Int J Legal Med (2007) 121:493–499
DOI 10.1007/s00414-007-0177-3

TECHNICAL NOTE

First successful assay of Y-SNP typing by SNaPshot minisequencing on ancient DNA

C. Bouakaze · C. Keyser · S. Amory · E. Crubézy · B. Ludes

Received: 31 October 2006 / Accepted: 7 May 2007 / Published online: 30 May 2007

Abstract In the present study, a set of 13 Y-chromosomal single nucleotide polymorphisms (Y-SNPs) selected for the identification of the most frequent Asian Y-haplogroups was included in an allele-specific primer extension assay. Single nucleotide polymorphism (SNP) genotyping was accomplished by co-amplification of these 13 DNA fragments within 2 multiplex PCRs followed by detection with 1 minisequencing reaction using the SNaPshot™ Multiplex kit and analysis of extension products by capillary electrophoresis. First developed on modern samples, the assay was optimized for the analysis of 11 ancient DNA (aDNA) samples from the Krasnoyarsk region (southern Siberia) that were dated from 5,500–1,800 years before present (YBP). SNP typing was successful for most of them, which were all assigned to Y-haplogroup R1a1 except one. These results show that SNPs are well-suited for the analysis of aged and degraded DNA samples. Moreover, we found that the SNaPshot minisequencing methodology is a convenient, robust, and efficient method for SNP typing. To our knowledge, this study reports the first successful investigation of Y-SNPs on aDNA samples. The potential use of Y-SNPs in both evolutionary and forensic fields is also discussed.

Keywords Y-chromosome · SNPs · SNaPshot · Single base extension · Ancient DNA · Southern Siberia · Y-haplogroup R1a1

Introduction

The analysis of single nucleotide polymorphisms (SNPs) located within the male-specific region of the Y-chromosome (MSY) has been widely accepted in molecular anthropology as a unique tool for evolutionary studies. Due to the specific distribution of Y-haplogroups among populations, Y-chromosomal single nucleotide polymorphism (Y-SNP) typing helps researchers to infer the origin, evolution, and history of humans by tracing back male patterns of migration from modern human populations [1]. However, better insights into this issue should be provided by comparing modern genetic data to those obtained by studying ancient human populations.

In forensic genetics, the usefulness of Y-chromosomal markers has been recognized for deficiency paternity testing cases and for human identification where the typing of autosomal short tandem repeats (STRs) fails to give interpretable profiles, e.g., analysis of sexual assault stains (male and female DNA admixtures) [2–5]. The analysis of Y-SNPs may significantly contribute to forensic investigation by providing information on the ethnic origin of a male DNA sample [6, 7]. Combined with conventional markers, Y-SNPs could be a powerful tool for mass disasters such as airplane crashes, tsunamis or terrorist attacks where people from various geographical areas are involved.

The overlap between the anthropological and forensic fields is that the biological material is often highly degraded and only available in low quantities. The analysis of such samples using conventional STR typing often fails to

Electronic supplementary material The online version of this article (doi:10.1007/s00414-007-0177-3) contains supplementary material, which is available to authorized users.

C. Bouakaze (✉) · C. Keyser · S. Amory · B. Ludes
Institute of Legal Medicine, EA3428,
Strasbourg, France
e-mail: caroline.bouakaze@ulp.u-strasbg.fr

S. Amory · E. Crubézy · B. Ludes
Anthropobiology Laboratory, CNRS, FRE2960,
Toulouse, France

resolve informative profiles, which is assumed to be due to the relatively large size of amplicons (150–400 base pairs, bp). Because the amplification of small fragments has been reported to increase the chance of successful typing of degraded DNA [8–10] and SNPs can be analyzed in amplicons shorter than 150 bp, they seem to be a tool of choice for the analysis of degraded samples. SNP typing has been first successfully employed for heavily degraded DNA collected after the September 11, 2001, World Trade Center (WTC) attack [11]. Seventeen thousand profiles were generated and helped alone to identify about 10 individuals with 10 more identifications made when SNP results were used to supplement partial STR profiles.

Among the various SNP typing methodologies available today [12, 13], we selected the SNaPshot minisequencing-based approach, which consists of the single base extension (SBE) of an unlabeled primer that anneals one base upstream to the relevant SNP with a fluorochrome-labeled dideoxynucleotide (ddNTP). The allele designation is then possible by separating extended products and detecting the fluorescence by capillary electrophoresis. Currently, various multiplex minisequencing-based assays have been successfully validated for the analysis of mitochondrial DNA [14–17], autosomes [18, 19], the Y-chromosome [20–24], Duffy and ABO group system [25, 26], and the melanocortin 1 receptor gene as indicator of red hair phenotype [27]. All these studies were consistent with the fact that this SNP typing methodology is robust, reliable, and extremely sensitive.

The aim of the present study was to evaluate the effectiveness of a minisequencing assay for the typing of Y-SNPs with low amounts of aged and degraded DNA recovered from human bone remains. For this purpose, we first selected Y-SNPs relevant for the ancient populations currently under study in our laboratory, namely, Mongolian, Yakut, Siberian, and Amerindian populations. Second, we developed an efficient multiplex assay working with minute amounts of DNA on modern samples. Finally, we optimized and validated the whole strategy by analyzing a set of 11 aDNA samples.

Materials and methods

Y-SNP selection

A set of 13 biallelic markers (M3, M9, M17, M45, M89, M173, M175, M216, M217, M242, 92R7, RPS4Y$_{711}$ (M130), and Tat (M46)) characterizing Asian and Amerindian haplogroups was chosen from the literature [28–32]. The Y-SNP haplogroup nomenclature used here is that recommended by the Y Chromosome Consortium (YCC) [1, 33]. The sequence variation for each SNP and the Y-haplogroup tree defined by them are represented in Fig. 1.

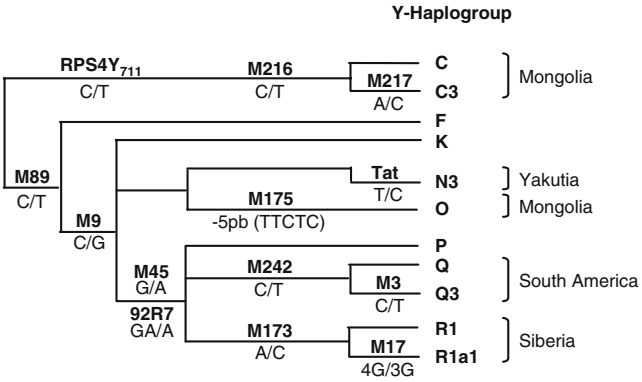

Fig. 1 Phylogenetic tree of the 13 Y-chromosomal binary polymorphisms analyzed in this study. Marker names are indicated *above* the lines and sequence variation *under* the marker name. The corresponding lineage name and the geographical region where it is predominantly found are shown at the *end* of each branch. The length of each one has no significance in terms of phylogenetic distance

Modern DNA samples

Modern DNA samples from five male individuals of different geographical origin (Europe, Yakutia, Mongolia) and one female, as negative control, were analyzed. Appropriate informed consent was obtained from these six subjects. Genomic DNA was extracted from blood or buccal swabs using a phenol–chloroform method, purified using the CleanMix Kit (Talent, Trieste, Italy), concentrated using YM-30 Microcons (Millipore, Bedford, MA), and finally, quantified by real-time PCR using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, AB, Foster City, CA) with the ABI PRISM® 7000 Sequence Detection System (AB) following the manufacturer's recommendations.

Design and validation of PCR and minisequencing primers

Despite the fact that primers for the amplification of all SNP loci were already described in the literature, most of them were redesigned so that the size of the amplicons ranged from 81 to 155 bp. The PCR and minisequencing primer design was performed according to the recommendations of Sanchez et al. [24]. PCR and minisequencing primer sequences are shown in Tables S1 and S2 of the electronic supplementary material (ESM), respectively. The minisequencing primers were 5′ tailed with a non-homologous sequence (5′-AACTGACTAAACTAGGTGCCAC GTCGTGAAGTCTGACAA-3′) [37] and poly-C, if necessary, to produce extension products ranging from 21 to 93 nt and differing in length from each other by 6 nt to allow sufficient separation by capillary electrophoresis.

PCR and minisequencing primers were tested in singleplex reactions with 1 ng standard male template DNA using conditions outlined by Sanchez et al. [24]. Amplification products were verified by direct sequencing.

Multiplex PCR amplifications

The 13 target sequences were amplified in 2 multiplex reactions (Table S1of the ESM), a 7-plex (Multiplex I) and a 6-plex (Multiplex II), in a 50 μl final volume composed of 1X PCR Gold Buffer (AB), 8 mM $MgCl_2$, 400 μM of each dNTP, 2 U of AmpliTaq Gold DNA polymerase (AB), and 1 ng of male template DNA. Concentrations of PCR primers in each reaction mix are specified in Table S1 of the ESM. The thermal cycling consisted of a first denaturation step at 94°C for 5 min followed by 33 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 65°C for 30 s with a final extension at 65°C for 7 min.

Removal of excess primers and dNTPs

Three PCR clean-up methods were tested on pooled PCR products:

1. Shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo*I) treatment: 15 μl pooled PCR product was treated with 5 U of SAP (GE Healthcare, Little Chalfont, UK) and 2 U of *Exo*I (GE Healthcare) by incubation at 37°C for 1 h followed by enzyme inactivation by heating at 75°C for 15 min.
2. UltraClean PCR Clean-up Kit (MoBio Laboratories, Carlsbad, CA): 50 μl of pooled PCR product was purified following the manufacturer's recommendations and finally eluted from the filters in 50 μl of sterile water.
3. Genopure ds™ (Bruker Daltonik, Bremen, Germany): 30 μl pooled PCR product was purified following the manufacturer's recommendations and finally eluted from magnetic beads in 10 μl sterile water.

Multiplex SBE reaction (minisequencing)

The multiplex minisequencing reaction was carried out in a 10 μl final volume containing 4 μl of SNaPshot™ Multiplex Ready Mix (AB), 20 mM ammonium sulfate, 3 μl cleaned pooled PCR products. Concentrations of primers in the reaction mix are specified in Table S2 of the ESM. Extension was performed for 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 30 s.

Removal of unincorporated ddNTPs

Extension products were treated with 1 U of SAP (GE Healthcare) by incubation at 37°C for 1 h followed by enzyme inactivation by heating at 75°C for 15 min.

Capillary electrophoresis and data analysis

For preparation of samples to capillary electrophoresis, 2 μl purified extension products were mixed to 17.6 μl Hi-Di™ Formamide (AB) and 0.4 μl of internal size standard GeneScan-120 LIZ™ (AB). After denaturation, samples were run on an ABI PRISM 3100® Genetic Analyzer (AB) using POP-4® (AB). Data were analyzed using the GeneScan™ v.3.7 software (AB) and automated allele calling was done by creating an optimized spectral matrix on the Genotyper™ v.3.7 software (AB) with the peak threshold set to a minimum of 100 relative fluorescence units (RFUs). Haplogroup assignment was performed according to the YCC2003 tree [1].

Male–female DNA mixture assay

The specificity of amplifications and the influence of contaminant female DNA were checked by analyzing mixtures of a fixed amount of male DNA template (1 ng) with a dominant amount of female DNA (6, 12, 24, 48, and 190 ng). The experiment was done with two different male and female samples.

Sensitivity test

The performance of the assay was tested by analyzing serial dilutions of male template DNA at concentrations of 10 ng/μl, 1,000, 800, 600, 400, 200, 100, 50, and 1 pg/μl. The same experiment was performed with PCR thermal cycling conditions detailed for multiplex amplifications and with increasing cycles from 33 to 37.

Ancient samples

Archaeological samples consisted of 11 femoral-diaphyseal bone fragments from unrelated male individuals. These samples originated from the Krasnoyarsk Museum, southern Siberia. One of them was attributed to the Afanassievo culture (3,500–2,500 B.C.), 3 to the Andronovo culture (2,500–1,500 B.C.), 6 to the Tagar culture (800–200 B.C.) and the remaining 1 to the Tachtyk culture (200 B.C.–200 A.D.). Removal of surface contamination and genomic DNA extraction were carefully done according to a published protocol [38].

Y-SNP typing of ancient samples

The same protocol as for modern DNA was applied to aDNA samples with the following modifications:

1. Multiplex amplifications were performed on 15 μl aDNA extract for 37 thermal cycles instead of 33. PCR

reaction clean-up was carried out using the Genopure ds™ kit (Bruker Daltonik).
2. The multiplex SBE reaction was performed on 3 μl purified PCR products and extension was performed for 30 cycles instead of 25.

Precautions against contamination

To ensure the accuracy and reliability of results obtained from ancient samples, all amplifications were performed at least two times from two independent DNA extracts. Additional measures taken to avoid contamination with modern DNA have been previously published [39].

Results and discussion

Y-SNP typing of modern DNA samples

The 13 Y-SNPs were analyzed by simultaneous amplification in 2 multiplex PCRs followed by allelic discrimination in 1 multiplex minisequencing reaction using the ABI PRISM® SNaPshot™ Multiplex Kit (AB) and capillary electrophoresis.

As recommended in the SNaPshot manufacturer's protocol, we first used the straightforward and fast *Exo*I and SAP treatment as a PCR clean-up method. All modern samples investigated were successfully typed. Signals for all SNPs were distinct except for marker M217 because of high background noise between 25 and 32 nt (Fig. S1a of the ESM). Assuming that these extraneous signals resulted from the extension of unremoved primer dimers, we tested two other purification methods. The use of the UltraClean PCR Clean-up Kit (MoBio Laboratories) was inefficient (Fig. S1b of the ESM), whereas the signal assigned to marker M217 was clearly distinguishable with Genopure ds™ (Bruker Daltonik) (Fig. S1c of the ESM). Moreover, the overall signal intensity was considerably increased up to 6,000 RFUs probably due to the low elution volume, which enabled sample concentration at the same time. Thus, the PCR clean-up step is of critical importance for correct typing using our strategy. Nevertheless, this phenomenon can be avoided by designing minisequencing primers with lengths above 30 nt so that result interpretation is unambiguous even in case of insufficient purification [22].

As expected, a single peak was observed for each SNP marker except for the ancestral state of marker 92R7, which was reported to be a paralogous sequence variant that originated from a duplication event [40]. In many cases, an extraneous green peak sized approximately 82 nt was observed (Fig. S1c of the ESM). The cause is unknown but it did not affect the allele designation process because no peak was expected to appear at this size and color. Moreover, there was always an artifactual peak sized approximately 60 nt in the blue channel when the ancestral allele of marker M17, namely, 4G, was observed but the causes are unknown.

Two phenomenons, the difference of peak height between the four dye lanes and the difference of migration according to the size of extension products, are observed in Fig. S1c of the ESM. According to the literature, the first one is attributed to the ratios of the fluorophore emission [19] and the second one can be explained by the fact that the mobility of shorter DNA molecules is influenced by the different masses of fluorochromes [22].

The specificity of Y-SNP amplification was tested using a negative control that contained female DNA. No peaks were observed on the resulting electropherogram except at the Tat locus where a green signal was clearly visible. This suggested the existence of a homologous sequence with an ancestral Tat allele. Previously, by sequencing Tat singleplex amplification products, we observed a weak amplification for a female sample. However, we did not consider it because PCR primers were selected from the literature where they were reported to be specific for the Y-chromosome. The sequence of the region surrounding the Tat locus was aligned to those of the GenBank database using nBLAST on the National Center for Biotechnology Information website and we found that this SNP locus is located within the ubiquitin-specific protease 9 gene (USP9Y). Because this gene has a homolog on the X-chromosome, USP9X, we assumed that the X-chromosome linked homologous sequence was co-amplified during the PCR. Thus, sequences of PCR and minisequencing primers were also BLASTed against the GenBank database and, indeed, they matched to the X-chromosome. However, it did not interfere with the Y-specific amplification because unambiguous results were obtained from a Yakut sample that bore the derived Tat allele. In addition, female DNA did not interfere with the amplification of Y-SNPs even for the Tat marker because same results were obtained with male template DNA only and male–female DNA mixtures (1:190).

A sensitivity test with decreasing amounts of DNA template showed that our strategy worked very well with DNA amounts ranging from 1 ng to 400 pg. The lower limit of detection was pushed back to 50 pg by increasing the amplification cycles from 33 to 37.

Y-SNP typing of aDNA samples

The effectiveness of this minisequencing-based SNP typing assay with low amounts of aged and degraded DNA samples was evaluated on 11 DNA samples recovered from ancient distinct human bones found in Siberia. The typing

was carried out at least twice for each sample and all replicates gave consistent results that always correlated with the YCC2003 tree [1]. Moreover, all extraction and PCR blanks were consistently negative, indicating that our results were unlikely to derive from contaminants in extraction and PCR processes. The typing always failed for 2 samples, the oldest sample belonging to the Afanasievo culture (3,500–2,500 B.C.) and 1 belonging to the Tagar culture (800–200 B.C.). This can be explained by the fact that the DNA content of these two bone samples was too low according to quantitation results (Table S3 of the ESM). Among the nine remaining samples, eight were assigned to Y-haplogroup R1a1 and one to Y-haplogroup C(xC3). This result will be further interpreted with regard to data obtained from typing of other genetic markers, namely, STRs and mitochondrial DNA, on the same aDNA samples (article in preparation). An example of an electropherogram obtained from an ancient sample belonging to Y-haplogroup R1a1 is shown in Fig. 2.

The yield of products was found to decrease with increasing PCR product size because it was generally the larger amplicon (marker M242, 155 bp) that was either weakly amplified or totally failed to amplify. Such an inverse dependence of the amplification efficiency on the size of the amplicons is typical of aDNA and results from damage and degradation processes. Moreover, it is important to note that this assay was first conceived with the intention to amplify all DNA fragments in a unique 13-plex PCR. Although it successfully worked with modern DNA template, no peaks were observed on electropherograms obtained with aDNA. This can also be explained by the degraded nature of aDNA. That is why we needed to divide our 13-plex PCR into a 7-plex and a 6-plex amplification reaction. It should be kept in mind that an increase in degree of multiplexing always implies a decrease in amplification efficiency.

To date, experiments to explore the suitability of a SNaPshot minisequencing-based SNP typing with low molecular weight DNA have either been done with artificially degraded DNA (e.g., sonicated DNA) or with forensic degraded samples with no indications about the age of the sample or the extent of DNA degradation. Because it can be predicted that without the repair mechanisms of living cells, DNA is spontaneously degraded in short DNA fragments over thousands years [41] and because the bone samples from which DNA was recovered in this study were approximately dated from 5,500–1,800 YBP, we assume that these aDNA samples were degraded and fragmented and present in minute amounts according to quantitation results (Table S3 of the ESM). Thus, to the best of our knowledge, the present study reports for the first time the efficiency of a Y-SNP typing using the SNaPshot minisequencing methodology with low amounts of aged and degraded DNA. This methodology was also found to be very robust because reproducible and reliable results were obtained from both modern and ancient samples that contained different quantities and qualities of DNA.

The fact that SNP typing can increase the success of obtaining genetic data from degraded DNA when used as a supplement to standard STR typing [42] was particularly well-illustrated in one case. For this sample belonging to the Tagar culture, only partial genotypes and Y-haplotypes were obtained from various independent extractions, whereas all replicates of Y-SNP typing were successful and assigned this individual to Y-haplogroup R1a1 (sample S25 on Table S3 of the ESM).

In conclusion, although further optimization is still needed, e.g., test a larger aDNA sample set and include additional SNP markers, the simple, robust, and efficient SNP typing assay developed in the present study should find an application in

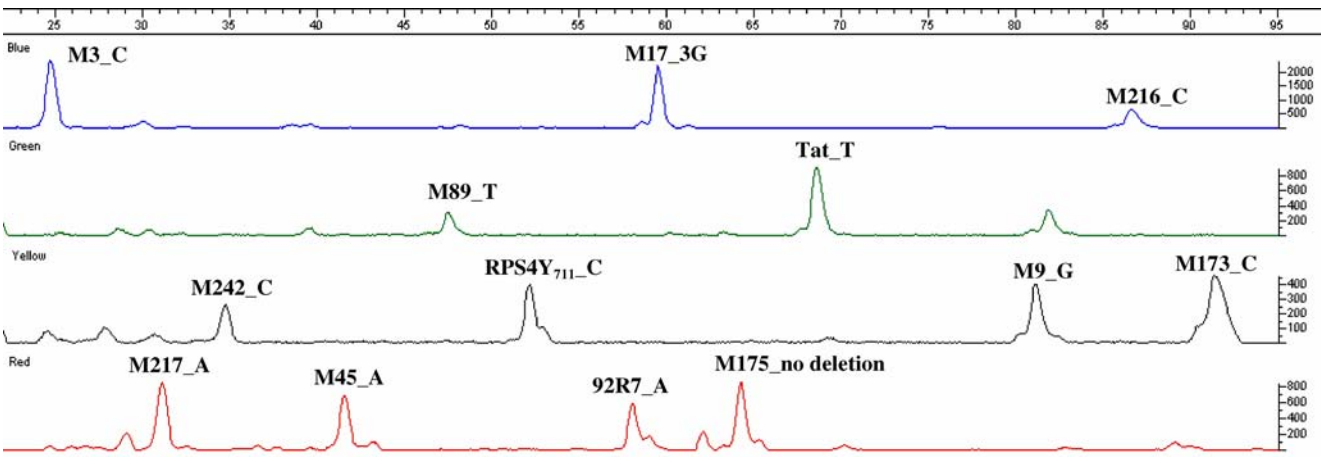

Fig. 2 Electropherogram obtained from an ancient male sample belonging to Y-haplogroup R1a1. Electropherogram obtained with approximately 450 pg aDNA of an individual belonging to Y-haplogroup R1a1. This plot, obtained using Genotyper v.3.7 software, shows the RFUs vs measured size (nt) of SBE products relative to the GS120 LIZ internal size standard

both evolutionary and forensic studies because both have to deal with low amounts of degraded DNA.

Acknowledgements We gratefully thank Sarah Romac for her valuable technical help.

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