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CASE REPORT

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Mitochondrial D-loop $(CA)_n$ repeat length heteroplasmy: frequency in a German population sample and inheritance studies in two pedigrees

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Abstract Sequence analysis of the human mitochondrial genome (mtDNA) has proven to be a valuable tool in forensic identity testing and the analysis of crime scene stains. In contrast to the very expensive sequencing technique, typing of different length variants can greatly facilitate screening of a large number of traces for their relevance during casework. Within the mitochondrial control region, a dinucleotide $(CA)_n$ repeat locus is present. To assess the discrimination power of this marker, we have determined $(CA)_n$ allele distribution and the frequency of heteroplasmy in a population sample of 2,458 Germans. The inclination to develop heteroplasmic mixtures $(CA)_n/(CA)_{n-1}$ was positively correlated with the number of CA repeats in the mtDNA. In addition, we have studied the inheritance patterns of $(CA)_n$ repeat sequence heteroplasmy in two pedigrees. In one pedigree, we also found a length heteroplasmy in the homopolymeric C-tract (nt 303–309). Our data show stable inheritance of heteroplasmy within the homopolymeric C-stretch, but rather unstable inheritance regarding the $(CA)_n$ repeat locus.

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Introduction

Sequence analysis of mitochondrial DNA (mtDNA) has proven to be a valuable complement to genomic DNA analysis in forensic identity testing and stain analyses [8, 9, 13, 15, 19, 28, 29, 31, 32]. Especially if DNA extraction yields quantities or qualities of genomic DNA not sufficient for typing of short tandem repeats (STRs), mtDNA analysis may be the method of choice due to the higher copy number of mitochondrial genomes in each cell. Although sequencing of the hypervariable regions 1 and 2 (HV1/HV2) is highly informative, other regions within the control region of the mitochondrial genome can be analyzed, in addition, to achieve a higher discrimination power [20, 22]. However, sequencing of the whole mitochondrial control region is a time and cost-consuming method. In forensic casework, large numbers of samples often have to be prescreened to determine their relevance in a case. Therefore, an easy-to-handle, fast, and cost-efficient technique is required.

One of the most informative mtDNA loci showing length variations is a $(CA)_n$ dinucleotide repeat within the human mitochondrial control region located between positions 514 and 523 [6]. Typing of individuals of different populations revealed a total of five different alleles, $(CA)_3$ to $(CA)_7$ [6, 11, 30]. In a population sample of 396 Germans investigated earlier [30], the majority (79%) showed five CA-dinucleotides $(CA)_5$, 11% of the population showed the $(CA)_4$ allele, 8% showed the $(CA)_6$ allele, and 2% showed the $(CA)_7$ allele. Although the power of discrimination (PD) is low (0.357), in some cases, typing of the $(CA)_n$ repeat region can be highly effective, especially when a rare constellation is present.

To further investigate this marker, data of a large population sample could be helpful. In a total of 2,458 individuals from Germany, most of them analyzed in the

context of a mass screening for police investigative purposes, several samples showed a heteroplasmic $(CA)_n$ repeat region. One of them exhibits, in addition, a heteroplasmy within the homopolymeric C-tract (nt 303–309). In two cases, it was possible to perform pedigree analyses with reference to four maternal relatives each.

Additionally, we report here the allele and heteroplasmy frequencies found in our population sample.

Materials and methods

DNA samples

In the context of a mass screening for police investigative purposes, oral cotton swabs from 2,332 males living in the area Saxony-Anhalt, Germany, were analyzed in the $(CA)_n$ repeat locus. As the samples were made anonymous, no personal data were available. Thus, a small percentage of related individuals in this rural population cannot be excluded. Additionally, 126 blood or buccal swabs donated from 126 unrelated persons from the same area were analyzed by sequencing of the entire D-loop. Hence, we investigated a total of 2,458 Caucasians. Two males from the latter group showed a $(CA)_n$ heteroplasmy. It was possible to perform pedigree analyses of maternal relatives. In pedigree I, five persons were analyzed (three brothers and their two cousins) using blood samples, buccal swabs, and hairs. In one of these persons, only buccal swabs and hair roots were available (see Fig. 1). In Pedigree II, five persons spanning two generations were analyzed using buccal swabs (see Fig. 2).

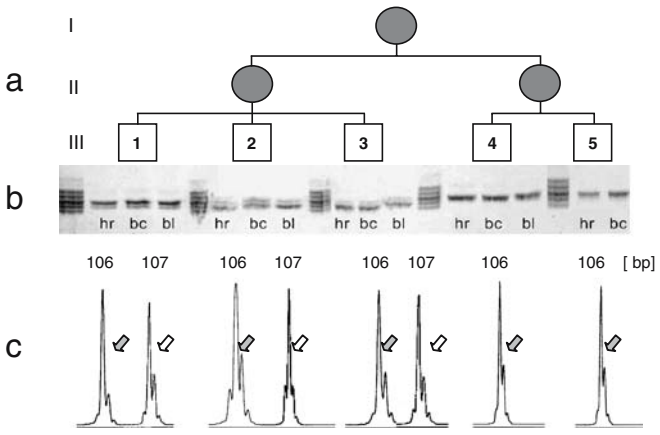

Fig. 1 **a** Pedigree of three brothers (III_1–III_3) and their cousins (III_4 and III_5). *Circles in grey* symbolize persons not available for investigation. **b** $(CA)_n$ repeat amplicon pattern demonstrating in a polyacrylamide gel after silver staining. Ladders are containing alleles $(CA)_3$–$(CA)_7$. DNA samples were extracted from hair roots (*hr*) buccal cells (*bc*), and blood (*bl*). **c** The gene scan peaks show the amplicons containing the homopolymeric C-tract. The plots labelled with a *dark arrow* refer to the amplification of the whole buccal cell DNA. *White arrows* point at plots showing the C-tract heteroplasmy in the $(CA)_4$ line. The indication of length in bp refers to the appropriate main peaks

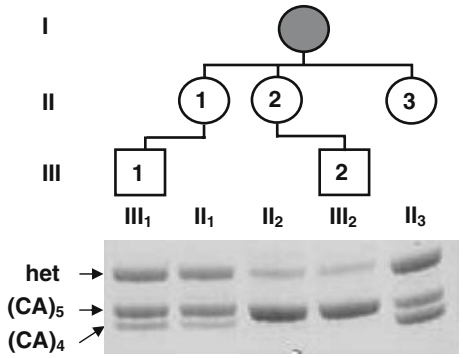

Fig. 2 **a** Pedigree II with amplicon pattern of three sisters (II_1–II_3) and their sons (III_1 and III_2). The *circle in grey* symbolizes a person not available for investigation. **b** The amplicon pattern shows the alleles $(CA)_4$/$(CA)_5$ and a heteroduplex (*het*). The *PCR* was carried out using DNA extracted from buccal cells

DNA extraction from buccal swabs and hair roots was performed using Nucleo Spin tissue kit according to the manufacturer's recommendations (Macherey-Nagel, Düren, Germany). For DNA extraction from blood samples, the Nucleo Spin blood kit (Macherey-Nagel) was used.

Amplification of the entire control region

One hundred twenty-six samples were analyzed by sequencing of the entire D-loop. Amplification was carried out with primer pair L15926/H00580 according to Orrego and King [24]. For sequencing, the RR Dye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA) was used, following the protocol of Parson et al. [25] using oligonucleotides L15990, L16364, and L00147 for sequencing to generate a continuous and overlapping sequence of the entire D-loop. The sequencing procedure followed the international guidelines for mtDNA typing [2, 35]. Sequences were aligned to the Cambridge reference sequence (CRS) [1]. All Primer sequences are given in Table 1.

Table 1 Sequences of PCR and sequencing primers

Primer Name	Primer Sequence
L15926	TAC ACC AGT CTT GTA AAC C
L15990	TTA ACT CCACCA TTA GCA CC
L16364	AGT CAA ATC CCT TCT CGT CC
L00147	CTG TCT TTG ATT CCT GCC TC
L00271	TCT GCA CAG CCA CTT TCC AC
L00484	CTC CCA TAC TAC TAA TCT CA
H00537	TGG TTG GTT CGG GGT ATG
H00356	GTG TTA GGG TTC TTT GTT TTT G
$H00510_{Sel\text{-}CA4}$	TTA GCA GCG GT GT GT GT GCA GG
H00580	TGA GGA GGT AAG CTA CAT A

Primer names (numbers) refer to the 3′-ends in accordance with CRS. "L" and "H" designate the light and the heavy strand, respectively. To highlight its selective function in primer $H00510_{Sel\text{-}CA4}$, the repeat structure has been underlined

Amplification of the $(CA)_n$ repeat locus

Amplification of $(CA)_n$ repeat and electrophoretical sizing was carried out as described previously [30] with minor modifications [reduction of polymerase chain reaction (PCR) cycles to 26] using the primers L00484 and H00537. PCR products were separated by polyacrylamide gel electrophoresis (PAGE). For cloning, primer L00484 was used in a 6-Fam-unlabelled version and for detection by capillary electrophoresis, in a 6-Fam-labelled version.

Selective amplification of the $(CA)_4$ repeat

Selective amplification of the $(CA)_4$ allele was carried out to separate heteroplasmic samples carrying an additional heteroplasmy in the homopolymeric C-tract. The amplification was performed using primer combination L00147/$H00510_{Sel\text{-}CA4}$ (see Tables 1 and 2) while the latter matches allele $(CA)_4$ but not allele $(CA)_5$. The matching/mismatching properties of $H00510_{Sel\text{-}CA4}$ are given in Table 2. PCR amplification was conducted for 25 cycles in a 25-µl reaction volume containing 2 ng DNA, 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1 U Hot Goldstar polymerase (Eurogentec, Seraing, Belgium), and 2.5 µl PCR buffer. Selective amplification of control templates $(CA)_4$ and $(CA)_5$ was achieved using a TGradient thermocycler (Biometra, Göttingen, Germany). In previous experiments using a temperature gradient in 1°C steps, we established an annealing temperature of 67°C to be optimal for selective amplification of allele $(CA)_4$. Hence, cycling conditions were as follows: 1 cycle of 95°C for 3 min, 25 cycles of 94°C for 60 s, 67°C for 60 s, 72°C for 90 s, and 1 cycle of 72°C for 10 min. To ensure a high reliability, homoplasmic $(CA)_4$ and $(CA)_5$ control templates were proceeded in parallel. Using the primers mentioned, the control template $(CA)_4$ gave a weak signal at 72°C. The $(CA)_5$ allele could not be amplified under such conditions (data not shown). Hence, a reliable selectivity has been demonstrated.

Amplicon length measurements and quantification of heteroplasmic components

$(CA)_n$ repeats

For typing $(CA)_n$ repeat alleles, L00484/H00537-generated amplicons were separated using PAGE and silver staining as described previously [30]. An allelic ladder was constructed from sequenced alleles $(CA)_3$–$(CA)_7$. The $(CA)_3$ which could not be found in our population originated from an African sample investigated earlier.

To quantify the allele ratio in heteroplasmic DNA samples, PCR products generated by primers L00484 and H00537 amplifying the $(CA)_n$ repeat region of the members of pedigree I (see Fig. 1) were cloned as described elsewhere. DNA was then extracted from 50 colonies and the $(CA)_n$ repeat region was amplified with the same primer pair. Thus, the allele ratio $(CA)_4/(CA)_5$ could be determined by counting the colonies.

Homopolymeric C-tract

Length variations of the homopolymeric C-tract were detected by PCR (primer combination L00271/H00356, Table 1) and direct sequencing with primer L00147 and by fragment length analysis using the ABI Prism 310 Genetic Analyzer and the GeneScan software (version 3.1) with the HD 400 internal lane standard (Applied Biosystems, Foster City, CA). Amplification was carried out for 28 cycles in a 25-µl reaction volume containing 2 ng DNA, 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer of the primer pair L00271/H00356, 1-U Hot Goldstar polymerase (Eurogentec, Seraing, Belgium), and 2.5 µl PCR buffer. The following cycle conditions were used: 1 cycle of 95°C for 3 min, 25 cycles of 94°C for 60 s, 57°C for 60 s, 72°C for 90 s, and 1 cycle of 72°C for 10 min.

Additionally, we generated, selectively, PCR products from one of the heteroplasmic components (allele $(CA)_4$) only. In a following amplification step using primer pair L00271/H00356, we produced amplicons which reflect the situation of the C-tract heteroplasmy within the $(CA)_4$ line.

Exclusion of artefacts

If a DNA sample contains PCR products by a carry-over contamination, heteroplasmy can be feigned. Therefore, in each case of heteroplasmy, the whole analysis including DNA extraction was repeated. In all cases, we could confirm our initial findings using this procedure.

Table 2 Matching/mismatching properties of primer $H00510_{Sel\text{-}CA4}$ for selective amplification of the $(CA)_4$ haplotype

$(CA)_4$ - template	5'-CTACCCAGCACACACACCGCTGCTAACC-3'
$H00510_{Sel\text{-}CA4}$	3'- GGACGTGTGTGTGGCGACGATT -5'
$(CA)_5$ - template	5'-ACCCAGCACACACACACCGCTGCTAACC-3'

Primer $H00510_{Sel\text{-}CA4}$ was designed to produce four mismatches to the $(CA)_5$ allele and one mismatch to the $(CA)_4$ allele, respectively. The underlined A within the $H00510_{Sel\text{-}CA4}$ primer represents a permanent mismatch locus, which does not disturb the $(CA)_4$ allele amplification but enhances amplification suppression of the $(CA)_5$ allele. Mismatch positions are highlighted in grey

Results

$(CA)_n$ allele distribution and heteroplasmy frequencies

The allele distribution of the $(CA)_n$ repeat in a population sample of 2,458 German individuals is demonstrated in Table 3. We found 257 (frequency: 0.105) individuals showing $(CA)_4$, 1,964 (0.799) showing $(CA)_5$, 186 (0.076) showing $(CA)_6$, 42 (0.017) showing $(CA)_7$, and 9 (0.004) showing $(CA)_8$. This summary includes heteroplasmic mtDNA between two neighbouring $(CA)_n$ alleles which were found in 34 of 2,458 individuals. Thus, the frequency of heteroplasmy was found to be 0.0138 (95% confidence interval (CI): 0.01–0.02). The inclination to develop heteroplasmic mixtures $(CA)_n/(CA)_{n-1}$ appears to be positively correlated with the number of CA repeats within the mtDNA (Table 3). In our overall allele frequency calculation, we put heteroplasmic individuals exhibiting the allele combination $(CA)_n/(CA)_{n-1}$ into the group of $(CA)_n$. Measuring the length by the electrophoretical method, 20 individuals were found to show non-integer alleles: allele 3.1 (two individuals), allele 4.1 (16 individuals), and allele 5.1 (two individuals), respectively. However, sequencing analyses show that these alleles were not caused by variations within the repeat area but rather by a C deletion at position 549 in the flanking region. Therefore, in this statistical overview, we have counted the apparent 3.1 allele as $(CA)_4$, 4.1 as $(CA)_5$, and 5.1 as $(CA)_6$, respectively.

Tissue specificity of heteroplasmy and inheritance studies

Two families with five test persons each were studied. In pedigree I (Fig. 1a), $(CA)_4/(CA)_5$ heteroplasmy could be observed in three brothers (persons III_{1-3}) but not in their cousins (persons III_{4-5}), which showed $(CA)_5$ exclusively (Fig. 1b). Quantification of the $(CA)_4/(CA)_5$ proportion by cloning of PCR products from blood or buccal cells affirmed the occurrence of $(CA)_n$ repeat heteroplasmy of the three brothers, but not of their cousins. The heteroplasmy ratios determined by cloning experiments are listed in Table 4. All analyzed members of pedigree I showed a C_7/C_8 length heteroplasmy within the homopolymeric C-stretch at positions 303–309 in blood, hair roots, and buccal cells. As could be shown not only by direct sequencing but also by amplicon length measurement, persons III_4 and III_5, which showed the $(CA)_5$ haplotype, also carried a C-stretch heteroplasmy. The investigation of the cousins revealed that in the $(CA)_5$ haplotype the C7 allele, which refers to the amplicon length of 106 bp, is predominant. Amplification of the C-stretch of the mixed $(CA)_4/(CA)_5$ templates of persons III_1, III_2, and III_3 produced heteroplasmic amplicons in which again the 106-bp peak (C7) was predominant. The question whether or not the $(CA)_4$ line was also affected by the same, C-stretch heteroplasmy was subsequently investigated in selectively amplified $(CA)_4$ haplotypes. Figure 1c demonstrates that in the three brothers (persons III_1, III_2, and III_3), C-tract amplicons produced from the selected $(CA)_4$

Table 3 (CA) alleles and heteroplasmy frequencies found in 2,458 German individuals

Alleles		n	Allele frequency	Proportion
	Variants	n	2,458	Heteroplasmy/ Homoplasmy (95% CI)
$(CA)_4$	$(CA)_{4/3\ heteroplasmy}$	0	0.105	0 (0.0001-0.01)
	$(CA)_{virtual\ 3.1}$	2		
	$(CA)_4$	255		
$(CA)_5$	$(CA)_{5/4\ heteroplasmy}$	6	0.799	0.0031 (0.001-0.007)
	$(CA)_{virtual\ 4.1}$	16		
	$(CA)_5$	1,942		
$(CA)_6$	$(CA)_{6/5\ heteroplasmy}$	18	0.076	0.11 (0.07-0.16)
	$(CA)_{virtual\ 5.1}$	2		
	$(CA)_6$	166		
$(CA)_7$	$(CA)_{7/6\ heteroplasmy}$	7	0.017	0.20 (0.1-0.36)
	$(CA)_{virtual\ 6.1}$	0		
	$(CA)_7$	35		
$(CA)_8$	$(CA)_{8/7\ heteroplasmy}$	3	0.004	0.50 (0.18-0.82)
	$(CA)_{virtual\ 7.1}$	0		
	$(CA)_8$	6		
Total		2,458		0.0138 (0.01-0.02)

haplotype also carried length heteroplasmy at positions 303–309. The amplicons produced from $(CA)_4$ haplotype show predominantly 107 bp, representing the C8 allele.

Three members of pedigree II also showed a $(CA)_4/(CA)_5$ heteroplasmy. Although the entire D-loop was checked using direct sequencing, no further heteroplasmic sites were found. Family members II_1, III_1, and III_3 offered two different amplification products in PAGE analysis. The larger amplicon of individuals II_1 and III_1, representing $(CA)_5$ allele, appeared to be about three times more abundant in the PAGE than the smaller fragment of the $(CA)_4$ allele. In contrast, within individual III_3, the ratio seemed to be more balanced. Family members II_2 and III_2 indicated another variant and seemed to be nearly homoplasmic for the $(CA)_5$ variant.

Discussion

In routine casework, a screening test for the $(CA)_n$ repeat locus (nt 514–523) was performed including 2,332 individuals, amend to detect an offender carrying a rare mtDNA haplotype including the $(CA)_6$ allele [33]. As the result of investigations on 126 unrelated persons carried out earlier, we were able to investigate two pedigrees exhibiting a $(CA)_4/(CA)_5$ heteroplasmy each. In pedigree I, the $(CA)_4/(CA)_5$ heteroplasmy was combined with a C-stretch heteroplasmy at positions 303–309. We jointly studied both phenomena.

In our population, we detected 34 individuals showing a CA_n/CA_{n-1} heteroplasmy. Hence, the frequency of length heteroplasmy in the German population (0.0138, 95% CI: 0.01–0.02) is slightly higher than that in a Korean population reported recently (three from 500; frequency: 0.006; 95% CI: 0.002–0.02) [11]. This finding is in agreement with the fact that the German population shows relative high frequencies of $(CA)_6$, $(CA)_7$, and $(CA)_8$ individuals. In our sample population, such alleles seemed to be prone to develop heteroplasmy (see Table 3).

Two families with five persons each were studied for both, the $(CA)_n$ and the homopolymeric C-stretch locus. Three of five individuals of pedigree I showed a $(CA)_4/(CA)_5$ heteroplasmy at positions 514-523. In contrast, the analyzed tissues of the two remaining persons showed a clear majority (or even a homoplasmy) of allele $(CA)_5$ (Table 4). In pedigree I, all members showed length heteroplasmy at positions 303–309 in blood and buccal swabs, if available (Fig. 1c). Hair roots showed length heteroplasmy at this position, too. Therefore, this pedigree demonstrates a stable inheritance of the heteroplasmic pattern of the homopolymeric C-stretch and a rather unstable inheritance in respect to the $(CA)_4/(CA)_5$ heteroplasmy.

Table 4 $(CA)_4/(CA)_5$ allele ratios in blood and buccal cells of members III_1–III_5 in pedigree I

Individual	III_1	III_2	III_3	III_4	III_5
	Proportion of alleles $(CA)_4/(CA)_5$				
Tissue					
Blood	14/36	30/20	31/19	0/50	–
Buccal cells	–	–	–	–	0/50

Quantification has been performed by cloning experiments and subsequent counting of the colonies

To check whether the C-stretch heteroplasmy in pedigree I was associated with one or both $(CA)_n$ repeat haplotypes, an investigation of selectively amplified PCR products was performed. Both persons III_4 and III_5 of pedigree I showed a homoplasmic $(CA)_5$ haplotype and a heteroplasmic C-stretch. The selectively amplified $(CA)_4$ haplotype of the three brothers offered a heteroplasmic C-stretch, too. Thus, we conclude that in pedigree I, the C-tract heteroplasmy is bound to both lines, the $(CA)_4$ and $(CA)_5$ lines. One possible explanation could be that the $(CA)_n$ heteroplasmy was lost during generations, while the C-tract heteroplasmy was carried more stable. No pedigree studies concerning the human mitochondrial $(CA)_n$ repeat locus are available so far. But several inheritance studies regarding the homopolymeric C-stretch have been published [3–5, 10, 14, 16, 18, 23, 26, 27, 34]. Generally, similar proportions of different haplotypes within maternally related individuals are a hint for a large bottleneck. In contrast, a fast change of haplotype within a maternal pedigree can be interpreted as a sign of a tight bottleneck. The studies published, so far, gave hints for both a slow segregation [3, 16, 23, 34] and a fast segregation [4, 5, 14, 18, 27]. Furthermore, even a combined occurrence of both forms within a maternal relationship has been observed [10, 17, 21]. Thus, the investigation of the two pedigrees presented here point out that the inheritance of $(CA)_n$ repeat heteroplasmy seems to be unstable, whereas, C-tract heteroplasmy can be transferred through many $(CA)_n$ repeat heteroplasmy. If this phenomenon could be confirmed in more pedigrees, a narrow bottleneck could be assumed and would explain the remarkable differences concerning the frequencies of these two heteroplasmic loci.

Members of pedigree II showed heteroplasmy at the $(CA)_n$ repeat locus only. In direct sequencing, the C-stretch at positions 303–309 was found to be homoplasmic for haplotype C7. Considering the $(CA)_n$ repeat region, three different ratios of the two observed haplotypes, $(CA)_4$ and $(CA)_5$, were found. This varying occurrence of $(CA)_4$ and $(CA)_5$ haplotypes within family members of pedigree II is concordant with the observation of a relatively unstable inheritance of a $(CA)_4/(CA)_5$ heteroplasmy in pedigree I.

It is remarkable that distinctive racial differences concerning the $(CA)_n$ frequencies exist and that $(CA)_5$ is the most frequent allele in all populations investigated [6, 11, 30]. Furthermore, $(CA)_4$ is common in Africans [30], East Asians, and Amerindians (unpublished data including more than 60 Japanese, Vietnamese, and Peruvians. Allele distribution was found to be similar to that of Koreans [11]). Heteroplasmy seems to be preferably bound to alleles with higher repeat numbers. This observation may reflect that $(CA)_4$ and $(CA)_5$ seems to be

more stable than longer alleles. Thus, it is surprising that within the Caucasian population, frequencies of alleles $(CA)_{6–8}$ were found to be high (0.05 up to 0.09). This observation is a hint that the mitochondrial $(CA)_n$ repeat locus can be regarded as quite similar to autosomal short tandem repeat (STR) loci because the mutation rate of STR was reported to be positively correlated to the geometric mean of the number of variable uninterrupted repeats [7].

It would be of interest to complete this overview by investigating a population sample of Australian Aborigines, which may have taken their own dispersal route along the borders of the Indian Ocean to arrive at their destinations about 60,000 years ago. This is comparable with the distance between the European and the Asian populations [12].

For forensic practice, we have to conclude that $(CA)_n$ heteroplasmy can appear or disappear during a few generations. Hence, when skeleton identification has to proceed via mtDNA typing, differences between bone samples and alleged relatives must be regarded as an exclusion marker of low relevance.

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