archaeometry

Archaeometry **55**, 4 (2013) 766–778 doi: 10.1111/j.1475-4754.2012.00707.x

DNA FROM HUMAN ANCIENT BACTERIA: A NOVEL SOURCE OF GENETIC EVIDENCE FROM ARCHAEOLOGICAL DENTAL CALCULUS*

C. DE LA FUENTE,[1,2] S. FLORES[2] and M. MORAGA[1,2]†

[1]*Programa de Genética Humana-ICBM, Facultad de Medicina, Universidad de Chile. Independencia 1027, Santiago, Chile*
[2]*Departamento de Antropología, Universidad de Chile, Ignacio Carrera Pinto 1045, Santiago, Chile*

We report a molecular methodology to obtain and analyse ancient bacterial DNA from archaeological dental calculus. Recent and archaeological DNA samples, as old as 4000 BP, were successfully extracted and amplified with species-specific PCR primers. We propose this approach in order to: detect the presence of specific bacterial species infecting past human populations; compare the composition of ancient oral microbiomes among human populations; and analyse the genetic variability and covariation of bacteria and human host populations. Genomic analysis of bacteria from dental calculus is a promising source of evidence for palaeopathological and micro-evolutionary studies, focused either on micro-organisms or their human hosts.

KEYWORDS: ANCIENT BACTERIAL DNA, ORAL MICROBIOME, MICRO-EVOLUTION, CO-EVOLUTION

INTRODUCTION

The human body is colonized by a myriad of micro-organisms, commensals and parasites, which were with our species even before the first migration from Africa. The dynamics between micro-organisms and humans depends on diverse factors such as pathogenicity, virulence, host susceptibility and ecological features related to infections (reviewed in Ashford 2000; Wirth *et al*. 2005). Such dynamics can influence processes of genetic change affecting micro-organisms and their host (i.e., co-evolution and parallel genetic drift), which allows the use of the genetic diversity of micro-organisms to infer aspects of the micro-evolutionary history of the ecological interaction itself, or as an approximation to the micro-evolutionary history of host human populations.

Several studies have shown a geographically structured covariation between the genetic diversity of micro-organisms and human hosts (Templeton 2007). These observations have motivated the use of viral and bacterial genomes as a source of indirect genetic markers, which means using the micro-organism genetic variation to infer past evolutionary events of the human host populations. This approach differs from the analysis of direct genetic markers or the analysis of genetic polymorphism from the human genome. However, inferences about the host evolution using indirect genetic markers need to take into account the mode of transmission, mutation rates, strength of vertical transmission and pathogenicity (Holmes 2004; Wirth *et al*. 2005).

According to current evidence, indirect genetic markers from viral genomes are suitable for inferring recent migration events, as has been shown by analysing molecular polymorphisms of

*Received 6 October 2012; accepted 18 June 2012
†Corresponding author: email mmoraga@med.uchile.cl

the JC virus (Agostini *et al*. 1997; Sugimoto *et al*. 1997; Pavesi 2005), the papilloma virus (Chan *et al*. 1992), the HTLV virus (Miura *et al*. 1994) and the hepatitis G virus (Pavesi 2001). On the other hand, analyses of *Helicobacter pylori* genetic variation in a phylogeographical framework have revealed more ancient events, covering the period from the origin of the infection of pre-modern human populations to the subsequent major migrations (Suerbaum and Josenhans, 2007; Castillo-Rojas *et al*. 2008; Yamaoka, 2009).

Recently, Nasidze *et al*. (2009) analysed the bacterial diversity from human oral microflora in order to identify species suitable for use as sources of indirect genetic markers. They described a broad diversity of microflora communities among human hosts, but a limited geographical structure of this diversity. The more ubiquitous genera were *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, *Haemophilus*, *Rothia*, *Porphyromonas* and *Fusobacterium*; these genera were identified as potential sources of genetic evidence of human micro-evolution. Using a different approach, Caufield (2009) evaluated the geographical distribution of *Streptococcus mutans*, which is part of the normal oral microflora and one of the principal cariogenic agents. Despite the strength of vertical transmission, the genetic markers analysed in this study did not consistently reflect the evolutionary history of their hosts; and because of this, Caulfield suggested the use of multiple loci to avoid this limitation.

Here, we propose to extend the analysis of genetic variation of oral bacteria to ancient populations, using dental calculus as a source of DNA. Tartar, or dental calculus, is formed on the surface of teeth due to the incorporation of crystals of calcium phosphate, principally hydroxyapatite, whitlockite, octacalcium phosphate and brucite, to the organic matrix of dental plaque (Liébana 1995; Lieverse 1999), and is composed of a diverse community of micro-organisms and polymers coming from the host and from the bacteria themselves (Li *et al*. 2004; Marsh 2005).

Due to its composition, dental calculus emerges as a potential material from which to obtain DNA from bacteria, which could be used as a source of indirect palaeogenetic markers as well as direct genetic markers for the analysis of oral microflora in the context of palaeopathology research. It should also be possible to obtain human DNA from dental calculus, as forensic studies have shown (Kawano *et al*. 1995), due to the incorporation of human cells (epithelial cells, leucocytes and macrophages) in calculus (Xie *et al*. 2010; Hunter *et al*. 2011).

Previous microscopic studies of ancient dental calculus have shown that general cell structure is maintained even in skeletons as old as 60 000 BP, in calculus samples from Neanderthals (Vandermeersch *et al*. 1994; Pap *et al*. 1995; Linossier *et al*. 1996). Cell walls from ancient samples dating to between 1000 and 500 BP are still able to produce Gram-positive reactions, which implies that the cell surface remains more or less intact (Linossier *et al*. 1996). Comparison between archaeological and living human dental calculus shows that morphotypes are very similar (Linossier *et al*. 1988, 1996; Vandermeersch *et al*. 1994; Pap *et al*. 1995), further supporting the idea that bacteria are well preserved over long periods of time. Nevertheless, DNA from bacteria associated with dental calculus has not yet been surveyed; only human mitochondrial DNA (mtDNA) from this source has been used for forensic purposes (Kawano *et al*. 1995). Recently, Preus and collaborators demonstrated the presence of DNA in the bacterial cytoplasm from ancient dental calculus, using specific monoclonal antibodies for thymine dimers and transmission electron microscopy (Preus *et al*. 2011).

This work constitutes the first report of molecular genetic evidence obtained from fossil bacteria associated with archaeological dental calculus. Here, we show the results of a simple protocol for successful extraction and amplification of bacterial DNA from contexts dated up to 4000 BP. In addition, the implications of a preliminary scan of oral microflora biodiversity from archaeological and current dental calculus are discussed.

MATERIALS AND METHODS

Sampling

A total of 38 samples of dental calculus (from 38 individuals), taken from historic burials and bioarchaeological collections, were analysed in this study. The calculus samples were mechanically extracted from osteological remains from nine archaeological sites or collections described in Table 1. These collections are held by the Faculty of Social Sciences, University of Chile, and the Museo Arqueológico R. P. Gustavo Le Paige (San Pedro de Atacama, II Región, Chile). In addition, samples from archaeological sites in southern Chile and Argentina were included.

Bioanthropological collections from Catarpe 2, Pica-8 and Tarapacá-40 had previously been retrieved from archaeological sites assigned to the Solor Phase (Late Intermediate Period), the Pica–Tarapacá cultural complex (Late Intermediate Period) and the Formativo complex (Tarapacá-40) of northern Chile, respectively. The Chono samples are from different archaeological sites from southern Chile, assigned to the indigenous group known as the Chono.

DNA extraction

DNA was extracted from approximately 10 mg of dental calculus using a modified protocol designed for the extraction of DNA from bones (Kemp *et al*. 2006). Dental calculus was extracted directly from dental pieces using a curette (a dental instrument with sharp edges, used to remove dental calculus) and placed in a 1.5 ml tube. The samples were treated with 500 µl of sodium hypochlorite at commercial concentration (4–6%) for 1 min, in order to eliminate surface contaminants, and then washed three times in ddH_2O to eliminate the sodium hypochlorite. The

Table 1 *The samples used in this study*

Sample	*N*	*Chronology*	*Location*	*Reference(s)*
Cementerio General	12	1960–1970	Región Metropolitana, Chile (33°28′S, 70°35′W)	–
Caserones-Tarapacá 40	4	1500–1000 BP	Región de Tarapacá, Chile (19°58′S, 69°33′W)	True (1980), Núñez (1982)
Pica 8	6	1000–500 BP	Región de Tarapacá, Chile (20°29′S, 69°19′W)	Zlatar (1984)
Catarpe 2	3	1000–500 BP	Región de Antofagasta, Chile (22°54′S, 68°12′W)	Schiappacasse *et al.* (1989), Uribe *et al.* (2004)
Chono	10	500 ± 70 BP*	Guaitecas–Peninsula of Taitao, Chile (43°52′S, 73°57′W)	Ocampo and Aspillaga (1984)
RB02	1	3741 ± 51 BP†	Site Río Bote 1, Santa Cruz, Argentina (50°15′S, 7143′W)	Franco (2008)
OB03	1	3565 ± 45 BP	Site Oreja de Burro 1, Santa Cruz, Argentina (52°07′S, 69°33′W)	L'Heureux and Barberena (2008)
IP11	1	4520 ± 60 BP	Site Ayayema, Madre de Dios Island, Occidental Archipelagos Patagonia, Chile (50°21′S, 75°20′W)	Legoupil and Sellier (2004)

*Dating from bark associated with human remains.

†Dating corresponds to individual RB01, found in the same stratigraphic level as RB02.

demineralization was performed using 450 µl of 0.5 M EDTA at room temperature and continuous rotation for 24 h. Subsequently, proteins were digested using 50 µl of 10% SDS, 20 µl of 1 M Tris-HCl pH 8.0 and 3 µl of proteinase-K (20 mg ml^{-1}) at 53°C for 16 h.

Protein and cell components were eliminated by two treatments of phenol:chloroform: isoamyl alcohol (25:24:1) and one extraction in chloroform:isoamyl alcohol (24:1). DNA was precipitated in one volume of 5 M ammonium acetate and two volumes of isopropanol for 16 h. Isopropanol was eliminated by centrifugation at 13 000 rpm for 15 min and the pellet was washed in 1 ml of 75% ethanol. Finally, DNA was resuspended in 50 µl of TE buffer.

To avoid sample contamination, all extraction procedures and polymerase chain reaction (PCR) set-ups were carried out in a laboratory devoted exclusively to ancient sample analysis; that is, one separated from those in which modern DNA is extracted and from the post-PCR laboratory. This laboratory has positive pressure (filtered air, HEPA quality, treated with ultraviolet light) and UV lamps on all working surfaces. Only disposable sterile plastic materials and DNA-free reagents were used. Reagents and samples were manipulated only below a laminar flow cabinet. Every set of extractions included six samples plus one blank extraction. All steps of DNA extraction and amplification were carried out at the Faculty of Medicine, University of Chile.

As an additional validation criterion for the results, we repeated three tartar samples from the Cementerio General; both sampling and DNA extraction were performed months after the first analysis.

Primer design and PCR amplification

The PCR primers were designed to produce amplicons 179 bp or less in length, considering the constraints imposed by degradation and fragmentation, which affect DNA over time (Willerslev and Cooper 2004). The primers were designed from bacterial genomes deposited in GenBank (http://www.ncbi.nlm.nih.gov/guide/) and the PCR parameters were determined using the program Primer3 Input (Rozen and Skaletsky 2000). Specificity was tested using PrimerBlast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For each species, different sets of genes were selected to be amplified.

PCR primers were designed for five species of bacteria (Table 2): *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Streptococcus gordonii* and *Streptococcus mutans*. The first four species were selected on the basis of their incidence in dental plaque (Kolenbrander *et al*. 2002; Duncan 2003; Avila *et al*. 2009; Hojo *et al*. 2009; Filoche *et al*. 2010). *S. mutans* was selected because it was identified in a previous microscopic study of dental calculus (Linossier *et al*. 1996). *S. gordonii*, *S. mutans* and *A. naeslundii* are Gram-positive, facultative anaerobic species, which play a role in the primary colonization of dental plaque (Kolenbrander *et al*. 2002). *P. gingivalis* and *F. nucleatum* are Gram-negative anaerobic bacteria. The former is associated with the development of pathological conditions such as periodontal disease, being part of the late colonization of oral plaque, while the latter acts as a bridge between early and late colonizers and has not been associated directly with pathologies (Kolenbrander *et al*. 2002). Details of the DNA fragments and PCR primers used for each species are summarized in Table 2. The reaction mix (25 µl total volume) contained: 0.2 µl of GoTaq HotStart enzyme (5 U/µl)(Promega), 5 µl enzyme buffer 5×, 2 µl of dNTPs (2.5 mM), 2 µl of $MgCl_2$ (25 mM), 1.25 µl of each primer (10 µM), 0.7 µl of BSA and 3 µl of the extracted DNA. The PCRs were performed with the following conditions: initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 s, annealing for 30 s using specific temperatures for each primer (see Table 2) and elongation at 72°C for 30 s, with a final elongation at 72°C for 5 min.

Table 2 *The DNA fragments amplified in this study*

Species	*Gene*	*Function*	*GenBank accession number*	*Primers*	T_m (°C)	*Size (bp)*	*Reference*
A. naeslundii	uref	Urease accessory protein	AF048783	F:5′cacacccactgacccttc3′ R:5′agcccctcggagaaggtc3′	59	179	Morou-Bermudez and Burne (1999)
S. gordonii	sspA	Surface protein coding gene associated with adherence	NC_009785	F:5′gtctgattggtggcctcatt 3′ R:5′tttgcagcatctgtttcagc 3′	52	166	Kolenbrander *et al.* (2002)
S. mutans	gbpA	Glucan-binding protein A, involved in virulence	NC_004350	F:5′cttcaccgctgtctttgtca3′ R:5′cgatggcaaacgtgtatcag3′	58	149	Matsumura *et al.* (2003)
F. nucleatum	fadA	Colonization and adherence to oral plaque	DQ012979	F:5′gcagtttctgcttcagcattt3′ R:5′tgcttgaagtctttgagctcttt3′	53	166	Han *et al.* (2005)
P. gingivalis	fimA	Biofilm synthesis and adhesion	AB195790	F:5′tgcgacgctatatgcaagac3′ R:5′tcttcaaaaccacgctgatg3′	52	166	Lo *et al.* (2010)

Negative extraction and PCR blank controls were included in all PCR procedures as monitors for potential contamination generated during the study.

Additionally, we have a database of bacterial sequences included in this study from the saliva of the first author (C. de la F.), who performed the procedures for the DNA extraction and PCR amplification. These sequences were compared with the sequences of ancient samples in order to evaluate any potential contamination.

DNA cloning and sequencing

In order to reveal whether two or more strains belonging to one bacterial species occur in the same sample, the amplified DNA fragments were cloned using Plasmid pGEM®-T (Promega) as the cloning vector, according to the instructions provided by the manufacturer. Ligation was performed at 4°C overnight. The amount of PCR product included in the ligation solution was 1:1 in relation to the amount of cloning vector. Transformation was performed by heat shock using competent cells JM109 (Promega), incubated at 37°C for 1.5 h. Ten colonies were sampled from each plaque for subsequent PCR and sequencing. Ligation success was evaluated by PCR amplification using plasmid primers (T7 and SP6), followed by evaluation of fragment sizes in agarose electrophoresis.

The PCR products from the extracted DNA were submitted for direct sequencing. Then, the PCR products were cloned and subsequently sequenced by Macrogen Inc. A BLAST search was performed in order to confirm the expected species-specific amplification according to the primer design.

Endogenous mtDNA amplification

Although the analysis of human mitochondrial DNA is not the objective of this study, we know that there are human cells included in dental calculus, and thus it should be possible to amplify endogenous human DNA. The characterization of the HV1 region of the mtDNA of the samples

may be used as a criterion of authenticity, as long as it is consistent with the population origin of the sample and different from that of the investigator who performs the analysis. A database is maintained of the mitochondrial DNA sequences of all researchers who enter the ancient DNA facilities.

A total of 327 bp mtDNA (positions 16055–16382) was sequenced using three sets of primers (Malhi *et al*. 2007) that amplified overlapping fragments of the mtDNA hypervariable region I. The length range of the PCR products was 202–212 bp. Amplification of the three DNA fragments was performed as described previously, except that the annealing temperature was changed to 51°C. The PCR products were sequenced by Macrogen Inc. in both directions.

RESULTS AND DISCUSSION

Of the 38 samples analysed, 16 showed amplicons for one to four bacteria (Table 3). Nine of those samples were from southern Chile, most of them revealing two to four bacterial species. Only three samples from northern Chile showed amplicons for *F. nucleatum* and *S. gordonii*. The remaining four samples showing positive PCR products were from the collection of the Cementerio General. *F. nucleatum* had the greatest number of positive results; it was detected in 14 samples. On the other hand, *S. mutans* was detected only in one sample. *P. gingivalis* and *S. gordonii* were identified in nine and 12 samples, respectively, while *A. naeslundii* was found in four. The saliva sample analysed (C. de la F.) amplified only for the bacteria *F. nucleatum*, *A. naeslundii* and *S. mutans*. Note that *A. naeslundii* and *S. mutans* only amplified in four and one samples of ancient dental calculus, respectively, while *S. gordonii*, which was well represented in the ancient samples, and *P. gingivalis* did not amplify from the saliva of the researcher.

Table 3 *Bacteria species identified from historic burials (Cementerio General) and archaeological samples: species identified are indicated with crosses (×)*

Sample	*Collection/site*	*A. naeslundii*	*F. nucleatum*	*P. gingivalis*	*S. gordonii*	*S. mutans*
B0095	Cementerio General	×	×	×	×	
B0219	Cementerio General		×		×	
B0233	Cementerio General		×	×	×	
B0339	Cementerio General		×	×		
B0495	Pica-8		×		×	
1819	Catarpe 2		×		×	
1825	Catarpe 2		×		×	
4025	Chono	×	×	×	×	
4033	Chono				×	
4093	Chono	×	×	×	×	
C27	Chono		×	×	×	
CH1	Chono		×		×	
CH3	Chono	×	×			×
CH4	Chono			×		
RB02	Río Bote 1		×	×	×	
IP11	Ayayema		×	×		
Total		4 (25%)*	14 (87.5%)*	9 (56.25%)*	12 (75%)*	1 (6.25%)*

The nucleotide sequences from this study are available in GenBank (accession numbers: JX398007–JX398072).

*The total number of amplicons by bacterium and their percentages were calculated on the basis of total number of samples with amplificons (16).

Our results are in line with a previous study that reported a high prevalence of *F. nucleatum* in dental plaque (Handfield *et al*. 2008). A preliminary study allowed us to identify this bacterial species in 39 samples (100%) from living individuals from Santiago (Chile), suggesting that success in PCR amplification is affected by DNA degradation over time in archaeological samples. However, diet and dental hygiene also probably affect the dental microbiome differentially among human populations. Differences in bacterial morphotypes have been reported among dental calculus samples from prehistoric populations, which have been explained as resulting from different dietary patterns (Linossier *et al*. 1988, 1996).

Also in those studies, dental calculus from Atacameño populations (northern Chile) was analysed, and showed a wide variation of bacteria at the morphological level and well-preserved cell walls. Those samples have similar datings to those from Catarpe-2, Pica-8 and Tarapacá-40; these three groups have been identified as farmers. However, in this study only three samples from northern Chile gave positive results, suggesting that in spite of the morphological conservation of the bacteria in this geographical area, ancient DNA from dental calculus is particularly affected. As other studies have shown, ancient DNA from arid and dry areas, such as those of northern Chile, are more susceptible to oxidative damage of ancient DNA, inhibiting the PCR reaction and therefore reducing the success of DNA extraction and amplification (Poinar and Stankiewicz 1999; Gilbert *et al*. 2003; Willerslev and Cooper 2004). The samples analysed in this study were from different collections than those analysed by Linossier and collaborators, but both sets of samples come from locations with similar environmental conditions.

Finally, 34 DNA sequences were obtained from four species of bacteria; three from *A. naeslundii*, 13 from *F. nucleatum*, eight from *P. gingivalis* and 10 from *S. gordonii*. These sequences were evaluated by using BLAST, confirming in all cases that they corresponded to bacteria and the expected fragment. The three replicated samples from the Cementerio General all yielded the same sequences, supporting the endogenous character of the amplified DNA. Neither the negative extraction controls nor the PCR controls gave amplification signals.

There are few nucleotide differences between the sequences obtained from *A. naeslundii*, *P. gingivalis* and *S. gordonii* and the published sequences, which suggests that the DNA fragments analysed are very conservative. In the case of *F. nucleatum*, we observed double peaks in some positions in the chromatograms, which is not surprising, since the co-infection of two strains of *F. nucleatum* has previously been reported, using molecular identification of subspecies (Kim *et al*. 2010). Also, the variable sites identified (double peaks) have been reported in published sequences in genomic databases (Han *et al*. 2005). Considering the above information and the fact that the amplification by PCR was performed in a multi-bacterial medium, it is unlikely that contamination or oxidative damage can account for this variability. To resolve the variants of this bacterium, the DNA fragments were cloned and subsequently sequenced. Two to four haplotypes were found per amplicon (Fig. 1).

We compared 78 sequences of *F. nucleatum* (supporting information, sheet 1) corresponding to seven published sequences (Han *et al*. 2005), 66 clones from the samples analysed in this study and five from the saliva of C. de la F. Three of the sequences of the researcher had unique mutations that were absent in the studied samples, while two were similar to published strains. Both the supplementary data table and the dendrogram constructed from it (see supporting information, sheet 1 and Fig. 2) show two principal groups: a first group composed mainly of clones from the Cementerio General and published strains; and a second group with a diverse composition, including published strains and clones of differing origins, including clones from both northern and southern Chile (Chono, Ayayema) and Argentina (Río Bote). Also, within the second group there were two clades composed exclusively of clones of the same sample, CH1

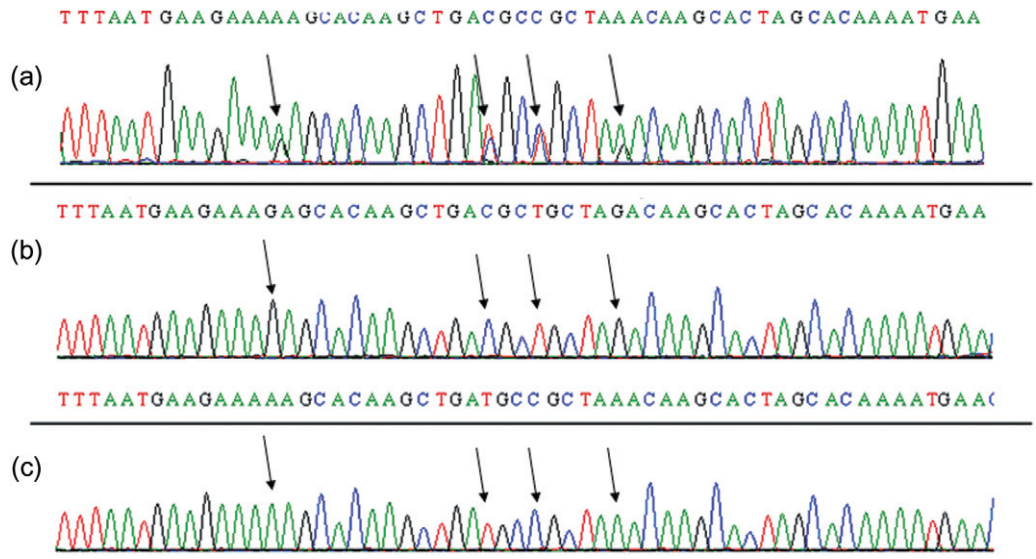
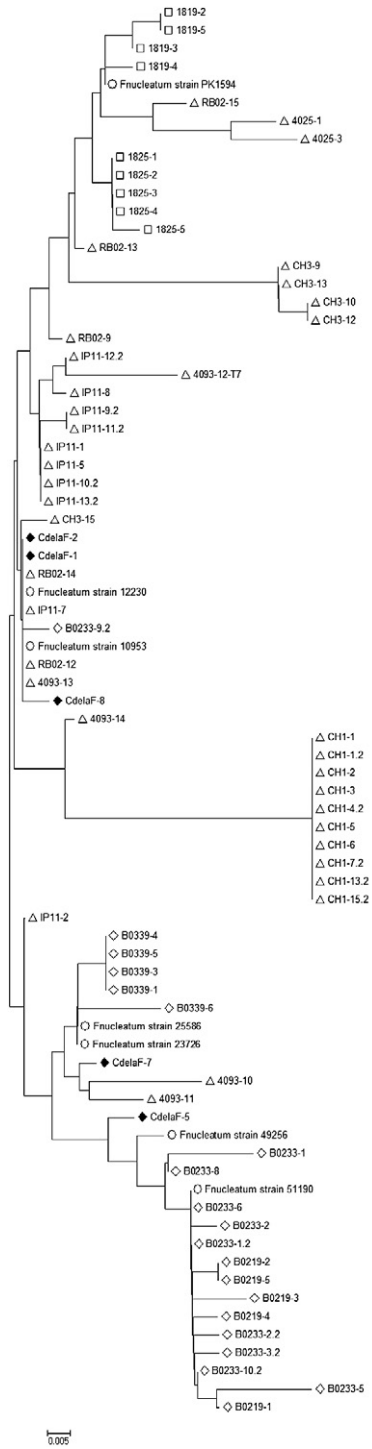


Figure 1 *Chromatograms from a fragment of* F. nucleatum *fadA gen. (a) A sequence obtained directly from a sample of dental calculus from the Cementerio General, showing four single nucleotide polymorphisms (SNPs) (indicated with arrows) and, therefore, rendering 16 possible sequences. (b, c) Sequences obtained from the cloning of the DNA fragment shown in (a), revealing that only two strains are responsible for the polymorphisms.*

and CH3, respectively. The marked differentiation of CH1 is explained by the high frequency of G>A changes (10 sites) observed in all clones, which is consistent with *post mortem* oxidative deamination of guanine or cytosines in the complementary strand (the most common *post mortem* DNA modification). By contrast, in CH3 the mutations observed were the C>T (sites 94, 108, 114, 179 and 181), T>C (sites 115 and 207), A>G (142) and G>A (102) transitions and one A>C transversion (site 143). The C>T transitions could be explained by *post mortem* deamination, except for those at sites 94 and 181, which could be original mutations. All other modifications could possibly be chemical damage (Willerslev and Cooper 2004).

In the other sequences, the majority of the changes are not attributable to *post mortem* DNA damage; their analysis supports the endogenous character of the bacterial DNA extracted.

As part of the controls for authenticity of the results, six samples were amplified with primers for human mtDNA (two Catarpe, two Chono and two Cementerio General); four of these amplified for fragments of hypervariable region 1 (one Catarpe, one Chono and two Cementerio General) (supporting information, sheet 2). The samples from Catarpe and Chono had the specific changes of subhaplogroup C1b (Tamm *et al*. 2007; Achilli *et al*. 2008), which is highly represented in South American Amerindian populations—the only difference was the presence of transition 16311C in Catarpe (the sequence of C. de la F. shares the same transition in HVI with this last sample). One of the samples from the Cementerio General belongs to a recently described variant of the Amerindian haplogroup D1, characterized by the transition 16187T (Bodner *et al*. 2012), while the other belongs to the macro-haplogroup R (T or J) of Indo-European populations (Torroni *et al*. 2006), in this case probably supplied by the European component of the mixed Chilean population. The presence of four different sequences consistent with the population origins of the samples confirms the endogenous character of the amplified DNAs, even though one of them is shared with C. de la F.


1819-2
1819-5
1819-3
1819-4
Fnucleatum strain PK1594
RB02-15
4025-1
4025-3
1825-1
1825-2
1825-3
1825-4
1825-5
RB02-13
CH3-9
CH3-13
CH3-10
CH3-12
RB02-9
IP11-12.2
4093-12-T7
IP11-8
IP11-9.2
IP11-11.2
IP11-1
IP11-5
IP11-10.2
IP11-13.2
CH3-15
CdelaF-2
CdelaF-1
RB02-14
Fnucleatum strain 12230
IP11-7
B0233-9.2
Fnucleatum strain 10953
RB02-12
4093-13
CdelaF-8
4093-14
CH1-1
CH1-1.2
CH1-2
CH1-3
CH1-4.2
CH1-5
CH1-6
CH1-7.2
CH1-13.2
CH1-15.2
IP11-2
B0339-4
B0339-5
B0339-3
B0339-1
B0339-6
Fnucleatum strain 25586
Fnucleatum strain 23726
CdelaF-7
4093-10
4093-11
CdelaF-5
Fnucleatum strain 49256
B0233-1
B0233-8
Fnucleatum strain 51190
B0233-6
B0233-2
B0233-1.2
B0219-2
B0219-5
B0219-3
B0219-4
B0233-2.2
B0233-3.2
B0233-10.2
B0233-5
B0219-1
0.005

CONCLUSIONS

At least two properties of bacteria from dental calculus may be suggested as potential contributions for studies of micro-evolutionary processes of human host populations. First, bacterial genomes have shown high mutation rates associated with short generation times, rendering more highly geographically structured populations than human populations (reviewed in Wirth *et al*. 2005; Nieberding and Olivieri 2007; Kitchen *et al*. 2008). In this case, the genes analysed in each bacterial species were very conservative, but useful for standardizing the DNA extraction technique and identifying each bacterium. Second, bacterial DNA from dental calculus can easily be obtained from differing archaeological contexts, avoiding massive destruction of dental and osteological remains (Kawano *et al*. 1995). This study demonstrates that DNA from bacteria associated with dental calculus is indeed a valuable source of genetic evidence, one that can easily be obtained.

Our approach allowed us to amplify bacterial DNA from dental calculus from diverse geographical and chronological contexts, ranging from current to archaeological populations dated around 4000 BP. We demonstrated that different bacterial species can easily be recovered from the ancient context; however, *F. nucleatum* seems to be more abundant, therefore being a more reliable target for indirect reconstruction of human micro-evolutionary processes. Additional analysis of the genetic variability of this species should be undertaken, in order to detect genomic regions rendering enough genetic and phylogenetic signals to be used as indirect markers. Especially in this species, we observed the simultaneous presence of two or more variants in the same individual, something that should be studied in greater depth in order to characterize these strains or variants, and to evaluate their variability at three levels; intra-individual, intra-population and among populations.

Genomic analysis of bacteria from dental calculus is a promising source of evidence for palaeopathological and evolutionary studies, focused either on micro-organisms or human hosts.

ACKNOWLEDGEMENTS

The authors are deeply grateful to Mark Hubbe (Museo Arqueológico R. P. Gustavo Le Paige) and Paulina Mena and her students from Central College (Pella, IA, USA), who provided valuable critiques on the original draft of the manuscript, as well as to the researchers and institutions that provided us with valuable samples: Museo Arqueológico R. P. Gustavo Le Paige (San Pedro de Atacama, II Región, Chile); the Department of Anthropology of the University of Chile (Faculty of Social Sciences); Nora Franco, CONICET Instituto Multidisciplinario de Historia y Ciencias Humanas (BA, Argentina); Luis Alberto Borrero, CONICET Instituto Multidisciplinario de Historia y Ciencias Humanas (BA, Argentina); and Flavia Morelo, Instituto de

Figure 2 (previous page) *The evolutionary relationships of 78 sequences from* F. nucleatum. *The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). The optimal tree, with sum of branch length = 0.51518136, is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 166 positions in the final data set. The phylogenetic analyses were conducted in MEGA4 (Tamura* et al. *2007).* ◇*, Cementerio General;* ◆*, C. de la Fuente;* ○*, sequences from the NCBI database;* □*, northern Chile;* △*, southern Chile.*

la Patagonia, Universidad de Magallanes (Pta. Arenas, Chile). Finally, we are indebted to our colleague Eugenio Aspillaga, who suggested that we initiate this work.

This research was supported by grants FONDECYT 11060442, FONDECYT 1100643 and Programa de Investigación Asociativa Conicyt, Anillo ACT-096 (www.cienciaymemoria.cl).

REFERENCES

Achilli, A., Perego, U. A., Bravi, C. M., Coble, M. D., Kong, Q. P., Woodward, S. R., Salas, A., Torroni, A., and Bandelt, H. J., 2008, The phylogeny of the four pan-American MtDNA haplogroups: implications for evolutionary and disease studies, *PLoS ONE*, **3**, e1764.

Agostini, H. T., Yanagihara, R., Davis, V., Ryschkewitsch, C. F., and Stoner, G. L., 1997, Asian genotypes of JC virus in Native Americans and in a Pacific Island population: markers of viral evolution and human migration, *Proceedings of the National Academy of Sciences, USA*, **94**(26), 14 542–6.

Ashford, R. W., 2000, Parasites as indicators of human biology and evolution, *Journal of Medical Microbiology*, **49**(9), 771–2.

Avila, M., Ojcius, D. M., and Yilmaz, Ö., 2009, The oral microbiota: living with a permanent guest, *DNA and Cell Biology*, **28**, 405–11.

Bodner, M., Perego, U. A., Huber, G., Fendt, L., Röck, A. W., Zimmermann, B., Olivieri, A., Gómez-Carballa, A., Lancioni, H., Angerhofer, N., Bobillo, M. C., Corach, D., Woodward, S. R., Salas, A., Achilli, A., Torroni, A., Bandelt, H. J., and Parson, W., 2012, Rapid coastal spread of First Americans: novel insights from South America's Southern Cone mitochondrial genomes, *Genome Research*, **22**(5), 811–20.

Castillo-Rojas, G., Cerbón, M. A., and López-Vidal, Y., 2008, Presence of *Helicobacter pylori* in a Mexican pre-Columbian mummy, *BMC Microbiology*, **8**, 119.

Caufield, P. W., 2009, Tracking human migration patterns through the oral bacterial flora, *Clinical Microbiology and Infection*, **15**, 37–9.

Chan, S. Y., Ho, L., Ong, C. K., Chow, V., Drescher, B., Dürst, M., ter Meulen, J., Villa, L., Luande, J., Mgaya, H. N., and Bernard, H. U., 1992, Molecular variants of human papillomavirus type 16 from four continents suggest ancient pandemic spread of the virus and its coevolution with humankind, *Journal of Virology*, **66**(4), 2057–66.

Duncan, M. J., 2003, Genomics of oral bacteria, *Critical Reviews in Oral Biology and Medicine*, **14**, 175–87.

Filoche, S., Wong, L., and Sissons, C., 2010, Oral biofilms: emerging concepts in microbial ecology, *Journal of Dental Research*, **89**, 8–18.

Franco, N. V., 2008, La estructura tecnológica regional y la comprensión de la movilidad humana: tendencias para la cuenca del río Santa Cruz, in *Arqueología del extremo sur del continente americano: resultados de nuevos proyectos* (eds. L. A. Borrero and N. V. Franco), 119–54, Ed. Instituto Multidisciplinario de Historia y Ciencias Humanas (CONICET), Buenos Aires.

Gilbert, M. T., Hansen, A. J., Willerslev, E., Rudbeck, L., Barnes, I., Lynnerup, N., and Cooper, A., 2003, Characterization of genetic miscoding lesions caused by *postmortem* damage, *American Journal of Human Genetics*, **72**, 48–61.

Han, Y. W., Ikegami, A., Rajanna, C., Kawsar, H. I., Zhou, Y., Li, M., Sojar, H. T., Genco, R. J., Kuramitsu, H. K., and Deng, C. X., 2005, Identification and characterization of a novel adhesin unique to oral fusobacteria, *Journal of Bacteriology*, **187**(15), 5330–40.

Handfield, M., Baker, H. V., and Lamont, R. J., 2008, Beyond good and evil in the oral cavity: insight into host–microbe relationships derived from transcriptional profiling of gingival cells, *Journal of Dental Research*, **87**, 203–23.

Hojo, K., Nagaoka, S., Ohshima, T., and Maeda, N., 2009, Bacterial interactions in dental biofilm development, *Journal of Dental Research*, **88**, 982–90.

Holmes, E. C., 2004, The phylogeography of human viruses, *Molecular Ecology*, **13**, 745–56.

Hunter, S. J., Easton, S., Booth, V., Henderson, B., Wade, W. G., and Ward, J. M., 2011, Selective removal of human DNA from metagenomic DNA samples extracted from dental plaque, *Journal of Basic Microbiology*, **51**, 1–5.

Kawano, S., Tsukamoto, T., Ohtaguro, H., Tsutsumi, H., Takahashi, T., Miura, I., Mukoyama, R., Aboshi, H., and Komuro, T., 1995, Sex determination from dental calculus by polymerase chain reaction (PCR), *Nihon Hoigaku Zasshi*, **49**(3), 193–8.

Kemp, B. M., Monroe, C., and Glenn Smith, D., 2006, Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts, *Journal of Archaeological Science*, **33**, 1680–9.

Kim, H., Lee, D., Chang, Y., Kim, M. J., Koh, S., Kim, J., Seong, J., Song, S. K., Shin, H. S., Son, J., Jung, M. Y., Park, S., Yoo, S. Y., Cho, K. W., Kim, D., Moon, S., Kim, D., Choi, Y., Kim, B., Jang, H., Kim, C. S., Kim, C., Choe, S.,

and Kook, J., 2010, Application of rpoB and zinc protease gene for use in molecular discrimination of *Fusobacterium nucleatum* subspecies, *Journal of Clinical Microbiology*, **48**, 545–53.

Kimura, M., 1980, A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences, *Journal of Molecular Evolution*, **16**, 111–20.

Kitchen, A., Miyamoto, M. M., and Mulligan, C. J., 2008, Utility of DNA viruses for studying human host history: case study of JC virus, *Molecular Phylogenetics and Evolution*, **46**, 673–82.

Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S., Palmer, R. J. Jr, and Colonizers, L., 2002, Communication among oral bacteria, *Microbiology and Molecular Biology Reviews*, **66**, 486–505.

Legoupil, D., and Sellier, P., 2004, La sepultura de la Cueva Ayayema (Isla Madre de Dios, archipiélagos occidentales de Patagonia), *Magallania*, **32**, 115–24.

L'Heureux, G. L., and Barberena, B., 2008, Evidencias bioarqueológicas en patagonia meridional: el sitio Orejas de Burro 1 (Pali Aike, provincia de Santa Cruz), *Intersecciones en Antropología*, **9**, 11–24.

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S., and Oppenheim, F. G., 2004, Identification of early microbial colonizers in human dental biofilm, *Journal of Applied Microbiology*, **97**, 1311–8.

Liébana Ureña, J., 1995, *Microbiología oral*, Interamericana-McGraw-Hill, Madrid.

Lieverse, A. R., 1999, Diet and the aetiology of dental calculus, *International Journal of Osteoarchaeology*, **9**(1), 219–32.

Linossier, A., Aspillaga, E., and Gajardo, M., 1988, Hallazgo de bacterias comensales de la cavidad oral en tártaro dental de restos óseos de indígenas chonos, *Revista Chilena de Antropología*, **7**(1), 123–8.

Linossier, A., Gajardo, M., and Olavarria, J., 1996, Paleomicrobiological study in dental calculus: *Streptococcus mutans*, *Scanning Microscopy*, **10**(4), 1005–13.

Lo, A., Seers, C., Dashper, S., Butler, C., Walker, G., Walsh, K., Catmull, D., Hoffmann, B., Cleal, S., Lissel, P., Boyce, J., and Reynolds, E., 2010, FimR and FimS: biofilm formation and gene expression in *Porphyromonas gingivalis*, *Journal of Bacteriology*, **192**(5), 1332–43.

Malhi, R. S., Kemp, B., Eshleman, J., Cybulski, J., Glenn Smith, D., Cousins, S., and Harry, H., 2007, Mitochondrial haplogroup M discovered in prehistoric North Americans, *Journal of Archaeological Science*, **34**, 642–8.

Marsh, P. D., 2005, Dental plaque: biological significance of a biofilm and community life-style, *Journal of Clinical Periodontology*, **32**, 7–15.

Matsumura, M., Izumi, T., Matsumoto, M., Tsuji, M., Fujiwara, T., and Ooshima, T., 2003, The role of glucan-binding proteins in the cariogenicity of *Streptococcus mutans*, *Microbiolology and Immunology*, **47**(3), 213–15.

Miura, T., Fukunaga, T., Igarashi, T., Yamashita, M., Ido, E., Funahashi, S., Ishida, T., Washio, K., Ueda, S., Hashimoto, K., Yoshida, M., Osame, M., Singhal, B. S., Zaninovic, V., Cartier, L., Sonoda, S., Tajima, K., Ina, Y., Gojobori, T., and Hayami, M., 1994, Phylogenetics subtypes of human T-lymphotropic virus type I and their relations to the anthropological background, *Proceedings of the National Academy of Sciences, USA*, **91**(3), 1124–7.

Morou-Bermudez, E., and Burne, R. A., 1999, Genetic and physiologic characterization of urease of *Actinomyces naeslundii*, *Infection and Immunity*, **67**(2), 504–12.

Nasidze, I., Li, J., Quinque, D., Tang, K., and Stoneking, M., 2009, Global diversity in the human salivary microbiome, *Genome Research*, **19**, 636–43.

Nieberding, C. M., and Olivieri, I., 2007, Parasites: proxies for host genealogy and ecology? *Trends in Ecology and Evolution*, **22**, 156–65.

Núñez, L., 1982, Temprana emergencia de sedentarismo en el desierto chileno: Proyecto Caserones, *Chungara*, **9**, 80–122.

Ocampo, C., and Aspillaga, E., 1984, Breves notas sobre una prospección arqueológica en los archipiélagos de las Guaitecas y de los Chonos, *Revista Chilena de Antropología*, **4**, 155–6.

Pap, I., Tillier A., Arensburg, B., Weiner, S., and Chech, M., 1995, First scanning electron microscope analysis of dental calculus from European Neanderthals: Subalyuk (Middle Paleolithic, Hungary), *Bulletins et mémoires de la Société d'anthropologie de Paris*, **7**, 69–72.

Pavesi, A., 2001, Origin and evolution of GBV-C/hepatitis G virus and relationship with ancient human migrations, *Journal of Molecular Evolution*, **53**(2), 104–13.

Pavesi, A., 2005, Utility of JC polyomavirus in tracing the pattern of human migrations dating to prehistoric times, *Journal of General Virology*, **86**, 1315–26.

Poinar, H. N., and Stankiewicz, B. A., 1999, Protein preservation and DNA retrieval from ancient tissues, *Proceedings of the National Academy of Sciences, USA*, **96**(15), 8426–31.

Preus, H., Marvik, O., Selvig, K., and Bennike, P., 2011, Ancient bacterial DNA (aDNA) in dental calculus from archaeological human remains, *Journal of Archaeological Science*, **38**, 1827–31.

Rozen, S., and Skaletsky, H. J., 2000, Primer3 on the WWW for general users and for biologist programmers, in *Bioinformatics methods and protocols: methods in molecular biology* (eds. S. Krawetz and S. Misener), 365–86, Humana Press, Totowa, NJ.

Saitou, N., and Nei, M., 1987, The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Molecular Biology and Evolution*, **4**, 406–25.

Schiappacasse, V., Castro, V., and Niemeyer, H., 1989, Los Desarrollos Regionales en el Norte Grande, in *Culturas de Chile. Prehistoria: desde sus orígenes hasta los albores de la conquista* (eds. J. Hidalgo, V. Schiappacasse, H. Niemeyer, C. Aldunate and I. Solimano), 181–220, Andrés Bello, Santiago.

Suerbaum, S., and Josenhans, C., 2007, *Helicobacter pylori* evolution and phenotypic diversification in a changing host, *Nature Reviews: Microbiology*, **5**(6), 441–52.

Sugimoto, C., Kitamura, T., Guo, J., Al-Ahdal, M. N., Shchelkunov, S. N., Otova, B., Ondrejka, P., Chollet, J. Y., El-Safi, S., Ettayebi, M., Grésenguet, G., Kocagöz, T., Chaiyarasamee, S., Thant, K. Z., Thein, S., Moe, K., Kobayashi, N., Taguchi, F., and Yogo, Y., 1997, Typing of urinary JC virus DNA offers a novel means of tracing human migrations, *Proceedings of the National Academy of Sciences, USA*, **19**(94), 9191–6.

Tamm, E., Kivisild, T., Reidla, M., Metspalu, M., Glenn Smith, D., Mulligan, C. J., Bravi, C. M., Rickards, O., Martinez-Labarga, C., and Khusnutdinova, E. K., 2007, Beringian standstill and spread of Native American founders, *PLoS ONE*, **2**, 1–6.

Tamura, K., Dudley, J., Nei, M., and Kumar, S., 2007, MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0, *Molecular Biology and Evolution*, **24**, 1596–9.

Templeton, A. R., 2007, Shared history of human and gut bacteria. Evolutionary togetherness: coupled evolution of human and pathogen, *Heredity*, **98**(6), 337–8.

Torroni, A., Achilli, A., Macaulay, V., Richards, M., and Bandelt, H. J., 2006, Harvesting the fruit of the human mtDNA tree, *Trends in Genetics*, **22**(6), 339–45.

True, D., 1980, Archaeological investigations in northern Chile: Caserones, in *Prehistoric trails of Atacama: archaeology of northern Chile* (eds. C. Meighan and D. True), 139–78, Monumenta Archaeologica 7, University of California Press, Los Angeles.

Uribe, M., Adán, L., and Agüero, C., 2004, Arqueología de los períodos Intermedio Tardío y Tardío de San Pedro de Atacama y su relación con la cuenca del río Loa, *Chungara*, **36**, Volumen Especial, 943–56.

Vandermeersch, B., Arensburg, B., Tillier A., Rak, Y., Weiner, S., Spiers, M., and Aspillaga, E., 1994, Middle Palaeolithic dental bacteria from Kebara, Israel, *Compte Rendus de l'Academie des Sciences, Paris*, serie II, **319**, 727–31.

Willerslev, E., and Cooper, A., 2004, Ancient DNA, *Proceedings of the Royal Society B: Biological Sciences*, **272**, 3–16.

Wirth, T., Meyer, A., and Achtman, M., 2005, Deciphering host migrations and origins by means of their microbes, *Molecular Ecology*, **14**, 3289–306.

Xie, G., Chain, P., Lo, C., Liu, K., Gans, J., Merritt, J., and Qi, F., 2010, Community and gene composition of a human dental plaque microbiota obtained by metagenomic sequencing, *Molecular Oral Microbiology*, **25**, 391–405.

Yamaoka, Y., 2009, *Helicobacter pylori* typing as a tool for tracking human migration, *Clinical Microbiology and Infection*, **15**, 829–34.

Zlatar, V., 1984, *Cementerio prehispánico Pica-8*, Universidad de Antofagasta, Antofagasta.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Supporting information 1: Clone sequences of *F. nucleatum*.
Supporting information 2: Mitochondrial DNA sequences obtained from dental calculus.