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ORIGINAL ARTICLE

‘Mitominis’: multiplex PCR analysis of reduced size amplicons for compound sequence analysis of the entire mtDNA control region in highly degraded samples

Cordula Eichmann · Walther Parson

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Abstract The traditional protocol for forensic mitochondrial DNA (mtDNA) analyses involves the amplification and sequencing of the two hypervariable segments HVS-I and HVS-II of the mtDNA control region. The primers usually span fragment sizes of 300–400 bp each region, which may result in weak or failed amplification in highly degraded samples. Here we introduce an improved and more stable approach using shortened amplicons in the fragment range between 144 and 237 bp. Ten such amplicons were required to produce overlapping fragments that cover the entire human mtDNA control region. These were co-amplified in two multiplex polymerase chain reactions and sequenced with the individual amplification primers. The primers were carefully selected to minimize binding on homoplasic and haplogroup-specific sites that would otherwise result in loss of amplification due to mis-priming. The multiplexes have successfully been applied to ancient and forensic samples such as bones and teeth that showed a high degree of degradation.

Keywords mtDNA CR · Mini-amplicons · Multiplex · Forensic science

Introduction

The analysis of mitochondrial DNA (mtDNA) has become a powerful tool in forensic casework, mainly due to its high copy number per cell, its stability against degradation, and its maternal mode of inheritance. The standard method is sequence analysis of the hypervariable segments of the mtDNA control region (HVS-I and HVS-II) that involve amplicons spanning more than 300 bp depending on the choice of the polymerase chain reaction (PCR) primers. After strong degradation processes, however, such amplicon sizes may not be available in the DNA extract. The trend to reduce the fragment length of an amplicon by redesigning PCR primers closer to each other as demonstrated with autosomal markers [1, 2] has also been applied to the successful amplification of highly degraded mtDNA [3]. The authors proposed a total of eight individual amplifications to cover the hypervariable regions HVS-I and HVS-II. The amount of extract to perform this number of PCRs may be limited in cases in which the DNA concentration is very low. Therefore, multiplex analysis of the short amplicons would be an effective alternative and this has already been demonstrated in part for HVS-I [4]. Here, a new protocol for multiplex PCR analysis of short amplicons that cover the entire control region is presented and thus provides increased discrimination power for even highly degraded samples.

Materials and methods

Extraction

Bone and teeth samples were extracted using the phenol/chloroform extraction according to [5]. The blood reference sample was extracted using the BioRobot M48 workstation (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and the Qiasoft M Operating System software (ver.2.0E001).

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C. Eichmann · W. Parson (✉)
Institute of Legal Medicine, Innsbruck Medical University,
Müllerstrasse 44,
6020 Innsbruck, Austria
e-mail: walther.parson@i-med.ac.at

Amplification and sequencing strategy of the entire mtDNA control region

The PCR primers were designed (Primer Express software version 5.0; Applied Biosystems, AB, Foster City, CA, USA) to avoid systematic loss of amplification due to mis-priming at positions that display homoplasic or haplogroup-specific mutations. For this purpose, a dataset of 5,173 full and partial control region (CR) sequences was taken from the EMPOP database, Release 1 [http://www.empop.org; 6]. Primers F15989 and F109 were taken from [3] and R599 from [7] (Table S1). Ten overlapping mini-amplicons (MA) were amplified in two different multiplex PCR assays and sequenced with the PCR primers to generate a consensus sequence displaying full double-strand sequence coverage (Fig. 1). Both multiplexes were amplified in a 50-µl assay including 1× BD Advantage 2 SA PCR Buffer (Clontech Laboratories, Mountain View, CA, USA), 0.25 mg/ml BSA (Serva, Heidelberg, Germany), and 200 µM each dNTP, 1× BD Advantage 2 Polymerase Mix (Clontech Laboratories). The concentration of the primers was tested using the NanoDrop ND-1000 Spectrophotometer (PEQLAB Biotechnologie, Erlangen, Germany) using standard instrument settings as recommended by the manufacturer. Amplification was performed on a Multicycler PTC2 40 Tetrad 2 (MJ Research, Waltham, MA, USA) comprising an initial denaturation step at 95°C for 2 min followed by 39 cycles at 95°C for 15 s, 57°C for 10 s, and 72°C for 10 s. PCR products were purified using ExoSAP-IT (Amersham, Bucks, UK) and sequenced using BigDye Terminator sequencing reagents (Version 1.1, AB). Sequencing was performed on a Multicycler PTC2 40 Tetrad 2 (MJ Research) comprising an initial denaturation step at 96°C for 60 s followed by 30 cycles at 96°C for 15 s, 50°C for 5 s, and 60°C for 4 min. The primer concentration used for the sequencing reactions was 0.5 µM for each primer. Sequencing reaction products were purified from residual dye terminators using Sephadex G-50 Fine (Amersham), according to the manufacturer's manual. DNA sequencing was carried out on a 3100 Genetic Analyzer (AB) using POP 6, 36-cm capillary arrays, and default instrument settings as recommended by the manufacturer. The data were analyzed using Sequencing Analysis Version 3.7 (AB) and Sequencher Version 4.7 (GeneCodes, Ann Arbor, MI, USA).

mtDNA quantitation

mtDNA genome equivalents were determined using a real-time quantitation PCR (rtqPCR) assay according to [8]. All rtqPCR assays were performed in a total volume of 20 µl and run on an ABI PRISM 7700 sequence detector (AB) according to [8]. Two mitochondrial fragments of 283 and 143 bp were quantified simultaneously in order to estimate the extent of degradation of the samples. Extraction blanks and negative and positive controls were carried through the complete amplification and sequencing processes.

Results and discussion

The primer design is generally crucial for the specificity and efficiency of the polymerase chain reaction [8]. It has been shown that the selection of primers for DNA regions with elevated mutation activity requires careful design to avoid systematic loss of amplification due to mis-priming [2]. The mitochondrial control region consists of a great variety of mutational hotspots, e.g., homoplasic mutations and hypervariable sequence stretches that display characteristic mutational patterns which are found throughout the worldwide mitochondrial phylogeny. In order to avoid unfortunate primer design that interacts with such mutational hotspots as well as haplogroup-specific positions that could result in decreased or failed amplification, we screened 5,173 full and partial CR sequences as listed in

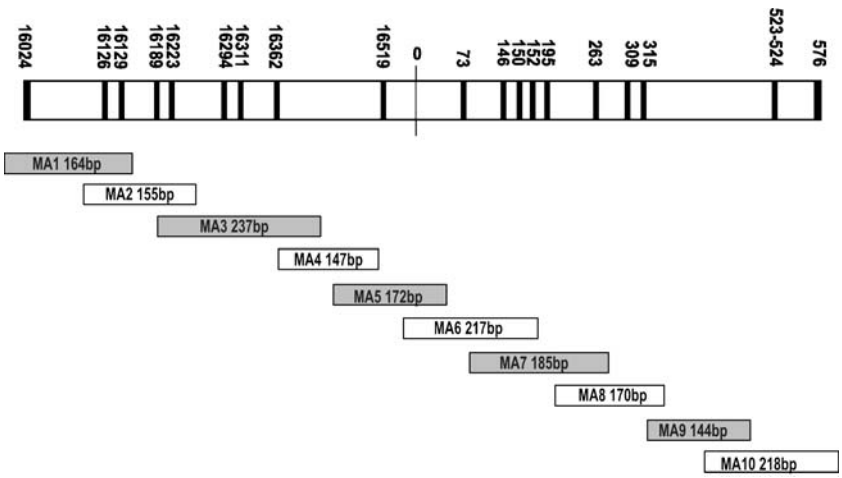

Fig. 1 Schematic representation of the amplification and sequencing strategy for the analysis of the mtDNA control region using mini-amplicons. *Gray bars* represent DNA fragments co-amplified in multiplex 1 and *white bars* represent mini-amplicons in multiplex 2. The 19 highlighted positions indicate mutations that occurred in at least 10% of the dataset (positions 263 and 315 included for orientation)

the EMPOP database, Release 1 (http://www.empop.org; [6]). These sequences display the variation found in West Eurasian, East Asian, Southeast Asian, and African haplotypes. With respect to the observed frequency, this set included 521 positions that showed between one and nine mutations, 214 positions displayed more than ten mutations, and 19 positions gave a level of polymorphism of at least 10% (Fig. S1). This frequency estimate served as a rough indicator for highly polymorphic mutations that need to be considered for the primer design, bearing in mind that the frequency values will vary on a slightly different haplogroup background. As a result, ten mini-amplicons were designed that fully cover the entire control region of which all but MA3, MA6, and MA10 gave amplicon sizes below 200 bp. Two PCR multiplexes were established by combining five non-overlapping mini-amplicons into a single reaction (Fig. 1).

We evaluated the performance of the two multiplexes in silico by screening a dataset of 1,544 mtDNA haplotypes [Romania—Ashkenazi (n=173), Romania—Csango (n=182), Dubai (n=249), Austria (n=273), Macedonia (n=199), Romania—Szekely (n=178), Thailand (n=190), Ulm—Germany (n=100)]. We found 102 polymorphic positions that fell within the binding regions of the 20 employed PCR primers (Table S2). To test their effect on the amplification success, DNA of 116 samples (including the 102 mutations) was amplified and the respective mini-amplicon was sequenced. In all but one case we obtained the expected sequences despite a primer-site mutation (cross-check with previous sequencing results). This haplotype (sample from Ulm, hg X2c) displayed two mutations within the binding region of primers F16094 and R16248 (positions 16108 and 16232) that led to loss of amplification. The results indicate that our primer design is generally stable; however, it cannot be completely excluded due to mutations at new or unexpected positions. In these cases, alternative primers need to be employed to successfully establish the haplotype.

A species cross-reactivity test was performed by testing the DNA of 20 different animal species (Table S3). In most cases, the tested DNA did not yield any amplification product, whereas some species gave successful sequences. This was, e.g., the case for the DNA of the rat (*Rattus norwegicus*), in which mini-amplicon 4 gave a sequence that was homologous to a fragment of the chromosome 1 in the rat (data not shown). As expected, the chimpanzee gave results for all mini-amplicons (Table S3) and therefore a full chimpanzee control region sequence that can, however, clearly be identified as such (data not shown). In contrast to STR analysis, where a positive signal of an animal species can potentially be mistaken as a human-specific allele, the sequence analysis of mtDNA fragments would always identify a non-human species by its nucleotide sequence (e.g., as a result of a BLAST search [9]).

The amplification parameters of the two multiplexes were optimized for a DNA template amount of 1,000 mtDNA genome equivalents. As a general observation, the mini-amplicons also gave successful results with lower DNA amounts, such as 50–200 mtDNA genome equivalents. We tested the method by typing 200-year-old bone and teeth samples that showed severe degradation as determined by real-time quantitative PCR with target amplicon sizes of 143 and 283 bp (Table 1). The two multiplexes gave full CR haplotypes for 13 of the 15 samples which gave no results with conventional HVS-I and HVS-II analysis. In four samples, some of the mini-amplicons (MA5, MA7, and MA10) needed repeated amplification in singleplex mode as the multiplex approach failed in these cases.

Conclusions

The multiplex amplification of size-reduced fragments resulted in a significantly increased success rate for sequencing the control region in highly degraded samples compared to conventional approaches. The amenability to multiplexing allows adoption for routine casework as the sample consumption is within the practical standards. The primers were carefully selected to avoid binding on hotspot mutations and haplogroup-specific sites within the mitochondrial control region. The risk of hitting a private

Table 1 Summary of the quantification results of the bone and teeth samples examined

Sample	283-bp fragment (mtGE/µl)	143-bp fragment (mtGE/µl)	Sequencing results
Bone 1	n.a.	30	+/−
Bone 2	n.a.	5,500	+
Bone 3	36	118	+/−
Bone 4	653	1,840	+
Bone 5	n.a	12	−
Bone 6	n.a.	5	−
Bone 7	n.a.	270	+/−
Tooth 1	n.a.	455	+/−
Tooth 2	236	628	+
Tooth 3	n.a.	2,196	+
Tooth 4	710	2,336	+
Tooth 5	332	1,833	+
Tooth 6	n.a.	2,987	+
Tooth 7	n.a.	2,120	+
Tooth 8	n.a.	8,385	+

Degradation was evaluated using two different primer combinations resulting in 283- and 143-bp amplicons. See [8] for details.
n.a. No rtPCR quantitation using the fragment size 283 bp, *mtGE/µl* mitochondrial genome equivalents/microliter, + full sequencing result, +/− partial sequencing result (singleplex amplification necessary), − no sequencing result

mutation that could result in failed amplification always needs to be considered and then alternative amplification primers need to be used. We further observed a slightly elevated contamination rate with the mini-amplicons compared to the traditional HVS-I and HVS-II protocol that highlights the requirement to carefully select working conditions when setting up the PCR [10].

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