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Analysis of mitochondrial DNA with an infrared automated DNA sequencer in a Tuscan population (Central Italy)

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Abstract

In our laboratory, we performed a study of the D-loop region of mtDNA in unrelated individuals, natives of Tuscany (Central Italy), using an Infrared Automated DNA Sequencer (LI-COR 4200). Taking advantage of the long read characteristics of the IR Automated Sequencer, we adopted a new strategy based on a single fragment amplification and sequencing, including the entire HVI and HVII regions. Preliminary results of the variability in the population we studied were also presented.

Keywords: Mitochondrial DNA; Infrared sequencer

1. Introduction

The D-loop region of the mtDNA provides a very attractive target for human forensic identification studies, especially in situations in which only reduced amounts of biological samples are available (hair shafts, faeces, combusted bodies, etc.). The hypervariable regions HVI and HVII are classically analysed with automatic sequencers for their high variability content. The use of mtDNA in casework is possible if a relevant database is available. Normally, the strategy for analysing the hypervariable regions is based on the separate amplification and sequencing of HVI and HVII regions. At the beginning of our experience with mtDNA sequencing, we adopted the strategy proposed

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by Holland et al. [1] and Pfeiffer et al. [2]. Subsequently, to construct a population database from a Tuscan population (Central Italy), we used a new protocol to save time and consumables. This strategy is based on the amplification of one long fragment of 1046 bp containing the whole D-loop region of mtDNA. The sequencing reaction was directly performed in forward and reverse directions by using an IR-based DNA Automatic Sequencer.

2. Materials and methods

2.1. DNA extraction

DNA samples were obtained from 55 unrelated healthy individuals, who came to our Centre for a genetic consultation. DNA was extracted by using the Puregene® system (Gentra) and the concentration was evaluated with a spectrophotometer at 260 nm.

2.2. PCR amplification

The PCR products are generated by using the Primer F15971: 5′-TTAACTCCACCATTAGCACC-3′ and Primer R448: 5′-TGAGATTAGTAGTATGGGAG-3′ suggested by Holland et al. [1].

PCR amplification was performed in a total volume of 50 μl: reaction buffer (75 mM of Tris–HCl pH 9.0 at 25 °C, 20 mM $(NH_4)_2SO_4$, 0.01% Tween), 1.5 mM $MgCl_2$, 200 mM of dNTPs, 1 μM of both forward and reverse primers. The amount of DNA was 1–3 ng for each reaction. 0.25 units of Red Hot Thermostable Polymerase (Advanced Biotechnologies) was added in each tube with the hot-start method.

The amplification conditions were: 95 °C for 2 min for the initial denaturation, then 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, for 30 cycles, with a final extension at 72 °C for 10 min.

The PCR product, 1046-bp long, was evaluated from a quantitative point of view on a 2% agarose gel (Nusieve 3:1).

2.3. mtDNA sequencing

The sequencing reaction mixes are prepared by using the ‘‘Thermo Sequenase DYEnamic Direct cycle sequencing kit’’ or the ‘‘Thermo Sequenase Fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP’’ (Amersham Pharmacia Biotech). The sequencing process was performed in both forward and reverse directions, by using appropriate internal labelled Primers (F15989: 5′-CCCAAAGCTAAGATTCTAAT-3′ and R381: 5′-GCTGGTGTTAGGGTTCTTTG-3′) [1], 5′ labelled with the new molecule IRDye™ 800 (LI-COR), supplied by MWG Biotech, Germany (Table 1). C-stretch sequences in both HVI and HVII regions were analysed as suggested by Szibor and Michael [3]. PCR conditions were the following: 95 °C for 5 min for the initial denaturation, then 93 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, for 30 cycles. At the end of the sequencing process 3 μl of stop solution was added to each tube.

Table 1
Sequence polymorphism in both variable regions of mtDNA from Tuscan individuals (Central Italy)

		HVI	HVII
Transition	T–C	77	37
	C–T	51	12
	A–G	16	79
	G–A	–	5
Transversion	A–T	2	–
	A–C	2	–
	C–A	1	–
Insertion	A	1	–
	C	–	1C=7; 2C=8
Deletion	A	–	1
	C	1	1
	G	–	1
Heteroplasmy		–	6

Sequencing was separated in four separate lanes of a 6%, 33-cm-long, denaturing polyacrylamide gel, using the LI-COR® automated DNA Sequencer. 1.5 μl of each sample was loaded by using a 48-sharkstooth comb with a Hamilton syringe. The gel was run at a constant power of 31.5 W, with a constant temperature of 50±1 °C. The image file was completed 5–6 h after the samples were loaded.

2.4. Data analysis

For the identification of sequences we used the dedicated Image Analysis™ computer program.

The FASTA format was obtained by using the Chromas program, available as shareware (http://bioinformatics.weizmann.ac.il/software/chromas), while the reference sequence of Anderson et al. [4] was employed for the alignment of sequences, with the Clustalw program (EMBL) (http://www.ebi.ac.uk./clustalw/).

3. Results and discussion

Our results show the possibility to analyse the whole D-loop of mtDNA with a more rapid and cheap strategy than previously reported, using IR technology. The sequences we obtained are strong and easy to interpret, using images which are similar to a manual autoradiogram (Fig. 1). In one gel it was possible to run together 11 different sequences, consequently being less time-consuming.

Amongst 40 sequences on both variable regions of mtDNA we observed 38 unique haplotypes.

Changes with respect to the reference sequence of Anderson et al. [4] were found 376 times, of which 151 (36%) were in the HVI region and 225 (64%) in HVII. The most frequent type of polymorphism we observed was transition A→G in the HVII region. We also found six heteroplasmic positions in HVII and one in HVI.

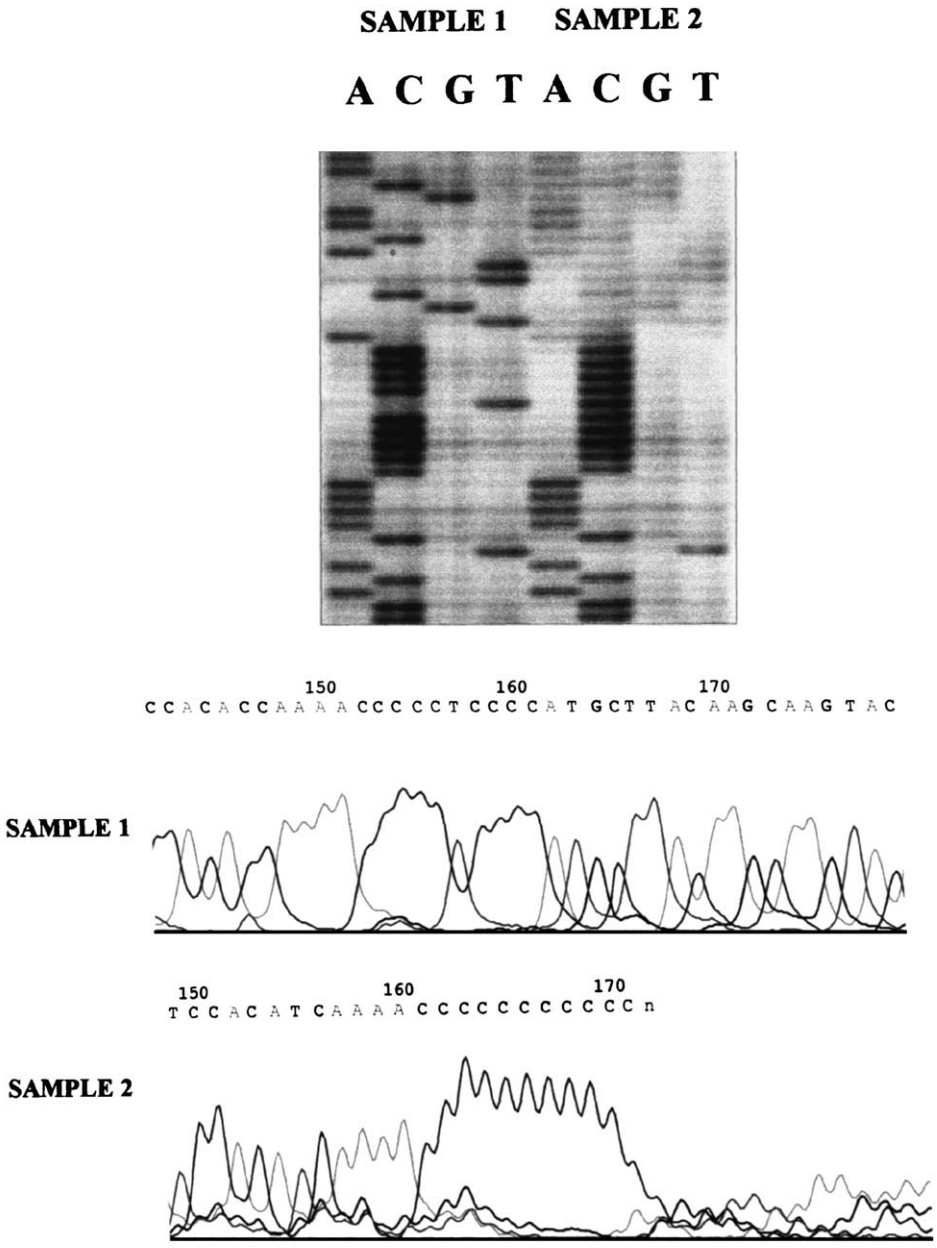

Fig. 1. mtDNA sequences of two subjects in autoradiogram-like and peak-like formats. In sample 2 a heteroplasmic condition is shown.

Sequencing the whole D-loop region, we found a minor mutation event between the HVI and HVII regions. Particularly the following positions contained a variation: 16519C, 16526A and 73G.

In our preliminary experience on simulated degraded samples, the technology we applied could be efficiently used also to investigate forensic samples in criminal casework.

References

[1] M.M. Holland, D.L. Fisher, R.K. Roby, J. Ruderman, C. Bryson, V.W. Weedn, Mitochondrial DNA sequence analysis of human remains, Crime Lab. Dig. 22 (1995) 109–115.

[2] H. Pfeiffer, B. Brinkmann, J. Huhne, B. Rolf, A.A. Morris, R. Steighner, M.M. Holland, P. Forster, Expanding the forensic German mitochondrial DNA control region database: genetic diversity as a function of sample size and microgeography, Int. J. Leg. Med. 112 (1999) 291–298.

[3] R. Szibor, M. Michael, Correct mitochondrial L-strand sequencing after C-stretches, Int. J. Leg. Med. 112 (5) (1999) 348–349.

[4] S. Anderson, A.T. Bankier, B.G. Barrell, M.H.L. De Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Scheier, A.J.H. Smith, R. Staden, I.G. Young, Sequence and organization of the human mitochondrial genome, Nature 290 (1981) 457–464.