Background sequence characteristics influence the occurrence and severity of disease-causing mtDNA mutations

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Abstract

Inherited mitochondrial DNA (mtDNA) mutations have emerged as a common cause of human disease, with mutations occurring multiple times in the world population. The clinical presentation of three pathogenic mtDNA mutations is strongly associated with a background mtDNA haplogroup, but it is not clear whether this is limited to a handful of examples or is a more general phenomenon. To address this, we determined the characteristics of 30,506 mtDNA sequences sampled globally. After performing several quality control steps, we ascribed an established pathogenicity score to the major alleles for each sequence. The mean pathogenicity score for known disease-causing mutations was significantly different between mtDNA macro-haplogroups. Several mutations were observed across all haplogroup backgrounds, whereas others were only observed on specific clades. In some instances this reflected a founder effect, but in others, the mutation recurred but only within the same phylogenetic cluster. Sequence diversity estimates showed that disease-causing mutations were more frequent on young sequences, and genomes with two or more disease-causing mutations were more common than expected by chance. These findings implicate the mtDNA background more generally in recurrent mutation events that have been purified through natural selection in older populations. This provides an explanation for the low frequency of mtDNA disease reported in specific ethnic groups.

Author summary

MtDNA mutations are a major cause of genetic disease. Many of these variants have recurred several times in different populations and on diverse haplogroup backgrounds, but the clinical presentation of mutations causing Leber Hereditary Optic Neuropathy (LHON: m.14484T>C, m.3460G>A, m.11778G>A) are strongly associated with a specific mtDNA haplogroup. This raises the possibility that many pathogenic mtDNA mutations are subject to the same effects. Here, our analysis of 30,506 human mtDNA sequences shows that the association between disease-causing mtDNA mutations and...
background mtDNA haplogroups is not only restricted to three disease-causing mtDNA mutations known to cause LHON. The frequent recurrence of the same mutations on a population clade, and the reduced frequency of European mtDNAs harboring two or more diseases-causing mutations, suggest that the population mtDNA background influences the risk of developing mtDNA mutations. Our analysis also shows that disease-causing mtDNA mutations also occur more frequently on younger mtDNAs. This implies that, once formed, the mutations are selected against. These findings indicate that the clinical interpretation of mtDNA variants should be performed within an ethnogeographic context.

**Introduction**

Human mitochondrial DNA (mtDNA) is exclusively maternally inherited and undergoes negligible recombination at the population level. As humans emerged from Africa and populated the globe, different sub-populations acquired single nucleotide variants (SNVs) that define geographically-restricted mtDNA ‘haplogroups’[1]. Some rare genetic variants have emerged as a common cause of inherited metabolic disease, affecting 1 in 10,000 of the population[2]. Many of these variants have recurred several times in different populations and on diverse haplogroup backgrounds, but the clinical presentation of mutations causing Leber Hereditary Optic Neuropathy (LHON: m.14484T>C, m.3460G>A, m.11778G>A) are strongly associated with a specific mtDNA haplogroup[3, 4]. This raises the possibility that many (but not all[5]) pathogenic mtDNA mutations are subject to the same effects. This has not been systematically studied to date, in large part because of the limited number of sequences available for analysis.

**Results**

**Data description and quality control**

30,506 mtDNA sequences were downloaded from GenBank ([S1 Table](#) and [S2 Table](#)). These included 3,852 sequences from macro-haplogroups L, 6,202 from M and 20,452 from N. In 17,815 of the 30,506 mtDNA sequences it was possible to identify all known appropriate haplogroup markers down to the sub-haplogroup level [6, 7] ([http://www.phylotree.org/tree/index.htm](http://www.phylotree.org/tree/index.htm)) (referred to as ‘sub-haplogroup tagged’ [S1 Table](#)). Next we compared the frequency of the variants to the remaining 12,691 sequences (referred to as non-sub-haplogroup tagged), stratifying for each macro-haplogroup (2,710 sequences from L, 2,619 from M, and 7,362 from N; [S1 Table](#)). The allele frequencies were strongly correlated between the two groups (R² = 0.842, p-value < 2.2e-16 for macro-haplogroup L; R² = 0.968, p-value < 2.2e-16 for macro-haplogroup M; R² = 0.988, p-value < 2.2e-16 for macro-haplogroup N, Person’s correlation test; [Fig 1A](#), [S1A Fig](#)).

Next we compared the allele frequencies in the 17,790 European haplogroup N(R) sequences to an independent sample of 9935 population controls from the Wellcome Trust Case Control Consortium [8]. Allele frequencies from the 59 mtDNA variants present in both datasets were highly correlated (R² = 0.953, p-value < 2.2e-16, Person’s correlation test; [Fig 1B](#)). We then compared 30,506 mtDNA sequences with 1,370 mtDNA sequences from 1000 Genome Project (see Methods), which includes mtDNA sequences from across the world (462 from macro-haplogroup L, 271 from M, and 637 from N). The allele frequencies of the mtDNA variants were highly correlated between two datasets for each macro-haplogroup (R² = 0.941, p-value < 2.2e-16 for macro-haplogroup L; R² = 0.989, p-value < 2.2e-16 for macro-haplogroup M; R² = 0.985, p-value < 2.2e-16 for macro-haplogroup N; Person’s correlation test; [Fig 1C](#)).

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Further evidence of quality control came from an independent comparison of the GenBank dataset to 1,125 published global human mtDNA sequences from the literature [9], where the ratios of non-synonymous variants (NS) and synonymous variants (SS) within major haplogroups were no different (p-value > 0.05, Fisher test; Fig 1D). Finally the allele frequencies of common disease-causing mutations were no different to previously published values determined through a population-based study in sequential healthy live-births in Europeans [10].
Fisher test; Fig 1E)[10], providing further evidence that our dataset is not significantly enriched with sequences derived from patients with inherited mtDNA diseases in the European population. A similar comparison was not currently possible for African and Asian mtDNAs. However, we saw no relationship the pathogenicity score of specific alleles and the difference in allele frequency and sub-haplogroup tagged vs. non-sub-haplogroup tagged mtDNAs in our data set (See Methods for an explanation of pathogenicity scores, S1B Fig). When taken together, these analysis (Fig 1A–1E), indicate that the 30,506 GenBank sequences are a representative sample of the background populations and not significantly enriched for rare population variants or pathogenic mutations identified through patients presenting with mitochondrial diseases.

Determining the disease caused mutations

The 30,506 mtDNA sequences were predicted to cause 10,166 unique single amino acid substitutions (L = 3,732, M = 4,887, N = 8,427, Fig 2A). There was no difference in the frequency distribution for mtDNA variants between the macro-haplogroups (p-value = 1, ANOVA test; Fig 2B). Next we determined which variants were likely to be pathogenic. Ninety of the 202 proposed pathogenic mtDNA variants listed on the ClinVar database[11] were found in the 30,506 mtDNA sequences studied here (S3 Table). Incorporating published and on-line evidence of pathogenicity (see Methods), the number of likely pathogenic mutations reduced from 90 to 57(S4 Table).

Pathogenicity of variants in macro-haplogroups L, M and N

We initially focused on the pathogenicity of all variants within protein-encoding genes. 2,709 variants were present in 30,506 sequences, including 34 disease-causing mutations (S4 Table). As expected, variants with a higher pathogenicity score were less frequent in the population, and disease-causing mutations with a higher pathogenicity score were less frequent in the population (Fig 2C). Likewise, as expected, the probability distribution of pathogenicity scores for disease-causing mutations was significantly greater than the overall distribution of pathogenicity scores in the entire population (mean = 0.59 for disease-causing mutations, mean = 0.37 for all possible variants; two-sample t-test, p-value < 2.2e-16, Fig 2C). However, we were surprised to see that disease-causing mutations on the macro-haplogroup N and M backgrounds had significantly higher pathogenicity score than those occurring on the macro-haplogroup L background (mean = 0.47 for L group, mean = 0.62 for M group, mean = 0.59 for N group; L versus M: p-value = 1.959e-11, L versus N: p-value = 7.992e-08, M versus N: p-value = 8.967e-07, two-sample t test, Fig 2D). These differences could not be accounted for by the frequency of specific mutations, which did not follow the same trend (S4 Fig). 83(2.2%) mtDNA sequences carried at least one disease-causing mutation in L group, 481 (7.8%) in M group and 1,057 (5.2%) in N group. Group M sequences had a higher proportion of sequences carrying disease-causing mutations 481 (7.8%), followed by group N sequences 1,057 (5.2%), with the lowest in group L 83 (2.2%) (L versus M: p-value = 1.39e-36, L versus N: p-value = 9.49e-19, M versus N: p-value = 1.43e-13, Fisher test, Fig 2E).

Based on the overall frequency of disease causing mutations, we estimated the likelihood of a second mutation occurring by chance on the same genome (Fig 2F). For macro-haplogroups L and M, the observed frequency of mtDNAs with two of more disease-causing mutations was no different to the chance co-occurrence. However, for macro-haplogroup N sequences, the number of mtDNA sequences harboring two of more disease-causing mutations was 6-fold lower than expected by chance (p-value = 5.96e-09, Fisher test; Fig 2F). These observations implicate the mtDNA sequence background in the likelihood of acquiring or retaining a disease-causing mutation.
Background sequence characteristics influence mtDNA mutations
We then determined the frequency of the 57 disease-causing mutations on the 'major' haplogroups. Twenty-five (43.9%) of the 57 diseases-causing mutations were present on more than one macro-haplogroup, and 34 (59.6%) were present on more than one major haplogroup (Fig 3A, S5 Fig), indicating recurrent mutation events. 23 (40.4%) of the pathogenic mutations were only found on one haplogroup. Of these, m.4136A>G, m.4317A>G, m.7444G>A, m.11696G>A, and m.13637A>G were recurrent mutations based on analysis of the entire mtDNA sequence. This suggests that specific mtDNA mutations recur on specific mtDNA haplogroups, implying that the background haplogroup predisposes to the original mutation event, or the subsequent fixation of the allele on a particular maternal lineage. On the other hand, m.5631G>A (L), m.10663T>C (L), m.8313G>A (M), m.12770A>G (M), and m.3890G>A (N) were only seen on one clade, consistent with a single founder event. This raises the possibility that these several 'disease-causing' mutations are actually rare sub-haplogroup markers and not directly pathogenic (eg m.4295A>G, m.6489C>A). Overall, we found statistical evidence of haplogroup clustering for 10 (29.4%) of the 34 recurrent disease-causing mtDNA mutations after correcting for the sample size within each comparison (p-value < 0.01, Fisher test; Fig 3B).

Mutational signature

We then determined the relative frequency of mutations within all 96 possible flanking nucleotide triplets[12]. Overall, across all macro-haplogroups we saw a greater prominence of C>T/A>G and T>C/A>G substitutions for all variants (p<2.2e-16, Fisher test; Fig 4A–4C), and for disease causing mutations (p<2.2e-16, Fisher test; Fig 4D–4F). In order to confirm the prominence of C>T and T>C mutations observed were not due to the selection of the rCRS [13] as the "reference" by which the mutations are classified, we realigned 30,506 mtDNA sequences to Reconstructed Sapiens Reference Sequence (RSRS) [14]. We observed similar patterns, with T>C and C>T substitutions frequently observed (S6 Fig), suggesting the profiles of mutational signatures were not driven by the alignment process and are independent of the reference sequence used in the analysis.

We also observed that the higher mutability of CpG dinucleotides in disease-causing mutations when compared to all variants (CpG%, p-value = 0.00017, Fisher exact test; S7 Fig and S5 Table). Although the methylation of mtDNA appears to be rare in somatic tissues [15], nuclear genome methylation changes substantially during embryonic and germ-line development [16], raising the possibility that methylation of mtDNA CpGs during early development contributes to the origin of de novo mtDNA mutations [17]. Intriguingly, macro-haplogroup M had highest CpG% for all variants (19.93%; L versus M, p-value < 2.2e-16 and M versus N p-value < 2.2e-16 Fisher exact test) and defined diseases-causing mutations (21.21%; L versus M, p-value = 0.00049; and M versus N, p-value = 1.06e-06). By contrast, macro-haplogroup L
Background sequence characteristics influence mtDNA mutations
had much lower CpG% than other two groups for disease-causing mutations (5.88%; L versus M \( p \)-value = 0.00049 and L versus N \( p \)-value = 0.15) (S7 Fig and S6 Table). These findings add further weight to the hypothesis that haplogroup-specific sequence context influences the acquisition of mtDNA mutations.

The age of mtDNA sequences

We then estimated the age of the each mtDNA sequence based on the major haplogroup classification, using the Rho statistic[18]. Overall, variants with higher pathogenicity scores were found on ‘younger’ (more recent) mtDNA sequences (Fig 5A and 5B, \( p \)-value < 2.2e-16, Wilcoxon Rank test). Disease-causing mutations were also found on younger mtDNA sequences (Fig 5A, \( p \)-value = 0.019, Wilcoxon Rank test). When we studied the same relationship after removing the main pathogenic allele from each sequence in silico (Fig 5C and 5D), we did not observe an association between the remaining sequence and the age of predicted age of the sequence. This indicates that the association between disease-causing variants and the age of the mitochondrial genome is driven by the pathogenic variant itself and not the entire mtDNA sequence. Although it is possible that Rho dating may be inaccurate in the absence of population expansions [19], these observations are in keeping with the effects of natural selection acting on specific pathogenic variants.

The pathogenicity of mtDNA tRNAs

Finally, we estimated the pathogenicity scores for 207 variants in tRNA genes [20, 21]. Seventy-four tRNA variants were present in 30,506 sequences, including 16 previously defined disease-causing mutations (S4 Table). Consistent with the pathogenicity scores for non-synonymous variants, all tRNA variants and disease-causing tRNA mutations with a higher pathogenicity score were less frequent in the population (Fig 6A). Likewise, the probability distributions for disease-causing tRNA mutations was significantly greater than the overall distribution of pathogenicity scores in the entire population (mean = 0.427 for disease-causing tRNA mutations, mean = 0.102 for all possible tRNA variants; \( p \)-value < 2.2e-16, two-sample t test; Fig 6A). Again, we observed that disease-causing tRNA mutations on macro-haplogroups N and M had significantly higher pathogenicity score than those occurring on macro-haplogroup L (mean = 0.36 for L group, mean = 0.51 for M group, mean = 0.43 for N group; L versus M: \( p \)-value = 0.010, L versus N: \( p \)-value = 0.005, M versus N: \( p \)-value = 0.079, two-sample t test; Fig 6B). However, there was no correlation between either all tRNA variants or disease-causing tRNA mutations with the ages of the mtDNA sequences (Fig 6C and 6D). This could reflect the limited number of tRNA variants in the data set, or that the effects of selection cannot be detected for homoplasmic tRNA gene variants. This is in keeping with animal data, where there is severe selection against protein coding variants within one or two generations, but not tRNA variants[22].

Discussion

Our analysis of 30,506 human mtDNA sequences shows that the association between disease-causing mtDNA mutations and background mtDNA haplogroups is not only restricted to three disease-causing mtDNA mutations known to cause LHON. The frequent recurrence of
Fig 4. The mutational signatures observed in 30,506 mtDNA sequences. Each signature is displayed according to the 96 substitution classification defined by the substitution class and sequence context immediately 3' and 5' to the mutated base. The probability bars for the six types of substitutions are displayed in different colors and labeled at the top of the graph. The mutation types are on the horizontal axes at the bottom of the graph. (A) All possible variants in macro-haplogroup L. (B) All possible variants in macro-haplogroup M. (C) All possible variants in macro-haplogroup N. (D) All diseases-causing mutations in macro-haplogroup L. (E) All diseases-causing mutations in macro-haplogroup M. (F) All diseases-causing mutations in macro-haplogroup N. The arrows highlight the variants in CpG dinucleotides.

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the same mutations on a population clade, and the reduced frequency of European mtDNAs harboring two or more diseases-causing mutation, both suggest that the population mtDNA background influences the risk of developing mtDNA mutagenesis. Although it is conceivable that this is due to the functional consequences of haplogroup variants, perhaps though the generation of increased oxygen free radicals, or the segregation of heteroplasmic variants, our analysis indicates that this is unlikely because we also saw an association of mutations with underlying sequence characteristics (such as CpG%) that are not directly linked to oxidative phosphorylation or heteroplasmy segregation.

Our analysis also shows that disease-causing mtDNA mutations also occur more frequently on younger mtDNAs. This implies that, once formed, the mutations are selected against. Although tempting to think that this pattern simply reflects an ascertainment bias related to the number of defined disease-causing mtDNA mutations in populations of European origin, the data suggests that this is not the case. Firstly, the majority of mtDNA mutations were not restricted to specific continental populations; and secondly a greater proportion of known disease-causing mutations were seen in the sequences derived from the Asian population (Fig 3 and S5 Fig). To our mind, the simplest explanation is that the more severe homoplasmic mutations have been removed from the older populations through natural selection. A similar pattern has been noted previously in older African mtDNA sequences[23], which have been exposed to the effects of purifying selection for a longer period of time. This further endorses the validity of our dataset and analysis. Thus, both a predilection to mutate and natural selection explain the associations between different mtDNA haplogroups and known-pathogenic
mutations, thereby explaining why different ethnic groups are more or less likely to present with a specific mtDNA disease. However, it is important to note that other population genetic effects could have influenced the current mtDNA landscape. For example, recent ancient mtDNA studies point to a European population bottleneck ~14,500 years ago[24], which could also explain the relative lack of evidence of selection in extant Europeans.

Our study was restricted to the major alleles within each mtDNA sequence, and not designed to study the effects of mtDNA heteroplasmy. The prospective analysis of mtDNA heteroplasmy and the segregation of heteroplasmic variants in the population adds a further complexity, but might cast further light on the mechanisms we propose. However, we did observe common heteroplasmic mtDNAs within the dataset across all populations (eg. m.3243A>G), and these variants followed the same overall patterns described here. In addition, no phenotypic data was available. Although a limitation, even without this information we can draw some conclusions. First, despite the well-established strong association between LHON families harboring m.3460G>A, m.11778G>A and m.14484T>C and mtDNA haplogroup J, at the population level, these variants are found across a broad range of mtDNA haplogroups in all three continental groups (Fig 3). In this study, m.14484T>C was found on multiple haplogroups, with an allele frequency up to 1.5% on haplogroup M(G). This contrasts sharply with the almost exclusive association of m.14484T>C with haplogroup J described in epidemiological studies of LHON[3, 4]. This strongly supports the view that haplogroup J modifies the
clinical penetrance of the m.3460G>A, m.11778G>A, and m.14484T>C mutations, and that
the association is not due to an increased mutation rate. Although we cannot be certain, this
indicates that the mutation has a reduced penetrance in certain contexts, perhaps related to
the nuclear genetic background or environmental factors. Whichever is the case, our findings
indicate that the clinical interpretation of mtDNA variants should be performed within an eth-
nogeographic context.

Materials and methods
mtDNA sequence source
30,619 human mtDNA sequences sampled from across the globe downloaded from GenBank
(https://www.ncbi.nlm.nih.gov/genbank/). 113 were removed due to poor sequence quality.
30,506 sequences were included in this study and aligned to Revised Cambridge Reference
Sequence (rCRS)[13] and Reconstructed Sapiens Reference Sequence (RSRS)[14] using
BLASTn (S1 Table).

Phylogenetic analysis
Haplogroup assignment was performed using HaploGrep 2[7]. Sequences were classified into
macro-haplogroups (L, M and N), major and sub-haplogroups based on the mtDNA phyloge-
netic tree[6] (http://www.phylotree.org/tree/index.htm) (S2 Table), including 3,852, 6,202
samples and 20,452 samples, respectively. Coalescence times were estimated using the ρ statis-
tic (average distance of the haplotypes of a clade from the respective root haplotype) accompa-
nied by a heuristic estimate of the standard error (σ) calculated from an estimate of the
genealogy[18].

Sequence quality control datasets
Sub-haplogroup tagged mtDNA sequences in GenBank database. We selected the
‘sub-haplogroup tagged’ mtDNA sequences it was possible to identify all known appropriate
haplogroup markers down to the sub-haplogroup level [6, 7] (http://www.phylotree.org/tree/
index.htm), including 1,142 sequences from macro-haplogroups L, 3,583 from M and 13,090
from N(S1 Table). Next we compared the frequencies of the variants to the remaining 12,691
sequences (non- sub-haplogroup tagged), stratifying for each macro-haplogroup (2,710
sequences from L, 2,619 from M and 7,362 from N; S1 Table). The allele frequency (AF) differ-
ence of each variant between two groups was calculated as log2 (ratio of frequencies from two
groups).

mtDNA sequences in 1000 genome project. mtDNA sequences from 2,182 samples were
downloaded from 1000 Genome Project (http://www.internationalgenome.org/). mtDNA
haplogroup of each sequence was predicted using HaploGrep 2[7] and mt-classifier[21]. We
excluded the low quality mtDNA sequences using the following criteria: 1) when <90% of the
known haplogroup markers were present for each mtDNA sequence; 2) when more than one
haplogroup prediction was reported; 3) when the haplogroups predicted by two prediction
tools were inconsistent. There were 1,370 mtDNA sequences, including 462 sequences from
macro-haplogroups L, 271 from M and 637 from N for the further QC analysis.

Other published mtDNA sequences. We compared the ratio of non-synonymous to syn-
onymous substitutions of 3,852 mtDNA sequences in macro-haplogroup L, 6,202 in M and
2,662 in N (nonR) from this study to 1,125 published global human mtDNA sequences (445
mtDNA sequences in macro-haplogroup L, 239 in M and 199 in N (nonR))[9], across the
entire mtDNA phylogenetic tree. For the 17,790 European haplogroup N(R) sequences we also
compared the frequency of 59 mtDNA variants to 9,935 population controls from the Wellcome Trust Case Control Consortium[8].

**Determining the disease caused mutations**

We determined which variants were likely to be pathogenic. We listed the variants which were present in 30,506 mtDNA sequences and also proposed pathogenic mtDNA variants on the ClinVar database[11]. We did not include data where two or more nucleotide variations and Indels were listed (S4 Table). We then reviewed published and on-line evidence of pathogenicity for each one of the 90 variants using the following criteria: (1) Reported more than once with mtDNA disease and/or (2) Documented heteroplasmy, with evidence of segregation with the phenotype at the clinical or biochemical level.

**Pathogenicity measure**

We followed the approach first described by Pereira et al. which was based on MutPred software applied to the 13 protein coding genes of mtDNA [25–27]. The details and a list of pathogenicity scores for 24,206 possible amino acid variations are available in Table S3 in Pereira et al. [27]. The pathogenicity scores of variants in tRNA genes were retrieved from the literature [20, 21].

**Mutational signature**

Mutational spectra were derived directly from the both rCRS [13] and RSRS mtDNA[14] reference sequences and alternative alleles at each variant site. The resulting spectra are composed of the relative frequencies of the six distinguishable point mutations (C:G>T:A, T:A>C:G, C:G>A:T, C:G>G:C, T:A>A:T and T:A>G:T). Each signature was displayed using a 96 substitution classification defined by the substitution class and the sequence context immediately 3’ and 5’ to the mutated base[12].

**Statistical analysis**

The p-values for all comparisons of mean values were calculated by using two-sample t tests or Wilcoxon Rank test as appropriate. Variant counts or fractions were performed using Fisher’s exact test. Pearson’s correlation coefficient (R²) and the significance were calculated using the correlation test. All statistical analyses were performed using R (v3.3) (https://www.r-project.org).

**Data availability**

The mtDNA sequences that support the findings of this study are available in Genbank: https://www.ncbi.nlm.nih.gov/genbank/. Accession numbers are supplied in S1 Table. No other data is generated specifically in this study.

**Supporting information**

S1 Fig. Correlation of allele frequency (AFs) of the variants between 17,815 sub-haplogroup tagged mtDNA sequences and 12,691 non sub-haplogroup tagged sequences in NCBI database. (a) The allele frequencies of variants in 12,691 non sub-haplogroup tagged sequences were highly correlated with 17,815 sub-haplogroup tagged sequences within each marco-haplogroup. AFs were shown in log2(AF/1-AF). (b) There was no relationship between the AF difference of sub-haplogroup tagged sequences(AF_tagged) and non sub-haplogroup tagged sequences.
(AF_non-tagged) with the pathogenic scores of variants in each macro-haplogroup.

(S2) Fig. Correlation of allele frequency (AFs) of the variants between 1000 Genome Project and 30,506 NCBI mtDNA sequences, AFs were shown in log2(AF/1-AF). The allele frequencies of variants in 1000 Genome Project were highly correlated with 30,506 NCBI mtDNA sequences within each macro-haplogroup. L, M and N groups were shown, respectively.

(S3) Fig. Correlation of allele frequencies (AFs) of non haplogroup makers between 1000 Genome Project and 30,506 NCBI mtDNA sequences, AFs were shown in log2(AF/1-AF). Macro-haplogroup L, M and N were shown in different colors, respectively.

(S4) Fig. Correlation of pathogenicity score between polymorphisms and disease-causing mutations. (a) The correlation of mean pathogenicity score between all variants of each mtDNA sequence and only disease-causing mutations of each mtDNA sequence. (b) The correlation of mean pathogenicity score between non-disease-causing mutations of each mtDNA sequence and only disease-causing mutations of each mtDNA sequence.

(S5) Fig. Comparison of the presence of specific disease causing mtDNA mutations in each macro-haplogroup. Columns left to right: mtDNA variant; mutations specific to macro-haplogroup L (blue); mutations specific to macro-haplogroup M (pink); mutations specific to macro-haplogroup N (green); mutations found on all three macro-haplogroups L,M&N (purple); mutations found on more than one macro-haplogroup (yellow); mutations found on one macro-haplogroup (violet). No = number of variants found in each category.

(S6) Fig. The categories of mutational signatures observed in 30,506 mtDNA sequences. 30,506 mtDNA sequences were realigned against Reconstructed Sapiens Reference Sequence (RSRS). The probability bars for the six types of substitutions (C>A, C>G, C>T, T>A, T>C and T>G) are displayed within each macro-haplogroup.

(S7) Fig. Comparison of the distribution of CpG% (%C>T) in macro-haplogroups. (a) Distribution of CpG% (%C>T) in all possible variants. (b) distribution of CpG% (%C>T) in disease-causing mutations.

(S1) Table. The mtDNA sequences IDs and haplogroup of each sequence used in this study. 17,815 of the 30,506 mtDNA sequences which was possible to identify all known appropriate haplogroup markers down to the sub-haplogroup level were labeled as sub-haplogroup tagged in the QC column.

(S2) Table. Sample size of each haplogroup and macro-haplogroup.

(S3) Table. The details of 202 reported pathogenic variations in ClinVar.

(S4) Table. The details of 57 defined disease-causing mutations in 30,506 mtDNA sequences.
S5 Table. **Comparison of the CpG% between all possible variants and disease-causing mutations for the entire population and each group.** The percentage of variants in CpG region and the p-value for Fisher’s exact test—all possible variants vs all disease-causing mutations in the entire population and each group were shown and calculated.

S6 Table. **Comparison of the CpG% between any two of three groups.** The p-value for Fisher’s exact test—each group vs the rest of groups were shown and calculated.

**Author Contributions**

**Conceptualization:** Patrick F. Chinnery.

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**Formal analysis:** Wei Wei.

**Funding acquisition:** Patrick F. Chinnery.

**Project administration:** Patrick F. Chinnery.

**Supervision:** Patrick F. Chinnery.

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**References**

1. Stewart JB, Chinnery PF. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. Nat Rev Genet. 2015; 16(9):530–42. https://doi.org/10.1038/nrg3966 PMID: 26281784.

2. DiMauro S, Schon EA, Carelli V, Hirano M. The clinical maze of mitochondrial neurology. Nat Rev Neurol. 2013; 9(8):429–44. https://doi.org/10.1038/nrneurol.2013.126 PMID: 23835535; PubMed Central PMCID: PMCPMC3959773.

3. Brown MD, Torroni A, Reckord CL, Wallace DC. Phylogenetic analysis of Leber’s hereditary optic neuropathy mitochondrial DNA’s indicates multiple independent occurrences of the common mutations. Hum Mutat. 1995; 6(4):311–25. https://doi.org/10.1002/humu.1380060405 PMID: 8680405.

4. Hudson G, Carelli V, Spruijt L, Gerards M, Mowbray C, Achilli A, et al. Clinical expression of Leber hereditary optic neuropathy is affected by the mitochondrial DNA-haplogroup background. Am J Hum Genet. 2007; 81(2):228–33. https://doi.org/10.1086/519394 PMID: 17668373; PubMed Central PMCID: PMCPMC1950812.

5. Torroni A, Campos Y, Rengo C, Sellitto D, Achilli A, Magri C, et al. Mitochondrial DNA haplogroups do not play a role in the variable phenotypic presentation of the A3243G mutation. Am J Hum Genet. 2003; 72(4):1005–12. https://doi.org/10.1086/373936 PMID: 12612863; PubMed Central PMCID: PMCPMC1180329.

6. van Oven M, Kayser M. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat. 2009; 30(2):E386–94. https://doi.org/10.1002/humu.20921 PMID: 18853457.

7. Weissensteiner H, Pacher D, Kloss-Brandstatter A, Forer L, Specht G, Bandelt HJ, et al. HaploGrep 2: mitochondrial haplogroup classification in the era of high-throughput sequencing. Nucleic Acids Res. 2016; 44(W1):W58–63. https://doi.org/10.1093/nar/gkw233 PMID: 27084951; PubMed Central PMCID: PMCPMC4987869.

8. Hudson G, Panoutsopoulou K, Wilson I, Southam L, Rayner NW, Arden N, et al. No evidence of an association between mitochondrial DNA variants and osteoarthritis in 7393 cases and 5122 controls. Ann Rheum Dis. 2013; 72(1):136–9. https://doi.org/10.1136/annrheumdis-2012-201932 PMID: 22964172; PubMed Central PMCID: PMCPMC3551219.

9. Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC. Effects of purifying and adaptive selection on regional variation in human mtDNA. Science. 2004; 303(5655):223–6. https://doi.org/10.1126/science.1088434 PMID: 14716012.
Background sequence characteristics influence mtDNA mutations

10. Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF. Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet. 2008; 83(2):254–60. https://doi.org/10.1016/j.ajhg.2008.07.004 PMID: 18674747; PubMed Central PMCID: PMC32495064.

11. Landrum MJ, Lee JM, Benson M, Brown G, Chao C, Chitipiralla S, et al. ClinVar: public archive of interpretations of clinically relevant variants. Nucleic Acids Res. 2016; 44(D1):D862–8. https://doi.org/10.1093/nar/gkv1222 PMID: 26852918; PubMed Central PMCID: PMC4702865.

12. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013; 500(7463):415–21. https://doi.org/10.1038/nature12477 PMID: 23945592; PubMed Central PMCID: PMC3776390.

13. Andrews RM, Kubbaca I, Chinnery PF, Lightowers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA [letter]. Nat Genet. 1999; 23(2):147. https://doi.org/10.1038/13779 PMID: 10508508.

14. Behar DM, van Oven M, Rosset S, Metspalu M, Loogvali EL, Silva NM, et al. A "Copernican" reassessment of the human mitochondrial DNA tree from its root. Am J Hum Genet. 2012; 90(4):675–84. https://doi.org/10.1016/j.ajhg.2012.03.002 PMID: 22482806; PubMed Central PMCID: PMC3322232.

15. Liu B, Du Q, Chen L, Fu G, Li S, Fu L, et al. CpG methylation patterns of human mitochondrial DNA. Scientific reports. 2016; 6:23421. Epub 2016/03/22. https://doi.org/10.1038/srep23421 PMID: 26996456; PubMed Central PMCID: PMC4800444.

16. Tang WW, Dietmann S, Irie N, Leitch HG, Floros VI, Bradshaw CR, et al. A Unique Gene Regulatory Network Resets the Human Germline Epigenome for Development. Cell. 2015; 161(6):1453–67. https://doi.org/10.1016/j.cell.2015.04.053 PMID: 26046444; PubMed Central PMCID: PMC4459712.

17. Ren L, Zhang C, Tao L, Hao J, Tan K, Miao K, et al. High-resolution profiles of gene expression and DNA methylation highlight mitochondrial modifications during early embryonic development. J Reprod Dev. 2017; 63(3):247–61. Epub 2017/04/04. https://doi.org/10.1062/jrdev.2016-168 PMID: 28367907; PubMed Central PMCID: PMC5481627.

18. Forster P, Harding R, Torroni A, Bandelt HJ. Origin and evolution of Native American mtDNA variation: a reappraisal. Am J Hum Genet. 1996; 59(4):935–45. PMID: 8806611; PubMed Central PMCID: PMC1914796.

19. Cox MP. Accuracy of molecular dating with the rho statistic: deviations from coalescent expectations under a range of demographic models. 2008. Hum Biol. 2009; 81(5–6):911–33. Epub 2010/05/28. https://doi.org/10.3378/027.081.0631 PMID: 20504206.

20. McFarland R, Elson JL, Taylor RW, Howell N, Turnbull DM. Assigning pathogenicity to mitochondrial tRNA mutations: when "definitely maybe" is not good enough. Trends Genet. 2004; 20(12):591–6. https://doi.org/10.1016/j.tig.2004.09.014 PMID: 15522452.

21. Rubino F, Piredda R, Calabrese FM, Simone D, Lang M, Calabrese C, et al. HmtDB, a genomic resource for mitochondrion-based human variability studies. Nucleic Acids Res. 2012; 40(Database issue):D1150–9. https://doi.org/10.1093/nar/gkr1086 PMID: 22139932; PubMed Central PMCID: PMC3245114.

22. Stewart JB, Freyer C, Elson JL, Wredenberg A, Cansu Z, Trifunovic A, et al. Strong purifying selection in transmission of mammalian mitochondrial DNA. PLoS Biol. 2008; 6(1):e10. https://doi.org/10.1371/journal.pbio.0060010 PMID: 18237233; PubMed Central PMCID: PMC2214808.

23. Howell N, Elson JL, Howell C, Turnbull DM. Relative rates of evolution in the coding and control regions of African mtDNAs. Mol Biol Evol. 2007; 24(10):2213–21. Epub 2007/07/24. https://doi.org/10.1093/molbev/msm147 PMID: 17624741.

24. Posth C, Renaud G, Mittnik A, Drucker DG, Rougier H, Cupillard C, et al. Pleistocene Mitochondrial Genomes Suggest a Single Major Dispersal of Non-Africans and a Late Glacial Population Turnover in Europe. Curr Biol. 2016; 26(6):827–33. Epub 2016/02/09. https://doi.org/10.1016/j.cub.2016.01.037 PMID: 26853362.

25. Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN, et al. Automated inference of molecular mechanisms of disease from amino acid substitutions. Bioinformatics. 2009; 25(21):2744–50. https://doi.org/10.1093/bioinformatics/btp528 PMID: 19734154; PubMed Central PMCID: PMC3140805.

26. Pereira L, Freitas F, Fernandes V, Pereira JB, Costa MD, Costa S, et al. The diversity present in 5140 human mitochondrial genomes. Am J Hum Genet. 2009; 84(5):628–40. https://doi.org/10.1016/j.ajhg.2009.04.013 PMID: 19426953; PubMed Central PMCID: PMC3081004.

27. Pereira L, Soares P, Radivojac P, Li B, Samuels DC. Comparing phylogeny and the predicted pathogenicity of protein variations reveals equal purifying selection across the global human mtDNA diversity. Am J Hum Genet. 2011; 88(4):433–9. Epub 2011/03/31. https://doi.org/10.1016/j.ajhg.2011.03.006 PMID: 21457906; PubMed Central PMCID: PMC3071914.