AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY 129:620–629 (2006)

Molecular Characterization of a Pre-Columbian Mummy and In Situ Coprolite

Stefania Luciani,[1] Gino Fornaciari,[2] Olga Rickards,[3] Cristina Martínez Labarga,[3] and Franco Rollo[1]*

[1]*Laboratorio di Archeo-Antropologia Molecolare/DNA Antico, UNICAM, I-62032 Camerino, Italy*
[2]*Dipartimento di Oncologia, Sezione di Paleopatologia, Università di Pisa, I-56126 Pisa, Italy*
[3]*Centro di Antropologia Molecolare per lo Studio del DNA Antico, Dipartimento di Biologia, Università degli Studi di Roma Tor Vergata, I-00133 Rome, Italy*

KEY WORDS ancient DNA; mtDNA; bacterial DNA; mummy; palaeopathology

ABSTRACT The history of *Homo sapiens* dispersal around the world and inherent interpopulation contacts and conflicts has given rise to several transitions in his relationships with the natural world, with the final result of changes in the patterns of infectious disease (McMichael [2001] Ecosystem Health 7:107–115). Of particular interest, in this context, is the contact between Amerindians and Europeans that started at the end of the 15th century, and the resulting exchange of microbes. We successfully recovered ancient DNA from a pre-Columbian mummy from Cuzco (Peru), radiocarbondated to 980–1170 AD, for which consistent mtDNA amplifications and sequences were obtained. The analysis of mtDNA revealed that the mummy's haplogroup was characteristic of Native American populations. We also investigated a sample of feces directly isolated from the intestines of the mummy, using a polymerase chain reaction system designed to detect the broadest spectrum of bacterial DNAs. The analysis of results, following a criterion of "paleoecological consistency" (Rollo and Marota [1998] Philos. Trans. R. Soc. Lond. [Biol.] 354: 111–119), demonstrated that some vestiges of the original microbial flora of the feces were preserved. In particular, we were able to identify the DNA of *Haemophylus parainfluenzae*, thus suggesting that this recently recognized pathogen was present in precontact Native Americans. Am J Phys Anthropol 129:620–629, 2006.

It is well-known that when Europeans began to explore the Americas at the end of the 15th century, they brought with them pathogenic microorganisms unknown to the inhabitants of those lands. The Spanish conquest introduced smallpox, measles, influenza, and probably typhus to the New World, with devastating results: during the 16th century, these diseases killed around 90% of the inadvertently infected Native populations. Afterwards, cross-continental trade and travel favored the transoceanic spread of bacterial and viral infections to nonimmune populations of both coasts, predominantly via sailors and secondarily via animals and insects that acted as carriers of diseases (Aufderheide and Rodríguez-Martín, 1998; McMichael, 2001). DNA analysis offers a potentially powerful tool to unravel the history of infectious diseases. Actually, the pre-Columbian presence of tuberculosis in the New World remained controversial for many years, until the sequencing of a segment of DNA unique to the *M. tuberculosis* complex in an extract of a sample from a 1,000-year-old southern Peruvian mummy settled the issue affirmatively beyond reasonable doubt (Salo et al., 1994). The scientific literature of recent years reported on several cases of ancient pathogens identified in archaeological human remains by DNA analysis. This, however, almost invariably refers to infections that produce identifiable skeletal and soft-tissue alterations such as, in addition to the above-cited tuberculosis, leprosy (Spigelman and Donoghue, 2001) and syphilis (Kolman et al., 1999); this allows the researcher to perform a paleopathological screening of the anthropological material prior to DNA analysis. To fully appreciate the impact of human migration on the spread of infectious diseases, however, one would also need to identify those infections that leave no obvious trace, or poor traces only, on bone or desiccated soft tissue. In addition, it should be considered that many microorganisms that behave as harmless commensals with respect to their host under normal conditions can turn into pathogens in response to a variety of causes, e.g., trauma, alteration of the host physiology, or reduction of the immune response. Rodríguez Martín (1994), for example, reported an epidemic of influenza that killed more than 20% of the aboriginal Guanche population of Tenerife (Canary Islands) following the first contacts with the Spanish conquerors in 1494. In this case, the lethality of the viral infection was definitely augmented by serious complications such as pneumonia and encephalitis. In past years, we extensively characterized, using a variety of approaches such as anatomical dissection, histology (Fornaciari et al., 1992), and molecular analysis (Ubaldi et al., 1998), a naturally mummified human

Grant sponsor: Ministero dell'Istruzione, dell'Università e della Ricerca.

*Correspondence to: Dr. Franco Rollo, Laboratorio di Archeo-Antropologia Molecolare/DNA Antico, UNICAM, I-62032 Camerino, Italy. E-mail: francougo.rollo@unicam.it

Received 4 August 2004; accepted 5 April 2005.

DOI 10.1002/ajpa.20314
Published online 9 December 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Characteristic restriction-site polymorphisms for each haplogroup

Restriction site	Primer sequence	Length of PCR product	Length of restriction fragment	Annealing temperature (°C)
*Alu*I-5176	L5151: CTACTACTATCTCGCACCTG H5252: CAAAAAGCCGGTTAGC	102	77/25	60
*Dde*I-10394	L10359: GTGTGGCCTATGAGTGACTAC H10466: ATTTATGTAAATGAGGGGCATTTGG	131	96/35	52
*Alu*I-10397	L10359: GTGTGGCCTATGAGTGACTAC H10466: ATTTATGTAAATGAGGGGCATTTGG	131	94/37	52
*Mnl*I 10871	L10810: TCCAAAAAACACATAATTTG H10900: GGGGAACAGCTAAATAGGTT	110	71/39	60

(female) kept, since the end of the 19th century, in the National Museum of Anthropology of Florence (catalogue no. 3076). Nothing is known about the archaeological context in which the mummy was found, the sole information available being that it came from Cuzco (Peru). In 2001, radiocarbon analysis of the remains by accelerator mass spectrometry (AMS) showed that the body is older than previously inferred on an ethnological basis, most likely (95.4% probability) dating between 980–1170 AD.

In the present study, we characterized the mitochondrial DNA (mtDNA) of the mummy in order to ascertain whether its haplotype belongs to one of the four primary founder Native American maternal lineages. In fact, the yields of non-Amerindian-specific mtDNA sequences would have been indicative of contamination. In addition, as the dissection of the mummy's large bowel had yielded a relevant amount of desiccated feces (a coprolite), we isolated DNA from samples of the coprolite and performed a search for bacterial DNA, utilizing polymerase chain reaction (PCR) primer pairs designed to bind to conserved portions of the 16S ribosomal RNA gene (16S rDNA). This approach, though more laborious than that of a search for a specific microorganism, allows one to detect a broad range of pathogenic (and apathogenic) bacteria in the absence of any skeletal or soft-tissue indication.

MATERIALS AND METHODS

Extraction of mummy DNA from bone

Fragments isolated from a cuneiform bone were powdered in a freezer/mill and divided into three aliquots. Between each extraction, the grinder was washed with 10% bleach. DNA extraction and concentration were performed using the following protocol (Rickards et al., 2001). About 500 μg of powder were washed overnight, with shaking, in 0.5 M EDTA, pH 8.0. After centrifugation, the supernatant was removed, and the remaining sample was incubated overnight at 37°C with EDTA 0.5 M (pH 8.0), 0.5% SDS, 50 mM Tris HCl (pH 8.0), and 200 μg proteinase K. After incubation, the digest was extracted twice, first with phenol-chloroform/isoamylic and then with chloroform/isoamylic, and the aqueous phase was concentrated by dialysis centrifugation using Centricon-30 microconcentrators (Amicon) to a 150-μl volume. The DNA was further purified with the silica binding method (GeneClean). One hundred and fifty microliters of DNA were mixed with 500 μl of 6 M sodium iodide solution and 5 μl of silica matrix, and left for 5 min on ice. After a spin, the supernatant was removed, and the silica pellet was washed three times with NaCl/Tris/EDTA/ethanol. The nucleic acid was eluted in 15 μl of sterile water and then stored at −20°C for at least 3–4 days before further DNA concentration and purification by silica matrix.

Negative controls for both extractions and PCRs were used (i.e., experiments in which no bone or template were added, respectively) in all sample processing.

MtDNA analyses

The first and second hypervariable segments of the mtDNA control region were sequenced between nucleotide positions (nps) 16,000–16,400, and 00030–00408, respectively, of the revised Cambridge Reference Sequence (CRS; Andrews et al., 1999), in the pre-Columbian mummy. Restriction fragment length polymorphism (RFLP) analysis of diagnostic mtDNA positions (Table 1) was carried out, and the mtDNA haplogroup was assigned to the sample following published criteria (http://www.stats.ox.ac.uk/~macaulay/founder2000/motif.html).

Also, the small region V between the cytochrome oxidase II and lysine transfer RNA genes (COII/$tRNA^{Lys}$), which has two tandemly repeated copies of a 9-base pair (bp) sequence (CCCCCTCTA), was studied (Wrischnik et al., 1987). 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 rather low frequencies among other populations (reviewed in Rickards, 1995). Within America, the deletion is characteristic of haplogroup B, which together with haplogroups A, C, and D, represent the four primary mitochondrial haplotype groups present in contemporary Amerinds.

D-loop PCR amplification and direct sequence analysis

The mtDNA hypervariable region 1 (HVS-I) and 2 (HVS-II) sequences of the mummy were reconstructed from four and two overlapping amplification products, respectively, applying the following strategy. A first, PCR was carried out using 1 μl of template, 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA), 10 × reaction buffer (100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl, and 1 mg/ml gelatin), 1 mM dNTPs, and 0.1 μM of each primer reported in Table 2. After an initial 5-min denaturation step, 25 cycles of amplification were performed, each cycle consisting of 30-sec steps, with denaturation at 94°C, annealing at 60°C, extension at 72°C, and a final extension step of 10 min at 72°C. Negative controls consisting of mock extractions (powdered tissue omitted) and PCR blanks without ancient DNA were undertaken along with the ancient samples to monitor

*TABLE 2. Primers used to amplify HVS-*I *and HVS-*II *of mtDNA control region*[1]

HVS-I	
L15997	5′-CACCATTAGCACCCAAAGCT-3′
H16142	5′-ATGTACTACAGGTGGTCAAG-3′
L16131	5′-CACCATGAATATTGTACGGT-3′
H16218	5′-TGTGTGATAGTTGAGGGTTG-3′
L16159	5′-TACTTGACCACCTGTAGTAC-3′
H16271	5′-GTGGGTAGGTTTGTTGGTATCCTA-3′
L16209	5′-CCCCATGCTTACAAGCAAGT-3′
H16401	5′-TGATTTCACGGAGGATGGTG-3′
HVS-II	
L29	5′-GGTCTATCACCCTATTAACCAC-3′
H285	5′-GGTTTGGTGGAAATTTTTTGT-3′
L172	5′-ATTATTTATCGCACCTACGT-3′
H408	5′-CTGTTAAAAGTGGAAATTTTTTGT-3′

[1] Base positions refer to CRS (Anderson et al., 1981; Andrews et al., 1999).

against contamination. To obtain sufficient quantities of DNA for sequence analysis, a second step of PCR was set up, using 1 μl of the amplification product in 50-μl reamplification reactions. Overlapping fragments were obtained which covered the entire HVS-I and HVS-II regions. Five microliters of the PCR products were electrophoresed in 1.5% agarose gels in TAE (0.04 M Tris/acetate, 0.001 M EDTA) buffer and visualized with ethidium bromide staining. Positive amplifications were then purified with ethanol precipitation. Three independent amplifications were carried out for each DNA extraction to confirm the mutations. Automated sequencing of all purified PCR products was performed by dideoxy terminator cycle sequencing following the recommended sequencing kit protocols, and sequence data were obtained using an ABI Prism 310 DNA sequencer (Applied Biosystems). The analyzed sequences were completely verified through full overlap of light and heavy strands.

Cloning of PCR products and clone sequencing

To estimate the rate of misincorporations due to template damage or *Taq* errors, different PCR amplifications using six pairs of primers (L15997/H16142, L16131/H16218, L16159/H16271, L16209/H16401, L29/H285, and L172/H408) were cloned and sequenced using a TOPO TA cloning kit (Invitrogen), following the supplier's instructions. Two microliters of the PCR product were ligated into a plasmid vector linearized with single 3′ deoxythymidine overhangs. The ligation occurred spontaneously within 5 min at room temperature. Two microliters of the ligation product were transformed into 100 μl of competent cells and grown in 250 μl of SOC medium before plating on Amp/X-gal agar plates. Recombinant white colonies were added to a 20 μl PCR mix that was incubated for 10 min at 94°C to lyse the cells and inactivate the nucleases. DNA was amplified for 25 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min). Inserts that yielded the expected size in an electrophoresed gel were purified, and 15 μl of each purified product were directly sequenced using a commercial DNA sequencing kit (Applied Biosystems), and analyzed by an ABI Prism 310 DNA sequencer (Applied Biosystems). For each amplification, 20 clones were screened.

Typing of coding region diagnostic mutations

Purified DNA was used to amplify the polymorphic sites defining Native American mtDNA lineages (Torroni et al., 1994, 1996; Richards et al., 2000). PCR reactions were performed on a Perkin-Elmer thermal cycler, with the different profiles and primers reported in Table 1. Negative controls (extraction blanks and PCR blanks) were performed in parallel to monitor contamination. To screen the restriction site markers defining the haplogroups, 20 μl of the respective unpurified PCR products were digested overnight at 37°C with 5 units of the appropriate endonucleases, electrophoresed on 12% polyacrylamide gel in 1 × TBE buffer (0.045 M Tris-borate, 0.001 EDTA), and stained with ethidium bromide. The specific mtDNA sites in the coding region were also screened by amplification, cloning, and sequencing of the surrounding region.

Detection of the COII/tRNA^{Lys} 9-bp deletion

The mtDNA segment encompassing the deletion region was analyzed using cycling parameters, primers, and conditions described by Rickards (1995). The presence of the 9-bp tandem repeats was verified by sequencing of the PCR products. A Gene Clean (Bio 101) kit was used to purify the PCR products and concentrate the template. Sequencing reactions were performed as described above, using an internal primer (Wrischnik et al., 1987).

Contamination control

Three independent DNA extractions from bone fragments of the mummy were performed in the Center of Molecular Anthropology for aDNA Studies of the Department of Biology, University of Rome Tor Vergata, following a series of standard practices to reduce laboratory contamination as much as possible, and with stringent criteria for authentication. To prevent introduction of exogenous modern DNA during ancient samples processing, laboratory rooms routinely ultraviolet (UV)-irradiated, and exclusively dedicated to aDNA analysis, were used. In particular, nucleic acid extractions and PCRs were performed in two physically isolated work areas, using dedicated reagents and equipment for each. PCRs were set up in a laminar flow cabinet with dedicated equipment. The instruments were treated with 0.1 M HCl, followed by extensive rinsing in UV-irradiated distilled H_2O. Aerosol-resistant barrier pipette tips, sterile gloves, face masks, and laboratory coats were employed. All equipment, disposables, and reagents were UV-irradiated before use to decontaminate them properly, and frequent bleaching of working surfaces was adopted.

All analysts who handled ancient samples were typed in order to exclude contamination. To check for concordance of results, duplicate testing of the ancient samples was also performed in the laboratory at UNICAM (Camerino, Italy).

Database search

The mtDNA consensus sequence of the mummy was phylogenetically classified within a scheme based on RFLP patterns of variation and associated D-loop diagnostic sequence motifs.

For comparisons with extinct and extant populations, matching data from the literature were collected from various Native American populations (the complete database is available at http://www.uniroma2.it/biologia/centri/rickards/antrodna.htm).

Analysis of bacterial DNA from the mummy's coprolite

For DNA extraction, a fragment of approximately 330 mg was aseptically removed from the coprolite and left overnight in an extraction medium composed of SDS, Na_2EDTA, Tris-HCl (pH 8.0), and phenol. It was subsequently milled in a mortar with a pestle, following further addition of the extraction medium. The homogenate was then extracted stepwise, using phenol, phenol/chloroform/isoamylic alcohol, chloroform/isoamylic alcohol, and ether, and the nucleic acid fraction was precipitated using ethanol at −20°C. The DNA was further purified using a Mermaid kit (Bio 101, Vista, CA). The final preparation was electrophoresed through a 2% (w/v) agarose gel to check the size of the DNA fragments, and the result was recorded by a digital camera equipped with a filter. PCR was performed in 50 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM each dNTP, 1 μM of each primer, 2 units of DyNAzyme II DNA Polymerase (Finnzymes OY, Espoo, Finland), and 1 μl DNA. "Universal" 29f and 98r primers for 16S rDNA, as described by Ubaldi et al. (1998), were employed. The thermal profile was set for 5 min at 94°C, followed by 40 cycles at 94°C (1 min), 60°C (30 sec), and 72°C (30 sec), and concluded by a step at 72°C for 10 min. To eliminate any types of contaminant DNA, the *Taq* polymerase and the reaction components were pretreated with 1 unit of DNase I (from bovine pancreas) for 30 min at room temperature. After incubation, the DNase I was inactivated by boiling for 10 min at 94°C. PCR products were visualized by UV light, following electrophoresis on a 4% (w/v) agarose gel (AquaPor HR GTAC, National Diagnostics, Atlanta, GA). For sequence analysis, PCR products were purified and then cloned using a pGEM-Teasy vector system (Promega Corp., Madison, WI). Recombinant plasmids were isolated using a Miniprep kit (Applied Biosystems), and insert size and DNA concentration were assessed by gel electrophoresis. DNA sequences were obtained using an ABI-Prism 310 automated DNA sequencer and the Big-Dye Terminator Cycle Sequencing Version 2.0 Ready Reaction kit (Applied Biosystems). Cycle sequencing products were purified by Centri-sep spin columns (Princeton Separations, Adelphia, NJ). Results were compared with the reference sequences in the GenBank database, using the National Center for Biotechnology Information (NCBI) BLAST search. To prevent cross-contamination, we routinely employ stringent precautions such as wearing of sterile gloves and clothing, pretreatment of mortars and pestles with HCl, and the use of UV-irradiated safety cabinets, dedicated micropipettes, gel trays, and tanks. Moreover, all DNA extractions and initial PCR amplification steps are performed in a DNA-free chamber, physically separated from the site of subsequent analysis, cloning, and sequencing. Traditional contamination controls are represented by mock extraction and blank amplification reactions. We routinely perform both these types of control. In our laboratory, however, monitoring of contaminant DNA does not stop at this point, as we clone and sequence any type of spurious band. In this way, we are able to detect low copy number contaminant DNA species which might escape detection by other means. In the case of the pre-Columbian mummy, this was unnecessary, as no spurious band was produced.

RESULTS

mtDNA analysis

As a first step, the ancient sample was characterized at the mtDNA level, using the strategy of control region sequencing. Since even in the best preservation conditions ancient DNA molecules tend to be damaged, amplification of long fragments is very difficult. Therefore, we decided to reconstruct the whole HVS-I and HVS-II mtDNA regions by amplification of four and two short overlapping fragments, respectively. Both DNA strands of each region were directly sequenced from the PCR products obtained from three independent amplifications. Duplicate extractions and successful sequence determinations were performed. Then, to detect possible nucleotide misincorporations during the amplification reactions, which could affect most of the molecules of the amplified products, overlapping fragments for both regions were amplified, each PCR product was cloned, and several clones were sequenced.

The ancient HVS-I and HVS-II consensus sequences, inferred from screening the clones, showed mutations with respect to the CRS identical to the substitutions found in the direct sequencing and that are known to be polymorphic among Native American populations (Fig. 1). On the basis of the transitions C to T at nt 16223, T to C at nt 16362, and A to G at nt 00073, the type was tentatively attributed to haplogroup D. The consensus sequence did not correspond to any of the DNA sequences of the molecular anthropologists involved in the study. This finding, together with the fact that blanks showed no signs of contamination during the study, also excluded laboratory contamination, hence supporting the authenticity of sequences retrieved.

To confirm the attribution of the sequence to haplogroup D, specific mtDNA sites were also analyzed. The mummy showed the motifs (−) 5176 *Alu*I (5178T), (+) 10394 *Dde*I (10398G), (+) 10397 *Alu*I (10400T), and (−) 10871 *Mnl*I (10873C). These mutations, together with the lack of the 9-bp deletion in the COII-$tRNA^{Lys}$ intergenic region, diagnostic of the B haplogroup, and with the control region sequencing profile, allowed us to establish that the sequence belonged to the D haplogroup.

Bacterial DNA analysis of the coprolite

Once analyzed by gel electrophoresis, the DNA fraction of the coprolite was shown to be extensively damaged, with a maximum fragment length $\leq$100 bp. A similar situation was previously found for 14 bioptic samples from the internal organs of the mummy (Ubaldi et al., 1998). Correspondingly, PCR systems designed to bind to 16s rDNA portions longer than 100 bp (e.g., 196 bp) failed to produce any amplification signals. On the other hand, the 29f/98r primer pair repeatedly gave yields on the gel to a sharp amplification band of approximately 100 bp in length, while no signal was produced by mock extractions or amplification blanks. Following plasmid cloning of the amplification product, we sequenced a total of 65 clones. A preliminary BLAST search in the GenBank database and a sequence analysis by multiple alignment showed that the clones clustered into 12 groups (A1, A2, B, CC090, H, CC086, P, R, CC005, CC124, U, and X). They are shown in Figure 2, with the corresponding consensus sequences. Further analysis allowed us to putatively identify the microorganisms cor-

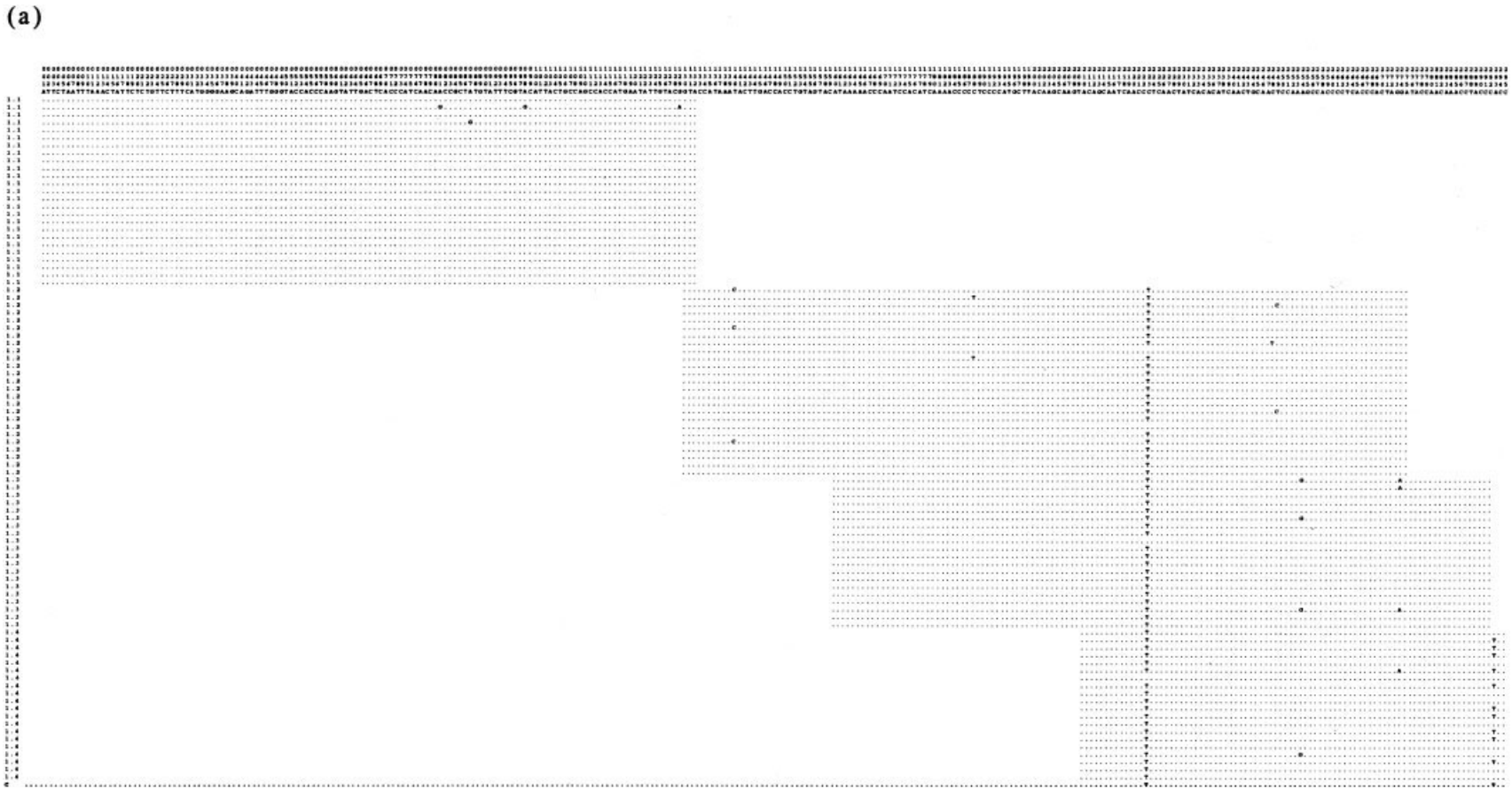

Fig. 1. (See legend page 625.)

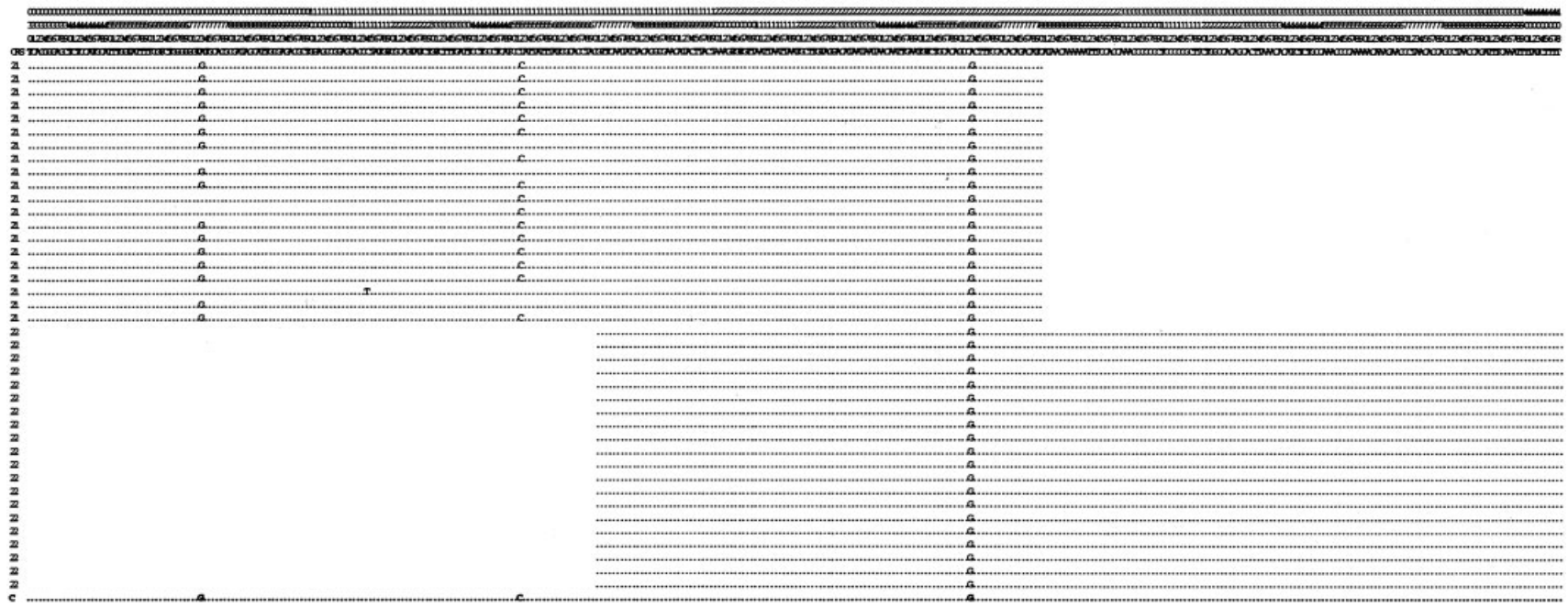

Fig. 1. Sequences from clones generated from different amplifications of mtDNA HVS-I (**a**) and HVS-II (**b**) (+16,000; Anderson et al., 1981; Andrews et al., 1999). Nucleotides identical to CRS (upper line) are represented by dots.

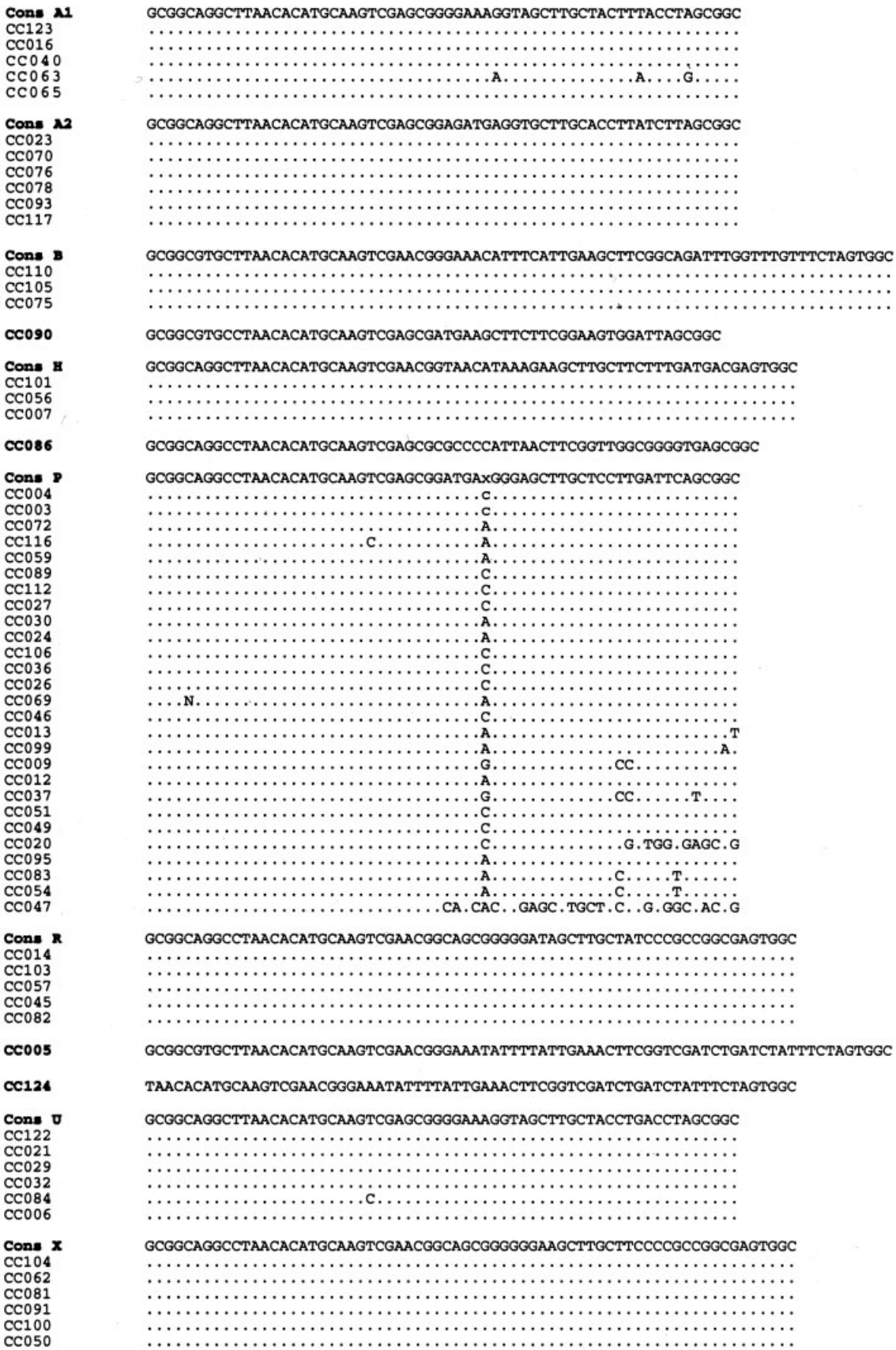

Fig. 2. Alignment by cluster of 65 16S rDNA sequences obtained by analysis of pre-Columbian coprolite. Corresponding consensus sequence is shown above each alignment.

TABLE 3. Identification for 10 consensus sequences from coprolite[1]

Consensus	No. of clones	Taxonomic identification[2]					Uncultured[3]	Base similarity
		Class	Order	Family	Genus	Species		
A1	5	Gamma subdivision		Moraxellaceae	*Acinetobacter*	*lwoffii*		61/62
A2	6	Gamma subdivision		Moraxellaceae	*Acinetobacter*			62/62
B	3						Feces	76/78
CC090	1	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*			60/60
H	3	Gamma subdivision		Pasteurellaceae	*Haemophilus*	*parainfluenzae*		68/68
P	27	Gamma subdivision		Pseudomonadaceae	*Pseudomonas*			61/62
CC005	1						Feces	77/78
CC124	1						Feces	67/68
U	6						Environment	62/62
X	6	Gamma subdivision		Enterobacteriaceae				63/64

[1] Only consensus sequences showing not more than two mismatches with GenBank reference sequence were considered.
[2] Query sequences match with those of cultivated bacteria: columns show lowest taxonomic level univocally determined through BLAST search. Note that identification level varies from sequence to sequence e.g., consensus X matches equally well with more than one genus, and thus univocal identification is possible only at family level. Consensuses A2, CC090, and P, on the other hand, are identified at genus level. Finally, consensuses A1 and H can be identified at species level.
[3] Query sequences match with those of bacteria known by DNA analysis only: in this case, column indicates type of sample from which reference sequence was obtained, as reported in database.

responding to 10 (A1, A2, B, CC090, H, P, CC005, CC124, U, and X) out of 12 consensus sequences (that were found to correspond to reference sequences with no more than two mismatches), as shown in Table 3.

DISCUSSION

mtDNA

The ancient Peruvian sequence was not observed to date in any extinct or extant population of our broad database of 1,500 Native American mitochondrial sequences.

The finding that the mummy's haplogroup is D perfectly agrees with the overall geographic distribution of the founding haplogroups in the extant Native populations of the Americas, which indicates the existence of opposing clines in the relative frequencies of mtDNA lineages. Indeed, the haplogroup to which the ancient sequence appears to belong is frequent among modern South American populations. In fact, haplogroup A displays a marked decrease in frequency with southerly latitude, whereas haplogroup B appears to be essentially confined to the central area of the continent. In contrast, haplogroups C and D exhibit a marked southward trend toward higher frequencies (Rickards et al., 1999). This pattern of variation was explained as the result of independent migrations from Asia accompanied by a reduction in genetic diversity of the Asiatic ancestors due to genetic drift, which occurred in each region and caused an increase in genetic diversification between populations.

Bacterial DNA

A fundamental issue in all studies devoted to the identification of DNA of ancient bacteria in human remains is how to discriminate between ancient and modern DNA. This is particularly important due to the ubiquitous nature of microbes.

Through the years, two criteria have been proposed:

1. Use of PCR systems designed on the basis of DNA sequences which are specific for the pathogenic forms of a certain bacterium, and absent in free-living isolates. Examples are given by the identification of *M. tuberculosis* (Dixon and Roberts, 2001) and *Yersinia pestis* (Drancourt et al., 1998) using, respectively, the insertion sequence IS6110, and the RNA polymerase beta-subunit-encoding gene (rpoB) and the virulence-associated plasminogen activator encoding gene (pla).
2. Application of the so-called criterion of "paleoecological consistency" (Rollo and Marota, 1998). According to this criterion, remains are first tested for their diagenetic state by analyzing the preservation of the original human mitochondrial DNA and the level of aspartic acid racemization. If the response is favorable, DNA preparations obtained from different bioptic samples are amplified using "universal" PCR systems addressed to the 16S rDNA sequence. Following cloning and sequencing of the amplified DNA, the ancient bacteria are putatively identified by database search. The results are finally evaluated for the (at least partial) consistency of the (putative) bacterial type and distribution with that of living persons. This criterion was applied to the study of the intestinal microflora of the Tyrolean Iceman (Cano et al., 2000) and to the present and past (Ubaldi et al., 1998) investigations on the mummy of the Museo Nazionale di Antropologia ed Etnologia of Florence.

We can remark that by no means are the two criteria conflicting. The first is to be preferred when the search concentrates on a single pathogen, normally on the basis of indications given by previous osteological or histological investigations. The second, on the other hand, overcomes the limitations posed by the first when no previous indication is available. Practically, the second criterion can help in answering a question as generic as, "Which microbes, or symbionts, opportunists or pathogens, were associated with a certain historical individual during his last days of life?"

The present investigation, and previous ones performed in our and other laboratories, show that indeed the Andean mummy (catalogue no. 3076) of the Museo Nazionale di Antropologia ed Etnologia of Florence meets the above-described criterion of paleoecological consistency. This conclusion stems from the following evidence:

1. The aspartic acid racemization level (D/L Asp) determined for the mummy's soft tissues is comparable to that of modern specimens (Ubaldi et al., 1998);
2. Remains of the original mitochondrial DNA are found; and
3. The concentration of bacterial DNA fragments along the intestinal tract of the mummy follows a "physiological" pattern, i.e., it is lower in the small intestine and substantially higher in the colon (Ubaldi et al., 1998).

Interestingly, of the 10 consensus sequences found to correspond to reference sequences in the database with no more than two mismatches (Table 3), six (A1, A2, CC090, H, P, and X) can be putatively identified at varying taxonomic levels (from species to family) with cultivated bacteria, while four (B, CC005, CC124, and U) correspond to uncultured microbes. According to the database, three sequences of the latter group (B, CC005, and CC124) were previously obtained exclusively following PCR amplification of the DNA isolated from modern fecal specimens. On the other hand, species belonging to some of the cultured taxa (*Enterobacteriacee*, *Pseudomonas*, and *Clostridium*) are commonly found in human feces, though they can also be found in other environments (Wilson and Blitchington, 1996; Suau et al., 1999). Among the putatively identified cultured bacteria, *H. parainfluenzae* is described in the microbiological literature as a human oropharyngeal commensal bacterium which is also found in approximately 25% of normal human feces (Nicoletti and Nicolosi, 1998). As suggested by the name, meaning "blood lover" in Greek, the whole genus *Haemophilus* consists of species that live in association with warm-blooded animals. Man, in particular, is the natural host of *H. influenzae*, *H. aegyptius*, *H. haemolyticus*, *H. ducreyi*, *H. parainfluenzae*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* (Krieg and Holt, 1984). In the past, the unfavorable growth characteristics of this organism and the poor specificity of traditional methods for species identification were responsible for inaccuracies in the diagnosis of infection caused by *H. parainfluenzae* and related organisms, leading to a substantial underestimation of their pathogenic role. More recently, also thanks to the application of molecular techniques, *H. parainfluenzae* was recognized as a cause of serious invasive diseases such as endocarditis (Hamed et al., 1994) and community-acquired pneumonia (Pillai et al., 2000). Finally, three sequences (A1, A2, and U) are putatively identified with bacteria (either cultured, such as *Acinetobacter*, or uncultured) that are not known members of the fecal flora. In conclusion, the analysis of the coprolite contributes toward confirming that the residual bacterial DNA meets, at least in part, the criterion of palaeoecological consistency. It should be pointed out that we never expect this criterion to be entirely fulfilled, for the obvious reason that we are dealing with human remains with a rather long taphonomic history. It is therefore conceivable that, through the centuries, some microbes from the outer environment entered the corpses, with the result of partially masking/erasing the traces left by the oldest ones. This is probably the case with the relatively large number of *Pseudomonas* (P) and *Acinetobacter* (A1, A2) sequences found.

CONCLUSIONS

The present study makes it possible to insert the ancient human mtDNA sequence within the phylogenetic scheme described for South America, excluding contamination with contemporary DNA, since all the experiments were performed by European researchers.

Fundamental to all speculations on the effects of European colonization on the New World is the detailed knowledge of the microbiological situation of indigenous populations prior to the era of geographic explorations. It has been claimed that the exchange of microbes between Amerindians and Europeans was virtually one-sided, as few serious infectious diseases tormented the former. This is currently explained by the fact that Amerindian populations were the descendants of populations from the Siberian region that had migrated through a partially sterilizing frozen environment (Torroni et al., 1993). In addition, the ensuing Central and South American civilizations had no large dense herds of wild animals to domesticate, in contrast to those of Europe and Africa. Hence, there were no ready herd-infection sources of potential human pathogens (McMichael, 2001). We show here that 400–500 years before the first contact of Inca populations with the Spaniards of Francisco Pizarro's expedition (1524), *H. parainfluenzae* was already associated with natives of the Andean region. On the basis of the present results, we can assume that this bacterium was most likely brought to the Americas by the migratory fluxes that took place through Beringia, starting approximately 30,000 years BP (Torroni et al., 1993). In a global paleo-epidemiological perspective, it seems to us of interest to remark that detailed molecular analyses performed, in our laboratory, on a fecal sample from the so-called Tyrolean Iceman (3350–3100 BC) using, also in this case, "universal" 16S rDNA PCR systems, failed to reveal any *H. parainfluenzae* sequences, though obviously this result cannot be taken as conclusive evidence that the bacterium was not present in Copper-Age Europe (Rollo et al., unpublished results).

Studies on the identification of *H. parainfluenzae* in ancient human remains would certainly benefit from a better knowledge of the evolutionary history of this microorganism and the possibility of performing strain differentiation. Unfortunately, only very recently has the phylogeny of the genus *Haemophylus* been the object of attention by specialists (Hedegaard et al., 2001; Nørskov-Lauritsen et al., 2005).These works, however, are mainly aimed at verifying the correspondence between traditional and molecular methods of bacterial classification, rather than reconstructing an evolutionary history of the pathogens. In addition, the techniques used in the recent past to differentiate among biotypes of *H. parainfluenzae* are either based on amino-acid metabolism (Taylor et al., 1992) or DNA fingerprinting based on the analysis of restriction-fragment patterns in the range of 1–3 kb (Kerr et al., 1993), and thus are totally unsuitable for the analysis of archaeological specimens. Other methods such as PCR-ribotyping (Privitera et al., 1998), based on identification of length polymorphisms in the

intergenic spacer regions of *Haemophilus* spp rDNA, were only able to distinguish between *H. influenzae* and *H. parainfluenzae*, but not to discriminate within species.

ACKNOWLEDGMENTS

Work in the authors' laboratories was supported by the Ministero dell'Istruzione, dell'Università e della Ricerca research project "Malattie e Regime di Vita Nell'Italia Centro-Meridionale dei Secoli XIII–XIX, Fonti Biologiche e Storico-Letterarie."

LITERATURE CITED

Anderson S, Bankier AT, Barrel BG, Debruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlichm DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457–465.

Andrews RM, Kubaka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. 1999. Reanalysis and revision of the Cambridge Reference Sequence for human mitochondrial DNA. Nat Genet 23:147.

Aufderheide AC, Rodr–guez-Martín C. 1998. The Cambridge encyclopedia of human paleopathology. Cambridge: Cambridge University Press.

Cano RJ, Tiefenbrunner F, Ubaldi M, Del Cueto C, Luciani F, Cox T, Orkand P, Kunzel KH, Rollo F. 2000. Sequence analysis of bacterial DNA in the colon and stomach of the Tyrolean Iceman. Am J Phys Anthropol 112:297–309.

Dixon RA, Roberts CA. 2001. Modern and ancient scourges: the application of ancient DNA to the analysis of tuberculosis and leprosy from archaeologically derived human remains. Ancient Biomol 3:181–193.

Drancourt M, Aboudharam G, Signoli M, Dutour O, Raoult D. 1998. Detection of 400-year-old *Yersinia pestis* DNA in human dental pulp: an approach to the diagnosis of ancient septicemia. Proc Natl Acad Sci USA 95:12637–12640.

Fornaciari G, Castagna M, Viacava P, Tognetti A, Bevilacqua G, Segura EL. 1992. Chagas disease in Peruvian Inca mummy. Lancet 339:128–129.

Hamed KA, Dormitzer PR, Su CK, Relman DA. 1994. *Haemophilus parainfluenzae* endocarditis: application of a molecular approach for identification of pathogenic bacterial species. Clin Infect Dis 19:677–683.

Hedegaard J, Okkels H, Bruun B, Kilian M, Mortensen KK, Nørskov-Lauritsen N. 2001. Phylogeny of the genus *Haemophilus* as determined by comparison of partial *infB* sequences. Microbiology 147:2599–2609.

Kerr GR, Forbes KJ, Williams A, Pennington TH. 1993. An analysis of the diversity of *Haemophilus parainfluenzae* in the adult human respiratory tract by genomic DNA fingerprinting. Epidemiol Infect 111:89–98.

Kolman CJ, Centurion-Lara A, Lukehart SA, Owsley DW, Tuross N. 1999. Identification of *Treponema pallidum* subspecies *pallidum* in a 200-year-old skeletal specimen. J Infect Dis 180:2060–2063.

Krieg NR, Holt JG. 1984. Bergey's manual of systematic bacteriology. Volume1. Baltimore: Williams and Wilkins.

McMichael AJ. 2001. Human culture, ecological change and infectious disease. Ecosystem Health 7:107–115.

Nicoletti G, Nicolosi VM. 1998. Dizionario di batteriologia umana normale e patologica. Salerno: Momento Medico.

Nørskov-Lauritsen N, Bruun B, Kilian M. 2005. Multilocus sequenze phylogenetic study of the genus *Haemophilus* with description of *Haemophilus pittmaniae* sp. nov. Int J Syst Evol Microbiol 55:449–456.

Pillai A, Mitchell JL, Stockley RA. 2000. A case of *Haemophilus parainfluenzae* pneumonia. Thorax 55:623–624.

Privitera A, Licciardello L, Gianninò V, Agodi A, Rappezzo G, Nicoletti G, Stefani S. 1998. Molecular epidemiology and phylogenetic analysis of *Haemophilus parainfluenzae* from chronic obstructive pulmonary disease exacerbations Eur J Epidemiol 14:405–412.

Richards M, Macaulay V, Hickey E, Vega E, Sykes B, Guida V, Rengo C, Sellitto D, Cruciani F, Kivisild T, Villems R, Thomas M, Rychkov S, Rychkov O, Rychkov Y, Cölge M, Dimitrov D, Hill E, Bradley D, Romano V, Cali F, Vona G, Demaine A, Papiha S, Triantaphyllidis C, Stefanescu G, Hatina J, Belledi M, Di Rienzo A, Novelletto A, Oppenheim A, Nørby S, Al-Zaheri N, Santachiara-Benerecetti S, Scozzari R, Torroni A, Bandelt HJ. 2000. Tracing European founder lineages in the Near Eastern mtDNA pool. Am J Hum Genet 67:1251–1276.

Rickards O. 1995. Analysis of the region V mitochondrial marker in two African-American communities of Ecuador and in their parental populations. Hum Evol 10:5–16.

Rickards O, Martínez-Labarga C, Lum JK, De Stefano GF, Cann RL. 1999. Mitochondrial DNA history of the Cayapa Amerinds of Ecuador: detection of additional founding lineages for Native American populations. Am J Hum Genet 65: 519–530.

Rickards O, Martínez-Labarga C, Favaro M, Frezza D, Mallegni F. 2001. DNA analysis of the remains of the Prince Branciforte Barresi family. Int J Legal Med 114:141–146.

Rodríguez Martín C. 1994. The epidemic of Modorra (1494–1495) among the Guanches of Tenerife. J Paleopathol 6:5–14.

Rollo F, Marota I. 1998. How microbial ancient DNA, found in association with human remains, can be interpreted. Philos Trans R Soc Lond [Biol] 354:111–119.

Salo WL, Aufderheide AC, Buikstra J, Holcomb TA. 1994. Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. Proc Natl Acad Sci USA 91:2091–2094.

Spigelman M, Donoghue D. 2001. Unusual pathological conditions in the lower extremities of a skeleton from ancient Israel. Am J Phys Anthropol 114:92–93.

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65:4799–4807.

Taylor DC, Cripps AW, Clancy RL, Murree-Allen K, Hensley MJ, Saunders NA, Sutherland DC. 1992. Biotypes of *Haemophilus parainfluenzae* from the respiratory secretions in chronic bronchitis. J Med Microbiol 36:279–282.

Torroni A, Schurr TG, Cabell MF, Brown MD, Neel JV, Larsen M, Smith DG, Vullo CM, Wallace DC. 1993. Asian affinities and continental radiation of the four founding Native-American mtDNAs. Am J Hum Genet 53:563–590.

Torroni A, Chen Y, Semino O, Santachiara-Benerecetti AS, Scott CR, Lott MT, Winter M, Wallace DC. 1994. mtDNA and Y-chromosome polymorphisms in four Native American populations from southern Mexico. Am J Hum Genet 54: 303–318

Torroni A, Huoponen K, Francalacci P, Petrozzi P, Morelli L, Scozzari R, Obinu D, Savontaus ML, Wallace DC. 1996. Classification of European mtDNAs from an analysis of three European populations. Genetics 144:1835–1850.

Ubaldi M, Luciani S, Marota I, Fornaciari G, Cano RJ, Rollo F. 1998. Sequence analysis of bacterial DNA in the colon of an Andean mummy. Am J Phys Anthropol 107:285–295.

Wilson KH, Blitchington RB. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl Environ Microbiol 62:2273–2278.

Wrischnik LA, Higuchi RG, Stoneking M, Erlich HA, Arnheim N, Wilson AC. 1987. Length mutations in human mitochondrial DNA: direct sequency of enzymatically amplified DNA. Nucleic Acids Res 15:529–554.