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SCIENCE DIRECT
Gene 368 (2006) 21–27
GENE
SECTION
EVOLUTIONARY GENOMICS
www.elsevier.com/locate/gene

Differences of sperm motility in mitochondrial DNA haplogroup U sublineages

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Received 3 June 2005; received in revised form 2 September 2005; accepted 27 September 2005
Available online 1 December 2005
Received by H. Musto

Abstract

We had previously shown that sperm from men harbouring haplogroup T mtDNAs swim less vigorously than those from haplogroup H. However, the biochemical basis of this motility was difficult to investigate because of the multiple mutations, the most important of which affected respiratory complex I for which there is no crystal structure. To more thoroughly study the relationship between mtDNA variation and differences in mitochondrial energy metabolism, we turned to the analysis of sperm baring haplogroup U mtDNAs. Haplogroup U is a monophyletic ancient and thus heterogeneous maternal lineage that is broadly distributed among European individuals. Several sublineages of haplogroup U were found to be associated with differences in sperm motility and vitality. These differences could be related to a highly conserved missense mutation in the mtDNA COIII gene (V91) and several equally conserved mutations in the cytochrome *b* (cytb) gene. Moreover, the lineages with the cytb mutations were substantially enriched in northern Europe, while those lacking these mutations were more prevalent in southern Europe. We suggest that some of these ancient conserved cytb missense mutations permitted our ancestors to adapt to cold by partially uncoupling mitochondrial oxidative phosphorylation (OXPHOS).

Keywords: Mitochondrial DNA; Haplogroup; Phylogeography; OXPHOS; Sperm motility

1. Introduction

Sequence polymorphisms in the human mitochondrial DNA (mtDNA) correlate markedly with the geographic origin of the indigenous populations. These geographically constrained mtDNA variants create clusters of related mtDNA haplotypes known as haplogroups (Wallace et al., 1999). Moreover, certain mtDNA haplogroups are frequently found in association with specific disease phenotypes, indicating that some of the regional mtDNA variants might be functionally significant (Brown et al., 1997; Torroni et al., 1997).

One method of detecting the functional effects of mtDNA polymorphisms is through their impact on sperm motility. The ATP that drives the sperm flagella is derived from the mitochondria located in the midpiece. Therefore, mutations in the mtDNA which increase or decrease ATP production will be reflected in increased or decreased sperm motility (Ruiz-Pesini et al., 1998; Troiano et al., 1998). This energy production assessment strategy was shown to be informative in a study comparing the sperm

Abbreviations: CO, cytochrome oxidase; cytb, cytochrome *b*; ETC, electron transport chain; HVSI, hypervariable segment I; ISP, Rieske iron-sulfur protein; LHON, Leber's hereditary optic neuropathy; mtDNA, mitochondrial DNA; mtPTP, mitochondrial permeability transition pore; ND, mitochondrial encoded complex I subunits; OXPHOS, oxidative phosphorylation; Qi (Qo), inner (outer) ubiquinone binding site; rRNA, ribosomal RNA; tRNA, transfer RNA; Uk, Uk subhaplogroup denomination is used instead K haplogroup.

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doi:10.1016/j.gene.2005.09.015

motility of European men harboring haplogroup H versus haplogroup T and demonstrating that T sperms were significantly less motile than H sperms (Ruiz-Pesini et al., 2000). Haplogroup T differs from H at seventeen nucleotide (nt) substitutions, three in the control region, six synonymous (S) protein gene variants, four rRNA and tRNA genes substitutions and four non-synonymous (NS) protein gene mutations. One of the NS variants, N150D/ND2, change the most conserved amino acid of all the ND2 polymorphic positions found in the human population (Ruiz-Pesini et al., 2004). Unfortunately, it has proven to be difficult to assess the functional significance of these haplogroup T variants since the crystal structure of complex I is not known, the functional significance of RNA mutations is difficult to assess (Florentz and Sissler, 2001), and synonymous or control region mutations could alter regulatory elements (Solakidi and Sekeris, 2003). Hence, we have not yet been able to make a direct link between sperm motility and specific functional mtDNA variants.

To more successfully correlate mtDNA genotype with cellular phenotype, we need to reduce the number of candidate variants by homogenizing the genetic background. Haplogroup U is monophyletic, ancient and thus heterogeneous, and broadly distributed throughout Europe, representing about 20% of the mtDNAs in Western Eurasian individuals (Torroni et al., 1996). Therefore, the European haplogroup U fulfills this criterion.

In the present study, we have discovered that different haplogroup U sublineages contain highly conserved COIII and cytb missense mutations. Moreover, the haplogroup U sublineages defined by these missense mutations show striking differences in their sperm motility and vitality. Therefore, ancient mtDNA haplogroup variants do appear to be functionally significant. Furthermore, these sublineages show differential latitudinal distribution, consistent with a role for some of these functional mitochondrial variants in climate adaptation (Ruiz-Pesini et al., 2004).

2. Material and methods

A neighbor-joining tree of 188 haplogroup U mtDNA coding sequences was built using MEGA2 (Kumar et al., 2001) (Fig. 1 in Appendix A). Eleven more recent sequences were manually added to the final tree (Fig. 1). Each mutation was positioned in this phylogenetic tree at the node that led to all haplotypes that shared the variant. For missense mutations, the mutant amino acid was defined as the one confined to the group of related haplotypes, while the wild type was the amino acid found in the rest of the global phylogeny. The standard amino acid conservation index (CI) was defined as the percentage of species from a list of 39 different animal species that have the wild type human amino acid at that position. This list included sequences from Mammals both Eutheria and Prototheria, representatives of the other vertebrate classes and *Drosophila*. (Ruiz-Pesini et al., 2004). The extended CI is the percentage of species with the wild type amino acid drawn from 10 widely divergent organisms (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Zea mays*, *Arabidopsis thaliana*, *Neurospora crassa*, *Saccharomyces cerevisiae*, *Rhodobacter spheroides* and *Paracoccus denitrificans*), some of them commonly used as experimental model to determine the physiological effects of pathological mtDNA mutations.

The functional consequence of missense mutations was evaluated by comparing the CI of the polymorphic amino acid with the average CI of 22 well characterized human pathogenic mutations [www.mitomap.org; (Ruiz-Pesini et al., 2004)]. The pathogenic mutations had CI of 93±13%. CIs within two standard deviations of this mean (the 95% confidence interval) were assumed to be potentially phenotypically relevant.

Analysis of seminal fluid and sperm motility, determination of the respiratory complex activities, and haplogroup characterization have been described (Ruiz-Pesini et al., 2000). Haplogroup U was divided into subhaplogroups by PCR/RFLP typing and by sequencing of the HVSI (Table 1 in Appendix A). Of the European haplogroup U studied, subhaplogroup U6 from North Africa (3 samples), U1 (2 samples) and non-defined haplotypes U (U*) (4 samples) were excluded. Several rare subhaplogroups in our sample

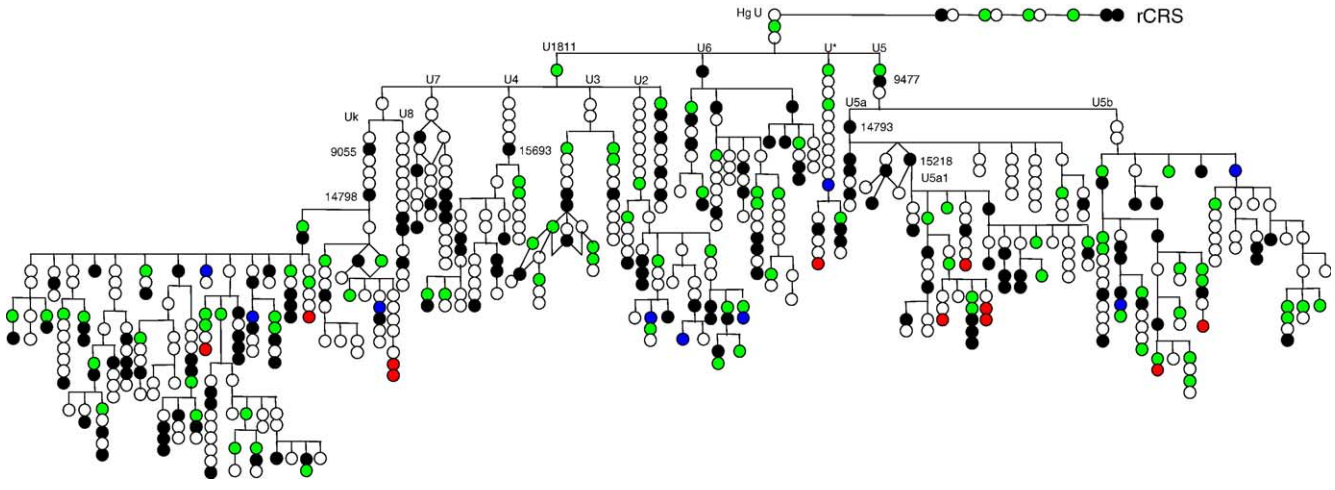

Fig. 1. Phylogenetic tree for the haplogroup U. Mutations described in the text are showed. Black, white, green, blue and red encode for non-synonymous, synonymous, RNA, non-coding in the coding region and pathological mutations. rCRS is the revised Cambridge reference sequence.

were grouped with phylogenetically related mtDNAs to provide sufficient numbers for statistical analysis.

Data from the literature was compiled on the European distribution of 1802 individuals harbouring haplogroup U mtDNAs subdivided for U subhaplogroups (see list of references in supplementary material). These studies encompass samples from Western, Central and Eastern Europe, both in North and South latitudes.

Fisher's exact tests (FET), ANOVA, Chi-Square and *t*-tests were performed by using the statistical program StatView. A *P* value lower than 0.05 was considered statistically significant.

3. Results

3.1. Subhaplogroups of U show differences in sperm phenotypes

The sequences of 199 haplogroup U mtDNAs encompassing 167 different mitochondrial coding region haplotypes were assembled and subjected to phylogenetic analysis. This permitted the positioning of all of the nucleotide substitutions found within haplogroup U mtDNAs into a branching tree (Ruiz-Pesini et al., 2004) (Fig. 1). European haplogroup U subdivided into two major subhaplogroups, U5 and U1811. Subhaplogroup U5 was founded by three nucleotide substitutions: nt 3197C/16S, nt 9477A/COIII and nt 13617C/ND5. The nt 13617C/ND5 variant is synonymous while that at nt 9477A in COIII is non-synonymous involving the replacement of a valine at codon 91 by an isoleucine (V91I). The CI of amino acid 91 in animals is 90%, comparable to the mean CI for pathological mutations (93%); while the CI for the full range of organisms from bacteria and yeast to man was 80%. Hence, this variant changes a highly conserved amino acid and is likely to be a functionally significant mutation (Fig. 2A). Subhaplogroup U1811 was founded by the substitution at nt 1811G in the 16S rRNA gene.

Semen samples were collected from 545 Spanish men and the mtDNA haplogroups determined. This revealed that 19.4% (106) belonged to haplogroup U (Ruiz-Pesini et al., 2000). Semen samples were evaluated for sperm motility and vitality, as well as a number of other seminal fluid parameters, and subdivided into U5 and U1811. In 97 semen samples sperm motility was quantitatively evaluated. Samples with <50% actively swimming (progressive) spermatozoa were considered to have low spermatozoa motility and classified as asthenozoospermic (AST). Samples with ≥50% progressive sperm were considered to have normal motility and were classified as non-asthenozoospermic (NAST) (WHO, 1992). By this criterion, haplogroup U5 had significantly more active spermatozoa than did subhaplogroup U1811. The percentage of progressive sperm was 46% for U5 and 31% for U1811 ($P=0.003$, *t*-test) (Fig. 2C), and the AST/NAST ratio for sperm samples from subhaplogroup U5 was lower than that for U1811 ($P=0.029$, FET) (Fig. 2B).

Sperm vitality was determined by dye exclusion. This parameter reflects the plasma membrane integrity and the

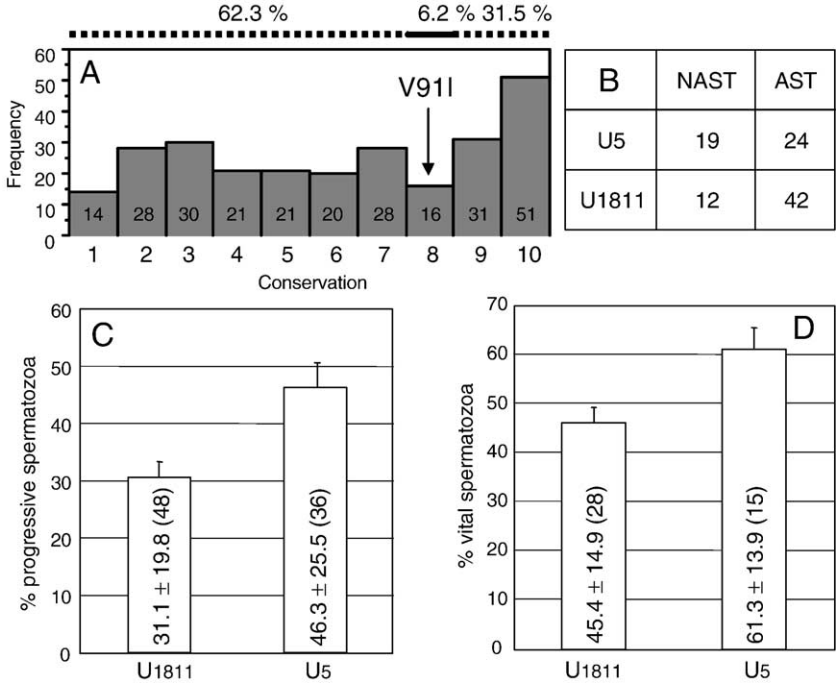

Fig. 2. Sperm motility and vitality variation within the subhaplogroups U. (A) Conservation of the COIII V91 amino acid. This position is conserved in 8 out of 10 (80%) species phylogenetically distant and commonly used in genetic studies (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Zea mays*, *Arabidopsis thaliana*, *Neurospora crassa*, *Saccharomyces cerevisiae*, *Rhodobacter spheroids* and *Paracoccus denitrificans*). This degree of conservation is higher than the conservation mean of 260 amino acid positions of the COIII protein that was 60.8%±30.2. Only 31.5% of the amino acid positions are more conserved than V91. (B) Sample distribution by motility phenotype and subhaplogroup U. AST and NAST encode for asthenozoospermic and non-asthenozoospermic individuals. Percentage of sperm motility (C) and vitality (D) in both subhaplogroups U. Numbers are means, standard deviations and sample sizes. Error bars represent the standard error.

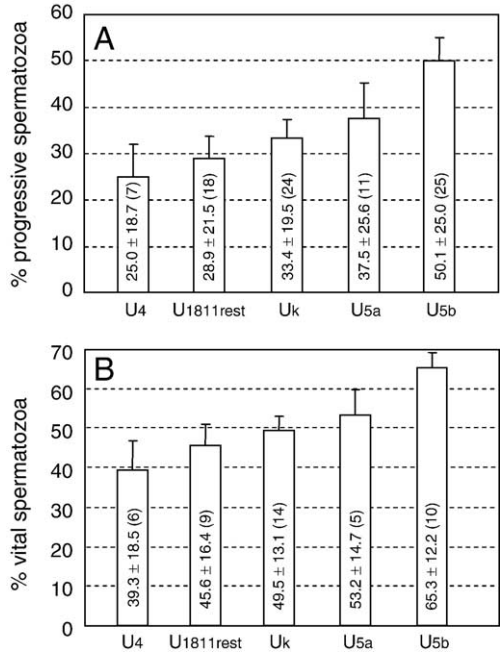

Fig. 3. Percentage of sperm motility (A) and vitality (B) in sublineages U. Numbers are means, standard deviations and sample sizes. Error bars represent the standard error.

membrane ATPase activities (WHO, 1992). Therefore, it can also be influenced by mitochondrial energy production. Again, the percentage of viable sperm was significantly higher in the U5 sperm samples (61%) than in the U1811 sperm samples (45%) ($P=0.002$, t-test) (Fig. 2D). Therefore, these parameters indicate that sperm from the U5 haplogroup harbouring the COIII V91I variant are more robust than those from the U1811.

To further investigate the genotypic basis for the phenotypic differences between U5 and U1811 spermatozoa, we characterized the potentially functional variants in subbranches of U5 and U1811. U5 subdivides into two primary branches: U5a and U5b (Achilli et al., 2005) (Fig. 1). U5a was founded by one cytb missense mutation: nt 14793G/H16R (CI=54%) and the most abundant U5a sublineage, U5a1 has another cytb mutation nt 15218G/T158A (CI=79%). The remaining U5 mtDNAs occupy a separate branch, U5b defined by two synonymous variants.

The U1811 lineage divides into several sublineages, including U4, Uk [previously defined as K haplogroup, (Torroni et al., 1996) and U1811rest, the later incorporating several small lineages. The U4 sublineage is founded by four synonymous variants and a cytb missense mutation at nt 15693C/M316T (CI=74%). The Uk sublineage is founded by four synonymous variants and two non-synonymous mutations: a cytb mutation at nt 14798C/F18L (CI=77%) and an ATP6 mutation at nt 9055A/A177T (CI=85%). The U1811rest sublineages lack highly conserved nodal missense mutations.

3.2. Haplogroup U sublineages differ significantly in sperm parameters

Comparison of the sperm motility and vitality between the different U sublineages revealed statistically significant differences in both motility ($F=3.37$, $P=0.013$) and vitality ($F=3.75$, $P=0.011$; ANOVA) (Fig. 3). The U5 mtDNA haplotypes with only the COIII mutation (U5b) showed the highest sperm motility and vitality: 50% motility and 65% viability. The U5a sublineage was 12% lower for both parameters. The sublineages of U1811 were all lower than the U5 lineages, again confirming that the COIII nt 9477G mutation has a positive effect on ATP production. Within the U1811 sublineages, the rank order of sperm motility and vitality and therefore probable mitochondrial ATP production was Uk,

Table 1
Seminal parameters in the U sublineages

	U4	U1811rest	Uk	U5a	U5b	*P*
Age (years)	34.2±4.0 (6)	32.8±5.6 (19)	31.7±9.0 (17)	33.3±4.4 (8)	33.1±6.2 (24)	0.933
Preanalytic time (min)	46.7±10.8 (6)	51.5±28.2 (10)	48.2±17.3 (15)	45.4±9.8 (6)	50.3±26.5 (10)	0.978
Seminal volume (mL)	4.1±3.3 (9)	3.5±2.0 (19)	3.3±2.0 (26)	3.2±1.5 (11)	3.1±1.5 (28)	0.742
Seminal pH	7.79±0.39 (7)	7.75±0.35 (10)	7.75±0.34 (19)	7.78±0.24 (9)	7.73±0.34 (13)	0.994
Prostatic markers						
Citric acid (mg/dL)	481±210 (6)	443±354 (8)	477±126 (14)	404±79 (5)	526±271 (8)	0.897
Zn (mg/dL)	9.4±4.1 (6)	11.3±8.6 (7)	9.7±2.8 (14)	7.4±1.3 (5)	10.3±6.6 (8)	0.772
Mg (mg/dL)	9.9±6.2 (6)	8.3±5.9 (8)	7.6±3.9 (14)	6.9±2.4 (5)	8.2±4.5 (8)	0.855
Ca (mg/dL)	33.5±5.6 (6)	32.9±15.7 (8)	33.5±10.4 (14)	33.3±11.0 (5)	37.2±20.0 (8)	0.967
Seminal vesicle marker						
Fructose (mg/dL)	341±92 (6)	296±135 (8)	325±117 (14)	207±113 (5)	248±63 (8)	0.168
Epididimal marker						
Carnitine (μM)	399±205 (6)	340±144 (6)	493±202 (14)	390±227 (5)	428±269 (8)	0.635
Cellular parameters						
Ejaculated sperm ($\times 10^6$)	96±60 (9)	160±174 (19)	135±129 (26)	117±89 (11)	186±217 (28)	0.543
Abnormal sperm (%)	72.0±15.2 (6)	50.0±14.4 (10)	50.6±16.8 (18)	40.6±18.2 (5)	56.8±22.1 (10)	0.046
Round cells (%)	21.2±11.0 (7)	13.4±10.9 (10)	24.5±27.9 (17)	7.4±3.3 (6)	12.6±11.7 (9)	0.256

Mean, standard deviation and sample size are given for each one. The *P* value was obtained by ANOVA.

U1811rest, and U4. U4 which harbours the cytb nt 15693C mutation had the lowest sperm motility and vitality, between 4% and 10% below those of Uk and U1811rest (Fig. 3). Uk with the cytb mutation but also a variant in ATP6 or U1811rest without mutations shows intermediate motility and vitality values.

Previously, the motility of sperm harbouring haplogroup T mtDNAs was found to be significantly slower than those with haplogroup H (Ruiz-Pesini et al., 2000). Comparing the present haplogroup U sublineages with H and T revealed that the motility of U5b sperm was significantly higher than that of haplogroup H ($P=0.031$, *t*-test) while the motility of U4 was lower than haplogroup T ($P=0.05$, *t*-test). Similarly, the vitality of subhaplogroup U5b sperm was significantly higher than that found in haplogroup H ($P=0.001$, *t*-test) while that of subhaplogroup U4 sperm was lower than T.

3.3. Sperm and seminal fluid variables not associated with mtDNA subhaplogroup

Most of the seminal parameters of the haplogroup U donors did not vary according to the subhaplogroup classification. These included age of the donor, time between sample collection and analysis, difference in ejaculated volumes, pH, and cellular composition of the semen (total number of ejaculated spermatozoa, percentage of round cells, and percentage of morphologically abnormal spermatozoa). This was also true for several biochemical parameters including markers for the prostatic function such as citric acid, zinc, magnesium and calcium concentrations; markers for seminal vesicle function including fructose concentration; and for epididymal function including carnitine concentration (Table 1). This observation discards any bias in the sampling of the U-harbouring semen samples assayed for this study.

To determine if the mtDNA polymorphisms correlated with differences in mitochondrial electron transport chain enzyme activity, we assayed sperm homogenates from 45 out of the 97 motility evaluated semen samples for complex I, complexes I +III, and for complex IV. No significant differences were found in specific activity in these assays.

3.4. European distribution of haplogroup U sublineages

If these variants in haplogroup U became established as an adaptation to the increased cold of the more northern latitudes, then they should be more prevalent in northern latitudes. Therefore, we surveyed the northern versus southern European distribution of the cytb missense mutation-containing haplogroup U sublineages.

Data from the literature was compiled on the European distribution of 1802 individuals harbouring haplogroup U mtDNAs (Table 2). This revealed a highly significant north–south geographic distribution of different haplogroup U sublineages ($\chi^2=109.6$, $df=5$, $P\leq 0.001$). For the U5 subhaplogroup, the U5a sublineage with cytb mutations was more prevalent in northern Europe (ratio=1.9), while the U5b sublineage without cytb mutations was more common in southern Europe (ratio=0.7). Of the U1811 subhaplogroup, Uk and U1811rest were equally distributed in both latitudes (ratio=0.9 and 1.0, respectively), but the U4 sublineage was enriched in the north (ratio=1.9). Moreover, the frequency of this subhaplogroup, as well as its proportion in the haplogroup U, increased eastwards, reaching maximum values in the populations of Northwest Siberia (Malyarchuk, 2004). Thus the combination of the cytb mutation-containing sublineages U4, Uk and U5a was in substantial excess in northern Europe.

4. Discussion

The uniquely robust motility of the sperm from U5 individuals correlates with the highly conserved COIII V91I variant at nt 9477A. It has been proposed that the COIII subunit maintains a rapid proton uptake into the D pathway and contributes to the conformation of the normal proton exit pathway (Hosler, 2004). The COIII V91I variant is located in the third transmembrane helix in contact with subunit I. Moreover, with its CI of 80%, in very divergent species, the V91I variant changes a more functionally constrained amino acid than the G9804A/A200T mutation (CI=70%). This mutation, when modelled in *P. denitrificans,* reduced growth yield, proton pumping stoichiometry, and the magnitude of membrane potential generated by the cytochrome oxidase (Mather and Rottenberg, 1998). Similarly, a replacement mutation at amino acid 203 in the yeast COIII gene, human amino acid 195, CI=70% abolishes respiratory growth and decreased the cytochrome *c* oxidase content (Meunier and Taanman, 2002).

The cytb missense mutations in sublineages U5a, U4 and Uk are comparably functionally important. Complex III pumps protons out of the mitochondrial inner membrane via the Q cycle and a reduction in the proton gradient would reduce the inner membrane potential and this has already been linked with a lower sperm motility (Troiano et al., 1998).

If these interpretations are correct it could account for the absence of significant differences in the specific activities of the

Table 2
Distribution of sublineages U according to the latitude

	U	Urest	U4	U1811rest	Uk	U5a	U5b
N	1214 (100)	52 (4.8)	235 (19.4)	130 (10.7)	280 (23.1)	309 (25.5)	208 (17.1)
S	588 (100)	78 (13.3)	59 (10.0)	63 (10.7)	156 (26.5)	79 (13.4)	153 (26.0)
N/S	1.0	0.4	1.9	1.0	0.9	1.9	0.7

Southeast and Mediterranean Europe is considered South (S) and North (N) is the rest of Europe. Percentages are given in brackets. U1811rest contains sublineages U1811 without well conserved missense mutations (U2, U3, U7 and U8). Urest contains subhaplogroups U without well conserved missense mutations (U1, U6 and U*). For references, see online material and supplementary bibliography.

ETC enzymes among the U sublineages, since these assays only test for the rate of electron flux through the complex and do not register changes in proton pumping efficiency.

Correlation of the amino acid substitution positions of the cytochrome *b* mutations with the crystal structure of complex III suggests that the potential adaptive variations are wholly functionally significant. The nt 15218G/T158A variant of U5a1 is located in the vise region of cytb that interacts with the hinge region of the Rieske iron-sulfur protein (ISP) (Crofts et al., 1999). The nt 14798C/F18L variant, defining Uk, is located in the cytb inner Q binding (Qi) site (Fisher and Rich, 2000) and affects the susceptibility to diuron, a respiratory chain inhibitor (di Rago and Colson, 1988).

If these variants in haplogroup U became established as an adaptation to the increased cold of the more northern latitudes, then they should be more prevalent in northern latitudes. Therefore, we surveyed the northern versus southern European distribution of the cytb missense mutation-containing haplogroup U sublineages.

Cytb mutations have been found at the base of several temperate and arctic mtDNA lineages, with cytb mutations being most prevalent in European mtDNAs (Wallace, 1994; Wallace et al., 1999, 2003; Mishmar et al., 2003; Ruiz-Pesini et al., 2004). Moreover, the cytb gene in European mtDNAs has a higher NS/S ratio than African mtDNAs, consistent with adaptive selection on this gene (Elson et al., 2004). Therefore, shifting the energy balance from primarily ATP production to increased heat production could explain the lower sperm motility and the predilection of these sublineages U to reside in colder climates and their northern distribution.

The functional importance of these and related nodal mtDNA mutations have been demonstrated for LHON. The more severe pathogenic mutations can cause blindness regardless of the background mtDNA haplotype. However, the milder LHON mutations mainly result in blindness when paired with the adaptive cytb haplogroup J variants at nt 14798C and 15257A (Brown et al., 1997; Torroni et al., 1997). These observations are consistent with blindness being manifest when the ATP production falls below some minimal threshold. In the case of the mildest pathogenic mutations, the reduction in ATP production is insufficient by itself to cause the phenotype. However, when combined with the reduced ATP producing capacity of the uncoupling adaptive mutations, the combined ATP deficiency is sufficient to develop the disease.

It has been also described that these proposed uncoupling mtDNA haplogroups are protective of Alzheimer's and Parkinson's diseases and associated with longevity (Ivanova et al., 1998; De Benedictis et al., 1999; Carrieri et al., 2001; Ross et al., 2001; Bonafe et al., 2002; Coskun et al., 2003; Niemi et al., 2003; van der Walt et al., 2003, 2004; Ghezzi et al., 2005; Pyle et al., 2005). These results have been interpreted as the uncoupling mutations keeping the ETC chronically more oxidized, thus decreasing mitochondrial ROS production and the probability of mtPTP activation and loss of neurons by apoptosis.

In summary, the results presented here show that several sublineages of haplogroup U were found to be associated with differences in sperm motility and vitality. These differences could be related to highly conserved mutations in the mtDNA COIII and several equally conserved mutations in the cytb. The lineages with the cytb mutations were substantially enriched in northern Europe, while those lacking these mutations were more prevalent in southern Europe. We suggest that some of these ancient conserved cytb mutations permitted our ancestors to adapt to cold by partially uncoupling mitochondrial OXPHOS but producing a lesser sperm motility.

Acknowledgements

This work was supported by NIH grants AG24373 and NS21328 and an Ellison Medical Foundation Senior Investigator Award awarded to DCW, and by the Spanish Fondo de Investigaciones Sanitarias (FIS). FMS was recipient of a CONACYT (Mexico) fellowship and MJLP, JAE and JM belong to Redemeth and Mitoespaña networks of FIS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.09. 015.

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