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ORIGINAL ARTICLE

Analysis of mitochondrial length heteroplasmy in monozygous and non-monozygous siblings

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Abstract The segregation of mitochondrial genomes and the inheritance of mitochondrial DNA are constant matters of debate. To obtain more information about this issue and to answer the question whether or not it is possible to distinguish mitochondrial DNA (mtDNA) samples from monozygous individuals by analysing heteroplasmic length variants, 290 monozygous and 121 dizygous twin pairs and 34 sets of multiples were studied by RFLP and partly by direct sequencing. A factor *D* describing the respective pattern of length variants in a given sample was also calculated. The results show that monozygous individuals exhibit a significantly lower median and closer distribution of *D* than non-monozygous siblings. Thus, a differentiation of mtDNA samples from monozygous twins by this trait is not possible. The high percentage of heteroplasmic individuals, the low median of the *D* values and the unexpectedly very similar distribution of length variants in monozygotic individuals support the existence of a relatively wide bottleneck or the assumption of a regeneration of length heteroplasmy following a tight bottleneck and agree with a random segregation of mtDNA genomes in dividing oocytes.

Keywords Monozygous · Dizygous siblings · Twins · Multiples · Mitochondrial DNA · Length heteroplasmy

Introduction

Length heteroplasmy in the mitochondrial genome is a common phenomenon, which occurs especially following monotonous runs of the same base, and which is very likely being caused by replication slippage of the DNA polymerase [14, 16]. Between nucleotide positions 303 and 310 within the hypervariable region II (HVII) of the human mitochondrial control region, such a homopolymeric cytosine stretch exists, in which a length heteroplasmy is frequently observed [2, 36].

Investigations in mother–child pairs [1, 5, 10, 18, 37], maternal relatives [26] and in different tissues of one individual [21, 24, 33, 36] revealed a sporadic instability of the HVII length heteroplasmy. In some cases, a change of the predominant length variants between generations could be observed [5, 10, 18, 26, 37].

To explain such changes of mitochondrial DNA (mtDNA) variants and the fact that the mitochondrial genomes seem to be very homogeneous despite the high mutation rate, the lack of recombination and the probably less efficient repair mechanism, the bottleneck theory was proposed. The bottleneck was assumed to occur during the stage of early primordial germ cell and primary oocyte [19, 20, 22], and this reduction of the original set of molecules can lead to a change of the composition of C-stretch variants in an offspring. The narrower the bottleneck, the smaller the number of segregational units. Thus, a reduction of the mtDNA copy number in early oogenesis in combination with random segregation leads to homoplasmy in one or only a few generations [20].

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In contrast to the variance between generations, a longitudinal study on cervical cells showed that the level of heteroplasmy remains stable during life [23]. Based on these observations—the potential interindividual variability of length heteroplasmy and the prospective lifelong stability within one tissue—the situation of heteroplasmy in monozygous (MZ) and non-monozygous (NMZ) siblings was brought into focus. Moreover, the question arose whether it is possible to distinguish mtDNA samples from monozygous siblings by length heteroplasmy profiles in forensic stain analyses.

To attempt to answer these questions, we examined 926 siblings by restriction fragment length analysis and 430 of these by additional direct sequencing of the mitochondrial control region.

Materials and methods

Samples

Blood samples or buccal swabs were taken from a total of 926 individuals with informed consent. Among these were 290 monozygous and 121 dizygous (DZ) twin pairs and 34 sets of multiple siblings (MS). These were monozygous triplets (“3+0”; 13 sets), dizygous triplets (“2+1”; 17 sets), trizygous triplets (“1+1+1”; two sets), and two sets of quadruplets (“2+2” and “1+1+1+1”, respectively). A subset of these donors was studied previously regarding their immune response to hepatitis A and B vaccination [15]. In the following multiple siblings that were not monozygous were termed as non-monozygous siblings.

Determination of the zygosity and analysis of mtDNA

DNA was extracted from peripheral blood using standard techniques as described previously [25] and from buccal cells using Chelex (Bio-Rad, Munich, Germany) [38]. To determine zygosity in twins and multiples, the PowerPlex 16 kit (Promega, Mannheim, Germany) was used.

Amplification and subsequent sequencing of mtDNA were performed as described previously [28] but with primers L15908/H16432, L16268/H159, L16450/H429 and L314/H611 for PCR and sequencing [29]. Also, restriction fragment length analysis of nucleotide (nt) positions 303–310 was performed as described earlier [27]. Internal (HD400, Applied Biosystems, Foster City, CA, USA) and external standards (mix containing sequenced amplicons with C-stretches of different length) were used. Separation of the products and data analysis was carried out on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems) using SeqScape v.2.5 and GeneMapper ID v. 3.2 software, respectively (Applied Biosystems).

Calculation of the distance measure *D*

To compare the degree of length heteroplasmy between individuals, a value *D* meaning “distance measure” was calculated as follows: for each sample which was subjected to restriction fragment length analysis, the number and area of C-peaks were assessed in the electropherogram as computed by GeneMapper software. Each peak of one sample was expressed as a percent age ratio of the sum of all peak areas of the respective sample. Only data showing similar signal heights were compared and peaks below 50 RFU were not taken into account. For each sibling pair, analogous C-peaks were compared by subtraction of their respective percent age ratios, and absolute values of the resulting numbers were added up. This sum of absolute values was then divided by the number of C-peaks observed in the respective sample. The resulting value was termed “distance measure” (*D*). An example for the calculation is given in Fig. 1. For multiples, pairwise *D* values were calculated for every possible combination of two siblings.

Statistical analysis of *D* values by Mann–Whitney *U* test and Wilcoxon signed-rank test (for dizygous triplets), and generation of box-and-whisker plots was performed using SPSS v.15 software (SPSS, Chicago, IL, USA).

Results

Direct sequencing of the mitochondrial control region

The complete control regions of 200 twin pairs and multiples (430 individuals) were sequenced. No sequence differences were observed between maternal relatives. Sequence data of one sibling of each respective maternal lineage were submitted to the mtDNA database EMPOP (www.empop.org). Concerning the C-stretches in HVII, siblings showed analogous patterns in their electropherograms in nearly all cases.

Restriction fragment length analysis

Twins

A total of 411 twin pairs (290 MZ, 121 DZ) were analysed. Table 1 shows the numbers of cytosines of the samples and their observed state of homoplasmy or heteroplasmy, and 113 of the MZ and 46 of the DZ twin pairs were homoplasmic (about 39%) and were excluded from further examination.

Calculation of *D* for heteroplasmic MZ and DZ twins resulted in a median of 0.51 for MZ and of 3.05 for DZ twins (Fig. 2a). *D* in DZ also showed a wider distribution than in MZ twins. Results were highly significant ($p<0.001$).

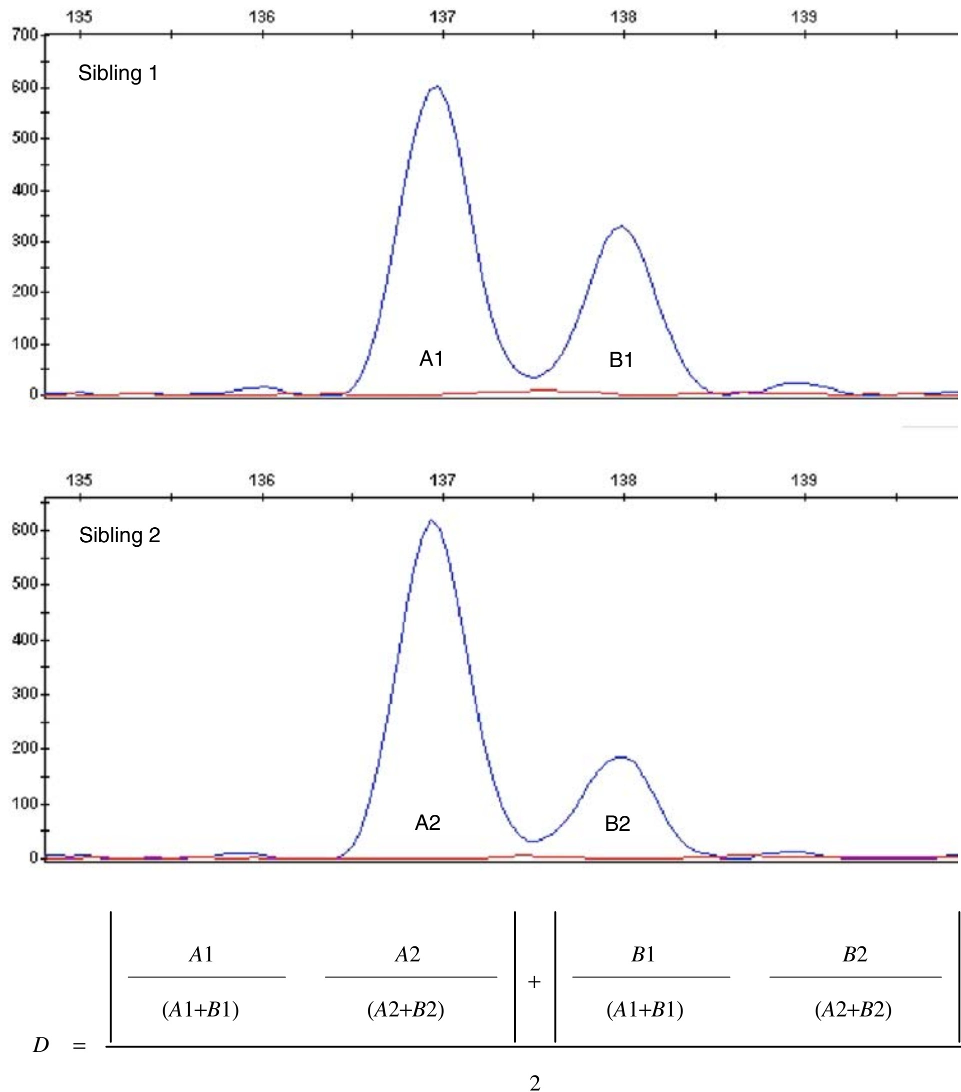

Fig. 1 Calculation of the distance measure D for two monozygous siblings showing a length heteroplasmy in HVII after RFLP analysis. Electropherograms show two length variants, i.e. a C8 (A1/2) and a C9 (B1/2) variant in both siblings. The formula for the calculation of D is given

Only five out of 252 heteroplasmic twin pairs showed obviously differing profiles of length heteroplasmy. An example is given in Fig. 3. All five cases concerned dizygous siblings and D was not calculated for these samples.

Multiples

A total of 34 sets of triplets and quadruplets were analysed. The state of homoplasmy or heteroplasmy and

Table 1 Most frequent numbers of cytosines between positions 303–310 (HVII) found in homo- and heteroplasmic samples of 445 monozygous (MZ) and dizygous (DZ) twins and in multiples

	Most frequent numbers of C-residues at positions 303–310					Transition at position 310	Differing results in RFLP analysis	Total
	C6	C7	C8	C9	C10			
MZ twins								
Heteroplasmic samples	0	20	116	38	0	3	0	177
Homoplasmic samples	3	107	3	0	0	0	0	113
DZ twins								
Heteroplasmic samples	0	6	56	7	0	1	5	75
Homoplasmic samples	0	42	4	0	0	0	0	46
Multiple siblings								
Heteroplasmic samples	0	8	12	5	0	1	0	26
Homoplasmic samples	0	7	1	0	0	0	0	8

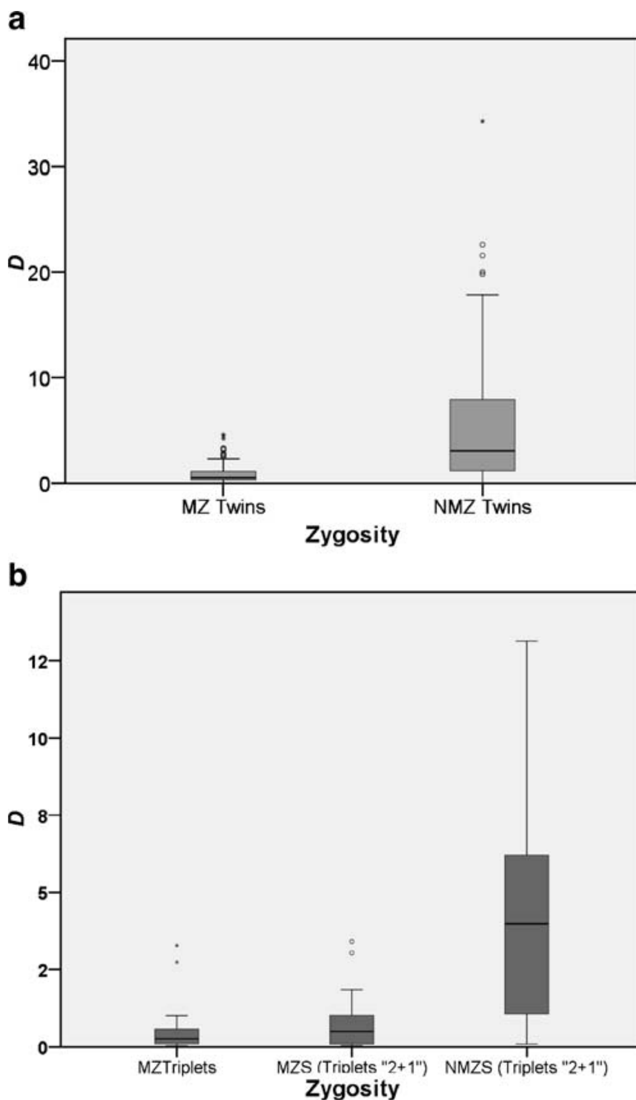

Fig. 2 *D* values calculated for 252 twin pairs (**a**) and for 23 sets of monozygous (MZ) and dizygous (DZ) triplets (**b**). The *boxes* comprise 50% of all data points—defined by the first and third quartiles—with a *black bar* indicating the median. *D* values of non-monozygous siblings are significantly higher than those of monozygous siblings ($p<0.001$). *Open circle*, Outliers. *Asterisk*, Extreme values. *MZS* Monozygous siblings, *NMZS* non-monozygous siblings

the number of observed cytosines in the samples are given in Table 1, and seven sets of triplets and one set of quadruplets were homoplasmic and thus excluded from further examinations.

Calculated *D* values showed a median for MZ multiples of 0.43 and for NMZ multiples of 4.26. *D* values of MZ triplets and of MZ siblings in non-monozygous sets of triplets were significantly lower than *D* values calculated for NMZ triplets/siblings ($p=0.001$; Fig. 2b). Again, *D* in NMZ multiples showed a wider distribution than in MZ multiple siblings.

Discussion

The C-stretch at positions 303–310 of the mitochondrial control region of 926 siblings was investigated using restriction fragment length analysis. Altogether, about 39% of the analysed samples were homoplasmic, and 61% showed length heteroplasmy at positions 303–310. The vast majority of the homoplasmic samples showed seven cytosines. In contrast, most of the heteroplasmic samples exhibited eight or more cytosines (see Table 1). This observation is in accordance with previous studies showing that a longer C-stretch is associated with a higher probability of heteroplasmy [6, 10, 26, 36], and that there seems to be a critical number of eight cytosines [6, 27].

For samples exhibiting length heteroplasmy, the distance measure *D* was calculated as described. The reliability and reproducibility of the RFLP technique for analysis of length heteroplasmy was discussed earlier [27]. Peak area values as provided by the GeneMapper software were used for calculation of *D*, as peak areas are a better reflection of the amount of the respective PCR fragment in a sample than only peak heights [11]. Nevertheless, analogous calculations using peak heights yielded similar results (data not shown). Also, repeated analysis of the same samples resulted in comparable electropherograms and thus similar values of *D* (data not shown).

Comparing sequence electropherograms as well as *D* values for the monozygous and non-monozygous twins and multiples (cf. Fig. 2a,b), the monozygous siblings showed more analogous electropherograms and patterns of C-stretch length variants. They also exhibited a lower median and a closer distribution of *D* values. Thus, a differentiation of monozygous individuals by C-stretch length variants is not possible. These results agree with the study of Detjen et al. [9], who sequenced the entire mtDNA genomes of four discordant monozygous twin pairs and found no sequence polymorphism and no differences in the distribution of the C-stretch variants. In an investigation of Bendall et al. [3] who sequenced the first hypervariable region of 180 twin pairs and found point heteroplasmy in four pairs (two MZ and two DZ), the monozygous twin pairs showed similar levels of heteroplasmy whereas the predominant mitochondrial haplotype differed within the dizygous twin pairs.

On the other hand, the significantly higher median and wider distribution of *D* values in non-monozygous individuals indicate that the C-stretch length variants in siblings originating from a single oocyte are more alike than in siblings stemming from different oocytes. This observation is concordant with the study of Marchington et al. [32] who described varying distributions of C-stretch lengths in different oocytes from a single individual.

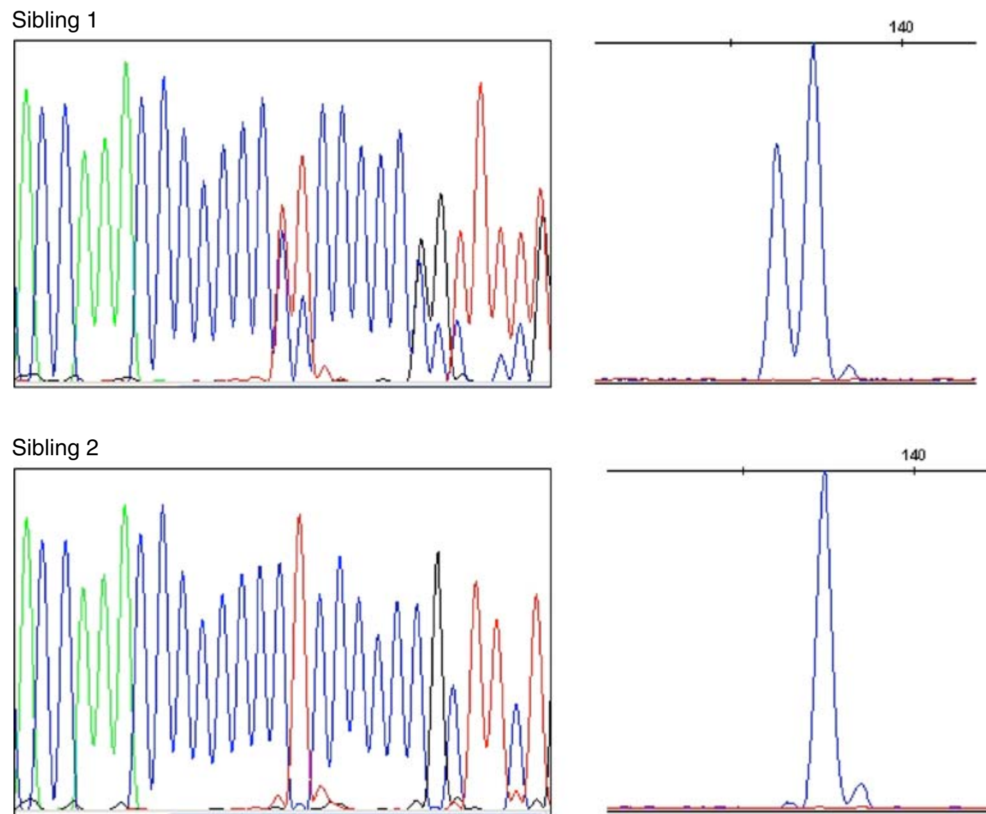

Fig. 3 Sequence electropherograms and RFLP analysis for a non-monozygotic sibling pair showing different distributions of length variants

The fact that different oocytes from one individual may show different mitochondrial length variants agrees with the bottleneck theory proposing a decrease of the number of mtDNA molecules during an early stage of oogenesis followed by an amplification of these founder molecules [7, 8, 13, 17]. The relatively high percentage of individuals showing length heteroplasmy at the HVII C-stretch as observed in this study thus supports the existence of a rather wide bottleneck.

Strikingly, all homoplasmic and nearly all heteroplasmic siblings examined in this study exhibited analogous patterns regarding the number of cytosines at nt 303–310. Only five out of 445 sets of siblings showed differing profiles of length variants (cf. Fig. 3) and in all five cases showing differing profiles of length variants dizygous twin pairs were affected. Considering the comparably high number of non-monozygous siblings with analogous patterns of C-stretch variants, a wider bottleneck seems to be rather common, whereas a small bottleneck is an infrequent event.

Regarding the relatively high percentage of individuals observed showing length heteroplasmy and the occurrence of analogous C-stretch patterns, another assumption can be made, which is the existence of a narrow bottleneck with a continuous formation of new heteroplasmic stretches. The influence of an as yet unknown “nucleus-specific factor” which controls the heteroplasmic mixture [31] would explain the significantly lower *D* values in monozygous twins in comparison with non-monozygous twins. Provided that such a mechanism exists, it would not be possible to draw any conclusions from the individual C-stretch variants as to the original situation in the oocyte.

The assumption of a regeneration of length heteroplasmy in each individual following a tight bottleneck was also proposed by Bendall et al. [3]. On the one hand, they observed an inheritance pattern of heteroplasmic point mutations that support the existence of a tight bottleneck. On the other hand, maternally related individuals showed identical proportions of different length variants surrounding the homopolymeric tract in HVI, indicating a wide bottleneck [2].

Another question is the distribution of the mtDNA genomes within a dividing oocyte: our study shows analogous patterns in all monozygous siblings. These findings still agree with the general opinion that segregation of mitochondria takes place randomly [34]. The human oocyte is estimated to contain about 100,000 copies of mitochondrial genomes [35]. Due to this high number of mtDNA genomes and presuming random segregation, one would expect a nearly equal distribution of the different length variants in monozygous siblings.

Approximately 7% of non-monozygous twins show different C-stretch variants. Following the theory of random segregation, one explanation for this observation in contrast to the completely equal patterns in MZ is that the lineages part from each other during oogenesis before the bottleneck event takes place, leading to one bottleneck per person in NMZ and one bottleneck per pair in MZ.

In monozygous siblings, *D* values between 0 and 4.6 were observed without exhibiting a correlation with individual age or the number of cytosines within the stretch (data not shown). In most of the monozygous siblings (160 out of 177), rather identical degrees of length heteroplasmy were observed (*D* values between 0 and 2). The rare observation of different degrees in length heteroplasmy (17 out of 177 with *D* between 2 and 4.6) might be explained by a different time of monozygous twinning.

One-third of monozygous twins separate within 4 days after conception, almost two-thirds between the fourth and the eighth day, and only a few twins separate later than that [12]. The earlier the fertilised ovum divides into two distinct embryos, the less they will share supportive structures as is shown by differences in formation of the amniotic sac, the chorion and placenta [30], and the earlier the twins are separated, the higher the probability of developing different levels of heteroplasmy.

In a bovine model [4], the mtDNA content decreased between the 2- and 4/8-cell stages but increased sharply between the morula and the blastocyst stages. For monozygous twins who develop before or within this amplification event, a much greater *D* value is plausible. For monozygous twins developing after this time, an uneven distribution of length variants at the time of twinning is possible, or, alternatively, an equal distribution followed by an uneven segregation of different variants during the development of one sibling through random genetic drift.

Very small *D* values of monozygous twins—meaning a very similar pattern of C-stretch variants—might be a consequence of monochorionicity. Monochorionic twins share the same placenta and due to placental vascular anastomoses blood and stem cells can be transferred from one twin to the other [12].

The significantly lower *D* values in monozygous twins can also be interpreted in a way that no or only little change within the hypervariable C-stretch occurs in the individual after the time of twinning.

In conclusion, this study shows that C-stretch length variants of HVII in monozygous siblings are more alike than those in non-monozygous siblings. Thus, a differentiation of DNA samples from monozygous twins by this trait is not possible. The high percentage of heteroplasmic individuals and the fact that only five out of 445 sets of siblings showed different patterns of length variants leads to the conclusion that a rather wide bottleneck or a regeneration of length heteroplasmy following a tight bottleneck has to be assumed. Another noticeable fact is the very close distribution, the low median of the *D* values and the complete absence of differing patterns in monozygous twins. These observations agree with the assumption of a random segregation of the mtDNA genomes in the dividing oocyte.

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References

1. Asari M, Azumi JI, Shimizu K, Shiono H (2008) Differences in tissue distribution of HV2 length heteroplasmy in mitochondrial DNA between mothers and children. Forensic Sci Int 175:155–159
2. Bendall KE, Sykes BC (1995) Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. Am J Hum Genet 57:248–256
3. Bendall KE, Macaulay VA, Baker JR, Sykes BC (1996) Heteroplasmic point mutations in the human mtDNA control region. Am J Hum Genet 59:1276–1287
4. Blakely EL, He L, Taylor RW, Chinnery PF, Lightowlers RN, Schaefer AM, Turnbull DM (2004) Mitochondrial DNA deletion in “identical” twin brothers. J Med Genet 41(2):e19
5. Brandstätter A, Niederstätter H, Parson W (2004) Monitoring the inheritance of heteroplasmy by computer-assisted detection of mixed basecalls in the entire human mitochondrial DNA control region. Int J Legal Med 118(1):47–54
6. Cavelier L, Jazin E, Jalonen P, Gyllensten U (2000) MtDNA substitution rate and segregation of heteroplasmy in coding and noncoding regions. Hum Genet 107:45–50
7. Cummins J (1998) Mitochondrial DNA in mammalian reproduction. Rev Reprod 3:172–182
8. Cummins JM (2001) Mitochondria: potential roles in embryogenesis and nucleocytoplasmic transfer. Hum Reprod Update 7:217–228
9. Detjen AK, Tinschert S, Kaufmann D, Algermissen B, Nürnberg P, Schuelke M (2007) Analysis of mitochondrial DNA in discordant monozygotic twins with neurofibromatosis type 1. Twin Res Hum Genet 10(3):486–495
10. Forster L (2004) Natural radioactivity and human mitochondrial DNA mutations in Kerala (India). Doctoral thesis, Medical Faculty of the Westphalian Wilhelm's University of Münster
11. Gill P, Sparkes R, Pinchin R, Clayton T, Whitaker J, Buckleton J (1998) Interpreting simple STR mixtures using allele peak areas. Forensic Sci Int 91(1):41–53
12. Gringras P, Chen W (2001) Mechanisms for differences in monozygous twins. Early Hum Dev 64:105–117
13. Hauswirth WW, Laipis PJ (1982) Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. Proc Natl Acad Sci U S A 79:4686–4690
14. Hauswirth WW, Van de Walle MJ, Laipis PJ, Olivo PD (1984) Heterogeneous mitochondrial DNA D-loop sequences in bovine tissue. Cell 37(3):1001–1007
15. Höhler T, Reuss E, Evers N et al (2002) Differential genetic determination of immune responsiveness to hepatitis B surface antigen and to hepatitis A virus: a vaccination study in twins. Lancet 28:991–994

16. Howell N, Smejkal CB (2000) Persistent heteroplasmy of a mutation in the human mtDNA control region: hypermutation as an apparent consequence of simple-repeat expansion/contraction. Am J Hum Genet 66:1589–1598
17. Howell N, Halvorson S, Kubacka I, McCullough DA, Bindoff LA, Turnbull DM (1992) Mitochondrial gene segregation in mammals: is the bottleneck always narrow? Hum Genet 90:117–120
18. Hühne J, Pfeiffer H, Brinkmann B (1998) Heteroplasmic substitutions in the mitochondrial DNA control region in mother and child samples. Int J Legal Med 112:27–30
19. Jansen RP, de Boer K (1998) The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. Mol Cell Endocrinol 145:81–88
20. Jenuth JP, Peterson AC, Fu K, Shoubridge EA (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nat Genet 14:146–151
21. Kirches E, Michael M, Warich-Kirches M et al (2001) Heterogeneous tissue distribution of a mitochondrial DNA polymorphism in heteroplasmic subjects without mitochondrial disorders. J Med Genet 38(5):312–317
22. Krakauer DC, Mira A (1999) Mitochondria and germ-cell death. Nature 40:125–126
23. Lagerström-Fermér M, Olsson C, Forsgren L, Syvänen AC (2001) Heteroplasmy of the human mtDNA control region remains constant during life. Am J Hum Genet 68(5):1299–1301
24. Lee HY, Chung U, Park MJ, Yoo JE, Han GR, Shin KJ (2006) Differential distribution of human mitochondrial DNA in somatic tissues and hairs. Ann Hum Genet 70:59–65
25. Lutz S, Weisser HJ, Heizmann J, Pollak S (1996) mtDNA as a tool for identification of human remains. Int J Legal Med 109:205–209
26. Lutz S, Weisser HJ, Heizmann J, Pollak S (2000) Mitochondrial heteroplasmy among maternally related individuals. Int J Legal Med 113:155–161
27. Lutz-Bonengel S, Sänger T, Pollak S, Szibor R (2004) Different methods to determine length heteroplasmy within the mitochondrial control region. Int J Legal Med 118:274–281
28. Lutz- Bonengel S, Sänger S, Heinrich M, Thomas Zacher T, Schmidt U (2007) Low volume amplification and subsequent sequencing of mitochondrial DNA on a chemically structured chip. Int J Legal Med 121(1):68–73
29. Lutz-Bonengel S, Sänger T, Parson W et al (2008) Single lymphocytes from two healthy individuals with mitochondrial point heteroplasmy are mainly homoplasmic. Int J Legal Med 122 (3):189–197
30. Machin G, Keith L (1999) Biology of twins and other multiple pregnancies. In: Machin G, Keith L (eds) An atlas of multiple pregnancy: biology and pathology. Parthenon, New York, pp 13–24
31. Malik S, Sudoyo H, Pramoonjago P et al (2002) Nuclear mitochondrial interplay in the modulation of the homopolymeric tract length heteroplasmy in the control (D-loop) region of the mitochondrial DNA. Hum Genet 110(5):402–411
32. Marchington DR, Hartshorne GM, Barlow D, Poulton J (1997) Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: support for a genetic bottleneck. Am J Hum Genet 60:408–416
33. Salas A, Lareu MV, Carracedo A (2001) Heteroplasmy in mtDNA and the weight of evidence in forensic mtDNA analysis: a case report. Int J Legal Med 114:186–190
34. Shoubridge EA, Wai T (2007) Mitochondrial DNA and the mammalian oocyte. Curr Top Dev Biol 77:87–111
35. Steuerwald N, Barritt JA, Adler R, Malter H, Schimmel T, Cohen J, Brenner CA (2000) Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. Zygote 8(3):209–215
36. Stewart JE, Fisher CL, Aagaard PJ et al (2001) Length variation in HV2 of the human mitochondrial DNA control region. J Forensic Sci 46(4):862–870
37. Turchi C, Buscemi L, Pesaresi M, Di Saverio M, Paoli M, Tagliabracci A (2003) Occurrence of heteroplasmy in related individuals. Int Congr Ser 1239(2003):553–556
38. Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10(4):506–513