An alternative strategy for whole mitochondrial genome amplification and sequencing suited for lower quality mtDNA

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Introduction

Amplification and sequencing of the entire mitochondrial (mt) genome becomes increasingly important in the fields of forensic DNA testing and phylogenetics, as control region sequencing and targeted coding region SNP typing are sometimes not enough for a clear haplogroup assignment.

Nowadays the amplification and sequencing of the mtDNA control region (CR) seems to be a routine exercise in some labs, while sequencing of the whole mt genome (~16.6kb) is more demanding, especially when the DNA quality of the samples of interest is low.

We earlier proposed a whole mitochondrial genome sequencing strategy [1], for which high quality DNA is required (**Figure 1**, left). The aim of this project was to evaluate an alternative approach for degraded or lower quality DNA samples that is based on the amplification of nine overlapping mitochondrial DNA fragments each ~2kb in size (**Figure 1**, right).

Materials & methods

Samples of interest were selected on the basis of the results of entire control region analyses which were performed under EMPOP recommendations [2], updated in [3]. Amplification of the nine mtDNA fragments (fragment A, B, C, D, E, F, G, H, and I; **Figure 1**, right) was performed in a final reaction volume of 25µL, using 5U Advantage Polymerase (Clontech), 2.5mg/mL BSA (St. Louis, Missouri), 2.5mM each dNTP (AB), 10µM each primer and an adequat amount of mtDNA genome equivalents. Thermal cycling conditions comprised one initial step of 95°C for 4 min, followed by 35 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 4 min, and finally an extension phase at 72°C for 10 min. The nine amplification primer pairs are listed in **Table 1**. The applied 96 forward and reverse sequencing primers were adopted from [4] and complemented by in-house primers.

Figure 2 illustrates the sequencing primer plate and respective primer names, according to their 5´ end. Cycle sequencing was performed with BigDye v1.1 (AB) and purification of sequencing products was done with Sephadex. Both procedures follow the recommended protocol. The consequent alignment of the 96 primers is presented in **Figure 3**.

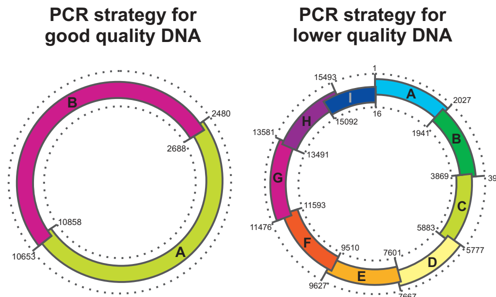

Figure 1. Schematic outline and comparison of the two mtDNA genome sequencing strategies mentioned including the localisation of the corresponding overlapping amplification products in the mt genome

Table 1. Sequences and nucleotide positions of the nine amplification primer pairs in the mt genome. Primer sequences taken from [5, 6, this study]

Primer of fragment	Primer sequence 5´- 3´	Primer position (nts)
A forward	GATCACAGGTCTATCACCCTA	1 - 21
A reverse	TTGGACAACCAGCTATCACCA	2027 - 2007
B forward	GCACACCCGTCTATGTAGCA	1941 - 1960
B reverse	TTCGATGTTGAAGCCTGAGAC	3948 - 3928
C forward	CCACACTAGCAGAGACCAAC	3869 - 3888
C reverse	GGCTGAGTGAAGCATTGGACT	5883 - 5863
D forward	GAAGCTGCTTCTTCGAATTTGC	5777 - 5798
D reverse	GGGCGTGATCATGAAAGGTG	7667 - 7648
E forward	CAAGTAGGTCTACAAGACGCT	7601 - 7621
E reverse	CTGATGCGAGTAATACGGATG	9627 - 9607
F forward	TACCACTCCAGCCTAGCCC	9510 - 9528
F reverse	TCGTAGGCAGATGGAGCTTG	11593 -11574
G forward	CGGCTATGGTATAATACGCCT	11476 - 11496
G reverse	AGCGATGAGAGTAATAGATAGG	13581 - 13560
H forward	CCTCACAGGTTTCTACTCCAA	13491 - 13511
H reverse	GAGGTCTGGTGAGAATAGTGT	15493 - 15473
I forward	GGCATTATCCTCCTGCTTGCAACTAT	15092 - 15117
I reverse	TGATAGACCTGTGATCCATCGTGA	16561 - 16

Figure 2. Sequencing primers applied in 96-well format. Primer names refer to the 5 prime end [4, this study]

Figure 3. Sequencher alignment of 96 sequence strands covering the coding region employing the alternative WGS strategy

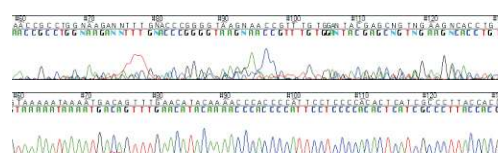

Figure 4. Raw data of sequencing primer F5318 from a low quality DNA sample; upper lane: sequence resulting from one ~8.5kb fragment; lower lane: sequence generated by the alternative sequencing strategy

Results

Our alternative approach of whole genome amplification and sequencing by the use of nine overlapping amplification products and 96 sequencing primers enabled the analyses of lower quality DNA, such as present in 5-10 years old DNA extracts.

In total, the entire mtDNA genome was amplified and sequenced in 39 samples from the South American continent that failed to give useful results with the previously described strategy [1]. The consensus sequence of all samples was based on full double-stranded sequence coverage and allowed for the unambiguous assignment of all base-calls including point and length heteroplasmy (**Figure 4**).

Discussion

The amplification and sequencing strategy of the full mitochondrial genome described herein is particularly relevant for lower quality DNA samples with the aim to enable reliable base-calling by redundant sequence analyses.

The careful assortment of primers contributed to a full double-stranded sequence coverage of the whole mtDNA genome (in combination with previously described CR sequencing strategies). We recommend this whole mt genome sequencing strategy to be applied on more challenging samples, where other whole mtGenome sequencing strategies failed to give conclusive results.

References

[1] Fendt L, Zimmermann B, Daniaux M, Parson W: Sequencing strategy for the whole mitochondrial genome resulting in high quality sequences. BMC Genomics 2009, 10:139

[2] Brandstätter A, Niederstätter H, Pavlic M, Grubwieser P, Parson W (2007) Generating population data for the EMPOP database - an overview of the mtDNA sequencing and data evaluation processes considering 273 Austrian control region sequences as example. Forensic Sci Int 166:164-175

[3] Parson W, Bandelt H-J: Extended guidelines for mtDNA typing of population data in forensic science. Forensic Sci Int Genet 2007, 1: 13-19

[4] Coble MD, Just RS, O'Callaghan JE, Letmanyi IH, Peterson CT, Irwin JA, Parsons TJ: Single nucleotide polymorphisms over the entire mtDNA genome that increase the power of forensic testing in Caucasians. Int J Legal Med 2004, 118:137-146.

[5] Bannwarth S, Procaccio V, Paquis-Fluckinger V: Rapid identification of heteroplasmic mitochondrial DNA mutations with mismatch-specific Surveyor Nuclease. Human Mutations 2005, 25:575-582.

[6] Gonder MK, Mortensen HM, Reed FA, de Sousa A, Tishkoff SA: Whole-mtDNA genome sequence analysis of ancient African lineages. Mol Biol Evol 2007, 24:757-768.

Acknowledgements

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