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SHORT COMMUNICATION

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Mitochondrial DNA heteroplasmy or artefacts — a matter of the amplification strategy?

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Abstract We compared two different PCR strategies for the amplification of mtDNA hypervariable region 1 (HV1) with regard to the detection and interpretation of point mutation heteroplasmy in human hair roots. We monitored the level of detected heteroplasmy using direct sequence analysis. PCR amplifications were performed in duplicate on each hair root, using 62 cycles of nested PCR versus 35 cycles of direct PCR. As a previous publication reported different sensitivities of heteroplasmy detection based on the number of PCR cycles used, we were interested in whether and how different PCR amplification strategies would impact sequence quality and the detection of point heteroplasmy. We identified 12 out of 93 hair roots as heteroplasmic (7 out of 31 persons) with direct PCR, whereas 2 of these heteroplasmic events could not be identified with the nested PCR approach. Generally, the quality of the sequence electropherograms in terms of background noise was significantly lower for the nested PCR amplification strategy, leading to ambiguous results in some of the nucleotide positions. Thus, the ability to clearly distinguish a genuine mixture of two nucleotides from background noise at a heteroplasmic position was substantially greater with direct PCR amplification, which generally resulted in higher quality sequence electopherograms.

Keywords mtDNA heteroplasmy · Nested PCR · Direct PCR · Hair roots · PHRED values · Forensic

Introduction

Heteroplasmy is the coexistence of two or more populations of mtDNA molecules with slightly different nucleotide composition in a single mitochondrion, cell, tissue or individual. According to recent studies this biological state is expected to be more common [1, 2, 3, 4] than has been postulated. However, the observation of point heteroplasmy is the exception rather than the rule in forensic analysis, mainly due to the fact that the established method of direct sequence analysis is a rather insensitive technique for the identification of a mixture of nucleotides at a single position. Thus heteroplasmy is restricted to the detection of apparent heteroplasmy as a consequence of these technical limitations.

The occurrence and the frequency of mtDNA control region (CR) point heteroplasmy is an important issue in forensic case work, especially for the investigation of hair samples. It has been demonstrated [5, 6, 7, 8] that hairs from a single individual may differ in their mtDNA nucleotide sequence as a consequence of the high mtDNA mutation rate and the characteristics of mtDNA transmission. The body of data as well as experimental knowledge on the detection of heteroplasmy is still growing. Recently, a paper on extremely high levels of heteroplasmy in hair [9] prompted substantial discussion in the forensic community and was the subject of two further publications [10, 11]. In these, among other points, the experimental design of the study was challenged. In particular, quality control measures, the amount of DNA used for amplification (20–80 ng) as well as the amplification strategy (62 cycles of nested PCR) were criticized.

In this study we evaluated these PCR conditions and compared the sequencing results to those obtained by a direct PCR approach. We were interested in whether the frequency of observed heteroplasmy would depend on the amplification strategy and/or the amount of DNA used. The visual interpretation of the sequencing result was supported by objective quality scoring with quantitative sequence analysis software.

Materials and methods

The HV1 region of the human mtDNA control region was sequenced from 93 single hair roots obtained from 29 unrelated individuals and 2 individuals related in maternal lineage (two brothers). A total of three roots were analysed from each individual (each root in a separate reaction).

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DNA extraction and quantitation

DNA was extracted from the hair roots using the GenoM-48 Robotic Workstation and the GenoPrep DNA from Tissue Kit (GenoVision, Oslo, Norway) according to the manufacturer's manual. DNA was resolved in Tris-buffer (10 mM, pH 9.0) and quantified fluorometrically. The minimum amount of genomic DNA measured was 0.4 ng/µl and the maximum was 7.6 ng/µl. As a mean value, we determined 2.1 ng/µl DNA (standard deviaton 1.7 ng/µl).

Primers and PCR strategy

We pursued two amplification strategies. In a first step of analysis, we followed the same amplification technique as described in [9], i.e. nested PCR applying 62 cycles overall and in a second step of analysis, we used direct amplification, as described in [12] using 35 cycles. Amplification and sequencing reactions were performed twice on each sample in which heteroplasmy was encountered, using alternative primers (L15989 and L16196 [13]). Extraction blanks and negative controls were carried through the complete amplification and sequencing process. PCR products were purified using ExoSAP-IT (Amersham, Bucks, UK) and sequenced using BigDye Terminator sequencing reagents (Version 2.0; Applied Biosystems ABI, Foster City, CA). Sequencing reaction products were purified from residual dye terminators using Sephadex G-50 Fine (Amersham) and Multiscreen filter plates (Millipore, Bedford, MA). DNA sequencing was carried out on an ABI Prism 3100 automated sequencer.

Statistical analyses

Prior to the alignment of the nucleotide sequences (Sequencher, GeneCodes, Ann Arbor, MI), base calls were computed applying the computer program PHRED (CodonCode, Dedham, MA). After calling bases, PHRED assigns a quality score to each base call ranging from 4 to 60, with higher values corresponding to higher sequencing quality. The quality scores are logarithmically linked to error probabilities, e.g. a PHRED quality score of 10 corresponds to an accuracy of the base call of 90%, whereas a PHRED quality score of 50 matches a correctness of the base call of 99.999%.

For qualitative statistical analyses, a random sample of 12 hairs from 12 unrelated individuals was taken from the study population. The PHRED values obtained were compared between the four sequence types generated for each hair: direct PCR-forward sequencing reaction (direct-for), direct PCR-reverse sequencing reaction (direct-rev), nested PCR-forward sequencing reaction (nested-for) and nested PCR-reverse sequencing reaction (nested-rev). The statistical evaluation consisted of the computation of mean values, standard deviations, skewness and medians of the PHRED values of a trimmed (380 bp) sequence spanning HV1. Medians, means and standard deviations for each of these groups were calculated using SPSS (Version 10.0 for Windows). For further statistical inference, the groups were tested for normal distribution (Shapiro-Wilk statistics for n =50) and the homogeneity of variance (Levene test). Having met these necessary assumptions, a one-way ANOVA and the Tukey-HSD post hoc test were performed.

Table 1 Average PHRED values and standard deviations obtained from sequences produced with direct and nested PCR

Sequence strategy	Mean value (PHRED)	No.	Standard deviation
Direct PCR (forward)	47.54	12	1.30
Nested PCR (forward)	44.55	12	1.49
Direct PCR (reverse)	43.01	12	1.86
Nested PCR (reverse)	41.40	12	2.21

Results and discussion

Sequence analysis of the mtDNA control region has become an essential tool in forensic genetics for the analysis of degraded material or samples containing little or no genomic DNA, notably hair shafts [14], which are an important and frequently discovered item at the scene of crime. Although technical improvements have significantly increased the rate of success in amplifying STRs from telogenic hair roots [15], the investigation using mtDNA is in some cases indispensable. Single observations rather than systematic studies, have shown that hairs are known to harbour heteroplasmic mtDNA variants [6, 7, 8, 16, 17], which result in the occurrence of heteroplasmy. Despite recommendations and interpretation guidelines [18, 19, 20], the correct assignment of point heteroplasmy based on the sequencing results remains the individual expert's responsibility. Given that forensic DNA laboratories use different PCR strategies, it would be interesting to investigate whether the PCR amplification scheme plays a role in the representation of point heteroplasmy.

Table 2 One-way ANOVA post hoc test (Tukey-HSD) showing the pairwise comparisons of the group means

Sequence strategy (I)	Sequence strategy (J)	Mean difference (I–J)	Significance	Confidence interval (95%)	
				Lower limit	Upper limit
Direct (for)	Nested (for)	2.99	0.001	1.08	4.90
	Direct (rev)	4.53	0.000	2.62	6.44
	Nested (rev)	6.14	0.000	4.23	8.04
Nested (for)	Direct (for)	–2.99	0.001	–4.90	–1.08
	Direct (rev)	1.54	0.152	–0.37	3.44
	Nested (rev)	3.15	0.000	1.24	5.05
Direct (rev)	Direct (for)	–4.53	0.000	–6.44	–2.62
	Nested (for)	–1.54	0.152	–3.44	0.37
	Nested (rev)	1.61	0.125	–0.30	3.51
Nested (rev)	Direct (for)	–6.14	0.000	–8.04	–4.23
	Nested (for)	–3.15	0.000	–5.05	–1.24
	Direct (rev)	–1.61	0.125	–3.50	0.30

Mean difference lists the differences between the sample means.
Significance lists the probability that the population mean difference is zero.
A 95% confidence interval is constructed for each difference. If this interval contains zero, the two groups do not differ.

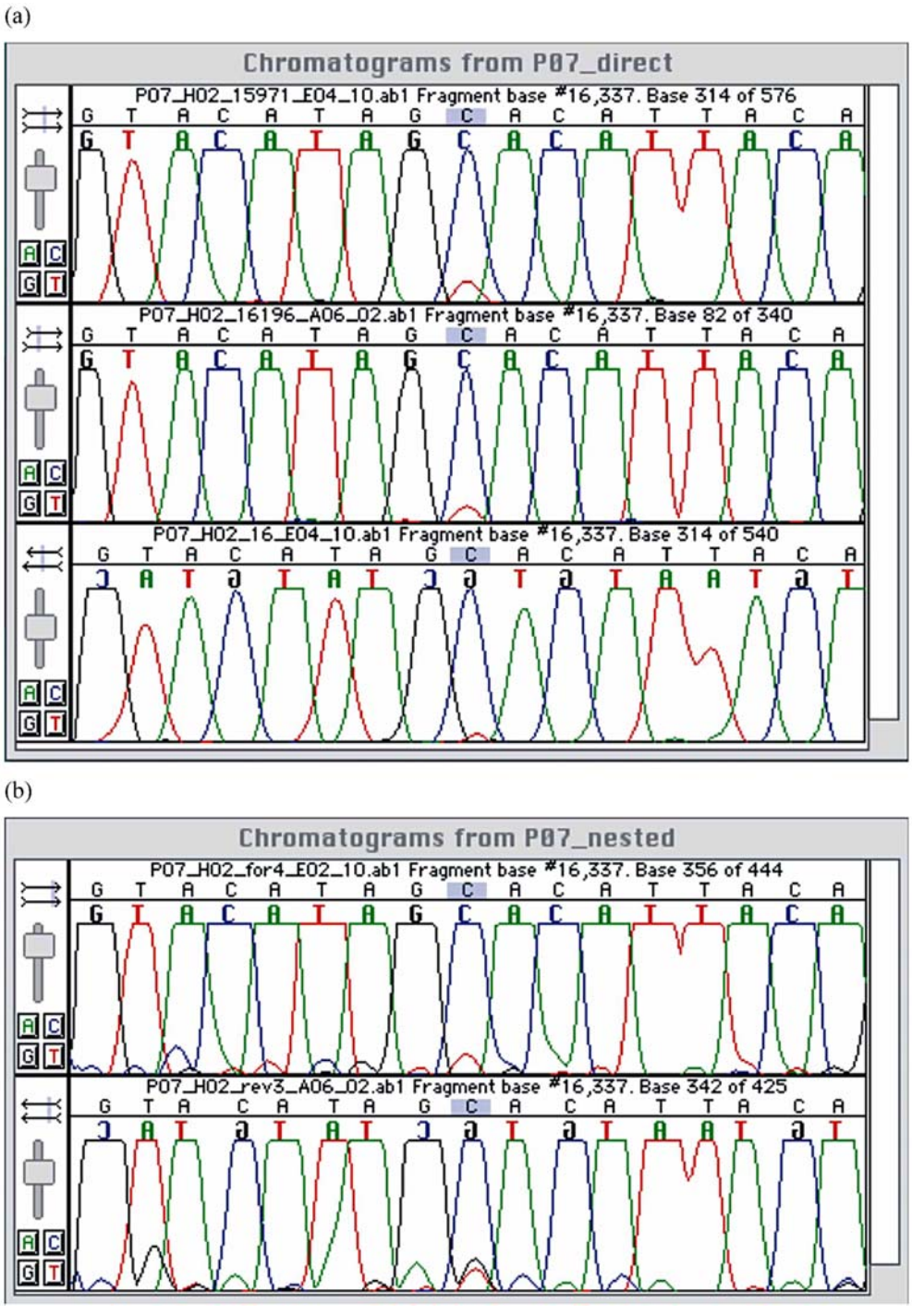

Fig. 1 Electropherograms obtained from direct **a** and nested **b** PCR. In **a** the C/T heteroplasmy at position 16337 is clearly recognisable derived from all 3 sequencing primers used, whereas in **b** the same heteroplasmic position cannot be identified due to an elevated level of background noise. Note that the slider for changing the scale of the traces is set near the top of the range, so that low level signal is better visualised

Sequence quality

Sequence analyses of all 93 hair roots tested led to full and correct HV1 sequences applying both amplification strategies, 62 cycles nested PCR and 35 cycles direct PCR. Extraction blanks and PCR negative controls remained clear. The statistical evaluation of PHRED values (as base-call averages over 380 bp sequences covering HV1) underscored the well-known fact that "L-strand sequencing" yielded higher sequence quality than "H-strand sequencing" (Tables 1 and 2, Figs. 1 and 2), which was demonstrated visually by much lower background noise. Additionally, and more interestingly, direct PCR resulted in higher PHRED values for both L-strand and H-strand sequencing in contrast to nested PCR. Averages within groups were normally distributed and variances between groups were homogeneous. Parametric analyses of variance (one-way ANOVA) pointed strongly to group differences. In order to determine which groups differed, post hoc comparisons (Tukey-HSD test) were performed. As depicted

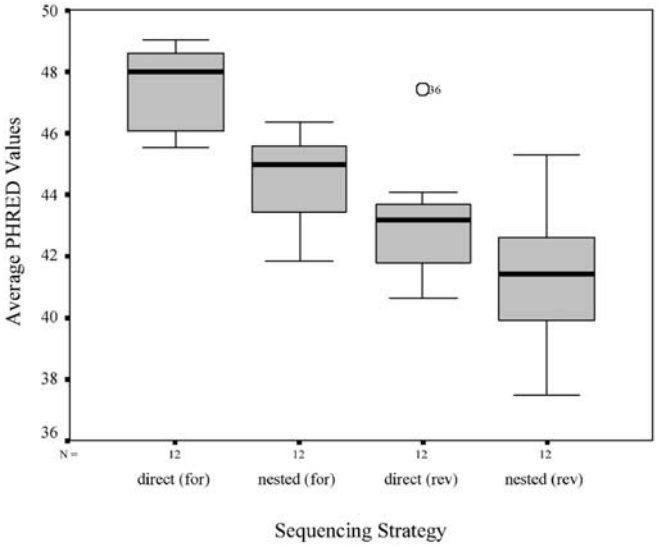

Fig. 2 PHRED values obtained by the four different sequencing reactions displayed in a box-and-whiskers plot. The horizontal line in the middle shows the median of the sample. The top and bottom of the boxes show the 75th and 25th percentiles, respectively. In this graph, the top and bottom of the whiskers show the maximum and minimum values. One outlier was observed for the direct reverse sequencing reactions. Sequence quality was higher after direct PCR compared to nested PCR

in Table 2, there is a significant quality difference between sequences obtained by direct L-strand sequencing (direct-for) and the other three methods. No significant difference was found between direct-rev and nested-for sequences. Interestingly, the test evaluated a significant quality difference between L-strand and H-strand sequences created with direct PCR, but no significant quality difference between nested forward and reverse reactions.

In summary, electropherograms obtained by direct PCR were of considerably higher quality, i.e. less background noise, than those obtained by nested PCR. This result is of practical importance for the detection and interpretation of low-level point heteroplasmy.

Heteroplasmy

Employing direct PCR, we found 12 out of 93 hairs to be point-heteroplasmic (Table 3). This result was confirmed by repeated amplification and sequencing with alternative primers. Performing the nested PCR approach, two of these were not identified to be heteroplasmic (Fig. 1, Table 3). This discrepancy is based on problems with the interpretation of the electropherograms obtained by nested PCR, which showed a substantially higher level of background noise compared with direct PCR. Thus, it was difficult to distinguish low levels of heteroplasmy from various types of sequence background signal for the nested PCR derived sequences.

Point heteroplasmy was detected reproducibly in 7 out of 31 individuals in the study population (Table 3), resulting in an overall incidence of 22.6% (95% CI 7.6–37.5).

Table 3 Heteroplasmic positions found in the HV1 segment of mtDNA of 7 individuals

Individual	No. of hairs analysed	No. of hairs where heteroplasmy was found (35 cycles/ nested: 62 cycles)	Heteroplasmic positions			
			16093	16153	16293	16337
05	3	2/2	Y			
07	3	1/0				Y
17	3	2/2	Y			
18	3	1/1		R		
23	3	1/1			R	
24	3	3/3	Y			
28	3	2/1	Y			

In the third column numerals before the dash correspond to the number of hairs where heteroplasmy was found with 35 cycles of PCR amplification, numerals after the dash meet the amount of hair where heteroplasmy was detected with nested PCR (62 cycles). IUPAC (International Union of Pure and Applied Chemistry) codes were applied for the designation of heteroplasmic sites (C~T=Y, A~G=R)

Among the 7 individuals, heteroplasmy was found at 4 different positions (16093, 16153, 16293, 16337). Heteroplasmy at position 16093, known as a mutation hotspot [21, 22], was observed in 4 individuals.

The higher number of cycles employed during nested PCR (62 cycles) did not result in an increase of observed heteroplasmy. On the contrary, the elevated sequence background masked low level heteroplasmy. Grzybowski [23] observed a significantly lower level of heteroplasmy after direct PCR (38 cycles) compared to nested PCR (62 cycles). He ascribed this result to the differing sensitivities of amplification techniques, a phenomenon which could not be reproduced in our study.

Amount of DNA

Another point of interest was the amount of DNA used for amplification. Budowle et al. [11] suggested that the large quantity of template DNA (20–80 ng) and additional amplification cycles (62 cycles) might be the basis for the unusual results observed by Grzybowski [9]. From a practical standpoint it is beyond doubt that this amount of DNA does not require such an extensive amplification protocol. Based on the results of our study, however, the excess amount of DNA was not related to the frequency of heteroplasmy observed in the samples. In a preliminary study (data not shown), we used the nested PCR approach to amplify HV1 in a dilution series of DNA (0.1 ng–100 ng DNA). Sequence analyses of the DNA dilutions did not reveal different base calls nor discrepant mixture ratios, furthermore the background noise level was comparable throughout the different dilutions of DNA. These results suggest that if there was any particular effect of the DNA amount on the quality of the sequence, it must have been

suppressed by the increased background level of the nested amplification scheme itself.

Conclusions

Generally, nested PCR techniques are more time-consuming and increase the costs of the laboratory process. In addition they cause logistical problems (pre-PCR and post-PCR areas) which can only be solved by assigning a separate laboratory area for the set-up of the second round of nested PCR. More importantly, the excess of PCR cycles and the handling of amplification products during the procedure introduce an increased risk of contamination. The findings of this study show that nested PCR is prone to elevated background noise, complicating correct nucleotide sequence interpretation and making the detection of low level point heteroplasmy practically impossible.

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References

1. Lutz S, Weisser HJ, Heizmann J, Pollak S (1999) Mitochondrial heteroplasmy among maternally related individuals. Int J Legal Med 113:155–161
2. Calloway CD, Reynolds RL, Herrin GL Jr, Anderson WW (2000) The frequency of heteroplasmy in the HVII region of mtDNA differs across tissue types and increases with age. Am J Hum Genet 66:1384–1397
3. 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
4. Tully LA, Parsons TJ, Steighner RJ, Holland MM, Marino MA, Prenger VL (2000) A sensitive denaturing gradient-gel electrophoresis assay reveals a high frequency of heteroplasmy in hypervariable region 1 of the human mtDNA control region. Am J Hum Genet 67:432–443
5. Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B (1995) Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. Biotechniques 18:662–669
6. Sullivan KM, Alliston-Greiner R, Archampong FIA, Piercy R, Tully G, Gill P, Lloyd-Davies C (1996) A single difference in mtDNA control region sequence observed between hair shaft and reference samples from a single donor. In: Genetic Identity Conference Proceedingsof the Seventh International Symposium on Human Identification. Promega Corporation, Madison, Wisconsin, pp 126–129
7. Wilson MR, Polanskey D, Replogle J, Di Zinno JA, Budowle B (1997) A family exhibiting heteroplasmy in the human mitochondrial DNA control region reveals both somatic mosaicism and pronounced segregation of mitotypes. Hum Genet 100: 167–171
8. Holland MM, Parsons TJ (1999) Mitochondrial DNA sequence analysis—validation and use for forensic casework. Forensic Sci Rev 11:21–50
9. Grzybowski T (2000) Extremely high levels of human mitochondrial DNA heteroplasmy in single hair roots. Electrophoresis 21:548–553
10. Tully G, Lareu M (2001) Letters to the Editor: Controversies over heteroplasmy. Electrophoresis 22:180–182
11. Budowle B, Wallard MW, Wilson MR (2002) Critique of interpretation of high levels of heteroplasmy in the human mitochondrial DNA hypervariable region I from hair. Forensic Sci Int 126:30–33
12. Parson W, Parsons TJ, Scheithauer R, Holland MM (1998) Population data for 101 Austrian Caucasian mitochondrial DNA d-loop sequences: application of mtDNA sequence analysis to a forensic case. Int J Legal Med 111:124–132
13. Szibor R, Michael L (1999) Letter to the editor: correct mitochondrial L-strand sequencing after C-stretches (W. Parson et al.). Int J Legal Med 112:348–349
14. Wilson MR, DiZinno JA, Polanskey D, Replogle J, Budowle B (1995) Validation of mitochondrial DNA sequencing for forensic casework analysis. Int J Legal Med 108:68–74
15. Hellmann A, Rohleder U, Schmitter H, Wittig M (2001) STR typing of human telogen hairs–a new approach. Int J Legal Med 114:269–273
16. Hühne J, Pfeiffer H, Waterkamp K, Brinkmann B (1999) Mitochondrial DNA in human hair shafts–existence of intra-individual differences? Int J Legal Med 112:172–175
17. 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
18. Bär W, Brinkmann B, Budowle B, Carracedo A, Gill P, Holland M, Lincoln PJ, Mayr W, Morling N, Olaisen B, Schneider PM, Tully G, Wilson M (2000) DNA Commission of the International Society for Forensic Genetics: guidelines for mitochondrial DNA typing. Int J Legal Med 113:193–196
19. Carracedo A, Baer W, Lincoln P, Mayr W, Morling N, Olaisen B, Schneider P, Budowle B, Brinkmann B, Gill P, Holland M, Tully G, Wilson M (2000) DNA Commission of the International Society for Forensic Genetics: guidelines for mitochondrial DNA typing. Forensic Sci Int 110:79–85
20. Tully G, Baer W, Brinkmann B, Carracedo A, Gill P, Morling N, Parson W, Schneider P (2001) Considerations by the European DNA profiling (EDNAP) group on the working practices, nomenclature and interpretation of mitochondrial DNA profiles. Forensic Sci Int 124:83–91
21. Meyer S, Weiss G, Haeseler A von (1999) Pattern of nucleotide substitutions and rate heterogeneity in the hypervariable regions I and II of human mtDNA. Genetics 152:1103–1110
22. Stoneking M (2000) Hypervariable sites in the mtDNA control region are mutational hotspots. Am J Hum Genet 67:1029–1032
23. Grzybowski T (2001) Letters to the editor: reply. Electrophoresis 22:180–182