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Genetic analysis of the skeletal remains attributed to Francesco Petrarca

David Caramelli [a,*], Carles Lalueza-Fox [b], Cristian Capelli [c], Martina Lari [a], María Lourdes Sampietro [d], Elena Gigli [a], Lucio Milani [a], Elena Pilli [a], Silvia Guimaraes [a], Brunetto Chiarelli [a], Vito Terribile Wien Marin [e], Antonella Casoli [f], Roscoe Stanyon [a], Jaume Bertranpetit [b], Guido Barbujani [g]

[a] *Dipartimento di Biologia Animale e Genetica, Laboratorio di Antropologia, Università di Firenze, via del Proconsolo 12, 50122 Firenze, Italy*
[b] *Secció Antropologia, Departament de Biologia Animal, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain*
[c] *Istituto Italiano di Antropologia, Dipartimento di Biologia Animale e dell'Uomo, Università di Roma "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy*
[d] *Unitat de Biologia Evolutiva, Universitat Pompeu Fabra, Dr. Aiguader 80, 08003 Barcelona, Spain*
[e] *Istituto di Anatomia Patologica, Università di Padova, via Gabelli 61, 35121 Padua, Italy*
[f] *Dipartimento di Chimica Generale e Inorganica, Chimica Analitica, Chimica Fisica, Università di Parma, Parco Area delle Scienze 17/A, 43100 Parma, Italy*
[g] *Dipartimento di Biologia, Università di Ferrara, via Borsari 46, 44100 Ferrara, Italy*

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Abstract

We report on the mitochondrial DNA (mtDNA) analysis of the supposed remains of Francesco Petrarca exhumed in November 2003, from the S. Maria Assunta church, in Arquà Padua (Italy) where he died in 1374. The optimal preservation of the remains allowed the retrieval of sufficient mtDNA for genetic analysis. DNA was extracted from a rib and a tooth and mtDNA sequences were determined in multiple clones using the strictest criteria currently available for validation of ancient DNA sequences, including independent replication. MtDNA sequences from the tooth and rib were not identical, suggesting that they belonged to different individuals. Indeed, molecular gender determination showed that the postcranial remains belonged to a male while the skull belonged to a female. Historical records indicated that the remains were violated in 1630, possibly by thieves. These results are consistent with morphological investigations and confirm the importance of integrating molecular and morphological approaches in investigating historical remains.

Keywords: Ancient DNA; Petrarca body; mtDNA; Sex determination

1. Introduction

Francesco Petrarca is one of the most famous figures that influenced Italian and European literature for centuries. He was born in Arezzo (Italy) on 20 July 1304, when the world was breaking out of the Dark Ages and bursting into the Renaissance. After his family was exiled from Florence, Petrarca spent most of his early years in France. Back in Italy, in 1327 Petrarca for the first time saw Laura, the 19-year-old wife of another man. His forbidden love went unreciprocated, but it did not stop him from becoming completely enthralled. In Petrarca's time, the Bubonic Plague was still rampant in Europe. In 1348, his precious Laura was struck with the plague and died. In life and death Laura inspired some of the greatest love lyrics of all time. To ease his pain, Petrarca continued to travel throughout the land, and finally settled around 1367 in Padua. He spent his remaining years in various religious services, finally dying on 18 July 1374 in Arquà.

Before his body reached its tomb, it spent 6 years interred in the cathedral at Arquà. In 1630 a drunken friar called Tommaso Martinelli, helped by four accomplices, broke into the tomb and took some bones, apparently for resale. Martinelli and his associates were arrested, tried and exiled, but the missing remains were never recovered. Could they have also inserted a

* Corresponding author. Tel.: +39 055 2743021; fax: +39 055 2743032.
E-mail address: david.caramelli@unifi.it (D. Caramelli).

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skull? But would someone cunning enough to switch a phoney skull not have replaced the other missing bones as well?

Due to its high copy number, rapid rate of evolution, and haploid, maternal mode of inheritance, mitochondrial DNA (mtDNA) offers certain advantages over autosomal DNA markers for the identification of human remains, in particular when the remains are of forensic, historical or prehistorical interest [1]. The high copy number, with several 100 mtDNA molecules per cell [2], means that with older remains there is a greater likelihood of success in analyzing mtDNA as opposed to nuclear DNA, simply because mtDNA is so much more abundant to start with. The rapid rate of evolution means that there is a correspondingly high probability of exclusion if the remains are not from the individual in question. For historical studies, living relatives often serve as sources of reference DNA. Because these relatives are often a number of generations away from the individual in question and identification based on nuclear DNA is essentially impossible. However, the haploid and maternal mode of inheritance of mtDNA means that any living maternal relative of the individual in question should have an identical mtDNA type, and can thus serve as a reference source. Even without a living reference mtDNA still offer the possibility to determine if the material in a grave is belongs to one or more maternally unrelated individuals. This is of particular interest when remains are from disturbed tombs and a single origin is questioned. DNA can confirm the geneological relationship among the remains making the conclusions based on single bones from the subsequent morphological analysis more reliable and robust.

2. Ancient DNA (a DNA) study

We followed the most stringent criteria proposed for ancient DNA studies [3–5]. Extreme precautions were taken to avoid contamination of samples with extraneous DNA. All DNA extractions and PCR involving the exhumed remains were carried out in a laboratory physically separated from the laboratory in which PCR cycling and post-PCR analysis was conducted. Disposable masks, gloves, and laboratory coats were worn throughout and were changed frequently. The ceramic vials and ball bearings used to pulverise the samples were rinsed with 10% bleach, followed by ddH_2O, and then UV-irradiated between uses. Dedicated reagents and pipettes were used, together with filter-plugged tips. Pipettes were UV-irradiated between uses. All DNA extractions and PCR reactions included negative controls. All steps of the analysis were replicated at least twice. In addition, samples were sent to the Barcelona laboratory, where the whole analysis was independently replicated. To test for preservation of other macromolecules as an indirect evidence for DNA survival [6,7] we estimated the degree of aminoacid racemization in each sample, using approximately 5 mg of tooth and rib powder and following the procedures described in Poinar et al. [6]. We quantified the amount of target DNA by real time (RT) PCR. After extraction, UNG treatments were performed in order to minimize post-mortem damage. PCR products were cloned, an average of 60 clones for each individual were sequenced, and the sequences thus obtained were aligned and compared across clones.

3. Materials and methods (Florence)

3.1. DNA extraction

Rib and teeth (molar) specimens, were obtained for DNA analysis in November 2003 after the exhumation of Petrarca's body from S. Maria Assunta church, Arquà Padua. To prevent contamination from prior handling, the outer layer of bone was removed with a rotary tool, while a tooth was briefly soaked in 10% bleach. After brushing and soaking both samples were irradiated (1 h under UV light). The tooth root and the rib were powdered and DNA was extracted by means of a silica-based protocol ([8], modified). At least two independent extracts were obtained from each remain. Multiple negative controls were included for each extraction.

3.2. Aminoacid racemization

About 5 mg of bone powder for each fragment was used in this analysis; the stereoisomers of aspartic, glutamic, and alanine amino acids were determined by high performance liquid chromatography [6]. The stereoisomeric D/L ratio observed for the three amino acids are D/L Asp 0.0479, D/L Glu 0.0104, D/L Ala 0.0092. The aspartic values are close to the proposed limit of 0.10 for DNA preservation [6].

3.3. UNG treatment

Uracil bases caused by the hydrolytic deamination of cytosines were excised by treating 10 μl of DNA extracted from both samples with 1 U of uracil-*N*-glycosylase (UNG) for 30 min at 37 °C. UNG reduces sequence artifacts caused by this common form of post-mortem damage, resulting in apparent C to T/G to A mutations and subsequent errors in the sequence results [9]. After this treatment, the extracts were subjected to the same PCR cloning and sequencing conditions as described above.

3.4. Quantification of DNA molecules

Real-time PCR amplification was performed using Brilliant® SYBR® Green QPCR Master Mix (Stratagene) in MX3000P (Stratagene), using 0.5 μM of appropriate primers (forward primer located at H16107 and reverse primer located at L16261). Thermal cycling conditions were 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 53 °C for 1 min and 72 °C for 30 s, followed by SYBR® Green dissociation curve step. Ten-fold serial dilutions of the purified and quantified standard were included in the experiment to create the standard curve and determine the number of initial DNA molecules in the samples.

3.5. Amplification of mtDNA

Two microliters of DNA extracted from the bone were amplified as follows: 94 °C for 10 min (Taq polymerase activation), followed by 50 cycles of PCR (denaturation, 94 °C for 45 s, annealing, 53 °C for 1 min and extension, 72 °C for 1 min) and final step at 72 °C for 10 min. The 50 μl reaction mix contained 2 U of AmpliTaq Gold (Applied Biosystems), 200 μM of each dNTP and 1 μM of each primer. The 360 bp long HVR-I was subdivided into three overlapping fragments using the following primer pairs: L15995/H16132; L16107/H16261; L16247/H16402. Each extract was amplified at least twice. Since overlapping primers were used throughout the PCR amplifications, it is highly unlikely that we amplified a nuclear insertion rather than the organelle mtDNA.

3.6. Cloning and sequencing

PCR products were cloned using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Screening of white recombinant colonies was accomplished by PCR, transferring the colonies into a 30 μl reaction mix (67mM Tris HCl (pH 8.8), 2 mM $MgCl_2$, 1 μM of each primer, 0.125 mM of each dNTP, 0.75 units of Taq Polymerase) containing M13 forward and reverse universal primers. After 5 min at 92 °C, 30 cycles of PCR (30 s at 90 °C, 1 min at 50 °C, 1 min at 72 °C) were carried out and clones

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with inserts of the expected size were identified by agarose gel electrophoresis. After purification of these PCR products with Microcon PCR devices (Amicon), a volume of 1.5 μl was cycle-sequenced following the BigDye Terminator kit (Applied Biosystems) supplier's instructions. The sequence was determined using an Applied BioSystems 3100 DNA sequencer.

3.7. "Long" amplificate detection

Appropriate molecular behaviour was also tested by amplification of longer mtDNA fragments (443 and 724 bp), which have been reported as very unusual for ancient DNA. PCR conditions were those described for mtDNA analysis above, primers used for 443 bp fragment were L15995 and H16401, while for 724 bp fragment primers used were L16247 and H00360.

3.8. Molecular sex determination

On each aDNA sample, six amplifications were performed by multiplex PCR. Starting from 2 μl of extracted DNA, two loci were amplified by multiplex PCR. The first intron of the amelogenin homologous gene and a small portion, 93 bp, of the SRY, sex determining region Y gene were co-amplified with the following thermal profile: 94 °C for 10 min (activation of AmpliTaq Gold, Applied Biosystems), 65 cycles (1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C) and a final extension step at 72 °C for 5 min. The fastest transitions available between each temperature step were applied using a MJ Research PTC 100 thermal cycler. The 25 μl reaction mix contained 2 U of Taq polymerase, 200 μM of each dNTP, 2.5 mM MgCl2, 9 pmol of each primer (XY1: 5′-CCC TGG GCT CTG TAA AGA ATA GTG-3′; XY2: 5′-ATC AGA GCT TAA ACT GGG AAG CTG-3′; SRY1: 5′-GCA CTT CGC TGC AGA GTA CCG A-3′; SRY2: 5′-ATA AGT ATC GAC CTC GTC GGA A-3′), and 2.5 μl of the relative 10× reaction buffer. Twenty-five microliters of each PCR product were controlled by electrophoresis, on a 5% HR agarose gel with a 2 h run at 50 V, then visualized with ethidium bromide.

4. Materials and methods (Barcelona)

Another tooth and a fragment of the same rib were sent to the Barcelona ancient DNA laboratory (University Pompeu Fabra) for independent replication. The specimens were washed with bleach before being ground to powder. Then, for each sample, DNA extraction was made following a procedure described elsewhere [10]. Briefly, the powder was incubated overnight with 0.5 M EDTA, digested with proteinase K and SDS and extracted with a phenol–chlorophorm protocol. One microliters of the extract was subjected to 25 μl PCR reactions, using standard HVR1 primers and bovine serum albumine (BSA) to circumvent inhibitory problems of the ancient samples. PCR reactions consist in 1.2 U of Taq DNA polymerase (Ecogen), 1× reaction buffer (Ecogen), 1.4 mg/ml BSA, 2.5–2.1 mM $MgCl_2$, 0.2 mM dNTP's and 1 μM of each primer. Forty amplification cycles (1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C) with an initial denaturalisation step at 94 °C during 5 min were used. The following primer pair were used: L16131/H16211 for the tooth and L16185/H16260 for the rib extract (in two different amplifications). The resulting PCR products were visualized in a 1% low-melting point agarose, excised from the gel and subsequently cloned with the pMOS Blue kit (Ammersham). Colonies that yielded the right insert size were posteriorly sequenced with an ABI 3100® DNA sequencer (Applied Biosystems), following the manufacturer's instructions.

5. Results and discussion

As summarized in the *Aminoacid racemization* paragraph, the proportion of the D enantiomer of Asp, Glu, Ala was good for both samples, suggesting that it was possible to obtain well-preserved DNA molecules from these specimens [6,11]. Therefore, we proceeded to extract the DNA from the two putative Petrarca samples.

Sporadic contamination is considered unlikely when the number of PCR template molecules (*target DNA*) is greater than 1000 [6]. Rt PCR results showed that sufficient DNA for amplification was present. We sequenced 60 and 65 clones for the rib and tooth samples, respectively (Fig. 1). By comparing sequences across clones nucleotide substitutions observed in only one clone were considered due to Taq-polymerase errors or cloning artifacts. The rate of *Taq* misincorporation, suggests that the DNA templates were damaged (1.9 substitutions every 1000 bp within the HVRI), with at least 80% of the clones showing the consensus nucleotide for each DNA fragment (Fig. 1). The C to T changes, observed in 4 out of 17 clones in nucleotide position 16,071 from the tooth sample, can be attributed to DNA damage from deamination of the original cytosine. Amplification of large DNA fragments, unusual for ancient DNA, was not observed, and the final consensus sequences of both samples made phylogenetic sense, i.e., did not appear to be a combination of different sequences (data not shown). The analysis of Petrarca remains was repeated in the Barcelona and the consensus sequences were identical to those obtained in Florence. Moreover, all persons who worked in this study were mtDNA genotyped (in Florence, in a specific Forensics Laboratory) to control if any of their sequences matched those obtained in this study. All these procedures, unusual in forensics cases, give strong credibility to the results provided.

Reproducible mtDNA sequences were obtained from the molar and the rib with no contamination observed in the extractions and PCR blanks. A total of 360 nucleotides of mtDNA sequence were obtained, corresponding to positions 16,024–16,384 of the published reference sequence CRS [12].

When we compared the consensus sequences from both samples the mtDNA sequences obtained from tooth and rib were dissimilar, they had four differences from each other. The tooth and rib differed from the published reference sequence [12] for three and one nucleotide positions, respectively (Fig. 1). The mtDNA sequence obtained from the rib (HVRI motif: 126 193 311) is a J2 and it was only described twice in a database of 6216 sequences [13], once in the Middle East and once in Italy (frequency 0.00032). The tooth mtDNA sequence, on the contrary, is more frequent; it is an H* (HVR I motif: 129), found 52 times in the same database (frequency 0.008365) among various sites in Italy ($N = 2$), Iberia ($N = 27$), the Middle East ($N = 4$) and Souss (North Africa) ($N = 1$).

Given the mtDNA results and the apparent good DNA preservation we decided to attempt molecular gender determination of the samples. Molecular sex determination is an unusual strategy in ancient DNA studies because the target is nuclear DNA that, due to single copy presence, can be very difficult to retrieve. Primers were designed to detect single copies of the amelogenin and SRY gene [14]. As expected, amplification results, as visualised on agarose gels, were less intense than the multicopy mtDNA amplifications (data not shown). DNA sex identification was carried out using two independent extracts and six PCR amplifications for both samples, and PCR products were obtained from all

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Fig. 1. (a) DNA sequences from the 60 clones analysed for the rib of the putative Petrarca sample. The first lines report the reference sequence with the numbering of the nucleotide positions. Nucleotides identical to the Cambridge reference sequence are indicated by dots. In the first column, clones are identified by a letter and 3 numbers: the letter refers to the laboratory (F: Florence; B: Barcelona); the first number indicates the extraction; the second number indicates the PCR. (b) DNA sequences from the 65 clones analysed for the tooth of the putative Petrarca sample. The first lines report the reference sequence with the numbering of the nucleotide positions. Nucleotides identical to the Cambridge reference sequence are indicated by dots. In the first column, clones are identified by a letter and 3 numbers: the letters refer to the laboratory (F: Florence; B: Barcelona); the first number indicates the extraction; the second number indicates the PCR.

the experiments (Table 1). Multiplex DNA sex identification indicated that the two bone samples belong to different individuals, the tooth belonged to a female and the rib belonged to a male. This result was in agreement with and supported the morphological analysis. Moreover, radio carbon analysis (Radiocarbon Laboratory of The University of Tucson, Arizona) indicated that the skull was two centuries older than bone sample (a rib) recovered from the postcranial remains. Therefore, it is unlikely that the two samples belong to the same individual or even to contemporary individuals. Sex identification of the skeleton bones was well defined by historical morphological data as a male individual. The reassembled skeleton bore evidence of injuries compatible with those mentioned by Petrarca during his lifetime. Skull sex determination is more difficult using anthropometry and anthroposcopy analyses, but a possible female origin, suggested that the skull and postcranial remains were from two individuals. The aDNA results are consistent with morphological investigations and confirm the importance of using both molecular and morphological approaches in investigating historical remains.

Table 1
Results of amelogenin-SRY amplifications

PCR	Rib (first extract)	Rib (second extract)	Tooth (third extract)	Tooth (fourth extract)
1	112-106-93	93	106	106
2	112-106-93	112-106-93	106	106
3	112-93	112	106	106
4	93	112-106-93	106	106
5	106-93	106-93	106	106
6	112-106-93	112-106-93	106	106

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