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Different methods to determine length heteroplasmy within the mitochondrial control region

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Abstract The first and second hypervariable regions of the human mitochondrial DNA control region contain two homopolymeric stretches of cytosine (nt 16184–16193 and nt 303–315, respectively). According to the Cambridge reference sequence these homopolymeric stretches are interrupted by thymine (T), at positions 16189 and 310, respectively. Monotonous runs of the same base have been suggested to be hot spots for mutations, probably caused by replication slippage, resulting in length heteroplasmy. This paper describes a rapid method based on restriction cleavage of labelled PCR products encompassing the homopolymeric tract in HVII to quantify the relative proportions of different length variants present in an individual. To compare the accuracy of this method, cloned PCR products from several heteroplasmic individuals have been additionally sequenced.

Keywords mtDNA · Homopolymeric tract · Length heteroplasmy

Introduction

Mononucleotide repeats are known as mutation hot spots, which are potentially due to slippage of the DNA polymerase during replication, resulting in DNA length heteroplasmy (Hauswirth et al. 1984; Hauswirth and Clayton 1985). The mitochondrial control region exhibits at least two homopolymeric tracts, where length polymorphisms can be frequently observed. Hypervariable region I (HVI) contains a homopolymeric tract between positions 16184 and 16193 (Bendall and Sykes 1995). Here, a mononucleotide stretch of cytosines (C) is interrupted at position 16189 by a thymine (T) residue (Anderson et al. 1981). Approximately 10–15% of samples were found to show a T to C transition at position 16189, producing an uninterrupted homopolymeric tract with 8–14 cytosine residues (Piercy et al. 1993; Bendall and Sykes 1995; Lutz et al. 1998; Parson et al. 1998).

In addition, length heteroplasmy has been observed at position 303–315 in the mitochondrial HVII segment (Greenberg et al. 1983). According to the Anderson sequence (Anderson et al. 1981) seven cytosine residues precede a thymine that is subsequently followed by five more cytosine residues. In contrast to the homopolymeric tract at HVI, where the thymine residue is often replaced by a cytosine, the thymine residue in HVII is less frequently missing. It is thought that the mechanism for HVII length heteroplasmy is the insertion of cytosines in the 303–309 area as well as replication slippage in the case of a thymine to cytosine transition at position 310 (Bendall and Sykes 1995; Stewart et al. 2001).

Sequencing electropherograms produced by the direct sequencing of PCR products with length heteroplasmy show a characteristic "out-of-phase" pattern downstream of the homopolymeric region (Bendall and Sykes 1995). In the hypervariable region II this phenomenon was observed in 8–69% of individuals analysed (Parson et al. 1998; Imaizumi et al. 2002; Brandstätter et al. 2004; Lee et al. 2004; Zupanic Pajnic et al. 2004). In most cases a predominant type can be identified (Parson et al. 1998). Interestingly the level of length heteroplasmy can vary among different tissues within the same individual (Pfeiffer et al. 2004) and among maternal relatives (Gocke et al. 1998; Hühne et al. 1998; Parson et al. 1998; Lutz et al. 2000; Brandstätter et al. 2004). To enhance the quality of sequence electropherograms downstream of a homopolymeric tract, the use of primer target sequences located within or near the homopolymeric DNA tract has been proposed (Szibor et al. 1997; Szibor and Michael 1999; Imaizumi et al. 2002; Rasmussen et al. 2002).

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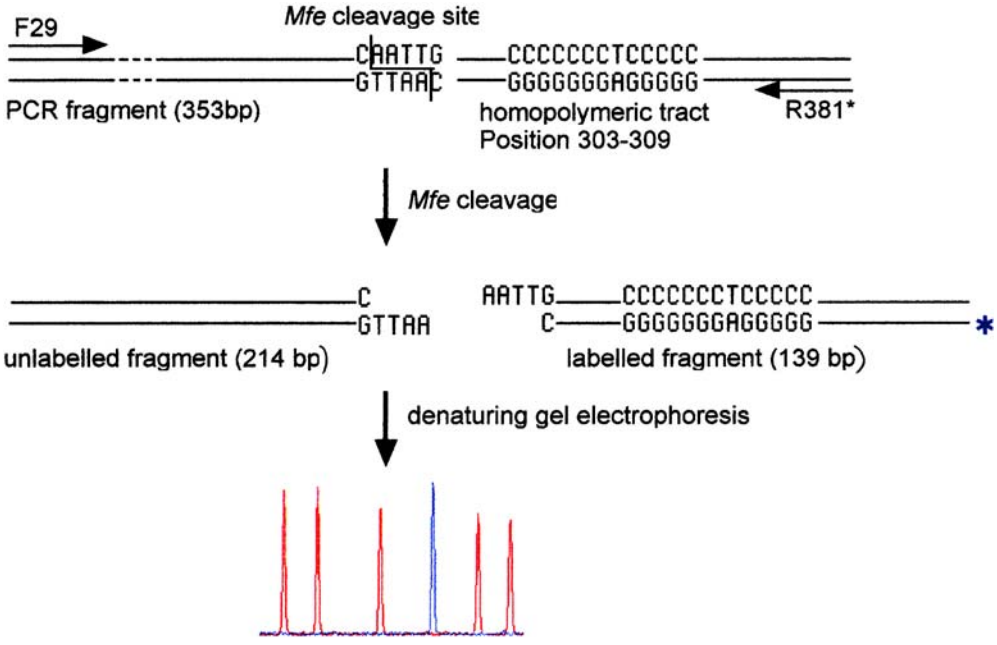

Fig. 1 Technical steps of heteroplasmy quantification by fluorescently labelled restriction fragment analysis. PCR primers F29 and R831 are indicated by horizontal arrows. Expected fragment lengths are given in parentheses. The fragment analysis electropherogram shows the 6-FAM labelled fragment (*blue*) and the ROX labelled size standard (*red*)

Table 1 Criteria for the classification of sequencing and fragment analysis samples in four groups which differ in the rate of length heteroplasmy at positions 303–309

Group	Description	Direct sequencing			Restriction fragment analysis	
		Sort and number of peaks at position 310	Quality of the sequence downstream position 310 (visually)	Ratio of quality values after and before the C-stretch	Number of peaks	Peak ratio
I	Purely homoplasmic	No peak below the T peak at position 310 exists	Sequence quality up- and downstream of position 310 is balanced	rseq≥1	Only a single peak at position 310 exists	
II	Slightly heteroplasmic	Minor fraction of one or more variants is represented by a small C-peak under the main T-peak at position 310	Sequence quality up- and downstream of position 310 differed slightly (poorer sequence quality following the C-stretch)	0.85≤rseq<1	One or two small peaks appear beside the main peak	The area of the minor peak(s) is maximum 1/10 of the amount of the main peak, $pa_{min} \leq 1/10\ pa_{max}$
III	Apparent heteroplasmic	Both a C-peak and a T-peak exists at position 310, whereas the minor peak shows more than 20% of the main peak area	Bias to a characteristic out of phase sequence downstream of the C-stretch	rseq<0.85	Beside the main peak at least one other peak	The area of the minor peak(s) is more than 1/10 of the amount of the main peak, $pa_{min} > 1/10\ pa_{max}$
IV	T to C transition with subsequent sequence dropout	Sequencing samples show a T to C transition at position 310 resulting in an uninterrupted homopolymeric tract	Out of phase pattern downstream of the C-stretch		Several peaks resembling the fingers of a hand	

r_{seq} Ratio of quality values after and before the C-stretch, r_{seq} $r_{QV(310–328.X)}/r_{QV(291–309.X)}$).
pa_{min}Area of minor peak.
pa_{max} Area of maximum peak.

Nevertheless, interpretation of length heteroplasmy still remains difficult, as heteroplasmy detection depends on the detection limit of the method used. Here we compare the efficiency of three heteroplasmy detection methods: direct sequencing, a rapid method employing fluorescently labelled restriction fragments and cloning and counting of PCR products.

Material and methods

Samples, DNA extraction and amplification

DNA samples from peripheral blood of 120 maternally unrelated individuals were analysed as previously described (Lutz et al. 1998; Pfeiffer et al. 2004). Nucleotides in the mtDNA sequences were numbered according to the Cambridge reference sequence (CRS; Anderson et al. 1981).

Direct sequencing

PCR products were directly sequenced as previously described (Lutz et al. 1998). Only high quality sequences lacking background signal were used. Sequences were aligned to the Cambridge reference sequence (Anderson et al. 1981) using Sequence Navigator software 1.0.1 and SeqScape software 2.0 (both: Applied Biosystems, Darmstadt) following international guidelines for mtDNA typing (Bär et al. 2000; Wilson et al. 2002).

Restriction fragment analysis

Amplification was accomplished using primer pair F29/R381 (Holland et al. 1995) except that R381 was fluorescently labelled with 6-FAM. The PCR-amplified products were then digested with *MfeI* restriction endonuclease (NEB, Beverly, MA) by mixing approximately 100 ng PCR product with 2.5 U of the restriction enzyme and 2 µl of the appropriate digestion buffer in a total volume of 20 µl. Of the enzyme treated and denatured PCR products, 1 µl was separated by capillary electrophoresis (Fig. 1). Electrophoresis and detection of the fluorescently labelled fragments containing the homopolymeric cytosine stretch were performed with an Applied Biosystems 310 Genetic Analyzer. Data interpretation was done using GeneScan 3.1.2 (Applied Biosystems, Darmstadt).

Cloning

PCR products were cloned into the pCR2.1 TOPO-vector (Invitrogen, Carlsbad, CA). Plasmids were isolated, purified and sequenced as previously described (Lutz et al. 2000). For each sample two independently generated PCR products were cloned to minimise effects of possible PCR errors and for each sample 40 clones (20 clones per PCR product) were sequenced.

Characterisation and quantification of length heteroplasmy

All samples were classified into four groups according to the proportion of length heteroplasmy (Table 1 and Fig. 2).

For sequencing data the quality values of several nucleotides of the mitochondrial light strand upstream and downstream of the cytosine stretch were determined using SeqScape software (Applied Biosystems, Darmstadt). Quality values (QV) for each nucleotide position are a measure of certainty of the base-calling algorithm and a per-base estimate of the base-caller accuracy. Higher values correspond to a lower chance of algorithm error. The per-base quality values are graded on a scale corresponding to $QV=-10\log_{10}(Pe)$, where Pe is the probability of error of the base-call. The QV range spans from 1 to 50, with 1 (probability of errors=79%) indicating low reliance and 50 (probability of errors=0.001%) indicating high reliance. Heteroplasmic positions show lower quality values than homoplasmic positions. For every sample an average quality value for positions 291–309.X and 310–328 was obtained and the ratio $r_{seq}=r_{QV(310-328)}/r_{QV(291-309.X)}$ was determined.

For restriction fragment analysis data the peak areas were determined using GeneScan software (Applied Biosystems, Darmstadt).

The criteria used to assign the analysed samples into four different groups are given in Table 1.

Exclusion of artefacts

To prevent artificial generation of length variations due to the single A 3'-overhang generated by *Taq* DNA polymerase during PCR, a proof-reading polymerase was used for amplification (*Pfu* DNA polymerase, Stratagene, Gebouw, CA). To further rule out amplification errors, two independently generated PCR products from every sample were used for direct sequencing and restriction fragment analysis and the results were compared.

For cloning, two independently generated PCR products from each sample were ligated into pCR2.1. Furthermore, randomly selected clones showing different lengths of the homopolymeric tract were amplified and directly sequenced. In addition, several of these amplified clone samples were ligated a second time, the resulting clones were then sequenced again to disclose any possible alteration in heteroplasmic state compared to the original sample.

The PCR reactions in preparation for all three methods (direct sequencing, restriction fragment analysis and cloning) were performed under identical conditions.

To assure accurate size analysis, HD400 (Applied Biosystems, Darmstadt) was used as an internal standard. Analysis revealed that the amplicon produced was located between the 120 bp and 150 bp standards. Furthermore the homoplasmic character of PCR products with different C-stretch lengths was demonstrated by cloning and capillary gel electrophoresis. This method proved capable of detecting individual PCR products from a mix containing amplicons of different length, used as external standard.

Results

Heteroplasmy quantification using direct sequencing

The homopolymeric cytosine-stretch (C-type) in HVII of 120 maternally unrelated humans was amplified and directly sequenced. Subsequently, the electropherograms were analysed between positions 303–309 and the predominant sequence type (number of cytosine residues between positions 303–309) was determined. Based on the quality of the nucleotides before and after the cytosine-stretch the samples were assigned into one of four groups (Table 2). The length of the homopolymeric stretches of cytosine identified have been expressed as Cx, were x is the length of the homopolymeric stretch of cytosine.

- Group I: 40% (48 out of 120) of the samples seemed to be purely homoplasmic. Most of these samples belonged to the sequence type C7 (46 out of 48).
- Group II: approximately 28.3% (34 out of 120) of samples contained a small number of other variants, but the sequence chromatogram downstream of the homopolymeric tract was unambiguous. These samples mainly represented the sequence type C8 (31 out of 34). The sequence type C9 was represented once, C7 twice.

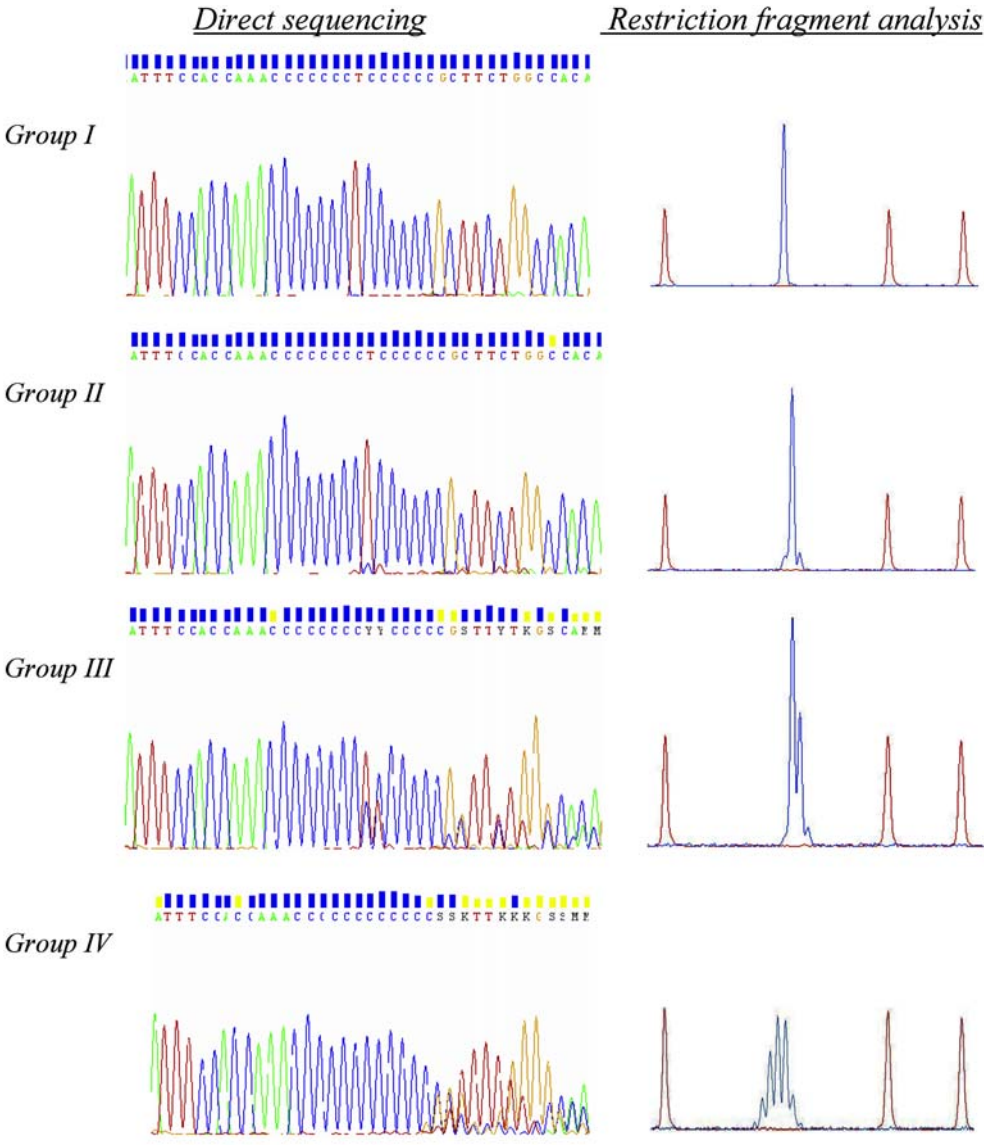

Fig. 2 Sequence and fragment analysis electropherograms of representative examples for the four groups, with sequencing results spanning nt 291–nt 328. The fragment analysis electropherograms show the 6-FAM labelled fragment(s) (139 bp+x, *blue*) and the ROX labelled size standard (allele size 120 bp, 150 bp and 160 bp, *red*)

– Group III: approximately 29.1% (35 out of 120) of samples exhibited a clear C-peak under the main T-peak and showed a blurred sequence downstream of the homopolymeric tract. In the majority of cases these samples represented sequence type C8 (21 out of 35) and C9 (13 out of 35). The sequence type C7 was detected once.
– Group IV: three samples showed a T to C transition at position 310.

Heteroplasmy quantification using restriction fragments

Further analysis was performed using fluorescently labelled restriction fragments of the homopolymeric cytosine-stretch in HVII. The restriction fragment electropherograms were analysed and the samples were assigned to one of the four groups based on measurements of the peak areas. Out of the 120 samples, 2 exhibited a C to T transition at position 242, which lies within the *Mfe* cleavage site and inhibited the restriction analysis. The results of the remaining 118 samples are shown in Table 3.

– Group I: 38.1% (45 out of 118) of samples were purely homoplasmic. These samples all belonged to the sequence type C7.
– Group II: 22.9% (27 out of 118) of samples contained a small number of other variants, with the electropherograms showing one or two smaller peaks beside the main peak. These samples were mainly represented by the sequence type C8 (21 out of 27). The sequence types C7 and C9 were each represented 3 times.
– Group III: in 36.4% (43 out of 118) of samples, next to the main peak another peak was detected with more than 10% of the main peak area. Out of 43 of these samples 32 belonged to the type C8, 11 out of 43 to the type C9. Neither the sequences C10 nor C7 were found.

Table 2 Classification of 120 analysed samples into 4 different groups, which differ in the rate of heteroplasmy level

Group	Number	C7	C8	C9	C10
		(predominant sequencing type)			
I	48	46	2	0	0
II	34	2	31	1	0
III	35	1	21	13	0
IV	3	nd	nd	nd	nd

The classification was based on the quality values of the nucleotides before and after the cytosine-stretch in direct sequencing.
nd Not detected.

Table 3 Classification of 120 analysed samples into 4 different groups, which differ in the rate of heteroplasmy level

Group	Number	C7	C8	C9	C10
		(predominant fragment analysis type)			
I	45	45	0	0	0
II	27	3	21	3	0
III	43	0	32	11	0
IV	3	nd	nd	nd	nd

The classification was based on the peak ratio in restriction fragment analysis.
nd Not detected.

– Group IV: according to the results obtained by direct sequencing, three samples showed several peaks, which was due to a T to C transition at position 310.

Heteroplasmy quantification by cloning

A representative collection of samples from the four groups was used to test the heteroplasmy level by cloning. The results are shown in Table 4.

Analysis of sample A, which by direct sequencing and restriction fragment analysis was determined to be C7 and representative of group I, yielded without exception C7 from 40 analysed clones. Sample B, which was assigned to group I after direct sequencing and to group II after restriction fragment analysis, was determined to be composed predominantly of the C8 sequence. Cloning analysis of 40 clones indicated that sample B contained one C9 sequence. Direct sequencing as well as restriction fragment analysis of samples C and D, revealed that the predominant sequences were C7 and C8, respectively. Both samples contained low levels of heteroplasmy. Analysis of 40 clones generated from each sample revealed that sample C contained both C7 and C8 sequences, and sample D contained C7, C8, C9, and C10 sequences. Samples E and F were assigned to group II after direct sequencing and group III after restriction fragment analysis. Further analysis of each sample showed that samples E and F had 31 and 37 clones of length C9, respectively, as well as sequences of length C8 and C10. Samples G and H contained an apparent heteroplasmy after direct sequencing and restriction fragment analysis. These two samples contained three (C8, C9, C10) and four (C7, C8, C9, C10) types, respectively.

Two clones, originating from sample G, were further amplified and cloned again, resulting in samples I and J. Both of the generated samples showed the same predominant type as the original sample, being C8 and C9, respectively.

Table 4 Cloning assay analysis of samples which show numerous homoplasmic and heteroplasmic sequence lengths in direct sequencing and fragment analysis.

Sample	Predominant sequence type	C7	C8	C9	C10		Total	Results Seq	Results FA
A	C7	40	0	0	0		40	I	I
B	C8	0	39	1	0		40	I	II
C	C7	34	6	0	0		40	II	II
D	C8	2	35	2	1		40	II	II
E	C9	0	2	31	7		40	II	III
F	C9	0	2	37	1		40	II	III
G	Inc (C8/9)	0	14	22	4		40	III	III
H	Inc (C8/9)	1	26	11	2		40	III	III
I (cs)	C8	0	40	0	0		40		
J (cs)	C9	0	0	40	0		40		
		C10[a]	C11[a]	C12[a]	C13[a]	C14[a]	Total	Results Seq	Results FA
K	Blurred	1	6	8	12	13	40	IV	IV

cs Clone samples which were amplified and cloned again.
Total Total number of analysed clone samples.
Results Seq Grouping by direct sequencing.
Results FA Grouping by fragment analysis.
Inc Inconclusive because C8 and C9 populations seem to be equally represented in direct sequencing.
Blurred Unclear signals beyond a T to C transition at position 310.
[a]Due to the T to C transition at position 310, the poly-cytosine stretch has been elongated, therefore the numbering system has been shifted for sample K.

Finally cloning of sample K, representing a sample with a T to C transition at position 310, provided clones with homopolymeric cytosine stretches of C10–C14 in length.

Examination of artefact probability

A number of different steps were taken to prevent the appearance of artefacts. With the direct sequencing and fragment analysis assays, two independent PCR products were analysed, and the results compared. All results were concordant and reproducible. In the case of the cloning assay, a minimum of two independently generated PCR products were cloned. Clones from independent PCR products from the same individual always produced almost identical amplicon length distributions. Furthermore, several of the cloned samples which had produced amplicons of different lengths, were amplified a second time, and then directly sequenced. Sequence electropherograms with no background noise were obtained, which was to be expected for amplification products of a single size. One C8 and one C9 clone sample were amplified and cloned a second time, to give 40 new clones from each original cloned sample. Subsequent sequencing analysis of the resulting clones gave unambiguous electropherograms, with all sequenced clones producing amplification products of a single length (Table 3, samples I and J).

However, mixing a number of DNA templates of different lengths generated a sequencing electropherogram identical to that produced by a HVII heteroplasmic individual (data not shown).

Discussion

Comparison between different heteroplasmy quantification systems: direct sequencing versus restriction fragment analysis

Classification by direct sequencing revealed 46 individuals of type C7, and 2 of type C8 in group I, with both of these C8 samples being homoplasmic. However, by using restriction fragment analysis the heteroplasmic character of 2 of the C8 samples was revealed. Therefore, the 2 C8 samples were assigned to group II and only C7 samples remained in group I after restriction fragment analysis. As restriction fragment analysis uncovered the heteroplasmic character of the 2 C8 samples, restriction fragment analysis seemed to be more sensitive than direct sequencing. The distribution of samples within groups II and III after analysis by either method confirms this assumption. Samples were re-classified into a more heteroplasmic group after consideration of the restriction fragment analysis results. Group II heteroplasmy in direct sequencing is difficult to detect if a low sequence background noise is present. Only in absolutely background-free electropherograms can the heteroplasmic level of group II be determined. Because the sequencing results of the heavy strand generally produced more background, a classification especially of group I and group II heteroplasmy is not possible. In general, low level length heteroplasmy is much easier to analyse when sequencing the mitochondrial light strand.

In contrast, it is easier to detect heteroplasmy in restriction fragment analysis electropherograms, and the overall proportions of each haplotype are more obvious. For group III samples, both the direct sequencing and restriction fragment analysis methods are equally suitable. In sequencing electropherograms, the sequence downstream of the heteroplasmic position is less readable. In restriction fragment analysis electropherograms more than one peak exists. For group IV samples, both methods are completely consistent and a T to C transition at position 310 is distinctive using either the direct sequencing or the fragment analysis method.

In addition to the different detection limits of the two methods, the data presented here suggest that a homopolymeric stretch of 7 cytosines seems to be much more stable than a stretch of 8 or more cytosine residues. This is evident in that both methods reveal that the quantity of C7 type DNA decreases with a concurrent increase in the proportion of heteroplasmic variants. On the other hand, the number of C8, C9 and C10 type DNA accumulates clearly with increasing heteroplasmy. According to previous reports, samples with a homopolymeric cytosine-stretch of eight or more cytosine residues show in general an increase in heteroplasmy (Stewart et al. 2001; Imaizumi et al. 2002; Lee et al. 2004).

Comparison between different heteroplasmy quantification systems: cloning versus direct sequencing and fragment analysis

The results of the cloning experiments are mostly in concordance with the results generated by both direct sequencing and restriction analysis. An example for a purely homoplasmic sequence is sample A. Using either direct sequencing or restriction analysis, sample A is characterised as a homoplasmic C7 type, using the cloning approach sample A showed exclusively C7 DNA in 40 sequenced clones. Sample B demonstrates the lower detection limit of the restriction fragment analysis method in contrast to direct sequencing. Sample B, which was classified into group I considering the sequencing electropherogram, was changed into group II after restriction fragment analysis results. Indicating its slightly heteroplasmic character, sample B revealed the presence of a single C9 stretch beside the more prevalent C8 stretch. In samples C, D, E and F this heteroplasmic character was found to be increased, and was identified by direct sequencing as well as, and more accurately, by restriction fragment analysis. For sample C, the analysis of 40 clones yielded 34 clones containing DNA stretches of C7, which corresponded to the predominant sequencing type in this sample, and 6 of length C8. In this sample the restriction fragment analysis clearly identified the heteroplasmic nature of sample C. The results of the appropriate restriction fragment analysis

show approximately these proportions. Similarly, cloning of samples D, E and F confirmed the predominant length of the homopolymeric cytosine stretch, and also revealed the relatively high heteroplasmy level of these samples. Samples G and H have no predominant length that could be detected for the homopolymeric cytosine stretch after either direct sequencing or restriction fragment analysis, as there seems to be an equal distribution between DNA stretches of C8 and C9. In contrast to this, the results from the cloning assay indicated that samples G and H were biased towards C8 and C9, respectively. Sample K, having a T to C transition at position 310, showed a blurred direct sequence, which resulted in no reliable data. The restriction fragment analysis suggested a Gaussian distribution of the different homopolymeric cytosine sequence lengths. Cloning analysis of sample K showed an increasing length in the homopolymeric cytosine sequence lengths in the range of C10–C14. Probably the number of sequenced clones was too small to reflect the Gaussian distribution. Altogether, the results derived from the cloning assay confirm that restriction fragment analysis is reasonably accurate, although cloning remains the most accurate method.

Probability of artefacts

Several different steps were taken to prevent the appearance of artefacts within this study. Firstly, instead of Taq polymerase, the proof-reading DNA polymerase *Pfu* was used. This enzyme also offered the advantage that it does not add single adenine residues, which would have disturbed the measurement of amplicon lengths. Possible amplification errors would have the greatest effect if they occurred within the first few PCR cycles. In order to detect the presence of any amplification errors, at least two independently generated PCR products were used and the results were compared for all three methods. Moreover, cloned samples, which were composed of amplicons of an identical length, were amplified, sequenced and cloned again. All results were in concordance with amplification products of a single length. The electropherogram from the direct sequencing analysis showed an unambiguous sequence, the fragment analysis electropherogram showed a single peak and the cloning assay results showed only cloned samples of a uniform length. These examinations of the results support the assumption that amplification errors within the first few cycles of PCR can be excluded as a reason for any observed length heteroplasmy at position 303–309.

Comparing the detection limit of the three tested methods

In comparing the detection limit of the three tested methods, the level of detection was found to increase in the order from direct sequencing of the mtDNA to restriction fragment analysis, to the cloning assay. The cloning assay was by far the most sensitive of the three tested methods, as it provided information on character and quantity of heteroplasmy variants. Given a sufficient number of clones, cloning was found to have the highest accuracy, due to its ability to identify the exact distribution of the different sequence variants. The disadvantage of cloning is that it is costly and time-consuming, compared to restriction fragment analysis. In our view, restriction fragment analysis is a sufficiently accurate alternative for most applications involving heteroplasmy analysis.

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