ramesses III and Unknown man E/Pentaware] (Hawass *et al.* 2012, e8268) were included for haplotype comparison.

Y-STR profile analysis:

The AmpFlSTR Yfiler PCR amplification Kit (Applied Biosystems, Foster City, California) was used to analyze the Y-chromosome specific haplotype profiles. The kit included sixteen human Y-chromosome DNA markers with sizes ranging between 99 bp and 324 bp, and a mean amplification size of 200 bp. All markers were tetranucleotide repetitive microsatellites with the exception of DYS392, DYS438 and DYS448, which displayed, respectively, trinucleotide, pentanucleotide and hexanucleotide repeat motifs. DNA templates were amplified according to the manufacturer's protocol with some modifications that involved decreasing the concentration of primers by 40% and increasing the AmpliTaq DNA polymerase concentration by 50%. Detection of the amplified PCR products was carried out using the ABI Genetic Analyzer 3130, Data collection Software v3.0 and GeneMapper ID v3.2 (Applied Biosystems).

The majority rule was employed for STR allele designation, where a predominant peak was generated at least 10 times [i.e. each exceeding the threshold of 50 relative fluorescence units] per mummy, using various bone samples from different body areas and monitored for slippage (Hawass *et al.* 2010, 638–647). A final consensus profile of each investigated mummy was constructed using the reproducible data of the generated partial profiles (Cowen *et al.* 2011, 400–406).

Amplification of the mitochondrial hypervariable region I, cloning and Sanger sequencing:

Specific primers were designed to amplify part of the hypervariable region I (HVRI) encompassing positions 16056 to 16409 (Table 2). These mitochondrial segments were amplified in a 50 μL PCR mix containing 1X AmpliTaq Gold® PCR Master Mix (Applied Biosystems), 20 pmol of each primer,

Mummy	Sex	Estimated age, y	Tomb	Status
Members of Tutankhamun Lineage				
Thuya	F	50-60	KV46	Great grandmother of Tutankhamun, mother of Tiye
Yuya	M	50-60	KV46	Great grandfather of Tutankhamun, father of Tiye
KV35EL (Tiye)	F	50	KV35	Grandmother of Tutankhamun, mother of KV35YL & Akhenaten
Amenhotep III	M	≥50	KV35	Grandfather of Tutankhamun, father of KV35YL & Akhenaten
KV35YL	F	25-35	KV35	Mother of Tutankhamun
KV55 (Akhenaten)	M	35-45	KV55	Father of Tutankhamun
Tutankhamun	M	19	KV62	Son of Akhenaten and KV35YL
Control Group				
TT320-CCG61065	M	≈30	TT320	Thought to be Thutmose I, unidentified 18th dynasty royal
Sitra-In	F	≈50	KV60 (original tomb KV20)	Royal wet nurse of Hatshepsut
Hatshepsut	F	≈50	KV60 (original tomb KV20)	Daughter of Thutmose I, early 18th dynasty royal

Table 1. Characterization of royal mummies under investigation

Name	Primer Pair	Sequence (5'-3')	Annealing Temperature	Product Size(bp)
m10	L-15931 H-16141	ACCAGTCTTGTAAACCGGAG ATGTACTACAGGTGGTCAAG	59ºC	250
m8	L-16056 H-16232	GAAGCAGATTTGGGTACCAC GCTTTGGAGTTGCAGTTGATGTGT	59ºC	220
m24	L- 16059 H-16111	AAGCAGATTTGGGTACCACCCA GGTACCGTACAATATTCATGGTG	TDP*62-57-52 (5,5,30)	97
m3	L-16118 H-16232	TACATTACTGCCAGCCACCAT GCTTTGGAGTTGCAGTTGATGTGT	62ºC	159
m11	L-16118 H-16302	TACATTACTGCCAGCCACCAT TGGCTTTATGTACTATGTAC	55ºC	225
m4	L-16118 H-16217a	TACATTACTGCCAGCCACCAT TGTGTGATAGTTGAGGGTTG	59ºC	140
m18	L-16126 H-16266	CTGCCAGCCACCATGAATATTG TAGGTTTGTTGGTATCCTAGTGG	60ºC	185
m23	L-16129 H- 16217b	GCCAGCCACCATGAATATTGTAC TGATGTGTGATAGTTGAGGGTTG	TDP62-57-52 (5,5,30)	134
m26	L-16129 H-16232	GCCAGCCACCATGAATATTGTAC GCTTTGGAGTTGCAGTTGATGTGT	64ºC	150
m12	L-16171 H-16232	CCACCTGTAGTACATAAAAACCCA GCTTTGGAGTTGCAGTTGATGTGT	63ºC	109
m6	L-16210 H-16302	CCCCATGCTTACAAGCAAGT TGGCTTTATGTACTATGTAC	52ºC	132
m5	L-16210 H-16347	CCCCATGCTTACAAGCAAGT ATGGGGACGAGAAGGGATTTG	62ºC	178
m17	L-16211 H-16266	CTCCCCATGCTTACAAGCAGTA AGGTTTGTTGGTATCCTAGTGG	60ºC	100
m22	L- 16226 H- 16290	GCAAGTACAGCAATCAACCCTC GTACTATGTACTGTTAAGGGTGG	TDP62-57-52 (5,5,25)	109
m7	L-16288 H-16409a	CACTAGGATACCAACAAACC GCGGGATATTGATTTCACGG	62ºC	161
m25	L- 16314 H-16409b	CCACCCTTAACAGTACATAGTAC GTGCGGGATATTGATTTCACGG	TDP62-57-52 (5,5,25)	140

*TDP – Touchdown protocol

Table 2. Mitochondrial primers used for PCR amplification

and aliquots of the extracted DNA. An alternative reaction mix, consisting of 100 mM dNTPs (25 mM each dNTP) and 0.125-0.5 µl of Herculase® II Fusion DNA polymerase solution with the 1X Stratagene buffer, was used for the aDNA samples that were not successfully amplified using the AmpliTaq Gold reaction mix. The amplification cycling conditions were: 94ºC for 5 minutes, then 35 cycles of 94ºC for 30 seconds, 52-63ºC for 30 seconds, and 72ºC for 30 seconds, followed by 72ºC for 10 minutes as a final extension, using a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems).

Before proceeding with the cloning steps, excision and purification of the gel bands of specific sizes were executed using the Qiaquick® Gel Extraction Kit according to the manufacturer's

instructions. Cloning of the PCR products was carried out using the TOPO TA Cloning® Kit and One Shot MAX Efficiency DH5α-T1 chemically competent *E. coli* cells (Invitrogen), following the standard protocols. Successful ligation and transformation were detected by the blue-white selection. Colony PCR was performed in a reaction mix of total volume 50µl, using the universal M13 primers, forward 5'-GTAAAACGACGGCCAG-3' and reverse 5'-CAGGAAACAGCTATGAC-3' (Eurofins MWG Operon). The cycling protocol was 94ºC for 10 minutes, and then 25 cycles of 94ºC for 1 minute, 55ºC for 1 minute, 72ºC for 1 minute, with a final extension at 72ºC for 10 minutes.

For Sanger sequencing, ExoSAP-IT® (USB) was used for DNA purification. Cyclic sequencing for the colony PCR was carried out using a reaction mix containing 10 pmol of each M13 primer and 1X BigDye® Terminator v3.1 Cyclic Sequencing Kit (Applied Biosystems). The cycling conditions were 96ºC for 1 minute, then 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes. Sodium dodecyl sulphate (Phytotechnology laboratories) was added to each PCR product, at a final concentration of 0.2% for further incubation at 98ºC for 5 minutes, then 25ºC for 10 minutes and Centrisep columns were used for the filtration of the product prior to sequencing. A Genetic Analyzer (ABI Prism3130, Applied Biosystems) was used for the electrophoresis of purified amplicons, according to standard protocols.

Diagnostic mutations in the mitochondrial sequences were determined using the majority rule. Sequence alterations that occurred with a frequency of more than 75% were defined as diagnostic mutations. Positions thought to be prone to post-mortem damage and artefacts were omitted from subsequent analyses (Gilbert *et al.* 2003, 32–47; Pusch & Bachmann 2004, 957–964; Gilbert *et al.* 2007, 1–10).

Bioinformatic analysis:

The Whit Athey's haplogroup predictor (Athey 2006, 34–39) [http://www.hprg.com/hapest5] was used to calculate percentages of the suggested Y-chromosomal haplotypes of the male mummies, (KV55/Akhenaten, Tutankhamun, Amenhotep III, Yuya, TT320-Cairo CG 61065) and to define the haplogroups of the ancient and modern control individuals.

Matrilineal inheritance of mtDNA and characteristic single nucleotide polymorphisms (SNPs) of the royal family under investigation were determined using Lasergene (SeqMan and EditSeq) version 8.0 (DNAstar, Madison, Wisconsin). Mitochondrial DNA haplogroups were determined using the van Oven & Kayser phylogenetic tree (http://www.phylotree.org – mtDNA tree 2014; van Oven & Kayser 2009, E386–E394)) and the Genebase mtDNA Haplogroup Reference Guide (www.genebase.com).

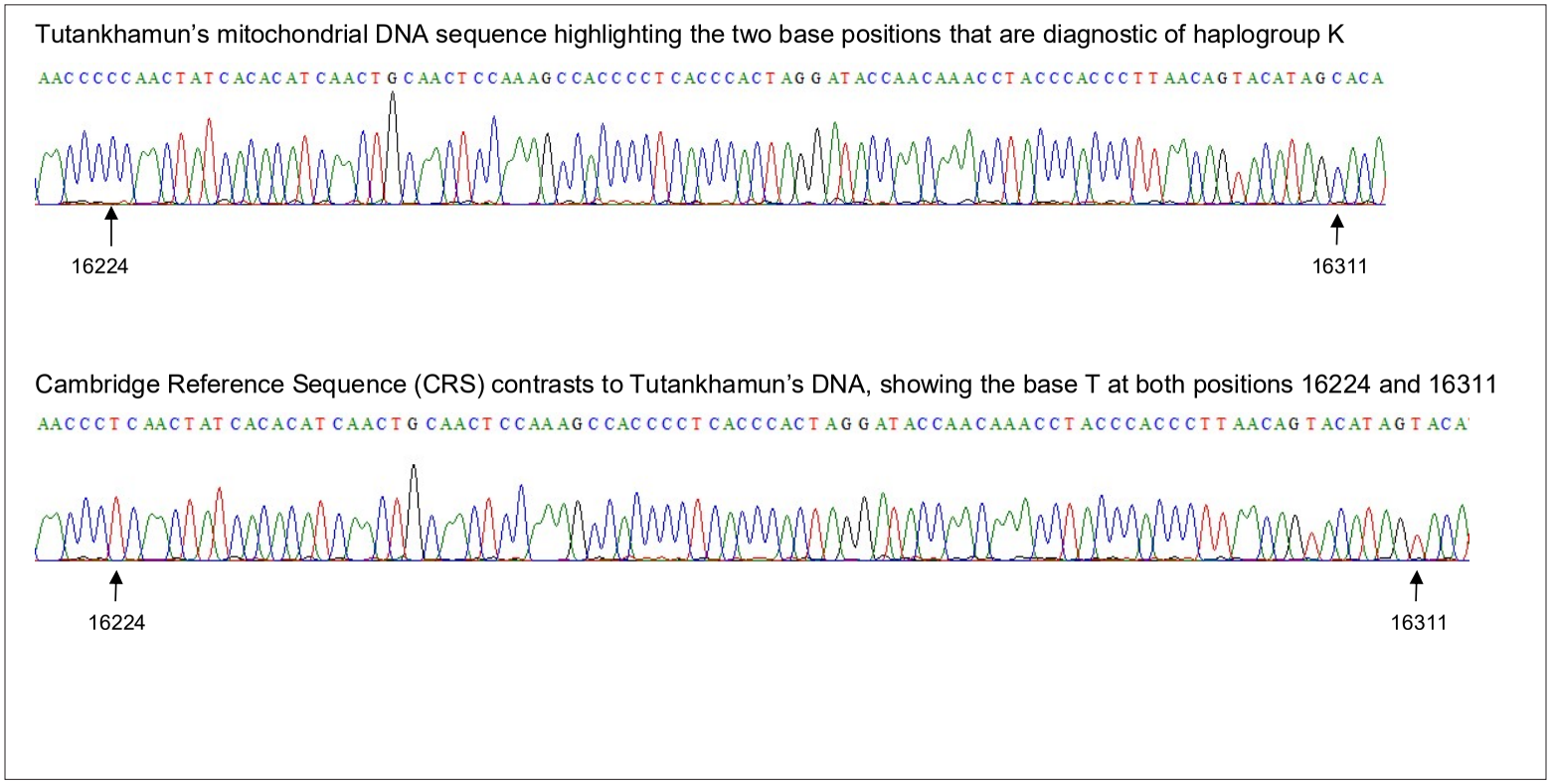

Fig. 1. Comparison of Tutankhamun's mitochondrial DNA sequence with the Cambridge Reference Sequence (CRS). Tutankhamun's mitochondrial DNA sequence highlighting the two base positions that are diagnostic to haplogroup K. Cambridge Reference Sequence (CRS) contrasts to Tutankhamun's DNA, showing the base T at both positions 16224 and 16311

a- Royal Y-chromosome Lineage Mummies

Sample No.	DYS 456	DYS 389I	DYS 390	DYS 389II	DYS 458	DYS 19	DYS 385a,b	DYS 393	DYS 391	DYS 439	DYS 635	DYS 392	YGATA H4	DYS 437	DYS 438	DYS 448	Predicted haplotype
Yuya	15	14	-	29	-	-	-	-	-	-	20	-	11	-	-	24	G2a*
Amenhotep III	15	13	24	30	16	-	-,14	13[a]	11	-	-	-	11[a]	14	-	-	R1b**
KV55 (Akhenaten)	15	13	24	30	16	14	11,14	13[a]	11	10	23	13	11[a]	14	12	19	R1b*
Tutankhamun	15	13	24	-	16	-	11,-	13[a]	11	-	23	-	11[a]	14	-	-	R1b**

b- Reference Mummies

Sample No.	DYS 456	DYS 389I	DYS 390	DYS 389II	DYS 458	DYS 19	DYS 385a,b	DYS 393	DYS 391	DYS 439	DYS 635	DYS 392	YGATA H4	DYS 437	DYS 438	DYS 448	Predicted haplotype[1]
TT320-CCG61065	15	12	19	-	18	13	-	9[a]	8	-	20	-	9[a]	15	-	-	L*
Ramesses III[b]	13	13	21	33	-	19	20	8	8	-	-	17	13	14	10	20	E1b1a*
Unknown ManE [b]	13	13	21	33	-	19	20	8	8	-	-	17	13	14	10	20	E1b1a*

c- DNA Controls

Sample No.	DYS 456	DYS 389I	DYS 390	DYS 389II	DYS 458	DYS 19	DYS 385a,b	DYS 393	DYS 391	DYS 439	DYS 635	DYS 392	YGATA H4	DYS 437	DYS 438	DYS 448	Predicted haplotype[1]
DNA 007	15	13	24	29	17	15	11,14	13	11	12	24	13	13	15	12	19	R1b***
Staff 1	16	13	24	29	15	14	11,11	12	11	12	23	13	12	15	12	19	R1b***
Staff 2	14	11	24	27	19	13	13,17	12	10	12	25	11	11	14	11	19	Q***
Staff 3	16	13	24	29	15	14	11,11	12	11	12	23	13	12	15	12	19	R1b***

Note: All female mummies were also tested with Y-chromosomal markers and did not yield any positive results.

[a] Data already published in Hawass *et al.* (2010)

* The haplogroup (hg) prediction was carried out through the website: www.hprg.com/hapest5/, by matching the concluded ancient Y-STR profiles versus those of the Mediterranean populations (meditp) as well as of the equal priors (eqp) selection (Athey 2006, 34-39). The predicted hg was suggested based on the probability that is more than 85%. The actual probabilities for the detected 4 ancient haplotypes were for the R1b hg (meditp: 99.9%; eqp: 99.6%), the G2a hg (meditp: 94.9%; eqp: 89.2%), the L hg (meditp: 92.9%; eqp: 98.4%) and E1b1a hg (meditp: 86.7%; eqp: 98.3%).

** Since the weights of the various alleles and loci in haplogroup prediction are apparently unequal, the Amenhotep III-KV55-Tutankhamun male lineage haplotype was predicted based on the complete KV55 data rather than the partial data of Tutankhamun and Amenhotep III which gave different probabilities. Amenhotep III partial profile gave (meditp: R1a: 55.5%, R1b: 31.5% and E1b1b: 12.5%; eqp: R1a: 84.1%, R1b: 9.3% and E1b1b: 5.4%), while that of Tutankhamun gave (meditp: R1b: 71.7%, R1a: 28% and E1b1b: 0%; eqp: R1a: 65.9%, R1b: 32.9% and E1b1b: 0%).

*** The contemporary staff samples were matched only to the Mediterranean populations selection and the 007 profile to the Northwest European selection. All probabilities exceeded 95%.

Table 3. Y-chromosome data of male mummies and DNA controls

Results

Y-chromosomal data:

The Y-chromosomal data of the 7 male mummies are collectively shown in Table 3. Those subjects included 4 mummies from the Tutankhamun family group, and the father-son pair from another dynasty. One complete (KV55-Akhenaten) and 6 partial profiles (Amenhotep III, Tutankhamun, Yuya, TT320, Ramesses III, Unknown Man E) were obtained for the tested male subjects. On the other hand, all female mummies repeatedly tested negative.

Using the Whit Athey haplogroup predictor, the most probable haplogroups obtained for the Tutankhamun family members were G2a for Yuya and R1b for KV55. Due to the incomplete profiles observed for Tutankhamun and Amenhotep III, analysis led to different probability figures, despite their concordant allele results. The haplogroup for these two mummies was thus predicted based on the full KV55 data, particularly since the relationships were confirmed through a previous study (Hawass *et al.* 2010, 638–647). The mummies that did not belong to the Tutankhamun lineage showed other haplotype probabilities (L and E1b1a). It should be noted that the identified R1b haplotype in Tutankhamun, KV55 and Amenhotep III was different from those detected in the kit control DNA 007 as well as in the staff members (1 and 3) as the profiles differed in several loci.

Mitochondrial data:

The mtDNA of 9 individuals was analyzed; 7 belonged to the Tutankhamun family (4 males, 3 females) and 2 females who were not (Table 4). Using PCR primers specifically designed to amplify short mtDNA fragments, partial analysis of the HVRI (16056–16409) of the D loop control region was done. Diagnostic mutations in the sequences from the tested mummies were determined using the majority rule. The total number of sequences obtained to characterize the HVRI region was 631. Considering the alignment data from Thuya (generation I in the family), 87 sequences were generated in total using 6 bone biopsies and 9 different primer combinations. Position 16224 was covered by 36 sequences from 10 different PCR products using 4 primer combinations. Of these 36 sequences, 32 showed a C and 4 showed a T residue. According to the majority rule and in the absence of contaminant sequences, this defines C to be the authentic base at mtDNA position 16224. Moreover, position 16311 was covered by 38 sequences in total and derived from 9 PCR products using 3 different combinations; 35 showed a C at this position, while 2 showed a T and 1 showed an A.

Likewise for Tutankhamun (generation IV), 79 sequences were obtained in total using 4 bone biopsies and 8 different primer combinations. Position 16224 was covered by 51 sequences in total from 10 different PCR products using 5 different combinations. Out of 51 sequences, 49 showed a C, while 2 showed a T. Position 16311 was covered by 51 sequences in total from 10 different PCR products using 3 different primer combinations. Out of these 51 sequences, 50 showed a C

a- Royal Mitochondrial Lineage Mummies

Mummy (tomb)	HVRI Diagnostic Mutation*, (Frequency, Experiments†)	Predicted Haplogroups#	Sequences per Alignment	Min/ Max Coverage	Coverage
Thuya (KV46)	16224 (89% - 4 exps) 16311 (92% - 3 exps)	K	87	4/57	Full
KV35EL (Tiye) (KV35)	16224 (90% - 5 exps) 16311 (100% - 2 exps)	K	74	3/43	Full
KV35YL (KV35)	16224 (88% - 4 exps) 16311 (100% - 3 exps)	K	72	6/55	Full
KV55 (Akhenaten) (KV55)	16224 (78% - 4 exps) 16311 (100% - 3 exps)	K	137	27/69	Full
Tutankhamun (KV62)	16224 (96% - 5 exps) 16311 (98% - 3 exps)	K	79	2/65	Full

b- Control Mummies

Mummy (tomb)	HVRI Diagnostic Mutation*, (Frequency, No. of Trials†)	Predicted Haplogroups#	Sequences per Alignment	Min/ Max Coverage	Coverage
Yuya (KV46)	16224 (80% - 2 exps) 16311 (89% - 3 exps)	K	64	2/58	Full
Amenhotep III (KV35)	16311 (100% - 1 exp)	H2b	80	17/47	Partial 16118-16409
Sitra-In (KV60)	None	K excluded	18	-	Partial 16118-16232
Hatshepsut (KV60)	None	K excluded	20	-	Partial 16118-16232

c- Staff Controls

Staff Member	HVRI Diagnostic Mutation	Predicted Haplogroups&
Staff 1	16189; 16192; 16223; 16292; 16355; 16519	L2a1
Staff 2	16069; 16126; 16193; 16300; 16309	J1d
Staff 3	16519	H (CRS)
Staff 4	16304; 16519	H5
Staff 5	16217; 16309	HV2
Staff 6	16218; 16519	H20
Staff 7	16069; 16126; 16145; 16187; 16217; 16261; 16300; 16311	J1b1
Staff 8	16051; 16132; 16172; 16189; 16219; 16278	U6
Staff 9	16069; 16126; 16214; 16231; 16305	J
Staff 10	16093; 16224; 16304; 16311	K1a
Staff 11	16129; 16309; 16318; 16362; 16397	U7

*Alterations observed in the mitochondrial DNA with a frequency higher than 75% were designated as diagnostic mutations.
#The phylogenetic tree (http://www.phylotree.org) and the Genebase mtDNA Haplogroup Reference Guide (www.genebase.com) were used to determine mtDNA haplotypes (Van Oven & Kayser 2009, E386-E394).
†"Experiments" indicate the number of primer combinations used to generate differently sized PCR amplicons which harbour the diagnostic mutation.
&Haplogroup determination for staff members was mainly based on the findings of Saunier *et al.* (2009, e97-e103) and Elmadawy *et al.* (2013, 338-341), in addition to general information from the van Oven & Kayser (2009, e386-e394) phylogenetic tree.

Table 4. Characterization of mitochondrial DNA of 18th Dynasty mummies

while only 1 showed a T. Using the same rule, this also defines C to be the authentic base at positions 16224 and 16311, respectively.

The two diagnostic mutations observed in Tutankhamun's DNA sequences (Fig. 1) are indicative of the mitochondrial haplogroup K according to the van Oven & Kayser (2009, E386–E394) phylogenetic tree (http://www.phylotree.org) and the Genebase mtDNA Haplogroup Reference Guide (www.genebase.com). The haplogroup data for the other individuals is listed in Table 4. Based on the occurrence of the two diagnostic mutations 16224 and 16311, six members of the

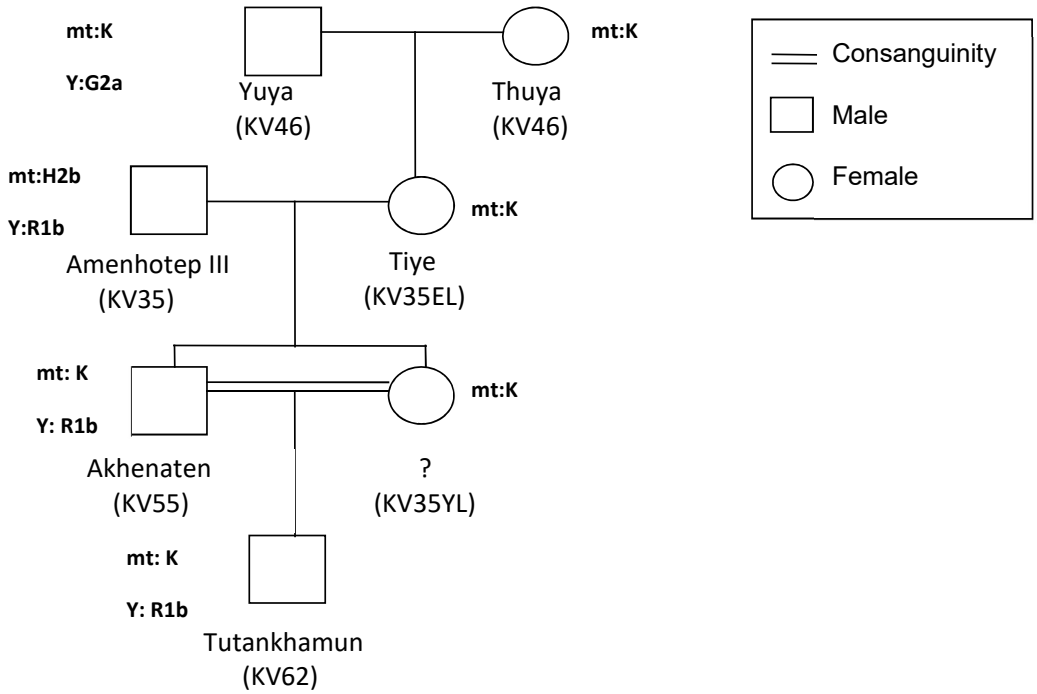

Fig. 2. Family pedigree depicting mitochondrial and Y chromosome haplogroups

Tutankhamun family group were characterized as haplogroup K, while one was characterized with haplogroup H2b. For the two KV60 reference mummies, a small PCR product encompassing 16118 to 16232 was amplified and sequenced but it did not show the 16224 diagnostic mutation associated with haplogroup K.

A pedigree representing the generated mitochondrial and Y-chromosome haplogroups of the investigated Tutankhamun family members is shown in Fig. 2.

Discussion

Implications from Y-chromosomal DNA analysis:

Haplogroup R1b

The proposed haplogroup for three of the investigated mummies, namely Tutankhamun, KV55 (Akhenaten) and Amenhotep III, was R1b. The R1b haplogroup is defined by the mutation M343 and thought to originate in the western Asian region (Wood *et al*. 2005, 867–876; Cruciani *et al*. 2010, 800–807; Myres *et al*. 2011, 95–101; Klyosov 2012, 87–105). R1b is not abundant in contemporary

Africa (Cruciani *et al*. 2010, 800–807), yet it is geographically widespread among the European populations making up to 50-60% of the genetic pool of the modern Europeans (Arredi *et al*. 2004, 338–345; Cinnioğlu *et al*. 2004, 127–148; Karafet *et al*. 2008, 830–838; Myres *et al*. 2011, 95–101; Klyosov 2012, 87–105). The introduction of this Y-chromosomal signature may be due to old introgression of lineages to the European gene pool from the Near East during the Neolithic spread of farming (Myres *et al*. 2011, 95–101). The phylogeographic analysis of the R1b haplogroup distribution provided strong support to the back-migration theory from Asia to Africa (Cruciani *et al*. 2010, 800–807). The R1b group was estimated to have an age of ca. 16,000 years before present (YBP) (Klyosov 2012, 87–105), and its suggested expansion time nearly approximates that of the G2a haplogroup (Cinnioğlu *et al*. 2004, 127–148). Less than 7% of the contemporary Egyptians share the R1b Y-chromosomal fingerprint (Cruciani *et al*. 2010, 800–807; El-Sibai *et al*. 2009, 568–581).

Haplogroup G2a

The great-grandfather of Tutankhamun, Yuya, carries a Y-chromosomal signature that could be assigned to the haplogroup G2a. Haplogroup G is an F-affiliated clade (Luis *et al*. 2004, 532–544; Wood *et al*. 2005, 867–876), and it is defined by the mutation M201 (Cinnioğlu *et al*. 2004, 127–148; Luis *et al*. 2004, 532–544; Wood *et al*. 2005, 867–876; Karafet *et al*. 2008, 830–838). This clade is not globally abundant, and its prevalence is mainly in the Middle East (highest in Druze), the Mediterranean basin and Caucasus Mountains where it exhibits its maximum frequency (Cinnioğlu *et al*. 2004, 127–148; Karafet *et al*. 2008, 830–838; Balanovsky *et al*. 2011, 18255–18259; Lacan *et al.* 2011, 2905–2920). The pattern of this haplogroup distribution in the Caucasus suggests a Near Eastern origin (Cinnioğlu *et al*. 2004, 127–148; Balanovsky *et al*. 2011, 18255–18259). The genetic share of the F-affiliated groups (G, H, I, J) is around 40% of the modern Egyptians, with G-M201 representing approximately 9% of the population (Luis *et al*. 2004, 532–544).

Haplogroup L

One of the male control mummies, an unidentified 18th Dynasty royal, previously thought to be Thutmose I (TT320-Cairo CG 61065), was proposed to belong to haplogroup L. This haplogroup is mainly defined by the M20 mutation, with additional markers to further confirm the clade definition (Sengupta *et al.* 2006, 202–221). It is found at low frequencies in some parts of the Middle East and Mediterranean Europe, and is mainly observed in southern, western and central Asia (highest in Indian subcontinent) (Kivisild *et al.* 2003, 313–332; Cordaux *et al.* 2004, 231–235; Sengupta *et al.* 2006, 202–221; Thanseem *et al.* 2006, 42; Karafet *et al*. 2008, 830–838). In Y-chromosome studies on modern Egyptians, no haplogroup L findings were reported (Arredi *et al*. 2004, 338–345; Luis *et al.* 2004, 532–544; El-Sibai *et al*. 2009, 568–581).

Haplogroup E1b1a

The father-son pair, Ramesses III and Unknown Man E/Pentawere, was found to have the haplogroup E1b1a (Hawass *et al.* 2012, e8268) that shows its highest frequencies in modern populations from

West Africa (~80%) and Central Africa (~60%). It is less than 10% among North Africans and nearly absent in East Africa, which is where it was hypothesized to have originated (Trombetta *et al.* 2011, e16073). Alternatively, an earlier origin of around 20,000-30,000 YBP, was hypothesized in West Africa due to its very high diversity and frequency in that location (Rosa *et al.* 2007, 124). Although the frequency of haplogroup E among contemporary Egyptians is 39.5%, that of the E1b1 subclade was only 1.4% (Cruciani *et al.* 2004, 1014–1022; Luis *et al.* 2004, 532–544; El-Sibai *et al*. 2009, 568–581).

Implications from mitochondrial DNA:

Haplogroup K

Haplogroup K most likely belonging to a Near Eastern lineage (Brandstätter *et al.* 2008, 191; Fernández *et al.* 2014, e1004401), is a subcluster of the macrohaplogroup U (Richards *et al*. 1998, 241–260; Kivisild *et al*. 1999, 1331–1334; Macaulay *et al*. 1999, 232–249; Maca-Meyer *et al*. 2001, 13). The age estimate of the K cluster is ~15,500–25,500 YBP (Richards *et al.* 2000, 1251–1276). The K haplogroup has been found to be present in 4.7% of contemporary Egyptian populations (Saunier *et al*. 2009, e97–e103).

Haplogroup H

The haplogroup of one of the studied mummies (Amenhotep III) was proposed as being H2b. Haplogroup H displays a sequence in the hypervariable region I (HVRI) identical to that of the Cambridge Reference Sequence (CRS) (Torroni *et al*. 1996, 1835–1850; Macaulay *et al*. 1999, 232–249; Richards *et al*. 2000, 1251–1276; van Oven & Kayser 2009, e386–e394). It is believed that the HVRI 16311 transition is an emerging subclade of the H-CRS and named as H2b (Roostalu *et al.* 2007, 436–448; Brandstätter *et al.* 2008, 191). The age estimate of cluster H in Europe can be dated to 19,200–21,400 YBP; however, it is older in the Near East and can be dated back to 23,200–28,400 YBP. Haplogroup H is substantially prevalent in Europe as it is in the Near East but with a lower frequency (Torroni *et al*. 1996, 1835–1850; Richards *et al*. 2000, 1251–1276; Tambets *et al*. 2000, 219–235; Malyarchuk *et al*. 2008, 1651–1658). This indicates that, although this cluster might have its roots in the Middle East, the significant evolution of this mitochondrial signature occurred in Europe (Richards *et al*. 1996, 185–203), probably in the late Upper Paleolithic expansions (14 500 YBP) (Richards *et al*. 1998, 241–260; Richards *et al*. 2000, 1251–1276). The frequency of haplogroup H was, respectively, 3.6 and 5.94% among modern Egyptians (Saunier *et al*. 2009, e97–e103; Elmadawy *et al.* 2013, 338–341).

Concluding remarks:

This study reconfirms and complements the previously published family pedigree of the King Tutankhamun (Hawass *et al.* 2010, 638–647). It also presents the plausible mitochondrial and Y-chromosomal signatures of the family members. The data suggest geographical and temporal

matching of the origins of Y-chromosomal and the mitochondrial signatures in the family to possibly Near Eastern lineages, with time coverage believed to be between ~14,500 and 28,400 YBP. A recent study by Schuenemann *et al.* (2017, 15694) has also shown the propensity of Near Eastern lineages among their ancient Egyptian sample coming from Faiyum, although these were from later ancient periods. A distinction between the geographical origins of the investigated late 18th Dynasty family and the succeeding 20th Dynasty pharaohs (Ramesses III and Unknown Man E/Pentawere) was also shown. The latter was estimated to be of African origin, not a Middle or Near Eastern one.

Due to its geographical location, Egypt could have over time created a melting pot of various gene pools. The genetic variation outside Africa reflects only a subset of the African gene pool (Underhill & Kivisild 2007, 539-564). Studies on today's Egyptian population samples have resulted in haplogroups that are predominantly European or west Eurasian (Stevanovitch *et al* 2003, 23–39; Saunier *et al*. 2009, e97–e103; Elmadawy *et al.* 2013, 338–341; Fadhlaoui-Zid *et al.* 2013, e80293). It was also reported that the modern Egyptian haplogroups in a study were more closely associated with non-African populations than with the West Africans (Pagani *et al.* 2015, 986–991). This accords with our findings that the same trend was indicated through the analysis of 18th Dynasty family members.

Conflict of Interest (COI):

The authors declare no conflict of interest to the submitted work.

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Bibliography

Arredi, Barbara, Estella S. Poloni, Silvia Paracchini, Tatiana Zerjal, Dahmani M. Fathallah, Mohamed Makrelouf, Vincenzo L. Pascali, Andrea Novelletto, and Chris Tyler-Smith
2004. "A Predominantly Neolithic Origin for Y-Chromosomal DNA Variation in North Africa." *The American Journal of Human Genetics* 75 (2): 338–345.

Athey, T. Whit
2006. "Haplogroup Prediction from Y-STR Values Using a Bayesian-Allele-Frequency Approach." *Journal of Genetic Genealogy* 2: 34–39.

Baca, Mateusz, and Martyna Molak
2008. "Research on Ancient DNA in the Near East." *Bioarchaeology of the Near East* 2: 39–61.

Balanovsky, Oleg, Khadizhat Dibirova, Anna Dybo, Oleg Mudrak, Svetlana Frolova, Elvira Pocheshkhova, Marc Haber, et al.
2011. "Parallel Evolution of Genes and Languages in the Caucasus Region." *Molecular Biology and Evolution* 28 (10): 2905–2920.

Brandstätter, A., B. Zimmermann, J. Wagner, T. Göbel, A.W. Röck, A. Salas, A. Carracedo, and W. Parson
2008. "Timing and Deciphering Mitochondrial DNA Macro-Haplogroup R0 Variability in Central Europe and Middle East." *BMC Evolutionary Biology* 8: 191.

Cavalli-Sforza, L. Luca, and Marcus W. Feldman
2003. "The Application of Molecular Genetic Approaches to the Study of Human Evolution." *Nature Genetics* 33 (3): 266–275.

Cinnioğlu, Cengiz, Roy King, Toomas Kivisild, Ersi Kalfoğlu, Sevil Atasoy, Gianpiero L. Cavalleri, Anita S. Lillie, et al.
2004. "Excavating Y-Chromosome Haplotype Strata in Anatolia." *Human Genetics* 114 (2): 127–148.

Cordaux, Richard, Robert Aunger, Gillian Bentley, Ivane Nasidze, S.M. Sirajuddin, and Mark Stoneking
2004. "Independent Origins of Indian Caste and Tribal Paternal Lineages." *Current Biology* 14 (3): 231–235.

Cowen, S., P. Debenham, A. Dixon, S. Kutranov, J. Thomson, and K. Way
2011. "An Investigation of the Robustness of the Consensus Method of Interpreting Low-Template DNA Profiles." *Forensic Science International: Genetics* 5 (5): 400–406.

Cruciani, Fulvio, R. La Fratta, P. Santolamazza, Daniele Sellitto, R. Pascone, Pedro Moral, Elizabeth Watson, Valentina Guida, E. B. Colomb, and B. Zaharova
2004. "Phylogeographic Analysis of Haplogroup E3b (E-M215) Y Chromosomes Reveals Multiple Migratory Events Within and Out Of Africa." *American Journal of Human Genetics* 74 (5): 1014–1022.

Cruciani, Fulvio, Beniamino Trombetta, Daniele Sellitto, Andrea Massaia, Giovanni Destro-Bisol, Elizabeth Watson, Eliane Beraud Colomb, Jean-Michel Dugoujon, Pedro Moral, and Rosaria Scozzari
2010. "Human Y Chromosome Haplogroup R-V88: A Paternal Genetic Record of Early Mid Holocene Trans-Saharan Connections and the Spread of Chadic Languages." *European Journal of Human Genetics* 18 (7): 800–807.

Elmadawy, Mostafa Ali, Atsushi Nagai, Ghada M. Gomaa, Hanaa M.R. Hegazy, Fawzy Eid Shaaban, and Yasuo Bunai
2013. "Investigation of MtDNA Control Region Sequences in an Egyptian Population Sample." *Legal Medicine* 15 (6): 338–341.

El-Sibai, M., D. E. Platt, M. Haber, Y. Xue, S. C. Youhanna, R. S. Wells, H. Izaabel, M. F. Sanyoura, H. Harmanani, and M. A. Bonab
2009. "Geographical Structure of the Y-Chromosomal Genetic Landscape of the Levant: A Coastal-Inland Contrast." *Annals of Human Genetics* 73 (6): 568–581.

Fadhlaoui-Zid, Karima, Marc Haber, Begoña Martínez-Cruz, Pierre Zalloua, Amel Benammar Elgaaied, David Comas, and Wolfgang Arthofer
2013. "Genome-Wide and Paternal Diversity Reveal a Recent Origin of Human Populations in North Africa." *PLoS ONE* 8 (11): e80293.

Fernández, Eva, Alejandro Pérez-Pérez, Cristina Gamba, Eva Prats, Pedro Cuesta, Josep Anfruns, Miquel Molist, Eduardo Arroyo-Pardo, and Daniel Turbón
2014. "Ancient DNA Analysis of 8000 B.C. Near Eastern Farmers Supports an Early Neolithic Pioneer Maritime Colonization of Mainland Europe through Cyprus and the Aegean Islands." *PLOS Genetics* 10 (6): e1004401

Fulton, Tara L.
2012. "Setting Up an Ancient DNA Laboratory." *Methods in Molecular Biology* 840: 1–11.

Gilbert, M. Thomas P., Hans-Jürgen Bandelt, Michael Hofreiter, and Ian Barnes
2005. "Assessing Ancient DNA Studies." *Trends in Ecology & Evolution* 20 (10): 541–544.

Gilbert, M. Thomas P., J. Binladen, W. Miller, C. Wiuf, E. Willerslev, H. Poinar, J. E. Carlson, J. H. Leebens-Mack, and S. C. Schuster
2007. "Recharacterization of Ancient DNA Miscoding Lesions: Insights in the Era of Sequencing-by-Synthesis." *Nucleic Acids Research* 35 (1): 1–10.

Gilbert, M. Thomas P., E. Willerslev, A. J. Hansen, I. Barnes, L. Rudbeck, N. Lynnerup, and A. Cooper
2003. "Distribution Patterns of Postmortem Damage in Human Mitochondrial DNA." *American Journal of Human Genetics* 72: 32–47.

Giles, R. E., H. Blanc, H. M. Cann, and D. C. Wallace
1980. "Maternal Inheritance of Human Mitochondrial DNA." *Proceedings of the National Academy of Sciences of the United States of America* 77 (11): 6715–6719.

González, Ana M., Oscar García, José M. Larruga, and Vicente M. Cabrera
2006. "The Mitochondrial Lineage U8a Reveals a Paleolithic Settlement in the Basque Country." *BMC Genomics* 7: 124.

Habicht, Michael E., A. S. Bouwman, and Frank Jakobus Rühli
2016. "Identifications of Ancient Egyptian Royal Mummies from the 18th Dynasty Reconsidered." *American Journal of Physical Anthropology.* 159: S216–S231.

Haak, Wolfgang, Oleg Balanovsky, Juan J. Sanchez, Sergey Koshel, Zaporozhchenko, Christina J. Adler, Clio S. I. Sarkissian, et al.
2010. "Ancient DNA from European Early Neolithic Farmers Reveals Their Near Eastern Affinities." PLOS Biology 11: e1000536.

Hawass, Z., S. Ismail, A. Selim, S. N. Saleem, D. Fathalla, S. Wasef, A. Z. Gad, et al.
2012. "Revisiting the Harem Conspiracy and Death of Ramesses III: Anthropological, Forensic, Radiological, and Genetic Study." *BMJ* 345: e8268.

Hawass, Zahi A., Yahia Z. Gad, Somaia Ismail, Rabab Khairat, Dina Fathalla, Naglaa Hasan, Amal Ahmed, et al.
2010. "Ancestry and Pathology in King Tutankhamun's Family." *Journal of the American Medical Association* 303 (7): 638–647.

Jobling, M. A., and C. Tyler-Smith
2003. "The Human Y Chromosome: An Evolutionary Marker Comes of Age." *Nature Reviews. Genetics* 4 (8): 598–612.

Karafet, Tatiana M., Fernando L. Mendez, Monica B. Meilerman, Peter A. Underhill, Stephen L. Zegura, and Michael F. Hammer
2008. "New Binary Polymorphisms Reshape and Increase Resolution of the Human Y Chromosomal Haplogroup Tree." *Genome Research* 18 (5): 830–838.

Keller, A., A. Graefen, M. Ball, M. Matzas, V. Boisguerin, F. Maixner, P. Leidinger, et al.
2012. "New Insights into the Tyrolean Iceman's Origin and Phenotype as Inferred by Whole-Genome Sequencing." *Nature Communications* 3: 698.

Khairat, Rabab, Markus Ball, Chun-Chi Hsieh Chang, Raffaella Bianucci, Andreas G. Nerlich, Martin Trautmann, Somaia Ismail, et al.
2013. "First Insights into the Metagenome of Egyptian Mummies Using Next-Generation Sequencing." *Journal of Applied Genetics* 54 (3): 309–325.

Kivisild, T., M. J. Bamshad, K. Kaldma, and M. Metspalu
1999. "Deep Common Ancestry of Indian and Western-Eurasian Mitochondrial DNA Lineages." *Current Biology* 9 (22): 1331–1334.

Kivisild, T. S., S. Rootsi, M. Metspalu, S. Mastana, K. Kaldma, J. Parik, E. Metspalu, et al.
2003. "The Genetic Heritage of the Earliest Settlers Persists Both in Indian Tribal and Caste Populations." *American Journal of Human Genetics* 72 (2): 313–332.

Klyosov, Anatole A.
2012. "Ancient History of the Arbins, Bearers of Haplogroup R1b, from Central Asia to Europe, 16,000 to 1500 Years before Present." *Advances in Anthropology* 02 (02): 87–105.

Lacan, M., C. Keyser, F. X. Ricaut, N. Brucato, J. Tarrús, A. Bosch, J. Guilaine, E. Crubézy, and B. Ludes.
2011. "Ancient DNA Suggests the Leading Role Played by Men in the Neolithic Dissemination." *Proceedings of the National Academy of Sciences of the United States of America* 108 (45): 18255–18259.

Lamers, Ryan, Shana Hayter, S., Carney D.Matheson,
2009 "Postmortem Miscoding Lesions in Sequence Analysis of Human Ancient Mitochondrial DNA". *Journal of Molecular Evolution* 68 (1): 40–55

Luis, J.R., D.J. Rowold, M. Regueiro, B. Caeiro, C. Cinnioğlu, C. Roseman, P.A. Underhill, L.L. Cavalli-Sforza, and R.J. Herrera
2004. "The Levant versus the Horn of Africa: Evidence for Bidirectional Corridors of Human Migrations." *The American Journal of Human Genetics* 74 (3): 532–544.

Maca-Meyer, Nicole, Ana M. González, José M. Larruga, Carlos Flores, and Vicente M. Cabrera
2001. "Major Genomic Mitochondrial Lineages Delineate Early Human Expansions." *BMC Genetics* 2 (1): 13.

Macaulay, V., M. Richards, E. Hickey, E. Vega, F. Cruciani, V. Guida, R. Scozzari, B. Bonne-Tamir, B. Sykes, and A. Torroni
1999. "The Emerging Tree of West Eurasian MtDNAs: A Synthesis of Control-Region Sequences and RFLPs." *American Journal of Human Genetics* 64 (1): 232–249.

Malyarchuk, B., T. Grzybowski, M. Derenko, M. Perkova, Vanecek, J. Lazur, P. Gomolcak, and I. Tsybovsky
2008. "Mitochondrial DNA Phylogeny in Eastern and Western Slavs." *Molecular Biology and Evolution* 25 (8): 1651–1658.

Myres, N. M., S. Rootsi, A. A. Lin, M. Jarve, R. J. King, I. Kutuev, V. M. Cabrera, E. K. Khusnutdinova, A. Pshenichnov, and B. Yunusbayev
2011. "A Major Y-Chromosome Haplogroup R1b Holocene Era Founder Effect in Central and Western Europe." *European Journal of Human Genetics* 19 (1): 95–101.

Pääbo, Savante, Hendrik Poinar, David Serre, Viviane Jaenicke-Després, Juliane Hebler, Nadin Rohland, Melanie Kuch, *et al*.
2004 "Genetic Analyses from Ancient DNA." *Annual Review of Genetics* 38: 645–679.

Pagani, L., S. Schiffels, D. Gurdasani, P. Danecek, Chen, Y. Xue, M. Haber, et al.
2015. "Tracing the Route of Modern Humans out of Africa by Using 225 Human Genome Sequences from Ethiopians and Egyptians." *American Journal of Human Genetics* 96 (6): 986–991.

Pakendorf, B., and M. Stoneking
2005. "Mitochondrial DNA and Human Evolution,"." *Annual Review of Genomics and Human Genetics* 6: 165–183.

Pusch, C. M., and L. Bachmann
2004. "Spiking of Contemporary Human Template DNA with Ancient DNA Extracts Induces Mutations under PCR and Generates Non Authentic Mitochondrial Sequences." *Molecular Biology and Evolution* 21: 957–964.

Richards, Martin, H. Corte-Real, P. Forster, V. Macaulay, H. Wilkinson-Herbots, A. Demaine, S. Papiha, R. Hedges, H.-J. Bandelt, and Bryan Sykes
1996. "Paleolithic and Neolithic Lineages in the European Mitochondrial Gene Pool." *American Journal of Human Genetics* 59 (1): 185–203.

Richards, Martin, Robert E. M. Hedges, and Bryan Sykes
1995. "Authenticating DNA Extracted From Ancient Skeletal Remains." *Journal of Archaeological Science* 22 (2): 291–299.

Richards, Martin, V. Macaulay, Hans-Jürgen Bandelt, and Bryan Sykes
1998. "Phylogeography of Mitochondrial DNA in Western Europe." *Annals of Human Genetics* 62 (3): 241–260.

Richards, Martin, Vincent Macaulay, Eileen Hickey, Emilce Vega, Bryan Sykes, Valentina Guida, Chiara Rengo, et al.
2000. "Tracing European Founder Lineages in the Near Eastern MtDNA Pool." *The American Journal of Human Genetics* 67 (5): 1251–1276.

Roberts, C., and S. Ingham
2008. "Using Ancient DNA Analysis in Palaeopathology : A Critical Analysis of Published Papers, with Recommendations for Future Work." *International Journal of Osteoarchaeology.* 18 (6): 600–613.

Roostalu, U., I. Kutuev, E. L. Loogväli, E. Metspalu, Kristiina Tambets, M. Reidla, E. K. Khusnutdinova, E. Usanga, T. Kivisild, and R. Villems
2007. "Origin and Expansion of Haplogroup H, the Dominant Human Mitochondrial DNA Lineage in West Eurasia: The Near Eastern and Caucasian Perspective." *Molecular Biology and Evolution* 24 (2): 436–448.

Rosa, Alexandra von, Carolina Ornelas, Mark A. Jobling, António Brehm, and Richard Villems
2007. "Y-Chromosomal Diversity in the Population of Guinea-Bissau: A Multi-Ethnic Perspective." *BMC Evolutionary Biology* 7: 7–124.

Saunier, J. L., J. A. Irwin, K. M. Strouss, H. Ragab, K. A. Sturk, and T. J. Parsons
2009. "Mitochondrial Control Region Sequences from an Egyptian Population Sample." *Forensic Science International. Genetics* 3 (3): e97–e103.

Scholz, Michael, and Carsten Pusch
1997. "An Efficient Isolation Method for High-Quality DNA from Ancient Bones." *Trends in Genet* 13: 249.

Schuenemann, Verena J., Alexander Peltzer, Beatrix Welte, W. Paul van Pelt, Martyna Molak, Chuan-Chao Wang, Anja Furtwängler, et al.
2017. "Ancient Egyptian Mummy Genomes Suggest an Increase of Sub-Saharan African Ancestry in Post-Roman Periods." *Nature Communications* 8: 15694.

Sengupta, S., L. A. Zhivotovsky, R. King, S. Q. Mehdi, C. A. Edmonds, C.-E. T. Chow, A. A. Lin, M. Mitra, S. K. Sil, and A. Ramesh
2006. "Polarity and Temporality of High-Resolution Y-Chromosome Distributions in India Identify Both Indigenous and Exogenous Expansions and Reveal Minor Genetic Influence of Central Asian Pastoralists." *American Journal of Human Genetics* 78 (2): 202–221.

Stevanovitch, A., A. Gilles, E. Bouzaid, Kefi, F. Paris, R. P. Gayraud, J. L. Spadoni, F. El-Chenawi, and E. Béraud-Colomb
2004. "Mitochondrial DNA Sequence Diversity in a Sedentary Population from Egypt." *Annals of Human Genetics* 68: 23–39.

Tambets, Kristiina, Toomas Kivisild, Ene Metspalu, Jüri Parik, Katrin Kaldma, Sirle Laos, Helle-Viivi Tolk, et al.
2000. "The Topology of the Maternal Lineages of the Anatolian and Trans-Caucasus Populations and the Peopling of Europe: Some Preliminary Considerations." In *Archaeogenetics: DNA and the Population Prehistory in Europe*, edited by Colin Renfrew and Katie Boyle, 219–235. McDonald Institute for Archaeological Research Monograph. Cambridge: Cambridge University Press.

Thanseem, Ismail, Kumarasamy Thangaraj, Gyaneshwer Chaubey, Vijay Kumar Singh, Lakkakula VKS Bhaskar, B. Mohan Reddy, Alla G. Reddy, and Lalji Singh
2006. "Genetic Affinities among the Lower Castes and Tribal Groups of India: Inference from Y Chromosome and Mitochondrial DNA.' *BMC Genetics* 7: 42.

Torroni, Antonio, Alessandro Achilli, Vincent Macaulay, Martin Richards, and Hans-Jürgen Bandelt
2006. "Harvesting the Fruit of the Human MtDNA Tree." *Trends in Genetics* 22 (6): 339–345.

Torroni, Antonio, K. Huoponen, P. Francalacci, M. Petrozzi, L. Morelli, R. Scozzari, D. Obinu, M. L. Savontaus, and D. C. Wallace
1996. "Classification of European MtDNAs from an Analysis of Three European Populations." *Genetics* 144 (4): 1835–1850.

Trombetta, Beniamino, Fulvio Cruciani, Daniele Sellitto, and Rosaria Scozzari
2011. "A New Topology of the Human Y Chromosome Haplogroup E1b1 (E-P2) Revealed through the Use of Newly Characterized Binary Polymorphisms." *PLOS ONE* 6 (1): e16073.

Tully, G., W. Bar, B. Brinkmann, A. Carracedo, P. Gill, N. Morling, W. Parson, and P. Schneider
2002. "Considerations by the European DNA Profiling (EDNAP) Group on the Working Practices, Nomenclature and Interpretation of Mitochondrial DNA Profiles." *Forensic Science International* 124 (1): 83–91.

Underhill, Peter A., and Toomas Kivisild
2007. "Use of Y Chromosome and Mitochondrial DNA Population Structure in Tracing Human Migrations." *Annual Review of Genetics* 41 (1): 539–564.

Van Oven, Mannis, and Manfred Kayser
2009. "Updated Comprehensive Phylogenetic Tree of Global Human Mitochondrial DNA Variation." *Human Mutation* 30 (2): E386–E394.

Wood, Elizabeth T., Daryn A Stover, Christopher Ehret, Giovanni Destro-Bisol, Gabriella Spedini, Howard McLeod, Leslie Louie, et al.
2005. “Contrasting Patterns of Y Chromosome and MtDNA Variation in Africa: Evidence for Sex-Biased Demographic Processes.” *European Journal of Human Genetics* 13 (7): 867–876.