FULL MT-GENOME SEQUENCING WITH MITOCHONDRIAL TILING PATH PRIMERS USING THE ION TORRENT PGM

C. Strobl[1], R. Lagacé[2], J. Chang[2], W. Parson[1,3]

[1]Institute of Legal Medicine, Medical University of Innsbruck, Innsbruck, Austria
[2]Thermo Fisher Scientific Inc., South San Francisco, CA, USA
[3]Forensic Science Program, The Pennsylvania State University, PA, USA

Introduction

Sequencing analysis of mitochondrial DNA (mtDNA) has proved particularly important in forensic applications addressing samples that are not amenable to the typing of highly polymorphic nuclear DNA markers. However, sequencing of only the non-coding control region is sometimes not sufficiently informative for acquiring the required discrimination power needed in some forensic investigations. With the availability of new sequencing technologies, analysis of the complete mitochondrial genome (mtGenome) has become feasible and is likely to evolve into a method of first choice. For challenging samples routinely encountered in forensic casework, such as hair shafts or teeth and bones, which suffer severe environmental stress, DNA quantity and/or quality sometimes is insufficient for conventional Sanger sequencing. The purpose of this study was to test the utility of an alternative technique, the so called mito tiling approach, relying on massively parallel (next generation) sequencing, to sequence the entire human mtGenome in 162 amplicons of about 175bp in length.

Materials and Methods

Our study included two hair shaft samples of different donors, eight tooth and two bone samples of skeletal remains from an early medieval graveyard in Volders (Tyrol, Austria) dated between the 5th/6th and 12th/13th centuries and a casework hair sample, 1,2cm in length, of which hair shaft and root were analyzed separately. Ancient samples were extracted as described in Bauer et al 2013 [1], DNA of hair samples was extracted using the Qiagen BioRobot M48 Robotic Workstation (Qiagen, Hilden, Germany) following the manufacturer's protocol. The quantity of mitochondrial DNA of the maximum available amount of extract ranged from 0 (no quant results) to 20964mtGE.

The chemistry for the mito tiling approach was provided within an Ampliseq Kit (Ion AmpliSeq Library Kit 2.0, Thermo Fisher) consisting of two PCR multiplex pools for library preparation, covering the whole mitochondrial genome (Fig. 1). We tested three alternative versions of the sequencing protocol, that differed in the sample pooling step after multiplex amplification: the “full” method where both amplification products are used individually for the next steps; the “conservative” method using the whole product of pool 2 transferred to the product of pool 1 and the “two-in-one method”, where half the volumes of both pools were combined in an extra well for the subsequent steps. After pooling samples, adapters were ligated and emulsion PCR was performed using the OneTouch 2 (Ion PGM Template OT2 200 Kit, Thermo Fisher) according to manufacturer's protocol. Samples where then sequenced with the Ion PGM using the newly introduced HiQ Sequencing Kit (designed for robust variant detection and to provide greater indel sequencing accuracy).

References

[1] Bauer CM, Niederstätter H, McGlynn G, Stadler H, Parson W: Comparison of morphological and molecular genetic sex-typing on mediaeval human skeletal remains. Forensic Science International Genetics 2013,7(6):581-586

[2] Eichmann C, Parson W: 'Mitominis': multiplex PCR analysis of reduced size amplicons for compound sequence analysis of the entire mtDNA control region in highly degraded samples. Int J Legal Med. 2008 Sep;122(5):385-8.

[3] van Oven M, Kayser M. 2009: Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat 30(2):E386-E394.

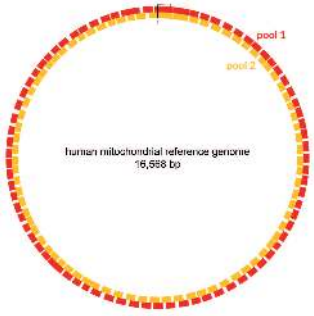

Figure 1: overlapping amplification products from two PCR primer pools (red: pool 1, yellow: pool 2)

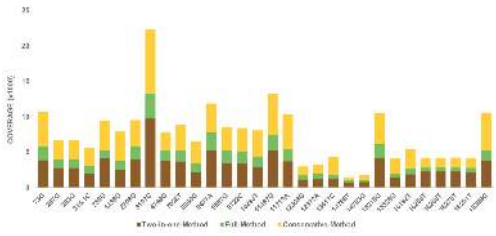

Figure 2: cumulative coverage values for the mtGenome haplotype of one hair sample, massively parallel sequenced after three different amplicon pooling methods

specimens	tissue	mean coverage whole genome	mean coverage mutations
sample_01	tooth	2502	490
sample_02	tooth	50	30
sample_03	tooth	44	22
sample_04	tooth	18	24
sample_05	tooth	26	17
sample_06	tooth	38	26
sample_07	tooth	47	23
sample_08	bone	3494	3326
sample_09	bone	20	10
sample_10	tooth	25	14

Table 1: mean coverage of the whole genome and single mutations of each sample

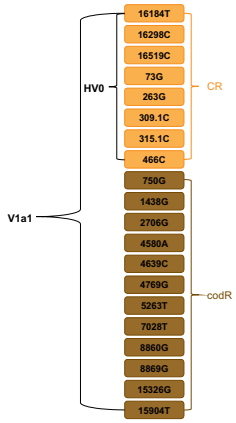

Figure 3: CR and mtGenome haplogroup assignments determined from a hair sample

Results

Comparison of three different mtDNA amplicon pooling protocols: For comparing the performance of the three different amplicon pooling protocols we sequenced DNA extracts of two hair shaft samples of different donors, both of known mtGenome haplotype. The three protocols resulted in identical haplotypes for both hair samples. We observed differences in coverage values with the “conservative” method exhibiting highest mean and minimal coverage values (Fig. 2) and the “two-in-one” method performing less effectively. However, the “two-in-one” method yielded sufficient sequence information to interpret the entire mtGenome haplotype and required lowest chemistry and sample amount compared to the other methods. This is why the “two-in-one” method was further used for additional experiments described in here.

Application of mtGenome mito path tiling assay to sequence ancient samples: The majority of the investigated ancient samples yielded useful mtGenome sequence information using the mito tiling path primers. Mean coverage values varied between coverage of 18 to 3494X per sample and 10 to 3326X per mutation (Table 1). From the ten samples analysed, two gave full profiles with minimal coverage values of 6 and 454. Five samples showed partial mtGenome haplotypes with 0.04 - 0.2% null coverage regions (7 to 39 positions). However, unambiguous haplogroup classification was possible in all these cases (Table 2). Some samples provided less DNA in the second round of extraction and showed the lowest mean coverage and highest zero coverage values between 1.1% (183 positions) and 5.9% (978 positions). The MPS haplotypes were compared to control region (CR) haplotypes obtained previously by Sanger-based sequencing (STS).

Application of the mtGenome mito path tiling assay to sequence a modern hair sample: A hair sample, 1.2 cm in size was dissected into shaft and root, which were analysed separately. STS analysis of the CR resulted in one full and a partial CR haplotype. The mito path tiling approach resulted in two identical full mtGenome haplotypes. The mean coverage values ranged from 3,994 for the root and 4,777 for the shaft, minimal coverage values varied between 72 and 76, respectively. Haplogroup assignment resulted in HV0 for the CR and V1a1 for the entire mtGenome [3] (Fig. 3).

Discussion

This study investigated the performance of a new PCR-based massively parallel sequencing method that targeted mtDNA amplicons in the size range of 175 bp (mito tiling path). This method proved to be useful for the application of severely degraded mtDNA observed in ancient teeth and bone samples as well as challenging hair samples. The application of the mito tiling path approach resulted in comparable data compared to Sanger-type sequencing data and was generally more successful in obtaining useful sequence data than an earlier developed CE-based approach. The development of even full mtGenome haplotypes from these degraded specimens was most intriguing and demonstrates the usefulness of such an approach to forensic research.

method	analysis date	sample									
		1	2	3	4	5[a]	6	7	8	9	10[b]
WGS-mito tiling	May 2015	H1b1	H1b1	U5b2a2b	H2a2a1	H2a2a1	K1a4	H5'36	HV1b1	H2a2a1	T1
CR-STS	Feb 2011	H1b	H1b	U5b2a2	H	T1a	K1a	H5	HV1	?	T1a

Table 2: Mitochondrial haplogroups determined from medieval skeletal remains, Control region data were determined using a mini-mulitplex Sanger-based approach [2] [a]new extraction from putatively same individual turned out to be a different sample [b]new extract yielded less DNA compared to first extraction

Acknowledgements: This study was partly supported by the FWF Austrian Science Fund (P 228800-B12). The authors would like to thank Cordula Berger and Anna König for hair extraction, Christiane Maria Bauer and Petra Kralj for bone extraction and Harald Niederstätter and Mayra Eduardoff for useful discussions.