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DNA analyses of the remains of the Prince Branciforte Barresi family

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Abstract The five skeletons found buried in the church of Militello di Catania, Sicily, were tentatively identified by morphological analysis and historical reports as the remains of Prince Branciforte Barresi, two of his children, his brother and another juvenile member of the family (sixteenth and seventeenth centuries). In order to attempt to clarify the degree of relationships of the five skeletons, sex testing and mitochondrial DNA (mtDNA) sequence analysis of the hypervariable segments I and II (HV1 and HV2) of control region were performed. Moreover, the 9 bp-deletion marker of region V (COII/tRNA ^{lys}) was examined. Molecular genetic analyses were consistent with historical expectations, although they did not directly demonstrate that these are in fact the remains of the Prince and his relatives, due to the impossibility of obtaining DNA from living maternal relatives of the Prince.

Key words Archaelogical specimens · Sex typing · Mitochondrial DNA · Hypervariable regions · Identification

Introduction

The official records of the fate of Prince Francesco Branciforte Barresi, a Sicilian arts patron and benefactor who lived between the sixteenth and seventeenth centuries, report that his mortal remains were buried in the chapel of San Benedetto church of Militello di Catania (Sicily) in 1622. In response to the desire of the local authorities to preserve the Prince's remains, in 1996 the paleoanthropological research team of the University of Pisa was engaged to assess the state of preservation of the corpse.

In the same chapel together with the Prince's coffin they found a secondary burial site containing bones all mixed together with the remains of four individuals, one adult and three juveniles. Historical records reported that the Prince was buried together with his brother, two of his three daughters and, probably, his grandson (Majorana 1990). Therefore, the four corpses were tentatively attributed to these individuals.

The remains of the Prince Branciforte family have been studied extensively by archaeological and osteological approaches. In our study we applied molecular sex identification and mitochondrial DNA (mtDNA) analyses on the DNA extracted from the ribs presumed to belong to each individual, in order to attempt a molecular identification of the five skeletons as applied in previous studies (Gill et al. 1994; Weichold et al. 1998). The first method was essential for determining the gender of the bone material from the juvenile individuals and for confirming whether the conclusions about the sex of the individual drawn from physical examination of the adult bones were correct.

The second method, mtDNA was applied to determine the maternal relatedness and, therefore, the degree of relationship among the five groups of skeletal remains. We followed this strategy because mtDNA is a more powerful tool in archaeo-anthropological investigations, as it can yield robust results even from difficult samples such as skeletal remains that may be too old or too decayed to allow chromosomal DNA analysis. Since mtDNA is usually present in high copy numbers in cells, it is more likely to survive for longer periods than single copy nuclear sequences. Moreover, the maternal mode of inheritance without recombination, and evolutionary rapid rate allow interpretation of phylogenetic trees that relate mtDNA types as genealogies reflecting maternal ancestry in recently differentiated populations (Brown 1985; Wilson et al. 1985; Cann et al. 1987; Wallace et al. 1987).

Two anthropologically informative regions of the human mitochondrial genome were analyzed: the fastest-

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changing regions, HV1 and HV2, within the control region (Vigilant et al. 1989) known to contain most of the sequence variability in the human mitochondrial genome (Vigilant et al. 1991; Lum et al. 1994; Rickards et al. 1999). This make them extremely useful for determining personal identification. Moreover, the small region V between the cytochrome oxidase II and the lysine transfer RNA genes, which has two tandemly repeated copies of a 9 bp (base pairs) sequence (CCCCCTCTA), was studied. A length mutation consisting of a deletion of one of the two repeat elements is often found in present day individuals of Asian origin and reaches fixation in some Polynesian islands, while it is present at low frequencies among other populations (for a review see Rickards 1995).

Materials and methods

Sample preparation and DNA extraction

Since different authors (Hagelberg et al. 1991; Herrmann and Hummel 1994) have observed a fairly good correlation between the external preservation of bone microstructure and the ability of a specimen to yield amplifiable DNA, microradiographs of femur cross-sections of each of the five skeletons were obtained and analyzed by SEM before proceeding with DNA extraction.

The first right rib of each individual of Prince Branciforte's family was removed by the project paleoanthropologist, who took precautions to minimize contamination and were then sent to Rome. A series of standard practices were followed to reduce laboratory contamination as much as possible. To prevent contamination from modern samples, a separate laboratory space routinely used for UV irradiation was set aside for conducting ancient DNA (aDNA) analyses. The areas for extraction and PCR were kept separate and all extractions were set up in a laminar flow cabinet with dedicated equipment. The instruments were treated with 1 M HCl, followed by extensive rinsing in UV-irradiated distilled H_2O. Equipment, disposables and reagents were UV-irradiated before use to decontaminate them properly.

Negative controls for both extractions and PCR were used (i.e., experiments in which no bone and no template were added, respectively) in all sample processing. The outer layer of the bones was scraped with a sterile razor blade to remove potential contamination from previous handling. Bone samples were soaked in a 10% solution of NaOCl, rinsed in double-distilled and UV-irradiated H_2O, and exposed to short-wave UV light for a few minutes. The method for DNA extraction described by Hagelberg (1994) was slightly modified: only one phenol/chloroform and two chloroform/isoamylic extractions were performed, since every step can cause further DNA damage mainly due to mechanical stress as well as DNA loss. The extractions were desalted and concentrated using Centricon 30 microconcentrators (Amicon, Danvers, Mass.) and the resulting products containing DNA were aliquoted and stored at –20°C for 3–4 days before PCR analysis.

To authenticate the results at least 2 independent extractions were performed for each individual, 4 amplifications were carried out for each extraction and 20 clones were screened for each amplification.

D-loop PCR amplification and direct sequence analysis

PCR amplifications of the two hypervariable segments of mtDNA control region (HV1 and HV2) were carried out in 50 µl volumes containing 2–5 ng of purified and concentrated DNA, 10 pM of each primer (L15996-H16401 and L29-H408; Vigilant et al. 1989), 40 µM dNTPs, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 1 mg/ml gelatin and 0.2–0.5 U Taq polymerase (Perkin Elmer, Foster City, Calif.). For PCR, 40 cycles were carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, Calif.) for 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. A denaturation step at 94 °C for 5 min at the beginning and an extension step at 72 °C for 10 min at the end of PCR were performed. The quality of the PCR amplifications was determined by electrophoresis on 2% agarose gels in TAE buffer.

Sequence data were obtained using either conventional methods or fluorescent dye labeling and an ABI PRISM 310 DNA sequencer (Perkin Elmer, Foster City, Calif.). For conventional sequencing asymmetric PCR (Gyllensten and Erlich 1988) was performed using a primer ratio of 1:50 and 30 cycles identical to the initial amplification. The products were purified with a Qiaquick PCR spin column (Qiagen, Hilden, Germany), or by the standard ethanol precipitation technique. Sequencing reactions were obtained using the dideoxy chain termination procedure of Sanger et al. (1977) with a Sequenase 2.0 reagent kit (US Biochemical, Cleveland, Ohio) according to the manufacturer's directions, using the limiting PCR primer and ^{35}SdATP. Electrophoresis was carried out in 6% polyacrylamide/7 M urea gels in 1xTBE buffer for 3 h at 40 mA, the gels were then dried and directly exposed to autoradiographic film. The analyzed sequences were completely verified through full overlap of light and heavy strands. For automated sequencing of double stranded purified PCR products, dideoxy terminator cycle sequencing was done using a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, Calif.) and following the recommended sequencing kit protocols.

Cloning and clone sequencing

Since neither primer dimers nor non-specific bands were visible by gel electrophoresis, amplification products were directly cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the supplier's protocol. In this method ligation of the plasmid vector, linearized with single 3′ deoxythymidine overhangs, with a PCR product containing 3′ deoxyadenosine overhangs is very efficient and occurs spontaneously within 5 min at room temperature.

Positive transformants were directly analyzed by PCR after selecting the colonies and resuspending them individually in 20 µml of the PCR reaction. After an incubation step at 94 °C for 10 min to lyse the cells and inactivate nucleases, 25 cycles of PCR were performed (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; final extension at 72 °C for 10 min), and the products visualized by agarose gel electrophoresis. From each positive PCR product 15 µml was directly sequenced using the DNA sequencing kit and analyzed by an ABI PRISM 310 DNA sequencer (Perkin Elmer, Foster City, Calif.).

Detection of the 9 bp deletion

The mtDNA segment encompassing the deletion region was amplified using the cycle parameters, conditions and primers described by Rickards (1995). The presence of the 9 bp tandem repeats was verified by direct sequencing of the double stranded PCR products. The Gene Clean (BIO 101, La Jolla, Calif.) kit was used to purify the PCR products and to concentrate the template. Sequencing reactions were performed as described above using an internal primer (Wrischnik et al. 1987).

Sex determination

Sex was determined by amplification of a segment of the X-Y homologous gene amelogenin using the primer system amelogenin A/B as described by Mannucci et al. (1993) and Gill et. al. (1994) except that 5–10 ng of total DNA was used. This method is usually applied for typing samples of a very degraded nature, since short X and Y-specific products of 106 and 112 bp, respectively are generated from a single primer pair.

It is worth stressing that sex determination and maternal relatedness were evaluated from blind experiments, since no informa-

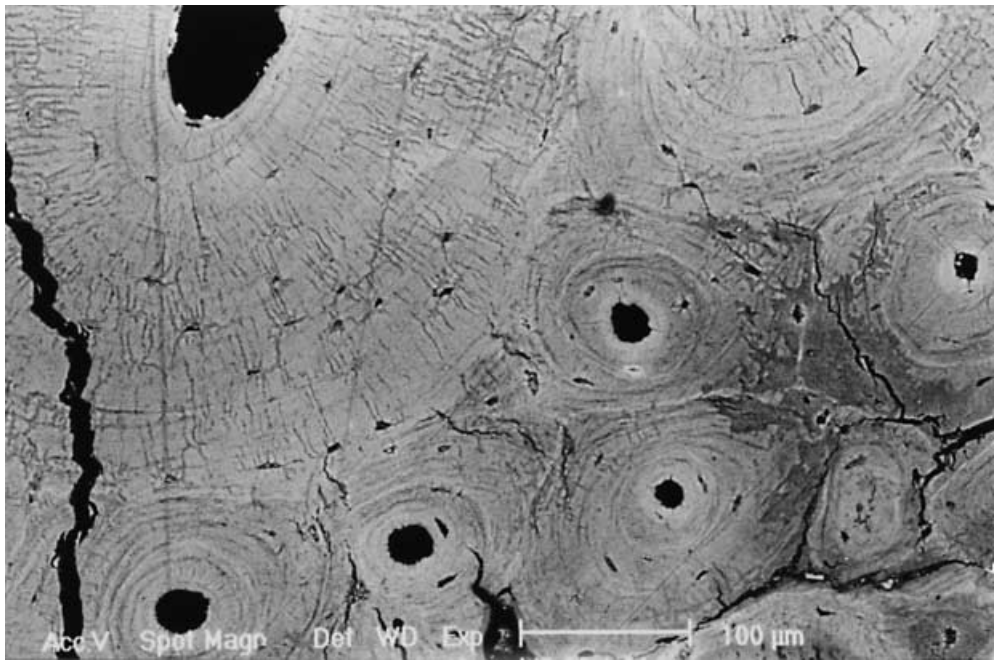

Fig. 1 SEM picture of a cross-section of a femur of Prince Branciforte. The bone microstructure is still perfectly preserved due to the wet and anaerobic conditions of the burial (Magnification bar in µm)

tion about either sex composition or degree of relationships of the Branciforte family was provided to the molecular anthropologists during the DNA processing.

Results and discussion

All the bones appeared to be in a good state of preservation on external macroscopic observation and microscopically, the microstructures were still perfectly preserved (Fig. 1), so that we could proceed with DNA extraction. Amplifiable DNA was successfully extracted from each bone sample. Although aDNA usually is not successfully amplifiable for long segments because of damage and degradation of the DNA (Pääbo 1989; Pääbo et al. 1989; Pääbo and Wilson 1991), the relatively recent age of the present archaeological material together with the good quality of preservation of the bones, allowed efficient amplification of mtDNA fragments up to 380 bp in length. As expected, experiments using primers that can detect single copies of the human amelogenin gene yielded less well amplified products than the multicopy mtDNA amplifications.

DNA sex identification was carried out using three independent extracts and PCR amplifications for each family member, and PCR products were obtained from all the experiments. Samples from one male and one female were analyzed in each independent experiment as controls. DNA sex identification indicated that the two adult individuals were males, in agreement with the morphological analysis, and that the three children, whose sex could not be unambiguously resolved by anthropometry and anthroposcopy, were females (Fig. 2).

The good quality of the extracted DNA made it unnecessary to amplify the whole HV1 and HV2 segments by nested PCR. Both DNA strands of hypervariable mtDNA control regions I and II were directly sequenced from the PCR products and a minimum of duplicate extractions and successful sequence determinations were performed on each specimen. To detect possible nucleotide misincorporations during the amplification reactions, which could affect most of the molecules of the amplified products, at least four independent PCR reactions were performed for each DNA extract. Each PCR product was then cloned and the sequence determined from multiple clones (at least 20 clones for each amplification). The quality of the sequences was comparable to that produced from fresh blood samples.

The 9 bp deletion marker turned out to be monomorphic, confirming its typical uninformativeness in European populations. In fact, at present the deletion seems to be rare in extinct and extant Italians and Europeans tested up to now (Rickards 1995; Rickards et al. 1998).

Table 1 gives the DNA sequences of the screened clones used to infer the HV1 and HV2 consensus se-

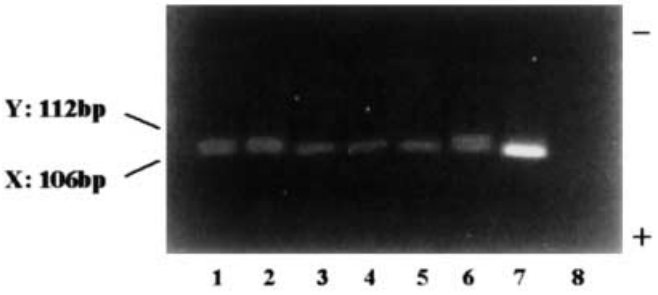

Fig. 2 Agarose gel electrophoresis of sex test PCR products using the primer system amelogenin A/B. *Lanes 1–5* questioned specimens, with *1* and *2* identified as male, *3*, *4* and *5* as female, *Lanes 6 and 7* positive control of male and female genomic DNA, respectively, *Lane 8* negative control

Table 1a Polymorphic sites of mtDNA D-loop sequences for HV1 from cloned PCR products. *Dots* indicate nucleotides identical to those in the reference sequence (*CRS*, Anderson et. al. 1981)

MtDNA types	HV1 Polymorphic sites										*N*[a]
Position	16067	16102	16113	16129	16278	16280	16343	16344	16355	16377	
CRS	C	T	A	G	C	A	A	C	C	C	
Prince, Male 1	.	.	.	.	.	.	.	.	.	.	
Type 1	.	.	.	.	.	.	.	.	.	.	152
Type 2	.	G	.	.	.	.	.	.	.	.	8
Male 2	.	.	.	.	.	.	.	.	.	.	
Type 1	.	.	.	.	.	.	.	.	.	.	136
Type 2	.	.	.	A	.	.	.	.	.	T	8
Type 3	.	.	.	A	.	.	.	.	.	.	16
Female 1	.	.	.	.	.	.	.	.	.	.	
Type 1	T	.	.	.	.	.	.	T	T	.	144
Type 2	T	.	.	.	.	.	G	T	T	.	16
Female 2	.	.	.	.	.	.	.	.	.	.	
Type 1	T	.	.	.	.	.	.	T	T	.	152
Type 2	T	.	G	.	.	G	.	T	T	.	8
Female 3	.	.	.	.	.	.	.	.	.	.	
Type 1	T	.	.	.	.	.	.	T	T	.	152
Type 2	T	.	.	.	T	.	.	T	T	.	8

[a] This column indicates the number of clones showing each mtDNA type. The figures were obtained from 2 independent DNA extracts, 4 amplifications from each extractions, and the screening of 20 clones from each amplification)

Table 1b Polymorphic sites of mtDNA D-loop sequences for HV2 from cloned PCR products

mtDNA types	HV2 polymorphic sites									*N*[a]
Position	0150	0189	0197	0214	0238	0252	0263	0337	0353	
CRS	C	A	A	A	A	T	A	A	C	
Prince, Male 1	.	.	.	.	.	.	.	.	.	
Type 1	.	.	.	.	.	.	G	.	.	152
Type 2	.	G	.	.	.	.	G	.	.	8
Male 2	.	.	.	.	.	.	.	.	.	.
Type 1	.	.	.	.	.	.	G	.	.	136
Type 2	.	.	.	.	.	C	G	.	.	8
Type 3	.	.	.	.	.	.	G	G	.	8
Type 4	.	.	C	.	.	.	G	.	.	8
Female 1	.	.	.	.	.	.	.	.	.	
Type 1	T	.	.	.	.	.	G	.	.	152
Type 2	T	.	.	G	.	.	G	.	.	8
Female 2	.	.	.	.	.	.	.	.	.	
Type 1	T	.	.	.	.	.	G	.	.	152
Type 2	T	.	.	.	C	.	G	.	T	8
Female 3	.	.	.	.	.	.	.	.	.	
Type 1	T	.	.	.	.	.	G	.	.	160

quences of the Branciforte family and two highly representative types, i.e., unique combinations of polymorphic sites, are evident. One type containing only the nucleotide substitution at bp 00263 when compared to the Cambridge reference sequence (CRS; Anderson et al. 1981) was identical in the two males. This mutation is very common both in Europe (about two-thirds of all the identified types) and in other areas of the world and was present in the three female skeletons as well. The other type containing five transition mutations was identical in the three females. Three sequences presented an extra C in a run of 7 Cs in the segment between bp 00303 and00309 and all

sequences showed the insertion of a C in a run of five Cs between the positions 00311 and 00315. Of the two insertion mutations, the first was detected in only some of the screened sequences, which seems to have been due to PCR artefacts, whereas the second could very likely have been due to the fact that the polynucleotide stretch between bp 00311 and 00315 is an insertion hot spot.

Neither of these two types matched with the DNA sequences of the paleoanthropologist and the molecular anthropologists involved in our study which excluded laboratory contamination. There were 12 clones which showed single base differences and 2 clones showed 2 base differences. These nucleotide substitutions were very likely due to damaged template DNA and to misincorporations during the amplification.

The polymorphic positions of HV1 and HV2 were confirmed by sequence analysis of the PCR products obtained with two overlapping primer sets each (data not shown). The phylogenetic analysis for verifying the reliability of the sequences, confirmed the clustering of the two identified mtDNA types into groups or haplogroups, each one referring to a monophyletic cluster of mtDNA types and defined by a set of associated polymorphisms, common in present populations of the Mediterranean area (Rickards and Martínez-Labarga, unpublished results; Comas et al. 1996). The first type belongs to haplogroup H, which is widely distributed among European and European-derived populations (Richards et al. 1996; Torroni et al. 1996). This type has been found at high frequencies not only in the general Italian population (32%) but also among Sicilians (29%). The other type is characterized as HV on the basis of the 16067–16344–16355 motif. The same haplotype has been recently identified in the Druze population, an ethnic minority living in Israel, Libya and Syria (Macaulay et al. 1999). It is worth mentioning, that strictly associated types were reported to have occurred in an individual of Sicilian maternal ancestry (Rickards and Martínez-Labarga, unpublished results), a Turk (Comas et al. 1996) and an Asian (Stoneking et al. 1991).

These experiments enabled us to confirm the sibling status of the two adult males on one hand and of two of the three children on the other, excluding any maternal relatedness between the two groups. Because of the strictly maternal mode of inheritance of mtDNA, these findings together with the results of sex identification seem to support the historical records, which indicate that the Prince was buried, following his will, together with his brother and two of his daughters. The third juvenile skeleton, tentatively attributed to the Prince's grandson but whose identity was not unambiguously established by historical records, showed an mtDNA profile identical to those of the Prince's two daughters. However, the molecular sex analysis did not prove the assumed identity of the third child, stressing the possibility that this skeleton does not belong to the son of Margherita Branciforte, married to Federico Colonna, but to another maternally related individual. Therefore the mtDNA haplotypes are consistent with the individuals expected to be in the grave, based on historical documentation.

Unfortunately, the living descendants of Prince Branciforte did not consent to give blood or hair samples in order to test whether they have the same mtDNA type and therefore it could not be conclusively demonstrated that the burials are of the Branciforte family, although the present findings seem to be very strong circumstantial evidence that the burials are as claimed.

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