Additional file 3

1. DNA amplification

Amplification of the HVSI from position 15914 - 9 (reference sequence GenBank V00654) was conducted using PCR primers that produce six overlapping fragments (hereinafter referred to as standard primer set). For 23 samples where amplification failed using the standard set, two alternate primer pairs were created that reduce amplicon length in favour of better amplification success. Primer sequences, positions, and annealing temperatures are given in Table 1. Figure 1 provides visualisation of the HVSI amplification strategy.

In 30 of 33 cases when a particular d-loop motif characteristic of this rare mtDNA clade was found (15,953 G and 16,255 C [1]), two additional amplicons outside the HVSI each covering a transversion (3,415 A and 10,717 T [1]) were amplified and sequenced. These additional fragments were also retrieved from eight previously published samples showing this motif and using remaining DNA extract (Ap7, God2, Mar10, Mf7, Sac3, Sac5, Sac7, Zag1 [2-4]). All of them, but none of the 14 other randomly chosen sequences from different haplogroups, show a full association with these transversions (see Table 2). We infer from this result that 15,953 G and 16,255 C alone can be considered as characteristic for haplogroup Q.

New primers were developed using the program PrimerSelect™ (DNASTAR Lasergene® 7.1 and 8).

Table 1: Mitochondrial primers.

3' end positions are given according to reference sequence V00654. The names indicate upper (U) and lower (L) primer. The standard primer set covering the targeted HVSI region in six overlapping fragments is in bold. Primers U1b and L2c are alternatives to U1 and L2 with shorter amplicon length. U8 is an alternative to U4 and replaced the latter in the course of this study because of its better performance. Primer pairs A8U/A7L and A9U/A8L are alternatives to U5/L5 with shorter amplicon length. Additionally, both primers were designed specifically not to amplify Sus scrofa DNA due to mismatches at the 3' ends. As Sus scrofa DNA is a possible lab contaminant because of its presence in various reagents [5, 6], these alternative primers were used in the rare cases when the rather unspecific primer pair U5/L5 had first resulted in Sus scrofa sequences.

Name	Sequence in 5'-3'-direction	3' end position	Primer length	Amplicon length	Annealing temperature	Reference
BosU1	**CCAAATATTACAAACACCACTAGCTAAC**	15913	28 bp			
BosL1	**GCATGGGGCATATAATTTAATGTACT**	16005	26 bp	91 bp		[4]
BosU1b	GCTAACATAACACGCCCATACAC	15930	23 bp	74 bp	56°C	this study
BosU2	**AATGTACATAACATTAATGTAATAAAGACA**	15992	20 bp			
BosL2	**TTGACATAATGTACTATGTACAGTCAATAA**	16077	30 bp	84 bp	52°C	[4]
BosL2c	TCATGTACTTGCTTATATGCATGG	16025	24 bp	32 bp	56°C	this study
BosU3	**CCATGCATATAAGCAAGTACATGA**	16048	24 bp			
BosL3	**CTCGTGATCTAATGGTAAGGAATATATA**	16131	28 bp	82 bp	52°C	[4]
BosU8	**GTACATTATGTCAAATTCATTCTTGATAG**	16121	29 bp	80 bp		this study
BosU4	ACTGTACATAGTACATTATGTCAAATTCAT	16112	30 bp			
BosL4	**CGGAGCGAGAAGAGGGATC**	16202	19 bp	89 bp	56°C	[4]
BosA8U	GTGAAACCAGCAACCCGCTA	16195	20 bp			
BosA7L	AATGAATTTTACCAGGCATCTGGTT	16249	25 bp	53 bp	56°C	
BosA9U	CCATAAACCGTGGGGGTCG	16242	19 bp			
BosA8L	TCTTCAGGGCCATCTCATCTAAAAC	16275	25 bp	34 bp	57°C	this study
BosU5	**TACCATGCCGCGTGAAACCA**	16184	20 bp			
BosL5	**TGAGATGGCCCTGAAGAAAGAA**	16272	22 bp	87 bp	56°C	
BosU6	**GGTCGCTATCCAATGAATTTTAC**	16260	23 bp			
BosL6	**ATGTGTGAGCATGGGCTGATTA**	10	22 bp	87 bp	56°C	[4]
BosQ1U	TGAATTCCCCTACCAATACCCTATCCTCTT	3385	30 bp			
BosQ1L	CATATCAAGCCTAGCCGTATACTCCATTCT	3421	30 bp	35 bp	60°C	
BosQ4U	CAGTTTGGCGACAACAGCCTTAAT	10681	24 bp			
BosQ4L	TTTTAACCATATGGCTCCTCCCTCT	10728	25 bp	46 bp	58°C	this study

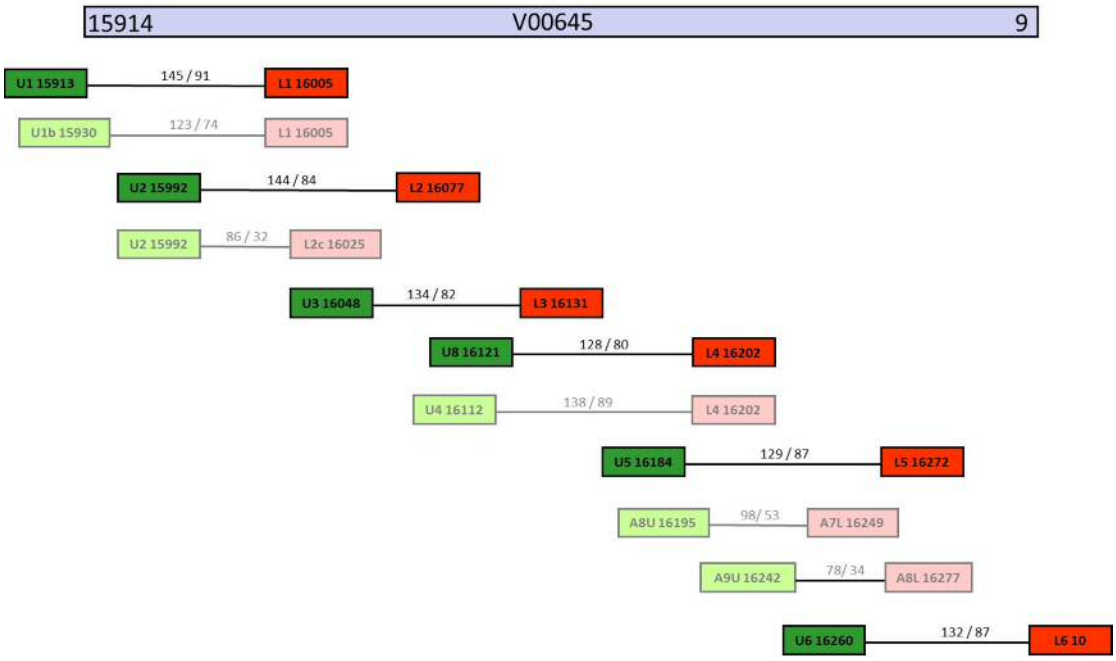
Lab Code	3.415	10.717	Haplogroup	Reference
V00654	***A***	***T***	*T3*	
Ap7	**T**	**A**	Q	Bollongino et al. 2006
God2	**T**	**A**	Q	Bollongino et al. 2006
Mar10	**T**	**A**	Q	Edwards et al. 2007
Mf7	**T**	**A**	Q	Bollongino et al. 2012
Sac3	**T**	**A**	Q	Bollongino et al. 2012
Sac5	**T**	**A**	Q	Bollongino et al. 2012
Sac7	**T**	**A**	Q	Bollongino et al. 2012
Zag1	**T**	**A**	Q	Bollongino et al. 2012
Ap15	**T**	**A**	Q	this study
Ap16	**T**	**A**	Q	this study
Ap17			T3	this study
Ber2			T3	this study
Bor3	**T**	**A**	Q	this study
Cav10	**T**	**A**	Q	this study
Cav11	**T**	**A**	Q	this study
Cav12	**T**	**A**	Q	this study
Dra2	**T**	**A**	Q	this study
Dra3	**T**	**A**	Q	this study
Dra4			T3	this study
Dra7	**T**	**A**	Q	this study
Dra8	**T**	**A**	Q	this study
Dra27	**T**	**A**	Q	this study
Dra30			T2	this study
Kan1			T3	this study
Kan2			T	this study
Kan3			T	this study
Kan5	**T**	**A**	Q	this study
Kan7	**T**	**A**	Q	this study
Kop1			T3	this study
Kop12	**T**	**A**	Q	this study
Kov22	**T**	**A**	Q	this study
Kov24	**T**	**A**	Q	this study
Kov25	**T**	**A**	Q	this study
Kov26	**T**	**A**	Q	this study
Kov42			T3	this study
Kov43	**T**	**A**	Q	this study
Kov47	**T**	**A**	Q	this study
Kov50	**T**	**A**	Q	this study
Kov53	**T**	**A**	Q	this study
Kov54	**T**	**A**	Q	this study
Oko7	**T**	**A**	Q	this study
Oko8			T3	this study
Ovc6	**T**	**A**	Q	this study
Ovc8	**T**	**A**	Q	this study
Ovc9	**T**	**A**	Q	this study
Pe18	**T**	**A**	Q	this study
Sam2	**T**	**A**	Q	this study
Sam11	**T**	**A**	Q	this study
Sam13			T2	this study
Uiv5			T2	this study
Uiv19			T	this study
Uiv20			T	this study

Table2: Q specific transversions.

Results for two additional positions outside the HVSI characteristic for haplogroup Q (positions 3,415 and 10,717 according to reference sequence GenBank V00654.

Figure 1: Mitochondrial primer scheme

Dark green and red bars indicate the position of the standard primer set according to the reference sequence (blue bar, GenBank V00645). Bright green and red bars indicate the position of the alternate primer pairs as specified in Table 1. Numbers within bars indicate the name of the primer and the 3' end position. Numbers on the connections indicate sequence length of the amplified region with/without primer sequence.



Amplification reactions were set up as shown in Table 3. At least three successful independent PCRs from two extractions were attempted.

Blank controls were processed during each PCR. The enzyme Uracil-N-Glycosilase was added to about 5% of the PCRs to cleave deaminated cytosine residues from particularly damaged samples.

Amplification was carried out in a Mastercycler gradient (Eppendorf). PCR products were visualized on an agarose gel by addition of ethidium bromide. PCR products were purified by incubation with the two enzymes Exonuclease I (2 U; Fermentas) and Shrimp Alkaline Phosphatase (0.3 U; Fermentas) for 45 min at 37°C, followed by heat inactivation for 15 min at 80 °C.

Table 3: PCR reagents and conditions.

Standard volumes and cycles are in bold. The protocol was sometimes modified in the given ranges to optimize amplification success. UNG was used only sporadically (see above).

Reagent	**Volume (µl)**	**Concentration (stock solution)**	**Final concentration**	**PCR parameters**	
PCR Gold Buffer (Applied Biosystems)	5-**6**	10 x	1 x – 1.2 x	**Initial activation**	6 min 90°C
$MgCl_2$ (Applied Biosystems)	4-**6**	25 mM	2 mM – 3 mM		
dNTP's (Qiagen)	1	10 mM	0.2 mM	**Denaturation**	40 sec 94°C
BSA (Roche)	**1**-2	20 mg/ml	0.4 µg/µl – 0.8 µg/µl	**Annealing**	40 sec (temp. see primers)
Primer, each (Biospring)	1	10 µM	0.2 µM	**Elongation**	40 sec 72°C
AmpliTaq Gold® (Applied Biosystems)	**0.5**-1	5 U/µl	2.5 U – 5 U		
UNG (Invitrogen)	0.5	1 U/µl	0.5 U		
DNA	0.5-14	not determined	not determined		
UV-irradiated HPLC-H_2O (Acros Organics)	add 50			**Cycles**	**50**-60

2. DNA sequencing

Cycle sequencing reactions were set up as shown in Table 4. Products were sequenced by capillary electrophoresis on an ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems) using POP-6™ polymer (Applied Biosystems).

Table 4: Cycle sequencing reagents and conditions.

Sequencing products were obtained using the BigDye® Terminator v.1.1 chemistry (Applied Biosystems). Volumes of PCR products were adjusted according to the intensity of their bands in the agarose gel.

Reagent	**Volume (µl)**	**Concentration (stock solution)**	**Final concentration**	**PCR parameters**	
Buffer	1.5	**5 x**	**0.75 x**	**Denaturation**	30 sec 92 °C
BigDye	1	**na**	**na**	**Annealing**	15 sec (temp. see primers)
Primers (Biospring)	1	**10 µM**	**1µM**	**Elongation**	2 min 30 sec 60°C
PCR product	1-4	not determined	not determined		
HPLC-H_2O (Acros Organics)	add 10			**Cycles**	25

3. Establishment of consensus sequences

Sequences were further analysed using the programs SeqMan™ and MegAlign™ (DNASTAR Lasergene® 7.1 and 8). At least three sequences obtained from independent PCRs from two independent DNA extractions per sample per primer pair were usually used to create a majority rule consensus sequence. If divergent positions occurred among the three sequences, we attempted to sequence further PCR products, at best obtained using UNG (see above), until the variable position was present in at least a 3:1 ratio.

Literature

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