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DIRECT PCR AMPLIFICATION OF THE HVSI REGION IN MITOCHONDRIAL DNA FROM BUCCAL CELL SWABS

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Abstract - Amplification of human mitochondrial DNA (mtDNA) has been widely used in population genetics, human evolutionary and molecular anthropology studies. mtDNA hypervariable segments I and II (HVSI and HVSII) were shown to be a suitable tool in genetic analyses due to the unique properties of mtDNA, such as the lack of recombination, maternal mode of inheritance, rapid evolutionary rate and high population-specific polymorphisms. Here we present a rapid and low-cost method for direct PCR amplification of a 330 bp fragment of HVSI from buccal cell samples. Avoiding the DNA isolation step makes this method appropriate for the analysis of a large number of samples in a short period of time. Since the transportation of samples and fieldwork conditions can affect the quality of samples and subsequent DNA analysis, we tested the effects of long-term storage of buccal cell swabs on the suitability of such samples for direct PCR amplification. We efficiently amplified a 330 bp fragment of HVSI even after the long-term storage of buccal cells at room temperature, +4°C or at -20°C, for up to eight months. All examined PCR products were successfully sequenced, regardless of sample storage time and conditions. Our results suggest that the direct PCR amplification of the HVSI region from buccal cells is a method well suited for large-scale mtDNA population studies.

Key words: mtDNA, HVSI, buccal swab, direct PCR

INTRODUCTION

Numerous reports have focused on the evaluation of different sampling procedures, storage time and conditions of samples on the quality and quantity of isolated DNA (Hansen et al., 2007; Lum and Le Marchand, 1998; Mulot et al., 2005; Quinque et al., 2006; Richards et al., 1993; Rogers et al., 2007). For sample collection, a buccal swab is the method of choice, since it has many advantages compared to other sources of human DNA such as peripheral blood, urine or saliva. This method is noninvasive, easy and quick and it doesn't require trained personnel to carry it out, it is readily accepted by subjects, sample storage and transportation at ambient temperature for a relatively long period of time is critical for field studies and the DNA quality is suitable for a variety of applications. The only disadvantage of this method is the lower DNA yield obtained from buccal swab samples compared to blood or saliva (Quinque et al., 2006).

PCR amplification is widely used in population analyses and genetic disease diagnosis (Garcia-Closas et al., 2001; Mulot et al., 2005; Quinque et al., 2006; Richards et al., 1993). Several studies have focused on direct PCR amplifications from different samples, including buccal cells, that circumvent DNA isolation but involve chemical, enzymatic or thermal cell lysis preceding the PCR (Čakić et al., 2001; Chomczynski and Rymaszewski, 2006; Li et al., 2011).

Amplification of human mitochondrial DNA (mtDNA) has been extensively used in human evolutionary and molecular anthropology studies (Ingman et al., 2000; Macaulay et al., 1999; Richards et al., 2000). mtDNA hypervariable segments I and II (HVSI and HVSII) have been proved to be a suitable tool in these genetic analyses due to the unique properties of mtDNA, i.e. apparent lack of recombination, maternal mode of inheritance, rapid evolutionary rate and high population-specific polymorphisms (Brown et al., 1979; Giles et al., 1980; Piganeau and Eyre-Walker, 2004).

The aim of this study was to optimize conditions for low-cost and efficient large-scale population mtDNA genotyping by using a direct PCR approach and omitting the DNA extraction step. We used buccal cells collected on swabs for direct PCR amplification of a 330 bp fragment of the first hypervariable segment (HVSI) of the control region of mtDNA. To the best of our knowledge there are no reports regarding the use of buccal cells for direct PCR amplification of the HVSI region and mtDNA genotyping. Since fieldwork conditions and transport of samples can affect their quality for DNA analysis, we wanted to evaluate the effects of the storage time of buccal cell samples on their suitability for direct PCR amplification. We tested the effects of a prolonged sample storage time on the PCR amplification of the mtDNA HVSI region. Our data confirmed that direct PCR on buccal cells can substitute the use of genomic DNA in PCR amplification of the HVSI region of mtDNA.

MATERIALS AND METHODS

Sample collection and storage

In this study we recruited 7 volunteers from the staff of the Institute of Molecular Genetics and Genetic Engineering. The subjects refrained from eating and smoking for 30 minutes before sample collection. Participants were asked to rinse out their mouths with tap water before sampling. For each subject, buccal cells were collected on two sterile cotton swabs by twirling the swab on the inner cheek for 30 s without touching the teeth. One swab was used for DNA isolation using the phenol-chloroform method (Cao et al., 2003), and the other was used for the resuspension of buccal cells in PBS and the direct PCR method. In order to assess the effect of storage time of the buccal swabs on the quality of the DNA extracts and their suitability for direct PCR reactions, we analyzed fresh buccal swabs as well as swabs stored at room temperature for 7 and 14 days (24°-26°C) before processing. 'Fresh sample' refers to swabs that were immediately processed with no delays.

In order to test the effect of long-term storage of buccal cells resuspended in PBS on the PCR amplification success rate, additional swabs were obtained from three subjects. The buccal cells were resuspended in PBS and kept for different periods of time at +4°C (2.5 months) or -20°C (8 months).

Sample preparation and PCR amplification

The top of the cotton swab was separated from the rest of the stick and placed in a tube containing 400 µl of phosphate buffered saline (PBS). After 20 min, the tube was vortexed and the swab was removed. The buccal cells were pelleted by centrifugation for 2 min at 13000 rpm, and 380 µl of supernatant was removed. The pellets containing buccal cells were then kept at 4°C or at -20°C and used for direct PCR amplification. The oligonucleotides L16110 (5' – ATTTCGTACATTACTGCCAG – 3') and H16401 (5' – TGATTTCACGGAGGATGGTG – 3') were used for PCR amplification of a 330 bp fragment of the mtDNA HVSI region. The PCR reaction mix contained 1 × buffer B (80 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.02% w/v Tween-20), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 1 µl of buccal cell suspension or 4 ng of genomic DNA and 1 unit of FIREPol® DNA Polymerase (Solis Biodyne) in a total volume of 25 µl. Amplifications were carried out using a 2720 Thermal Cycler (Applied Biosystems). The buccal cell PCR reactions were heated for 2 min at 97°C, cooled for 3 min at 55°C and this thermal lysis step was repeated three times. During the 55°C step for 5 min, FIREPol® DNA Polymerase was added to the reaction, followed by a PCR program that comprised of denaturation at 94°C for 5 min, 35 cycles of

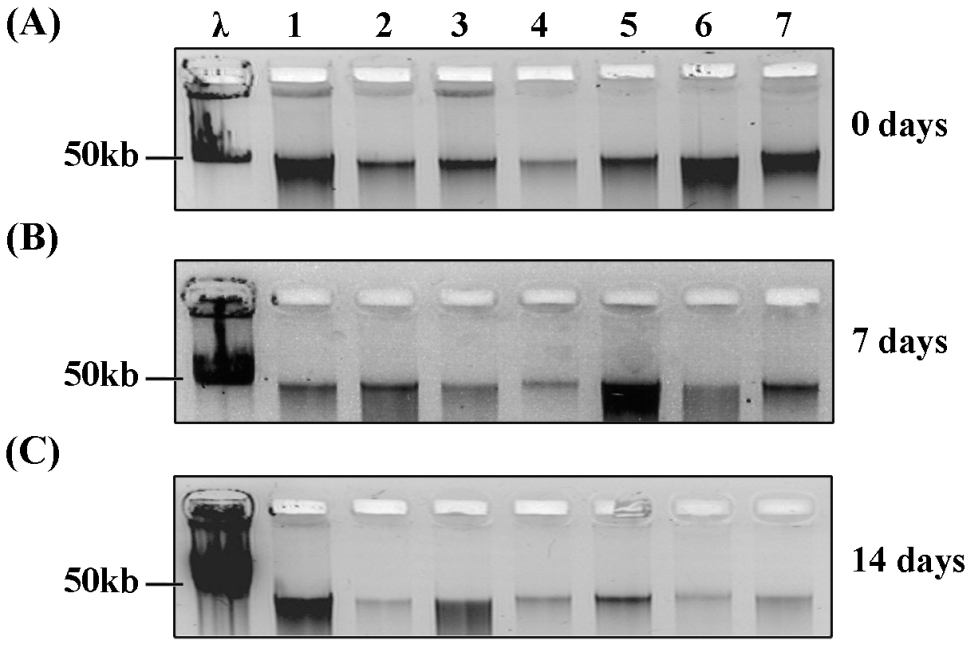

Fig. 1. Electrophoretic analysis of genomic DNA extracted from buccal cells collected from swabs that were processed immediately (A), after 7 days at room temperature (B), or after 14 days at room temperature (C). Lane λ: molecular weight marker λDNA; Lanes 1-7: genomic DNA extracted from buccal cells.

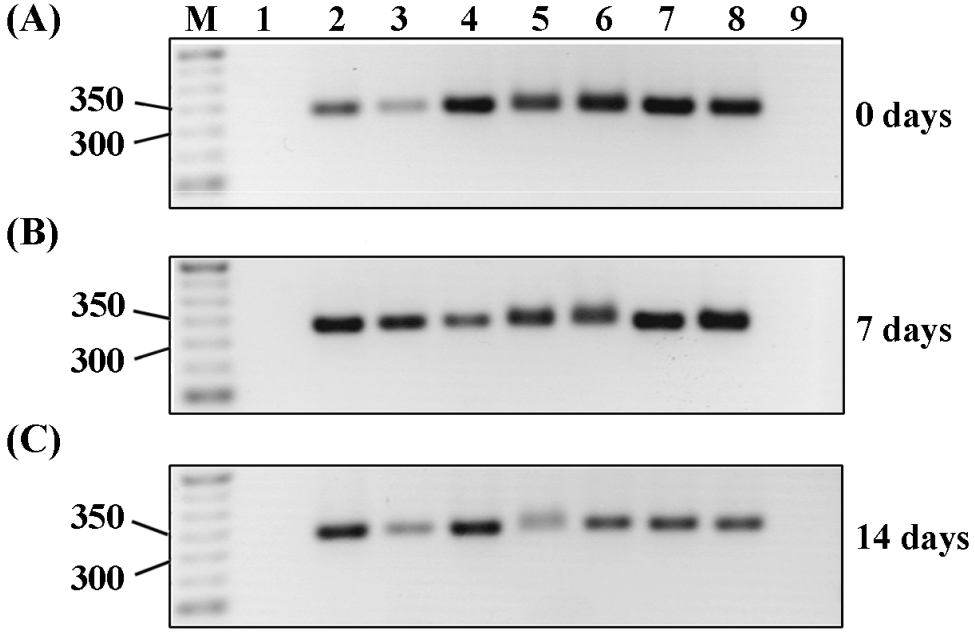

Fig. 2. The effect of long-term storage of buccal swabs at room temperature on direct PCR amplification. A 330 bp fragment of the HVSI region was amplified directly from the buccal cells collected from swabs that were processed immediately (A), after 7 days at room temperature (B) or after 14 days at room temperature (C). Lane M: O'RangeRuler™ 50 bp DNA Ladder (Fermentas); Lanes 1 and 9: negative PCR controls; Lanes 2-8: direct PCR products from buccal cells.

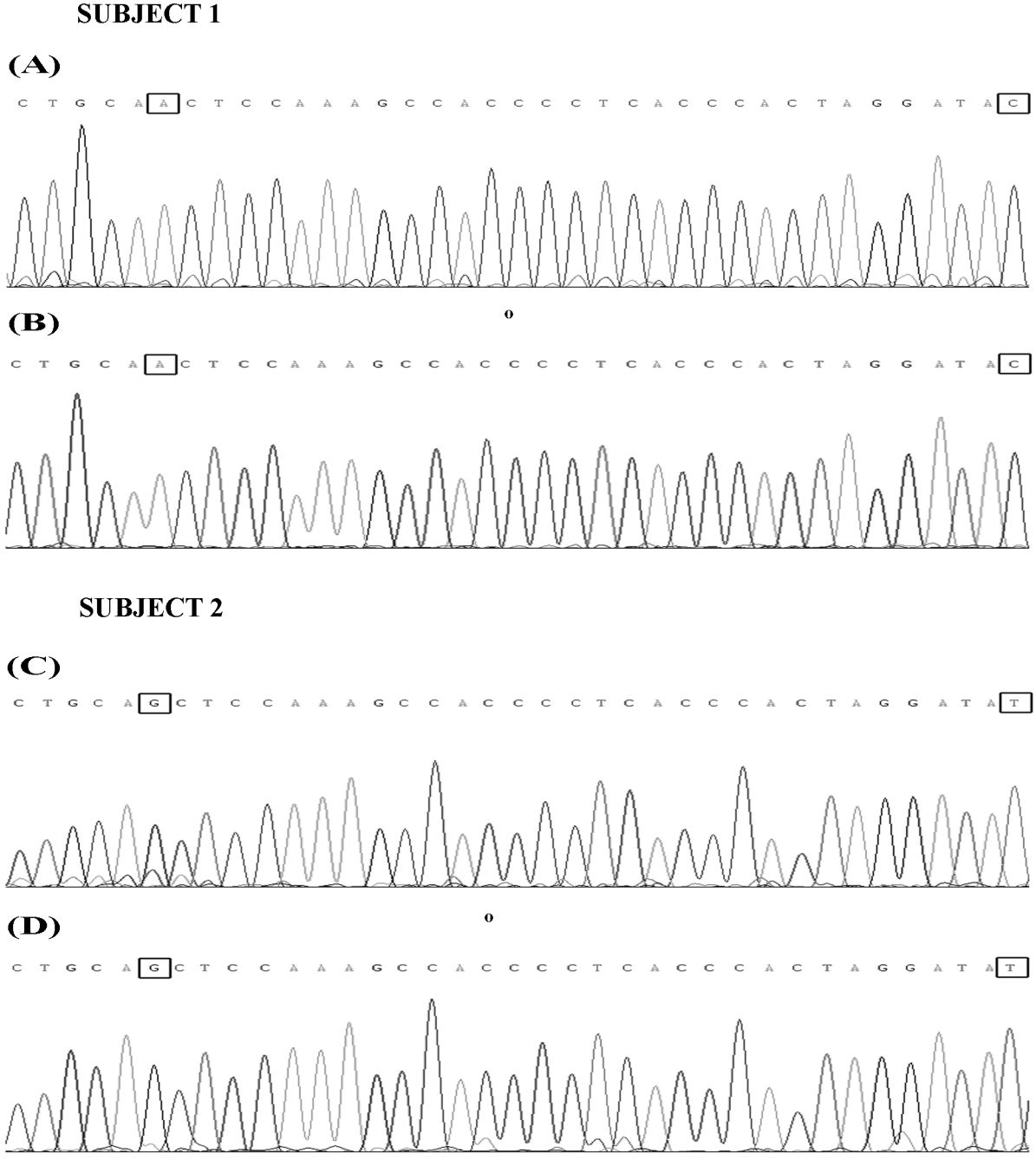

Fig. 3. Part of the mtDNA HVSI sequence. (A) and (C) - sequences from PCR products amplified on genomic DNA; (B) and (D) - sequences from PCR products amplified directly from buccal cells. (A) and (B) – Subject 1; (C) and (D) – Subject 2; Boxed nucleotides represent single nucleotide polymorphisms (SNPs) detected within the HVSI region of analyzed subjects.

94°C for 35 s, 56°C for 35 s, 72°C for 35 s and a final extension for 10 min at 72°C. One-fifth volume of the amplified product was analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

DNA extraction

Genomic DNA from the buccal swabs was isolated using the phenol-chloroform method as described (Cao et al., 2003) with minor modifications. Briefly, the top of the cotton swab was separated from the stick and incubated in 300 µl of digestion buffer (100 mM Tris-Cl, pH 8.0; 5 mM EDTA, pH 8.0; 1% SDS, 500 µg/ml of freshly added proteinase K) overnight at 37°C on a rotator. On the following day, the samples were incubated for 30 min at 53°C in a water bath. The swabs were then removed and DNA was extracted with an equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol solution (25:24:1). This step was repeated twice. After extraction with an equal volume of chloroform-isoamyl alcohol, the DNA was precipitated with 1/10 volume of 3 M sodium-acetate pH 5.2 and 2 volumes of ice-cold absolute ethanol. The pellet was washed twice with 70% and absolute ethanol. Finally, the DNA was resuspended in 100 µl of ddH_2O and stored at -20°C. The concentration and purity of the DNA was checked using a NanoVue spectrophotometer (GE Healthcare). DNA integrity was checked by electrophoresis on a 0.8% agarose gel, followed by visualization with ethidium bromide staining.

Sequencing

Amplified PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Sequencing reactions were performed in a 2720 Thermal Cycler (Applied Biosystems) using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). For sequencing of PCR products, the same primers as for the amplification were used. The sequencing reaction was performed in a total volume of 8 µl with the following components: 2 µl of BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix, 1 µl of 3.2 µM sequencing primer, 10-20 ng of purified PCR product as a template and ddH_2O. The conditions for cycle sequencing were as follows: 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. After the sequencing reaction, residual dye terminators were removed by ethanol precipitation. Capillary electrophoresis of the sequencing products was carried out on a 3130 Genetic Analyzer (Applied Biosystems). Analysis of the DNA sequencing data was performed using DNA Sequencing Analysis Software v5.2 Patch 2 (Applied Biosystems). The obtained sequences were analyzed using the BLAST program from the public database NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

RESULTS AND DISCUSSION

Numerous reports have evaluated different sampling procedures, storage times and storage conditions of buccal cell samples on the quality and quantity of isolated DNA (Lum and Le Marchand, 1998; Mulot et al., 2005; Quinque et al., 2006; Richards et al., 1993; Rogers et al., 2007). Since the transport of samples and fieldwork conditions can affect the quality of samples for DNA analysis, we wanted to test the effect of a prolonged storage time of buccal swabs at room temperature on DNA extracts' quality and their suitability for PCR. For that purpose, genomic DNAs were prepared from buccal cells either immediately after collection or after storage at room temperature for 7 and 14 days (Table 1). The average DNA yields per swab were 3.91 (0 days), 3.98 (7 days) and 4.29 µg (14 days) (Table 1). The purity of genomic DNA was estimated by OD260/OD280 ratio. As presented in Table 1, no significant differences in the amount and purity of isolated DNA were found for different storage times. This is in agreement with results obtained for DNA yields of cytobrush buccal samples that were stored for up to 7 days at room temperature (Mulot et al., 2005). On the other hand, there are reports in which an increase in DNA yield was observed in buccal samples with a prolonged storage time before processing, which could be explained by bacterial growth (Garcia-Closas et al., 2001) and the presence of a large proportion of bacterial DNA in the samples. However, we did not notice an increase

Table 1. Yields and quality of DNA from buccal swabs kept under different storage times (0, 7 and 14 days) at room temperature before extraction and the success rate of PCR amplification of the HVSI region

	Swab storage time at room temperature before processing (in days)		
	0	7	14
Average DNA yield per swab and range (in parenthesis) in μg	3.91 (1.54-8.3)	3.98 (1.25-8.1)	4.29 (0.87-7.9)
Mean OD260/OD280 ratio ± SD and range (in parenthesis)	1.68±0.15 (1.45-1.88)	1.64±0.16 (1.37-1.88)	1.67±0.19 (1.32-1.88)
PCR amplification of 330 bp fragment of HVSI from genomic DNA	+	+	+

Table 2. The effect of long-term storage of buccal swabs at room temperature and buccal cells in PBS at -20°C or at +4°C on direct PCR amplification of a 330 bp fragment of the HVSI region

Sample group	Swab storage time at room temperature before processing (in days)	Storage time of buccal cells in PBS at +4°C (in months)	Storage time of buccal cells in PBS at -20°C (in months)	Direct PCR amplification of 330 bp fragment of HVSI from buccal cells
1	0	/	/	+
2	7	/	/	+
3	14	/	/	+
4	0	2.5	/	+
5	7	2.5	/	+
6	14	2.5	/	+
7	0	/	8	+

in the amount of isolated DNA in our samples with prolonged storage time at room temperature.

The total amount of DNA isolated from all the tested buccal swabs was variable (Table 1) and this was confirmed by electrophoretic analysis of the extracted buccal cell DNA (Fig. 1). This could be explained by sampling variations resulting in a different number of buccal cells being collected for DNA extraction from each subject. Compared to λ DNA as a molecular weight marker, a high molecular weight DNA was obtained from all samples (Fig. 1). The yields of high molecular weight DNA from the buccal swabs that were processed immediately after collection were higher compared to the swabs that were processed with delays of 7 and 14 days (Fig. 1, compare A to B and C). Nevertheless, the DNA isolated from all samples proved to be suitable for PCR amplification of a 330 bp fragment of the HVSI region of mtDNA (Table 1). These results are in line with other reports where the storage of unprocessed specimens (buccal cells collected by mouthwash) at room temperature or at 37°C for 1 week did not affect the suitability of the extracted DNA for PCR amplification (Hayney et al., 1996; Lum and Le Marchand, 1998).

A number of studies have focused on direct PCR amplifications from different samples such as whole blood, buffy-coat, saliva, liver, hair, buccal cells or bacteria that circumvent DNA isolation but involve chemical, enzymatic or thermal cell lysis prior to PCR (Čakić et al., 2001; Chomczynski and Rymaszewski, 2006; Li et al., 2011; Mercier et al., 1990). We wanted to test the use of buccal cells from swabs that were

stored at room temperature for different periods of time for direct PCR amplification of the HVSI region of mtDNA without previous DNA extraction. For this purpose, we used buccal cells from fresh swabs as a control, as well as buccal cells from swabs that were stored at room temperature for 7 and 14 days before processing (Sample groups 1, 2 and 3, Table 2). These buccal cell samples subjected to direct PCR amplification of the mtDNA HVSI region yielded a specific PCR product with the expected size of 330 bp (Fig. 2 A, B and C, lanes 2-8 and Table 2). As presented in Fig. 2, the observed variations in the yields of PCR products could be attributed to inter-individual sampling variations resulting in different numbers of collected buccal cells used for direct PCR. Nevertheless, the yield and quality of the obtained PCR products were sufficient for further analysis.

In order to verify the specificity of amplifications, PCR products obtained from sample group 3 (Fig. 2 C, lanes 2-8) were sequenced. Analysis of these sequences using the BLAST program from the public database NCBI confirmed that the obtained PCR products were results of HVSI amplification of the human mtDNA (data not shown). It is important to note that a single buccal cells sample was sufficient to perform at least 20 independent PCR reactions. The obtained results indicate that direct PCR can be applied in fieldwork conditions, especially when a large number of samples needs to be analyzed in short period of time and that the buccal samples are suitable for direct PCR analysis even after 14 days storage at ambient temperature.

Next, we wanted to test the feasibility of direct PCR amplification using buccal cells that were stored in PBS for several months at +4°C or at -20°C. Precisely, buccal cells collected on swabs that were stored at room temperature for different periods of time (fresh, 7 and 14 days) were resuspended in PBS as described in section Materials and Methods and kept at +4°C for 2.5 months before they were subjected to PCR amplification (Sample groups 4, 5 and 6, Table 2). Additionally, we performed PCR on prepared buccal cell samples that were kept at -20°C for 8 months (Sample group 7, Table 2). All the samples subjected to direct PCR amplification of a 330 bp fragment of HVSI yielded a specific PCR product (data not shown, overview given at Table 2).

Finally, we examined whether long-term storage of prepared buccal cell samples (buccal cells in PBS) affected their suitability for mtDNA genotyping. Precisely, we tested whether the HVSI region of mtDNA can be reliably sequenced using PCR products directly amplified from buccal cells from sample groups 6 and 7 (Table 2). The sequence alignments revealed that sequences obtained from direct PCR on the buccal cells are identical to previously obtained sequences from PCR on genomic DNA for the same subjects (Fig. 3). Representative sequencing results for two subjects with different single nucleotide polymorphisms (SNPs) in the HVSI region of mtDNA are presented at Fig. 3.

In conclusion, we demonstrated that buccal cells resuspended in PBS can be used as an alternative for purified genomic DNA in PCR reactions. In addition to the noninvasive nature of buccal swab sampling, the preparation of buccal cells for PCR presented in this report is fast, low-cost and well suited for large-scale analysis of mtDNA variations. Furthermore, we have shown that long-term storage of buccal swab samples at different temperatures prior to direct PCR on the buccal cells does not affect their suitability for PCR amplification of the HVSI regions of mtDNA. Since this method can be applied to the PCR amplification of any particular mtDNA fragment, it could also prove useful in the detection of the pathogenic mutations that are associated with different mitochondrial diseases (Goto et al., 1990; Wallace et al., 1988).

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