Mitochondrial DNA sequence characteristics modulate the size of the genetic bottleneck

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Abstract

With a combined carrier frequency of 1:200, heteroplasmic mitochondrial DNA (mtDNA) mutations cause human disease in ~1:5000 of the population. Rapid shifts in the level of heteroplasmy seen within a single generation contribute to the wide range in the severity of clinical phenotypes seen in families transmitting mtDNA disease, consistent with a genetic bottleneck during transmission. Although preliminary evidence from human pedigrees points towards a random drift process underlying the shifting heteroplasmy, some reports describe differences in segregation pattern between different mtDNA mutations. However, based on limited observations and with no direct comparisons, it is not clear whether these observations simply reflect pedigree ascertainment and publication bias. To address this issue, we studied 577 mother–child pairs transmitting the m.11778G>A, m.3460G>A, m.8344A>G, m.8993T>G/C and m.3243A>G mtDNA mutations. Our analysis controlled for inter-assay differences, inter-laboratory variation and ascertainment bias. We found no evidence of selection during transmission but show that different mtDNA mutations segregate at different rates in human pedigrees. m.8993T>G/C

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segregated significantly faster than m.11778G>A, m.8344A>G and m.3243A>G, consistent with a tighter mtDNA genetic bottleneck in m.8993T>G/C pedigrees. Our observations support the existence of different genetic bottlenecks primarily determined by the underlying mtDNA mutation, explaining the different inheritance patterns observed in human pedigrees transmitting pathogenic mtDNA mutations.

**Introduction**

First described in 1988, point mutations of mitochondrial DNA (mtDNA) have emerged as a major cause of maternally inherited human disease (1,2). Pathogenic mtDNA mutations causing a severe multisystem phenotype are usually heteroplasmic, with a mixture of mutated and wild-type mtDNA present in the same individual. The percentage level of mutated mtDNA largely determines whether a biochemical defect is expressed on a cellular level, and the inherited level of heteroplasmy correlates more or less with the severity of the clinical phenotype (3).

Rapid intergenerational shifts in the level of mtDNA heteroplasmy levels were first observed in Holstein cows (4,5) and subsequently documented in human pedigrees transmitting pathogenic mtDNA mutations. A restriction in the number of mitochondrial genomes repopulating the female germ line (the mtDNA bottleneck) is thought to explain this phenomenon, supported by observations in mice (6,7). Although the initial analysis of human pedigrees implied differences in the mtDNA bottleneck between different families (8), the analysis of aggregate data did not support earlier reports (9). Limited data analysis pointed towards a random genetic drift mechanism acting on all heteroplasmic mtDNA mutations (9), but in some instances, there appeared to be evidence of selection in favour of mutated genomes (10), even after minimising the effects of ascertainment bias. These conflicting data lead to two fundamental unanswered questions: do all mtDNA mutations behave the same during inheritance, and is there selection for or against different levels of heteroplasmy? Clarifying the underlying trends is key to providing reliable recurrence risks for patients with mtDNA mutations: m.11778G>A (n = 117), m.8344A>G (n = 96), m.8993T>G/C (n = 117) and m.3423A>G (n = 128), citations shown in the Material and Methods); and 45 new unpublished mother–child pairs [m.8344A>G (n = 9), m.8993T>G/C (n = 2) and m.3243A>G (n = 34)] measured in two centres. Given that there was no obvious difference between the two data sets (Fig. 3), we merged all of the data and minimized ascertainment bias by analysing separately data with and without the clinically affected probands. The final data set included 467 mother–child pairs for the corrected data. m.3243A>G was analysed before and after correcting for the known decrease in leucocyte heteroplasmy levels for this specific mutation using the published approach (14). For this correction, only individuals with a heteroplasmic level of <95% were included to avoid pairs where the mother or offspring values corrected to >100%. This reduced the sample size of m.3243A>G from 137 to 99 pairs. The entire data set was used to determine the likely size of the bottleneck parameter, b, using the model described previously (12,13), incorporating the laboratory assay and laboratory site as covariates. Bayesian statistical analyses were performed using JAGS (15).

For each mutation, the average change in heteroplasmy was not significantly different from zero, consistent with no selection for or against the mtDNA mutations during transmission. The posterior differences in bottleneck strength, b, are shown in Figure 4. As predicted from the simulations, the bottleneck strength was overestimated (i.e. b is low, a narrower bottleneck) when we included pairs ascertained through a clinically affected proband. However, after the exclusion of affected probands, we observed a difference in the strength of the bottleneck parameter, b, estimated for different mtDNA mutations. m.8993T>G/C showed the largest difference,
closely followed by m.3460G>A. A two tailed Bayesian hypothesis test was used to determine whether the bottleneck parameter, b, was significantly stronger for m.8993T>G/C than for m.11778G>A, m.8344A>G and m.3460G>A. This test gave estimated posterior probabilities of 0.031, 0.044 and 0.54. The bottleneck parameter distribution for the uncorrected m.3243A>G data was distorted, likely due to the known confounding effect of changing heteroplasmy levels with age (Fig. 4, far right hand panel) (14). We therefore used age-corrected data in the Bayesian hypothesis test comparing the bottleneck parameter, b, for m.8993T>G/C to m.3243A>G, which revealed an estimated posterior probability of 0.001. No other tests reached statistical significance at the 5% level.

Discussion

Our analysis accounted for several sources of potential variability, including different analytical techniques to measure heteroplasmy levels, different laboratories, differences between individual pedigrees themselves and differences in maternal age (16). Having minimised the likely effects of ascertainment bias, we observed different rates of heteroplasmy segregation during maternal transmission between mtDNA mutations. Patients harbouring the m.8993T>G/C mutation showed more rapid segregation of heteroplasmy levels than any of the other mutations. Although this trend has been suspected for some time (17), the analysis we present here provides the first direct comparison and a formal demonstration that m.8993T>G/C behaves differently to other pathogenic mtDNA mutations. Given that the size of the mitochondrial genetic bottleneck is the primary factor determining the rate of segregation during transmission, our findings indicate differences in the behaviour of the mtDNA bottleneck based on the underlying mtDNA genotype.

The more rapid segregation observed in m.8993T>G/C pedigrees is consistent with earlier reports of major shifts in heteroplasmy observed in pedigrees transmitting this mutation (17,18). This explains why severely affected children are often the only affected individuals in families transmitting this mutation, and why their mothers are usually asymptomatic. Conversely the less dramatic rates of segregation seen for other mtDNA mutations explain why a wide range of heteroplasmy values are seen in different family members, with oligo-symptomatic individuals transmitting the mutation in larger pedigrees, with multiple moderate and severely affected family members (19,20). Our findings also provide a potential explanation for the relative frequency of different pathogenic mutations in epidemiological studies (for example, m.3243A>G being much more common than m.8993T>G/C, despite the same background frequency of healthy carriers in the population with low heteroplasmy levels (21,22). It should be noted, however, that this discussion relates to the observed statistical trends, and although uncommon, rapidly segregating families with m.3243A>G (23) and slowly segregating families with m.8993T>G/C have been described (24,25) and are also consistent with our findings.

Studies in mice have shown a dramatic reduction in the amount of mtDNA within single cells at an early stage in mammalian germ cell development (6,7). Mathematical models predict that subtle differences in the size of the mtDNA genetic bottleneck at this critical period will have a dramatic impact on the rate of segregation of mtDNA heteroplasmy (26). Given emerging evidence that the amount of mtDNA within cells can be influenced by the mtDNA sequence (27,28), it is plausible that the different mtDNA mutations we have studied cause differences in the amount of mtDNA within the developing germ line (29), either through a replication advantage or as a compensation for the lower rates of oxidative phosphorylation and higher levels of reactive oxygen species production (28,30). The differences in mtDNA level would lead to differences in the rate of segregation through the genetic bottleneck. Alternatively, selection either for or against a particular mutation would lead to differences in the rate of segregation (31). First described by population genetic theory (13,32), the effective bottleneck size, Ne, can be defined like the effective population size during genetic drift. Studies in isolated reduced populations have shown that selective pressures can influence Ne, without directly influencing the true population size. The apparent difference in bottleneck size between the different mtDNA mutations could thus reflect the selection pressure and need not necessarily be caused by a difference in the actual amount of mtDNA during germ cell development. Although it will be fascinating to determine the underlying molecular mechanisms, this will not alter our conclusions, or the relevance of our findings for women transmitting the mtDNA mutations we have studied.

These findings have important implications for our understanding of the recurrence risks of heteroplastic mtDNA diseases, for the prevention of mtDNA disease using conventional techniques such as pre-natal and pre-implantation diagnosis, and also suggest that therapies aimed at manipulating a germ cell mtDNA content could influence the underlying rate of segregation of pathogenic mtDNA mutations in humans. On the one hand, increasing the mtDNA content in germ cells could slow down segregation and thereby prevent the expression of severe disease in subsequent generations. On the other hand, factors known to reduce mtDNA content could lead to more rapid segregation of heteroplasmic alleles increasing the probability of generating germ cells with very low heteroplasmy levels (as well as very high heteroplasmy levels, which may not be viable at an early stage in pregnancy). Finally, environmental factors leading to selection pressure could also alter the behaviour of the genetic bottleneck, as described earlier. Given the recent observation of widespread low-level mtDNA heteroplasmy (33), these reductions in mtDNA content could lead to the emergence of pre-existing pathogenic variants, previously present at a very low level in the female germline. With recent evidence that common genetic polymorphisms of mtDNA can influence mtDNA levels (28), it is conceivable that geographic, ethnic and even inter-familial differences in segregation occur, influenced by non-synonymous single base-pair substitutions on the background mtDNA haplotype. This could explain differences in the rates of segregation of the same pathogenic mtDNA mutation, which have been described in different families, and also influence the segregation of low-level heteroplasmy, providing a potential mechanism for the association of mtDNA haplogroups with common late-onset human diseases (34).

Materials and Methods

Heteroplasmy levels in mothers and offspring

We studied the transmission of mtDNA heteroplasmy in both published and new unpublished pedigrees.

Ascertainment of published data

Published pedigrees transmitting m.11778G>A, m.3460G>A, m.8344A>G, m.8993T>G/C and m.3423A>G were identified through a systematic review of the literature. We included all papers where the age, clinical status and laboratory methods were clearly recorded, and there was at least one heteroplasmic individual. These parameters, along with the laboratory location,
1. Simulate a population of heteroplasmic mothers (red), each with two offspring (blue) using an established bottleneck model (see text).

2. Determine the likelihood that a mother is clinically affected and thus ascertained based on her heteroplasmy level.

3. Identify mother-offspring pairs through the affected mother, \(A_{AM}\).

4. Remove the affected mothers from the original distribution. Bold colors = ascertained data, light colors = non-ascertained data.

5. Determine the likelihood that a child is clinically affected and thus ascertained based on the heteroplasmy level.

6. Identify mother-offspring pairs through the affected child, \(A_{AC}\).

7. Identify mother-offspring pairs through the unaffected other child, \(A_{OC}\).
were incorporated in subsequent analyses. We identified 77 publications, containing blood DNA heteroplasm levels from 532 mother–child pairs transmitting 5 common heteroplasmic mitochondrial DNA mutations: m.11778G>A (n = 117 mother–child pairs) (35–45), m.3460G>A (n = 74) (35,37,43,46–51), m.8344A>G (n = 96) (52–62), m.8993T>G/C (n = 117) (74,62–84) and m.3423A>G (n = 128) (20,54,85,86,87,88,90,91,92,93,94,95–105).

Unpublished data
Unpublished pedigrees transmitting the m.8344A>G (n = 9 mother–child pairs), m.8993T>G/C (n = 2) and m.3423A>G (n = 34) mutations were identified from the following accredited diagnostic laboratories: Newcastle, UK; Maastricht, NL; Milan and Bologna, Italy; Bergen, Norway and Munich, Germany. Genomics DNA was analysed in two laboratories: Newcastle, UK; and Maastricht, NL.

Quantification of heteroplasm: Newcastle
Heteroplasm was measured by quantitative pyrosequencing. Pyromark Assay Design Software v.2.0 (Qiagen) was used to design primers for template generation, one of which contains a biotinylated tag (*BIO), and the pyrosequencing reaction (Seq). For m.3243A>G: F-*BIO-TAAGGCTACTTTCAAAAGCC, R-GCGAGTGAATGGGTACAATGAG, Seq-ATGCGATTACCGGGC; for m.8344A>G: F-*BIO-CATGCCCATCGTCCTAGAAT, R-CTGTTATGGGCTTTGGTGTAGG, Seq-TAAGTTAAAGATTAAGAGA; and for m.8993T>G/C, F-AGG, Seq-TAAGTTAAAGATTAAGAGA, and mutant clones, which when mixed at the correct proportions mimicked a range of heteroplasm levels between 0 and 100% for each mutation. Each mixed sample was assessed for mutation pyrosequencing assay was determined by generating wild-type and mutant clones, which when mixed at the correct proportions mimicked a range of heteroplasm levels between 0 and 100% for each mutation. Each mixed sample was assessed for mutation proportion.

Primer sequences
| Mutation       | Forward Primer                      | Reverse Primer                      | Sequence                              |
|----------------|-------------------------------------|-------------------------------------|---------------------------------------|
| m.11778G>A     | F-TAAGGCTACTTTCAAAAGCC             | R-GCGAGTGAATGGGTACAATGAG            | Seq-ATGCGATTACCGGGC                   |
| m.3460G>A      | F-CATGCCCATCGTCCTAGAAT             | R-CTGTTATGGGCTTTGGTGTAGG            | Seq-TAAGTTAAAGATTAAGAGA               |
| m.8344A>G      | F-CATGCCCATCGTCCTAGAAT             | R-CTGTTATGGGCTTTGGTGTAGG            | Seq-TAAGTTAAAGATTAAGAGA               |
| m.8993T>G/C    | F-AGG                               | R-CTGTTATGGGCTTTGGTGTAGG            | Seq-TAAGTTAAAGATTAAGAGA               |

Figure 1. Overview of the inheritance model. Simulations used to determine the potential effects of ascertainment bias through a mother, and affected child, or an unaffected child. Mothers = red; offspring = blue; unaffected offspring = green. Darker distributions represent ascertained pedigrees; lighter distributions represent non-ascertained pedigrees. The probability of a family being recruited depends on an individual developing the disease, which in turn is correlated with the heteroplasm level. The probability that an individual is recruited, \( p_i \), based on a distribution that is zero below a level of heteroplasm \( d_i \), and one above heteroplasm \( d_i \) with a linear increase between these points. The probability of being recruited with a heteroplasm of \( \delta_i + d_i/2 \leq 0.5 \), reflecting the increased likelihood of developing symptoms and thus presenting clinically. \( A_{AM} \) = families ascertained through an affected mother; \( A_{OC} \) = families ascertained through an affected child; \( A_{AC} \) = families ascertained through an affected child, only but including the transmission from the mother to the other child, thus mimicking the effect of deleting probands from the ascertainment of real pedigrees.

Figure 2. Modelling the effects of ascertainment bias on simulated pedigrees transmitting mtDNA heteroplasm. Frequency distribution histograms for the difference between maternal and offspring heteroplasm levels (\( \Delta M = 0 \)) for three different values of the bottleneck strength (bottleneck parameter, \( b = 0.7 \) a weak bottleneck; \( b = 0.2 \) a strong bottleneck); and for three different methods of ascertainment: sampling the families where there was an affected mother—(ascertainment - affected, AAM, red); sampling the families where there was an affected child—(ascertainment - affected child, \( A_{AC} \), blue); and sampling the families through an affected child, only but including the transmission from the mother to the other child—(ascertainment - other child, \( A_{OC} \), green). The grey histogram gives the simulated distribution before any ascertainment, thus corresponding to data without any ascertainment bias. Each row reflects different thresholds heteroplasm values required to cause disease. The parameter \( d \) models the threshold for disease resulting from the pathogenic mutation. The range given for \( d \) is the range of heteroplasmies where the probability of disease increases linearly from 0 (lower level) to 1 (upper level) (see legend to Fig. 1 for details of the simulation model).

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Figure 3. Relationship between the level of mtDNA heteroplasmy in mothers and offspring for five pathogenic mtDNA mutations. Maternal and offspring heteroplasmy levels are displayed as a proportion. Red symbols = affected proband, green symbols = mother of the proband, blue = other relatives. Circles = meta-analysis data, triangles = new pedigree data measured at Centre 1, squares = new pedigree data measured at Centre 2. m.3243A>G corrected refers to heteroplasmy values after a correction for the known decline in heteroplasmy levels with age [14].

Figure 4. Differences in the size of the mitochondrial DNA genetic bottleneck of five pathogenic mtDNA mutations. Violin plots show the probability density for a given bottleneck strength parameter, b, calculated from actual heteroplasmy measurements in human pedigrees shown in Figure 3. All = all of the mother–child pairs. No probands = mother–child pairs remaining after the exclusion of an affected proband, thus minimising ascertainment bias. 'm.3243A>G corrected' refers to heteroplasmy values after a correction for the known decline in heteroplasmy levels with age [14].
load using pyrosequencing and used to generate a standard curve (Supplementary Material, Fig. S1).

**Quantification of heteroplasmy: Maastricht**

Heteroplasmy was measured by semi-quantitative restriction fragment length polymorphism (RFLP) analysis using mutation-specific restriction enzymes. Primers used for m.3243A>G were as follows: CAACCTTAGATTATACCCACACACAATTTATTATACCCACAC (forward) and TTTGGTGGGTTAAGCATT (reverse); for m.8344A>G: TCTGGTGAATATTTCTCC (forward) and TAGTTATTGCTGCCCATCTGTAAGGCCTTGTTG (reverse); and for m.8993T>C/G: CACAGCTACCCCCCTATCC (forward) and TCATTATGTGTTGGTGCAG (reverse). For each forward primer, an unlabelled and FAM-labelled primer was available, polymerase chain reaction (PCR) was performed on the GeneAmp PCR System 9700 (Perkin–Elmer Applied Biosystems) in a total volume of 50 µl, containing 1X PCR buffer (Invitrogen) and 1 U of Taq DNA polymerase (Invitrogen), 2 mm MgCl2 (Invitrogen) and 0.1 mm dNTP (Pharmacia). Unlabelled forward (15 pmol) and reverse primer (3 pmol) sets were used in the first round. First round PCR started with 5-min denaturation at 94°C followed by 2 cycles of 1-min denaturation at 92°C, 45-s annealing at 53°C and 45-s elongation at 72°C, followed by a final elongation step of 7 min at 72°C. 15 µl of first round amplification product was adjusted to 50 µl with second round PCR mix, containing 1X PCR buffer (Invitrogen) 1 U of DNA polymerase, 2 mm MgCl2 (Invitrogen) and a fluorescently labelled forward primer (15 pmol). One final amplification cycle was performed of 5-min denaturation at 94°C, 1-min annealing at 53°C and 7-min elongation at 72°C. The labelled second round PCR product (15 µl) was digested in the appropriate digestion buffer in a total volume of 50 µl containing 10 U HaeIII (10 U/µl; Roche) for the m.3243A>G mutation, 10 U BglII (10 U/µl) for the m.8344A>G mutation and 10 U HpaII (10 U/µl; Roche) for the m.8993T>C/G mutation. The m.3243A>G-amplicon contains an additional HaeIII restriction site as an internal control for restriction enzyme digestion completion. For each amplification, a sample with known mutation load was included as reference. After digestion, samples were purified using a QIAquick PCR purification kit (Qiagen). Samples were analysed by capillary electrophoresis on an ABI Prism 3730 Genetic Analyser followed by GeneScan analysis. To calculate the mutation load, the area of the mutation peak was divided by the sum of the peak area of the wild-type and mutation peak. Each forward primer contains a biotinylated tag. PCR products were purified by Taq DNA Polymerase (Invitrogen) according to manufacturer’s protocol. The PCR fragments were digested by mutation-specific restriction enzymes: Hae III (10 U/µl) Roche for m.3243A>G; Bgl I (10 U/µl) Biolabs for m.8344A>G and Hpa II (10 U/µl) Roche for m.8993T>C/G. Digestion products were purified (Qiagen Qiaquick PCR Purification). Fragments were separated and analysed by capillary electrophoresis (automated DNA Sequencer 3730, Applied Biosystems) to determine mutation load.

**Simulation of mtDNA heteroplasmy inheritance**

An overview of the simulations is shown in Figure 1. First, we generated a population of simulated mothers with different heteroplasmy levels. We then modelled the inheritance of heteroplasmy from each of these mothers to two offspring (see below). Next we determined the likelihood of the individuals being clinically affected. We then sampled the simulated families based on the following ascertainment criteria, in the following order:

1. Sampling the families where there was an affected mother—(AADC). Here, we included the transmission from the mother to both offspring.
2. Sampling the families where there was an affected child—(AOC), but the mother was not affected. Here, we also included the transmission from the mother to both offspring.
3. Sampling the families through an affected child, only but including the transmission from the mother to the other child—(AOC).

This sampling algorithm mimicked the different ways that mother–child pairs could be identified in a study of real human pedigrees, and the resulting difference in distributions for the three different pairs (Supplementary Material, Fig. S2) resembles those from the real data from the human pedigrees shown in Figure 3.

We then compared the sampled mother–child pairs with the original simulated pedigrees to determine whether the ascertainment method influenced the observed distribution of heteroplasmy transmissions.

**Simulating the transmission of mtDNA heteroplasmy (the mtDNA genetic bottleneck)**

We modelled the mtDNA genetic bottleneck using established model derived from measurements of heteroplasmy made in human oocytes (12,13). Here, the bottleneck is modelled as an infinite population of mtDNA molecules that was instantaneously reduced to the minimum bottleneck size N for g generations, before expanding back to a large size. We simulated the transmission of mtDNA heteroplasmy of a neutral allele from the mother to two offspring through the bottleneck by approximating with a beta distribution with parameters (1−p)b/(1−b) and β = (1−p)b/(1−b), where b is the drift parameter b = exp(−g/N). A large b indicates a weak (or wide) genetic bottleneck. When g is large compared with N, b approaches 0, corresponding to a very strong (or narrow) bottleneck. Using this model, we generated a series of simulated heteroplasmy transmissions from mothers starting with a range of heteroplasmy values.

**Statistical methods**

Changes in heteroplasmy between mother and child were modelled using the same beta distribution as for the simulation, using a hierarchical model with a different bottleneck parameter b for each mtDNA mutation. Measurements of heteroplasmy were assumed to be normally distributed about the true values, with censored at 0 and 1, and with an error rate qi for publication i, to allow for different measurement technologies. Writing mi for the observed maternal heteroplasmy for mother i, and qi for the heteroplasmy for child j of mother i, where j = 1, . . . , ni and ni is the number of children for Mother I,

\[
m_i \sim N(m_i, \sigma_i^2),
\]

\[
o_j \sim N(n_j, \sigma_j^2), \quad \text{and}
\]

\[
\eta_j \sim \text{Beta}\left(\frac{\mu_i b_{m_i}}{1 - b_{m_i}}, \frac{(1 - \mu_i)b_{m_i}}{1 - b_{m_i}}\right)
\]

where \(b_h = \exp(-g/N_i)\), \(m_i\) gives the mutation for Mother i and \(r_i\) gives the publication for mother i. To test for systematic increases or decreases in the level of heteroplasmy (i.e. selection), an additional model was built. This allowed for changes in the expected heteroplasmy in the offspring, by multiplying the expected child heteroplasmy, \(\mu_i\), by a random factor, \(s_i\), which models selection by increasing or decreasing the average heteroplasmy value. The JAGS statistical package (15) was used to make inferences about the strength of the bottleneck using the statistical model above. A Bayesian framework was used with uniform priors between 0
and 1 for the $b_k$, and broad priors for the error parameters $\sigma_k^2$. The model and all JAGS and R scripts are available through the following URL: github.com/ijwilson/mtBottleneck.

Bayesian comparison of bottleneck sizes

Maternal heteroplasmy values were corrected for age as described (14). Bayesian hypothesis tests of $b_1$ versus $b_2$ were calculated using the empirical joint posterior distributions of $b_1$ and $b_2$ from JAGS (15) and using $\max(2b_1 > b_2, b_2 > 2b_1)$. The JAGS script is available through the following URL: github.com/ijwilson/mtBottleneck.

Supplementary Material

Supplementary Material is available at HMG online.

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