Extraction and Electrophoresis of DNA from the Remains of Mexican Ancient Populations

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1. Introduction

Ten years ago, the first reports of human genome sequencing were published in Nature and Science (Venter et al., 2001; Sachidanandam et al., 2001; Lander, 2011). This was very exciting and expectations for the application of genome sequencing technology were high. In the past decade, the cost of sequencing has gone down several orders of magnitude, making it a more accessible technology for research studies. The medical value of comprehensive genome sequencing is now becoming apparent: for example, the genetic cause of a rare and debilitating vascular disorder was solved by genome sequencing at NIH (Jasny and Zahn, 2011; Lander, 2011). It is also possible to solve the genetics of individual Mendelian disorders thereby relating phenotype to genotype. In addition, better treatments for diseases such as cancer, metabolic disorders, inflammation, neurodegeneration or diabetes are expected to be found through studies involving genome sequencing (Lander, 2011). Sequencing also has been used to query variation in populations worldwide, and sequences are now available from extinct hominids as well as from thousands of other species (Rasmussen et al., 2010; Krause et al., 2010; Reich et al., 2010; Balter, 2010; Rasmussen et al., 2010). We expect to know very soon what variation exists among individuals at almost all sites in the genome. This is a great opportunity for population genetics to reconstruct the entire genealogical and mutational history of humans (Callaway, 2011), to understand the evolutionary and genetic forces that affected every region of the genome, to determine disease mutations present in human populations, to elucidate the genetic bases of cognitive and physiological adaptations, and/or to determine the demographic events that led to the colonisation of the earth.

The question remains: what is the relationship between morphological features and ancient deoxyribonucleic acid (aDNA)? The evolutionary processes that generated modern species

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and populations are commonly inferred through the analysis of morphological and genetic markers in addition to analyses of contemporary organisms to create tentative reconstructions. To confirm this indirect evidence, it is necessary to check the reconstructions against the fossil records. Nevertheless, the comparison has been made possible now by analysing morphological characters, and the application of recent advances in deoxyribonucleic acid (DNA) sequencing technologies for aDNA are now allowing the genetic record to be generated. This new technology let us focus not only on single genetic loci, such as mitochondrial DNA (mtDNA), but it made possible to obtain whole genome sequences of extinct species and populations (Lander et al., 2011), our closest extinct relatives the Neanderthal (Green et al., 2010), and the extinct hominid group from Siberia, the Denisovans (Reich et al., 2010).

The field of aDNA was initiated more than twenty years ago (Higuchi et al., 1984; Cooper et al., 1992; Greenwood et al., 1999) and research efforts continue to grow and expand into new areas (Stoneking and Krause, 2011). The first aDNA studies demonstrated the inefficiency of bacterial cloning to amplify small sequences recovered from the skins of animals and human mummies (Higuchi et al. 1984; Pääbo, 1985) and showed that DNA was at very low concentrations of short damaged fragments. However, these studies are considered very important because they will elucidate population origins, migrations, relationships, admixture and changes in population size, essentially revealing the demographic history of the human population.

It is now accepted that DNA is preserved in ancient samples under a wide range of depositional environments (Willerslev and Cooper, 2005). Although the DNA of a deceased organism degrades rapidly, part of it may survive for more than 100,000 years under favourable conditions, such as cold, stable temperatures and a dry environment (Pääbo et al., 2004). Fortunately, the development of new technologies has made possible the recovery and manipulation of these molecules as well as the genetic characterisation of these samples. Because this DNA is degraded the analysis is complicated, nevertheless, the new sequencing technology makes it possible to obtain historical information. In addition, the presence of polymerase inhibitors makes DNA amplification exceedingly difficult. Research in this area shares a common problem with forensics and other approaches requiring analyses of museum and non-invasively collected specimens; the amount of endogenous DNA available in the samples is limited. In addition, when working with human samples it is also possible to have contamination from contemporary human DNA. Careful adherence to currently established procedures is necessary to avoid such contamination (Deguilloux et al., 2011).

Because aDNA contains the information of our past its analysis is of high importance. Here, we will review a variety of methods for extraction, purification, amplification and sequencing of aDNA segments informative for genetic population studies. Future prospects for the potential direction of ancient DNA research will be discussed. Furthermore, contributions to migratory theories will also be analysed based on population diversity, taking into account ancient mtDNA studies.

Although there is new technology to determine the sequence of nuclear DNA, we will focus on mtDNA analysis. mtDNA analysis has been very useful to extensively examine human population history throughout the world because of its relatively rapid rate of mutation, lack of recombination and maternal inheritance. Mitochondrial DNA sequence variations at the hypervariable regions HVI and HVII will be described and their importance in

population genetic studies will be discussed. Technical differences between DNA extraction procedures for ancient bones and mummy tissue will also be described. Molecular phylogenetic analysis, haplotype and haplogroup determination through software will also be defined and examined.

2. Procedures to study ancient DNA

There have been several aDNA extraction protocols suggested over the years. The first method was purification based on phenol/chloroform extraction, alcohol precipitation (Kalmár et al., 2000; Munoz et al., 2003; Hagelberg and Clegg, 1991; Hänni et al., 1995) and silica binding (Höss and Pääbo, 1993; Yang et al., 1998). In addition, other methods have been suggested, such as using Chelex (Faerman et al., 1995), centricon filters (Anzai et al., 1999), Dextran Blue (Kalmár et al., 2000), decalcifying bone with EDTA (Hagelberg and Clegg, 1991; Hänni et al., 1995; Yang et al., 1998) and hybridisation and magnetic separation (Anderung et al., 2008). The methods most commonly used now combine EDTA decalcification and silica purification (Yang et al., 1998; Krings et al., 1997; Anzai et al., 1999). It is evident that many different techniques have been used, demonstrating that no single procedure has clear advantages. Based on our experience, the selected method is a function of the sample characteristics, including considerations for the origin of the sample, from the skeleton or a mummy.

2.1 Samples

Samples from this study include bones pertaining to pre-Hispanic populations from different periods of time (200 to 1500 years before present). Bone samples of two individuals from Monte Albán, Oaxaca, one from Teotihuacán and a tissue portion from the mummy Pepita were used in the examples presented in this study. To work with the ancient Mexican samples, we made a written agreement with the "Intituto Nacional de Antropologia e Historia" (Mexico). Research on ancient unidentifiable human remains is excluded from the requirement of ethics review by the Research Ethics Boards.

Sampling should be conducted as soon as the bones appear in excavation, and gloves, mask and coat must be used to prevent contamination from excavators. This is not always possible because some samples were collected before these studies were initiated. Samples also have to be deposited directly in hermetic sterile tubes and frozen at -70°C. These practices prevent the introduction of contaminant DNA during the sample collection. In addition, it is also very important to manipulate the sample in a sterile clean room, to use bleach and ultraviolet light to degrade potential contaminants and to keep strict physical separation of modern DNA work from aDNA (Miller et al., 2008, Cooper and Poinar, 2000).

2.2 Ancient DNA extraction

All DNA purification and PCR experiments were carried out under sterile conditions in separate dedicated rooms. Samples were handled wearing protective clothing from collection to DNA isolation, and the laboratory equipment and reagents are maintained DNA-free. The laboratory managing the ancient samples has a high-pressure system to filter the incoming air and a laminar flow hood as well as UV light irradiation and bleach were used to clean of every surface to avoid contamination (Knapp et al., 2011).

DNA purification was preceded by a decontamination step to eliminate surface exogenous DNA when samples were collected and manipulated by unknown people. Each sample was washed with bleach followed by a water rinse and UV light irradiation for 30 min on each face. Some authors suggest removing the surface of the bone, however this procedure may also contaminate the inside of the bone if it has some kind of porosity. When bones were collected as soon as they appeared during the exaction with the necessary equipment to prevent contamination from excavators (i.e., gloves, mask and coat), it was not necessary to treat the sample with the decontamination steps (Deguilloux et al., 2011) and the potential to damage template or impede the efficiency of PCR was therefore avoided. Overall, there is no way to guarantee complete removal of contaminant DNA through decontamination procedures, but these practices are used to eliminate as much contamination as possible.

Bone powder was generated by grinding in a mortar with pestle until a fine powder was obtained, when bone quantities were around 1 g. When the weight of the samples was ≤ 0.5 mg, the bone sample was ground under liquid nitrogen with a sterile screw cylinder modified from those suggested by Thomas M. G. and Moore L. J., (1997). The powder (0.250-0.500 g) was transferred into a sterile 15 ml tube and was suspended in 2 ml of extraction buffer (0.01 M Tris-HCl, 0.1 M EDTA and 0.2% SDS pH 8.0), and the tubes were capped and sealed with Parafilm. After incubation with gentle agitation for 1 h at 37°C, 1 mg/ml proteinase K was added, and the sample was incubated at 50°C for 2 h. A blank extraction treated identically to the experimental samples throughout the procedure was included to monitor for contamination during the DNA extraction process. Finally, the samples were centrifuged at 5,000×g for 5 min, and the supernatants were extracted using phenol-chloroform-isoamyl alcohol (24:24:1) organic extraction (Maniatis, et al., 1989; Munoz et al., 2003; Hughes et al., 2006). Subsequently, the aqueous phase was concentrated by precipitation by the addition of 0.1 volumes of 3 M sodium acetate at pH 5.0 and 2.5 volumes of ethanol. After mixing, the sample was incubated at -78°C overnight and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was decanted, and the precipitate was rinsed with 70% ethanol. After drying the pellet at ambient temperature in a sterile area, the pellet was resuspended in 100 µl of high quality sterile water. Alternatively, the aqueous phase can be concentrated using Amicon® Ultra-0.5 30 kDa columns (Millipore, Billerica, USA), in a final volume of 40 µl.

Another method to extract the aDNA is by binding to silica: the powered sample (0.250 g) was suspended in 1 ml of extraction buffer (0.01 M Tris-HCl, 0.5 M EDTA pH 8.0) and after incubation at 37°C for 16 h, the suspension was incubated at 56°C for 3 h and centrifuged at 5,000xg for 2 min. The supernatant was transferred into 3 ml of binding buffer (5 M GuSCN, 0.025 M NaCl, 0.010 M Tris-HCl pH 8.0) in a 15 ml sterile conical tube and adjusted to pH 4.0 by adding 30% HCl in 25 µl aliquots. Then, the solution is passed through a QIAquick (Qiagen) silica column. The column was rinsed twice with the washing buffer (50% ethanol, 0.125 M NaCl and 0.010 M Tris and 0.001 M EDTA, pH 8.0) and dried for 15 min. Finally, aDNA was eluted from the column with 100 µl of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8).

Extracted DNA was kept in aliquots of 25 µl at -70 ^{o}C.

Ancient DNA can also be extracted by the Chelex-100 method: Extraction of DNA using Chelex1-100 (Bio-Rad Laboratories, CA, USA) was performed with 5% Chelex-100 in sterile H$_2$O using the protocol described by Walsh et al. (1991). Briefly, 200 µl of DNA extracted by

phenol-chloroform-isoamyl alcohol (24:24:1) was boiled at 94 °C for 10 min with 5% Chelex-100 and centrifuged, and an aliquot of the supernatant was taken as the template for the PCR experiment.

2.3 Amplification of DNA from pre-Hispanic samples

Analysis in Native Americans of mtDNA by PCR amplification and high-resolution restriction analysis with 14 endonucleases (Torroni, et al., 1992; Torroni, et al., 1993; Torroni, et al., 1994a,b; Richards et al., 1996) identified four major mtDNA lineages or haplogroups (A-D). These haplogroups of Asian ancestry, each defined by specific polymorphisms, together encompass 96.9 % of the mtDNA observed in modern Native Americans. Each lineage is characterized by specific mtDNA marker: the 9-bp deletion in the COII/tRNAlys region (haplogroup B); a HaeIII restriction site gain at nucleotide position 663 of the reference sequence (haplogroup A) (Anderson et al., 1981); a HincII restriction site loss at nucleotide 13259 (haplogroup C); and an AluI restriction site loss at nucleotide 5176 (haplogroup D) (Wallace et al., 1985; Schurr et al., 1990; Torroni et al., 1992; Wallace and Torroni, 1992). Sequence data indicate a correspondence between each marker and particular hypervariable region I (HVI) mutations (Horai et al., 1993; Bailliet et al. 1994). Consequently, the mtDNA amplification of the specific region has to be performed to characterize the Native Americans (ancient and contemporary) populations. Primers to amplify HVRII were also included, although we did not included any example, because analysing the HVRII region is not as informative as the HVRI.

Enzymatic amplification by PCR was performed as described previously (Munoz et al., 2003; Campos, et al., 2011) using heat-resistant *Thermus aquaticus* (Taq) DNA polymerase (FINNZYMES), or Platinum® Taq High Fidelity (Invitrogen). The PCR parameters were as follows: 2.5 U of hot start DNA polymerase, 1X buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.25 mg/ml bovine serum albumin (BSA) and 0.2 µM of each primer in a total volume of 25 µl, and 5 µl of the aDNA template. The primers used to amplify and sequence human mitochondrial DNA were as follow:

HVR I:
L15975-15996 5'-CTCCACCATTAGCACCCAAAGC-3';
H16401-16420 5'-TGATTTCACGGAGGATGGTG-3' (Vigilant et al., 1989);
L16140-16159 5'-TACTTGACCACCTGTAGTAC-3';
H16236-16255 5'-CTTTGGAGTTGCAGTTGATG-3' (Wilson et al., 1995);
L15989-16008 5'-CCCAAAGCTAAGATTCTAAT-3';
H16130-16152 5'-AGGTGGTCAAGTATTTATGGTAC-3' (Eichmann and Parson, 2008);
L16094-16122 5'-TCGTACATTACTGCCAGYC-3';
H16228-16248 5'-GTTGCAGTTGATGTGTGATAG-3' (Eichmann and Parson, 2008);
L16190-16209 5'-CCCCATGCTTACAAGCAAGT-3';
H16380-16398 5'-CAAGGGACCCCTATCTGAG-3' (Poinar et al., 2001);

HVR II:
L8-29 5'-GGTCTATCACCCTATTAACCAC-3';
H408-429 5'-CTGTTAAAAGTGCATACCGCC-3' (Vigilant et al., 1989)

Haplogroup A:
L610-633 5'-TGAAAATGTTTAGACGGCCTCACA-3';

H712-730 5′-CCAGTGAGTTCACCCTCTA-3′ (Parr et al., 1996).
Haplogroup B:
L8196-8215 5′-ACAGTTTCATGCCCATCGTC-3′;
H8297-8316 5′-CTGTAAAGCTAACTTAGCAT-3′ (Wrischnik et al., 1987);
Haplogroup C:
L13198-13213 5′- GCAGCAGTCTGCGCCC -3′;
H13384-13403 5′- ATATCTTGTTCATTGTTAA -3′ (Lorenz and Smith, 1996)
(1996)
Haplogroup D:
L5101-5120 5′-TAACTACTACCGCATTCCTA-3′;
H5230-5249 5′-TGCCCCCGCTAACCGGCTTT-3′ (Stone and Stoneking, 1993)

All amplifications were carried out in a GeneAmp® PCR System 9700 thermocycler with the following profile: 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 59°C (haplogroups A, D and RHVs) or 55 °C (haplogroups B and C), and 1 min at 72°C, with a final extension of 10 min at 72°C. At least one PCR blank was amplified alongside each batch.

The PCR products were visualised on 2% agarose gels with ethidium bromide, and all positive products were purified using the QIAquick kit (Qiagen) and sequenced using the BigDye® Terminator v3.1 kit (Applied Biosystems) in an ABI PRISM 310 genetic analyser.

2.4 Data analysis

Phylogenetic analysis. The sequences of the pre-Hispanic PCR products from the HVI segment were aligned with representative Amerindian mtDNA control-region sequences (GenBank accession numbers: AY195760, EU719927, EU719811, EU719679, EU720004, EU720308, EU720078, EU719797, AY195749, EU720177, EU720123, EU719764, HQ012155, EU720242, HQ012184, HQ012164, HQ012134, AY195772, EU720073, EU720339, AY195759, EU720071, EU720336, EU720102, EU720202, HQ012188, HQ012198, EU720029, HQ012255, HQ012254, HQ012253, GQ449339, EU034320, AY195748, AF214088, DQ973581, AF478614) and two ancient sequences from a prehistoric Oneota population (Stone and Stoneking, 1998) using the Clustal W program (Thompson et al., 1994). Then, the phylogenetic tree was constructed with the Jukes-Cantor method, and the distances were obtained from a neighbour joining algorithm. Finally, the tree was optimised for maximum likelihood, using Hy-Phy software (Kosakovsky-Pond et al., 2005).

Haplotype network analysis. The median-joining (ε=0) networks (Bandelt et al., 1999) of haplotypes were constructed using the Network package, v4.5.1.0 (Fluxus Engineering). Sequences used were those described for phylogenetic analysis. This method is for constructing networks from recombination-free population data.

3. Examples of ancient DNA extraction procedures

An example of DNA extraction from a pre-Hispanic sample is depicted in Figures 1 and 2. Figure 1 displays the contaminants with different colours during the phenol-chloroform-isoamyl alcohol procedure. Figure 2 shows the DNA extracted by the phenol-chloroform-isoamyl alcohol technique from 0.25 g of two powdered bone samples from the same individual. In this figure, we observe the sample contaminants that are one of the major

obstacles to these studies because they inhibit the Taq polymerase. The contaminants, such as Maillard products of reducing sugars (Pääbo, 1989) and humic acids with phenolic

groups, were observed by fluorescent stain in blue while the DNA degraded (which results in a smear pattern) is stained in pink by ethidium bromide (Figure 2, panel A). Figure 2, panel B shows the second sample in lane 3, which displays only contaminants. The DNA was not apparent. These compounds can be partially eliminated using kits such as the Amicon® Ultra-0.5 30 kDa columns. These results show variation in DNA yields between extracts taken from different samples of the same bone, even when using the same extraction method. We attribute such differences to heterogeneity within the bones.
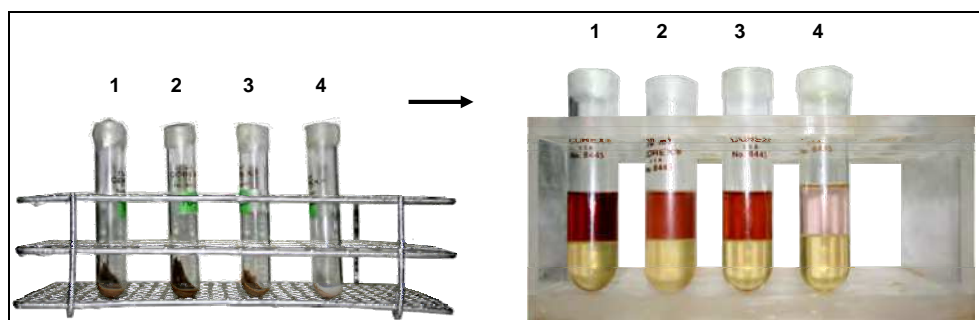
Fig. 1. Extraction of aDNA of bone samples from four different pre-Hispanic samples using the phenol-chloroform-isoamyl alcohol technique.
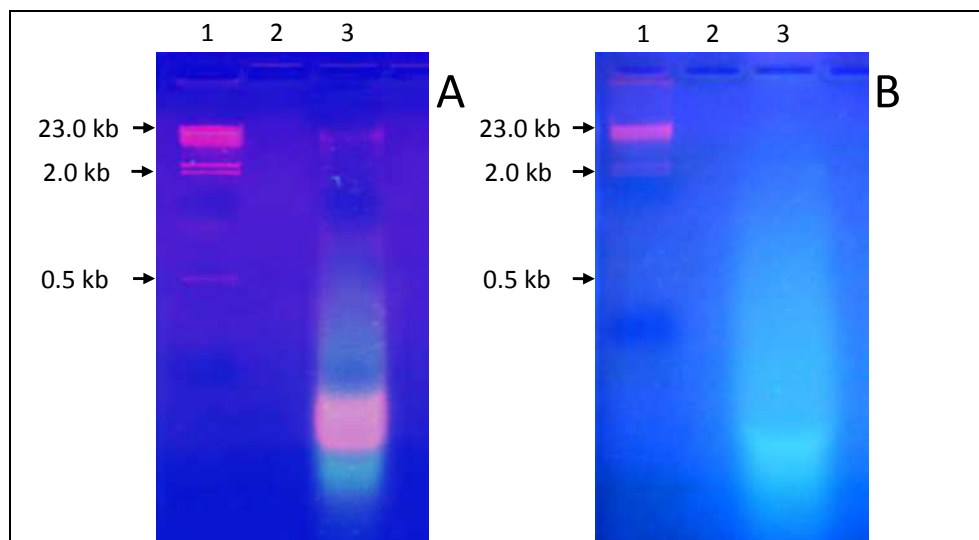
Fig. 2. Extraction of DNA of two independent samples (A, B) from the same pre-Hispanic individual by the technique of phenol-chloroform-isoamyl alcohol and ethanol precipitation. Lanes 1, molecular weight markers of *HindIII*; Lanes 2, no-sample; lane 3, DNA extracted from sample 1 of the Mexican pre-Hispanic population from Monte Alban.

Because DNA concentration is only possible with limited precision and concentrations of standard dilution series change over time in storage, we evaluated the relative performance of the DNA during PCR amplification using serial dilutions of the extracted DNA starting from 5 µl. Using this method, we were able to dilute the inhibitors of the Taq DNA polymerase.
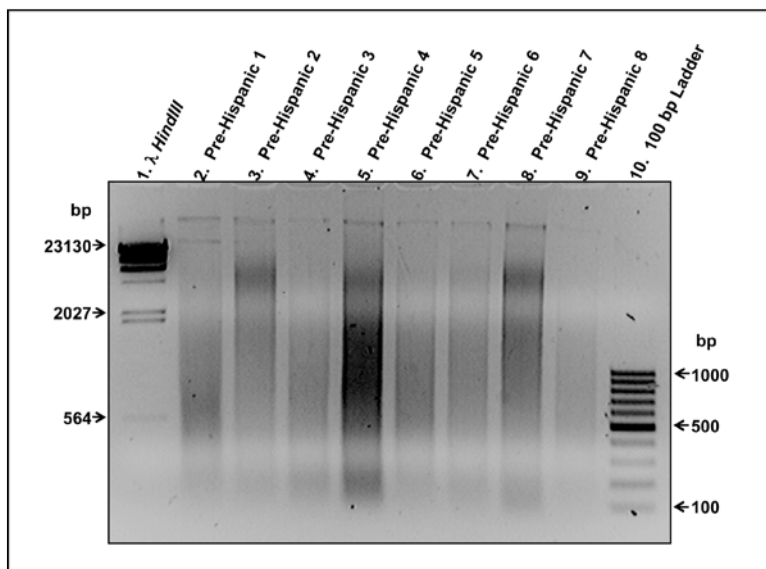

Fig. 3. Extraction of DNA of pre-Hispanic samples by the silica technique. Lanes 1 and 10, molecular weight markers of 23 kbp and 100 bp, respectively. Lanes 2 to 9, DNA extracted from different samples of Mexican pre-Hispanic populations.

Silica gel was also used to purify aDNA, results are shown in Figure 3. Each lane of this figure (2-9) displays aDNA extracted from 0.25 g of different pre-Hispanic bone samples. Lanes 1 and 10 show molecular weight markers. The quantity of Taq polymerase inhibitors is not evident, although we know that all ancient samples contain some of these inhibitors in different concentrations.

4. PCR performance

To study the effects of the Taq polymerase inhibitors, we added decrease quantities of the DNA extracted by the phenol-chloroform-isoamyl alcohol technique (shown in Figure 1) to the amplification reaction of the hypervariable segment I (15975-16420) using contemporary DNA. Figure 4, lane 1 displays the 100 bp molecular weight marker, lane 2 the negative control, lane 3 the PCR product of the contemporary DNA with the aDNA without dilution, lanes 4, 5, 6 and 7 show aDNA diluted 1:1, 1:2, 1:4 and 1:8, respectively, added to the PCR reaction mix and lane 8 contemporary DNA without any addition (positive control). Contaminated DNA allowed positive amplifications when aDNA was diluted at least 1:4 (Figure 4, lane 6). Therefore, an additional method to obtain the PCR product from the aDNA is by sample dilution.
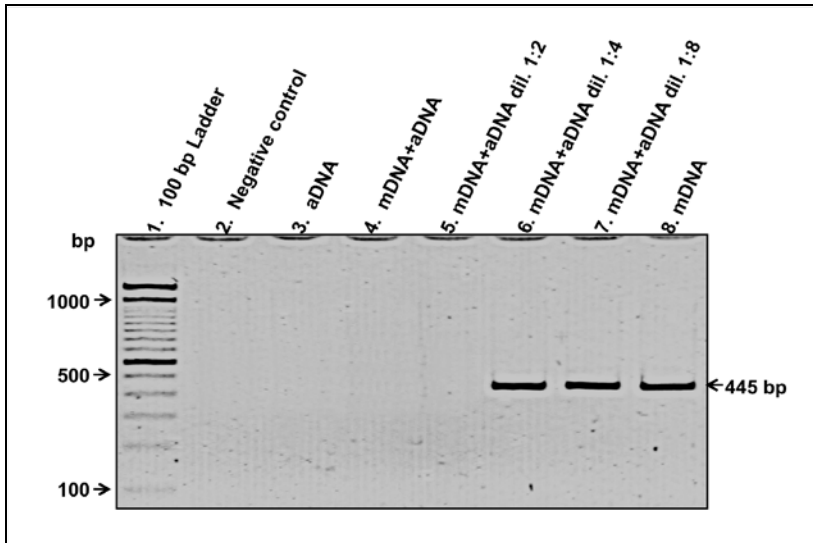
Fig. 4. Inhibition of the mitochondrial DNA hypervariable segment I amplification via inhibition of Taq polymerase by aDNA contaminants.
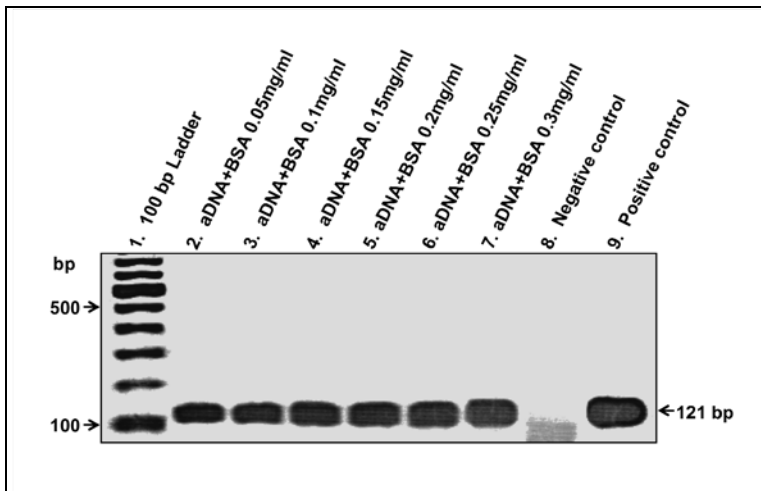

Fig. 5. Positive effect of bovine serum albumin (BSA) on the PCR performance of aDNA extracted by the phenol-chloroform-isoamyl alcohol procedure.

Sometimes aDNA dilution is not enough to obtain the PCR products, so we tested the effect of BSA addition by increasing the concentration of BSA in the reaction from 0.1 to 0.25 mg/ml. Figure 5 shows the positive effect of BSA on the PCR of aDNA extracted by the phenol-chloroform-isoamyl alcohol procedure. Increased amplification was observed in the PCR experiments when BSA was added in increasing concentrations (Figure 5, lanes 2-7, BSA at a concentration of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mg/ml, lane 8, negative control and

lane 9, positive control). Based on these results, we added 0.25 mg/ml BSA to all PCR experiments.
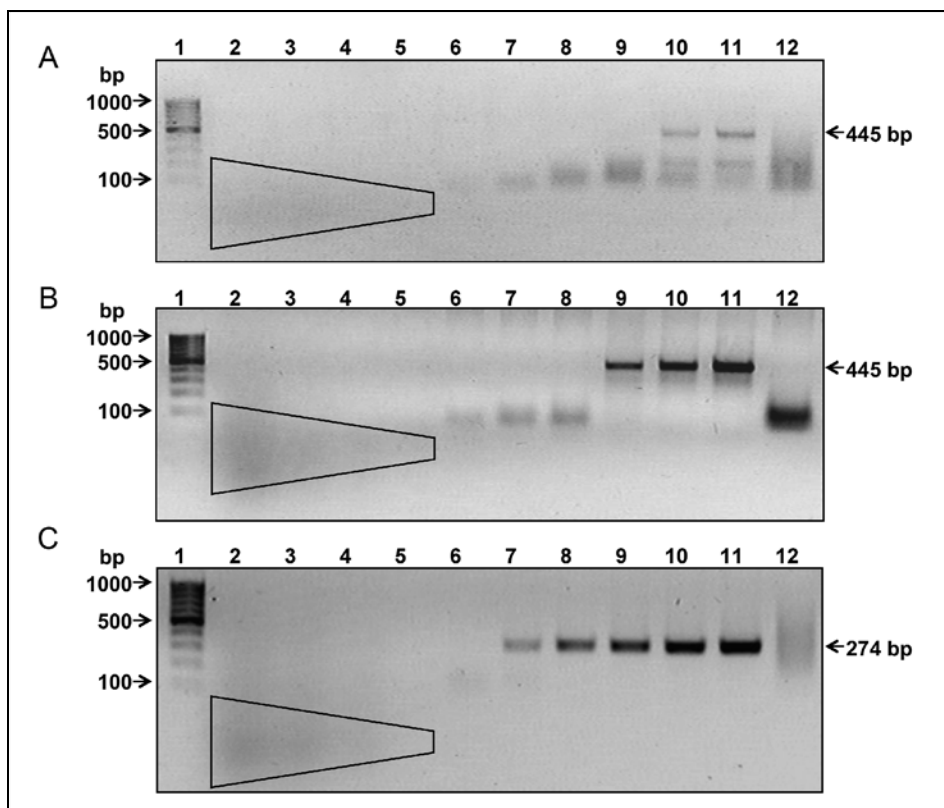

Fig. 6. Inhibition of the mitochondrial DNA hypervariable segment I amplification by inhibition of the Taq polymerase with aDNA contaminants and the effect of PVP.

Soils with high organic contents have humic acids with phenolic groups that denature biological molecules by bonding to N-substituted amides or oxidise to form a quinone that bonds to DNA or proteins (Young et al., 1994). Because aDNA contains these Taq polymerase inhibitors from soil, we tested the effect of Polyvinylpyrrolidone (PVP) during DNA extraction as has been suggested previously (Young et al., 1994; Rohland and Hofreiter, 2007). In addition, to make sure that PVP did not inhibit the PCR experiment, the reagent was added directly to the amplification mix. Figure 6 shows that 2% PVP added during the DNA extraction had a positive effect on DNA amplification. PCR amplification of contemporary DNA containing different dilutions of aDNA (1:1, 1:2, 1:3, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128; Figure 6, lanes 3 to 10, respectively) and 0.25 mg/ml BSA is shown in Figure 6, panel A, and amplifications using the same conditions in the presence of 2% PVP during aDNA extraction is shown in Figure 6, panel B. The use of 2% PVP during aDNA extraction resulted in amplification at an aDNA dilution of 1:64, in contrast to the aDNA sample without PVP in which amplification is only observed at 1:128 dilution or beyond.

The HVI mtDNA segment of 445 bp was amplified in Figure 6, panels A and B with the primers L15975-15996 and H16401-16420. When the primers L15975-15996 and H16228-16248 were used, the PCR product is shorter (273 bp), and the presence of PVP makes evident the PCR fragment at a dilution of 1:16 (Figure 6, panel C). In addition, PVP at a concentration of 0.4% in the PCR experiment did not inhibit amplification, as was previously published (Young et al., 1993). The molecular weight marker is in lane 1; positive control with contemporary DNA alone is in lane 11; and the negative control with no DNA is in lane 12. Nevertheless, the positive effect was not evident in all aDNA bone samples, likely because the amount of Taq polymerase inhibitors is different in each sample.

Contemporary DNA is very easy to amplify. However, when working with aDNA, the PCR reaction efficiency is greatly reduced. For example, in Figure 7, we show a PCR-amplified fragment of mDNA using the human-specific primers L15975-15996 and H16236-16255 where 4 of the 7 bone samples from the pre-Hispanic populations displayed the PCR product (Figure 7, lanes 2 to 8). Positive and negative controls are shown in lanes 9 and 10, respectively, and the molecular weight marker is shown in lane 1.
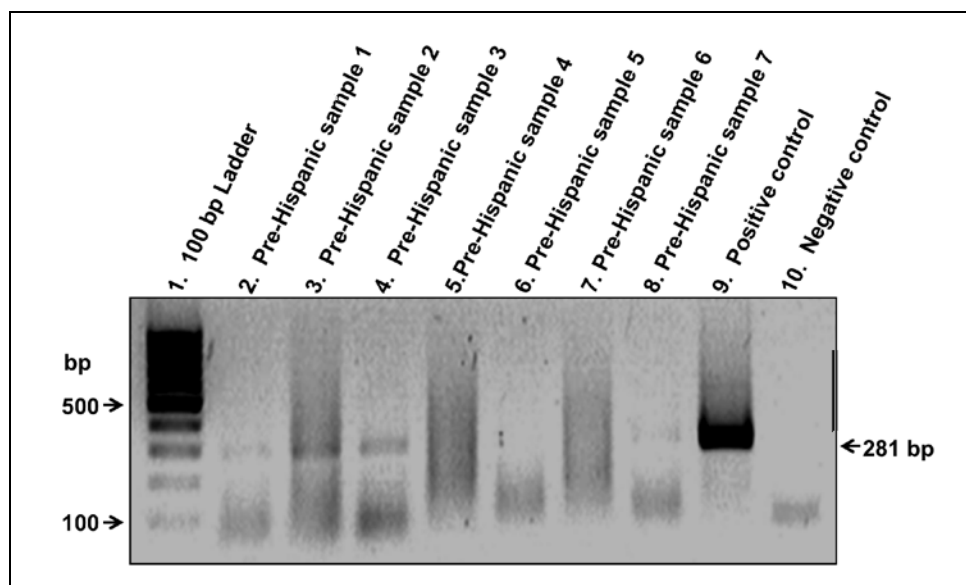

Fig. 7. Agarose gel showing the amounts of PCR-amplified product obtained after DNA extraction with the phenol-chloroform-isoamyl alcohol procedure.

Using the procedure indicated in the methods section, we purified and amplified the DNA from 14 bone samples of pre-Hispanic Native Americans to type them for haplogroup A described for Amerindians. The PCR products obtained were digested by the restriction enzyme *HaeIII*. Haplogroup A was detected in the 10 samples typed (Figure 8, lanes 2-11). Partial restriction digestion was observed in all of the ancient PCR products. This finding suggests the presence of the Amerindian polymorphism; however, we must sequence these amplification fragments or use real-time PCR to confirm the presence of the specific polymorphism because the partial restriction observed.
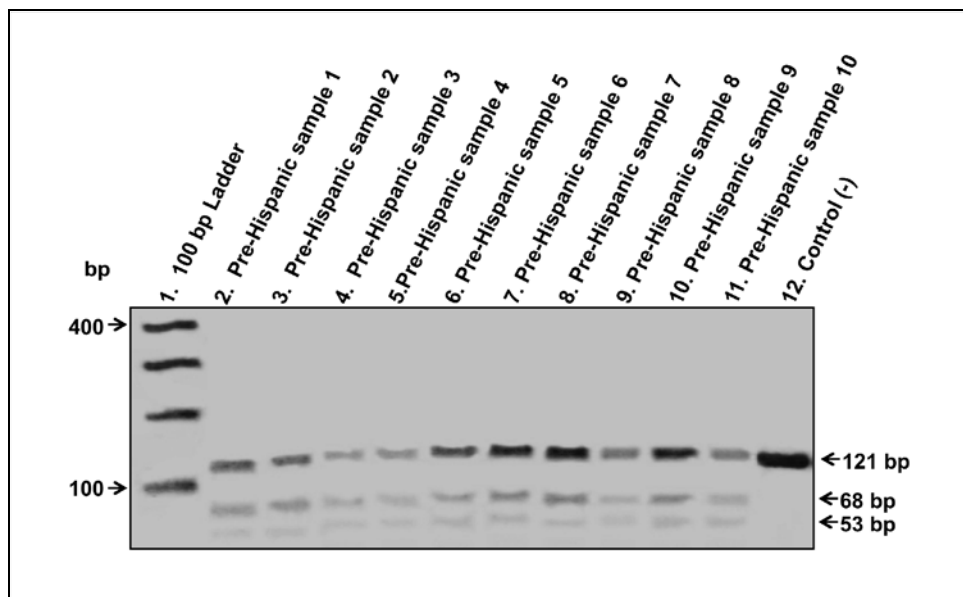
Fig. 8. Agarose gel showing the amounts of PCR-amplified product obtained after DNA extraction with the phenol-chloroform-isoamyl alcohol procedure and digested with the restriction enzyme *HaeIII*. Primers for amplification were specific to type haplogroup A.

Amplification of DNA extracted by Chelex was tested in the samples from pre-ceramic bones (Figure 9). Although experiments that compared the phenol-chloroform and Chelex method concluded that the Chelex method was simple and fast, inhibitory substances had not been eliminated in most of the cases (Kalmár et al., 2002). In our experience, DNA extracted by the phenol-chloroform method followed by Chelex treatment may improve DNA purification. Nevertheless, the silica method was better overall in our experience. The amplification products are observed at DNA dilutions of 1:30 in all samples, as shown in Figure 9.

Figure 10 displays the PCR amplification fragments using the specific primers L15975-15996 and H16236-16255 producing a fragment of 281 bp in panel A and L16140-16159 and H16380-16398 producing a fragment of 259 bp in panel B. We compared the amplification of aDNA extracted by the silica procedure and phenol-chloroform-isoamyl alcohol. Our results showed that aDNA extraction with the silica procedure was better than the phenol-chloroform-isoamyl alcohol method in this specific sample from pre-Hispanic populations because the amplification was observed exclusively in samples in which the DNA was extracted by the silica method. However, this may not be the case for all types of samples, and it is important to consider that when one method does not give good results, other methods may be useful.

Figure 10 shows aDNA from pre-Hispanic samples extracted by the silica gel method (lanes 2 and 3) compared with the phenol-chloroform-isoamyl alcohol procedure followed by concentration of aDNA with filter units (Centricon®) (lanes 4-5).

Ancient DNA was added to the PCR mix without any dilution (lane 2), diluted 1:10 (lane 3); aDNA phenol-chloroform-isoamyl alcohol extracted (lane 4); same procedure but diluted 1:10 (lane 5); washing buffer of the Centrifugal filter units (Centricon®) that were used to purify and concentrate aDNA in the phenol-chloroform-isoamyl alcohol purification procedure (lane 6) and diluted 1:10 (lane 7); negative control without DNA (lane 8); positive control with contemporary DNA (lane 9); and no sample (lane 10).
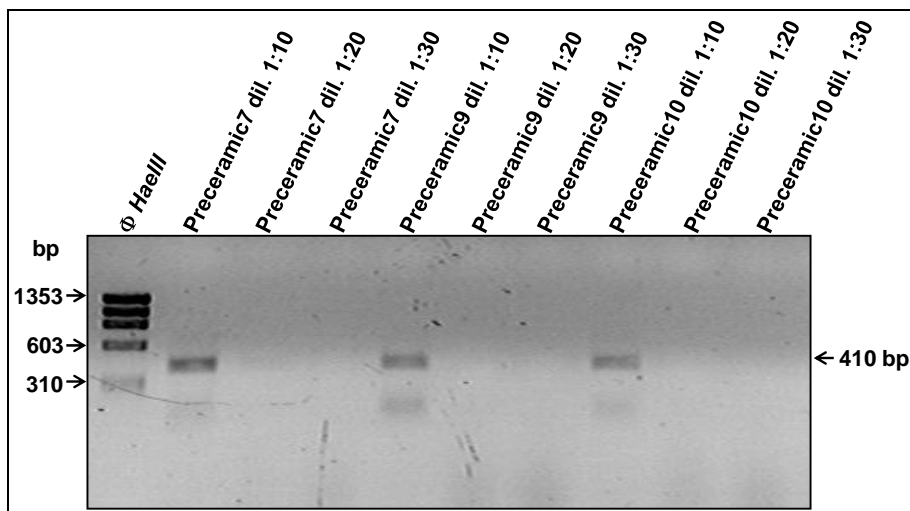

Fig. 9. Amplification of mtDNA HVI segment of 410 bp with specific primers (L15975-15996 and H16380-16398) using aDNA from pre-ceramic samples extracted with phenol-chloroform-isoamyl alcohol procedure followed by treatment with Chelex. Lanes 1 and 10, molecular weight markers φ. Lanes 2-4 pre-ceramic 7 diluted 1:10; 1:20 and 1:30; lanes 1-7, pre-ceramic 9 same dilutions as in lanes 2-4; lanes 8-10, pre-Ceramic 10, same dilutions as lanes 2-4.

When extracting DNA from small, degraded forensic samples or degraded ancient samples, the final concentration of DNA is usually too low for subsequent amplification. Consequently, we concentrated the aDNA samples extracted by the phenol-chloroform-isoamyl alcohol with filter units (lanes 2-3 and 4-5). Figure 10 shows clearly how aDNA was amplified using the DNA that was concentrated with the filter units. Furthermore, the washing buffer did not show any amplification, confirming in part that we do not have DNA contaminating our assays.

Next, we wanted to test all these methods with tissue from different mummies and determine the differences in using internal tissue and skin. From our results, we observe that when the mummy tissue is compact and from an internal organ the quantity of aDNA is very high compared with that obtained from bone samples. In addition the aDNA from the internal tissue was better as far as content is concerned. We had the opportunity to obtain DNA from the internal tissue of the mummy called Pepita that was intact and had no contamination by contemporary DNA. We were able to amplify the HVI segment using the specific primers for a mtDNA fragment of 445 bp (L15975-15996 and

H16401-16420, Figure 11, lane 8) and a fragment of 281 bp (L15975-15996 and H16236-16255, Figure 11, lane 1) or to amplify the specific second segment of HVI (L16140-16159 and H16401-16420, lane 5). The aDNA from this mummy was very well conserved. We have previously published DNA extraction from Mexican mummies with different origin and age (López-Armenta et al., 2008; Bustos-Ríos et al., 2008; Herrera-Salazar et al., 2008).
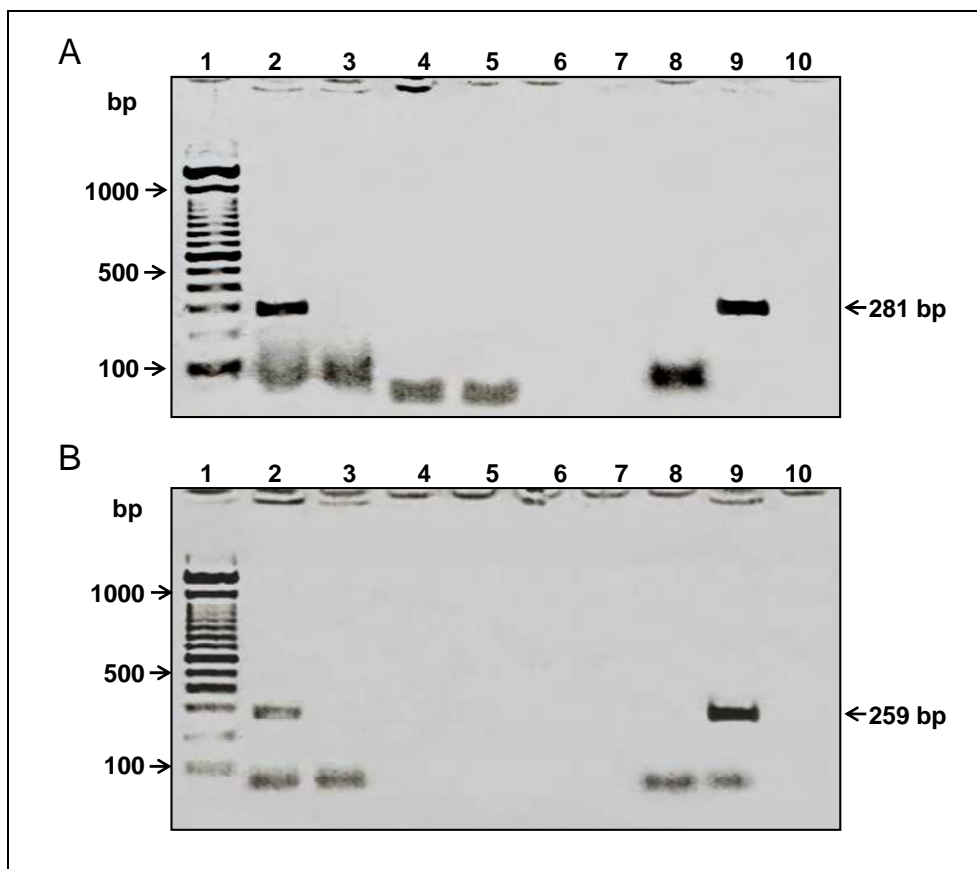

Fig. 10. Amplification of mtDNA HVI segments with the specific primers L15975-15996 and H16236-16255 producing a fragment of 281 bp (A); and L16140-16159 and H16380-16398 producing a fragment of 259 bp (B).

To examine the relationships between mtDNA lineages found in ancient and contemporary Native Americans, phylogenetic trees were constructed with the Jukes-Cantor method, and the distances were obtained from a neighbour joining algorithm and optimised for maximum likelihood, using Hy-Phy software (Kosakovsky-Pond et al., 2005). A total of 290 bp (nucleotides 16104–16394) of the HVI common to all sequences were used for these

analyses. Sequences from Monte Alban and Teotihuacán from this study as well as those from the Oneota population were clustered in the haplogroup D linage.
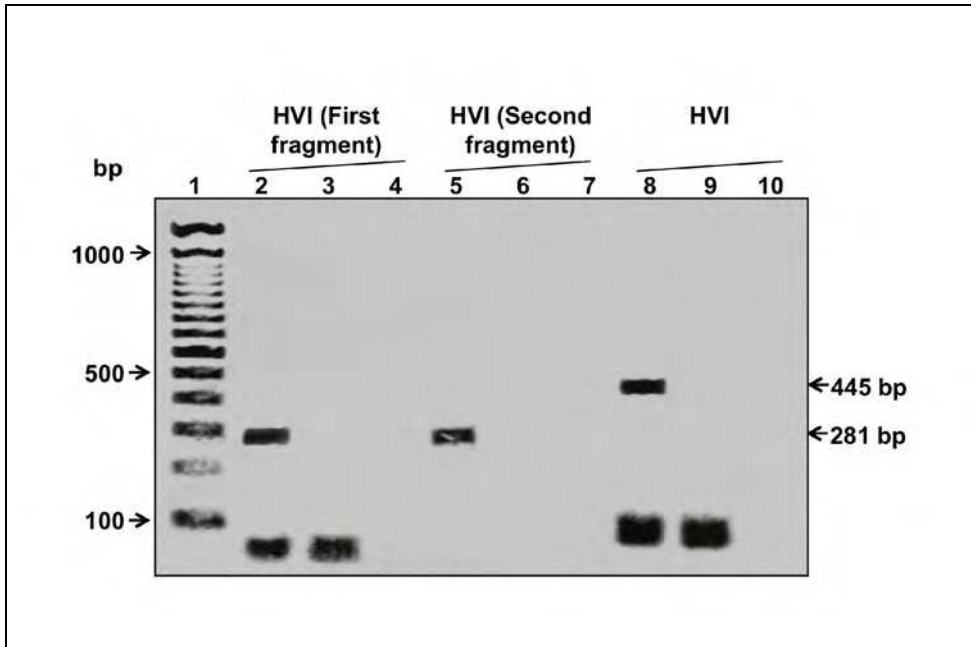

Fig. 11. Amplification of aDNA extracted from a Mexican mummy.

Haplotype network analyses were carried out on 290 bp of the mtDNA HVI from nucleotides 16104 to 16394. These networks were constructed using the Network package, v4.5.1.0 (Fluxus Engineering). These analyses included sequences from our own work and from other authors. The accession numbers of the sequences included in this network analyses were mentioned in the data analysis section. The pre-Hispanic DNA sequences included two ancient sequences from the prehistoric Oneota population (Stone and Stoneking, 1998), two sequences from Monte Alban, Oaxaca, Mexico and one from Teotihuacán, Mexico. Interestingly, the haplotype from the Oneota sequence may be derived from the Teotihuacán haplotype. The sequences from Monte Alban were grouped in the same haplotype as the more frequent haplotype from Native American populations. These results showed the potential to know the relationship among all Mexican pre-Hispanic populations or other populations as well as some haplotypes that were lost through the time.

It is important to mention that we never observed contaminant fragments with the specific HVR-1 mutations carried by the excavators or the geneticists. Therefore we are confident that following the procedures recommended by previous authors and our laboratory generates authentic sequences. Problems arise when the samples come

from museums or collections where the researcher does not know how they were managed. In these conditions, additional controls are recommended for all of the procedures.
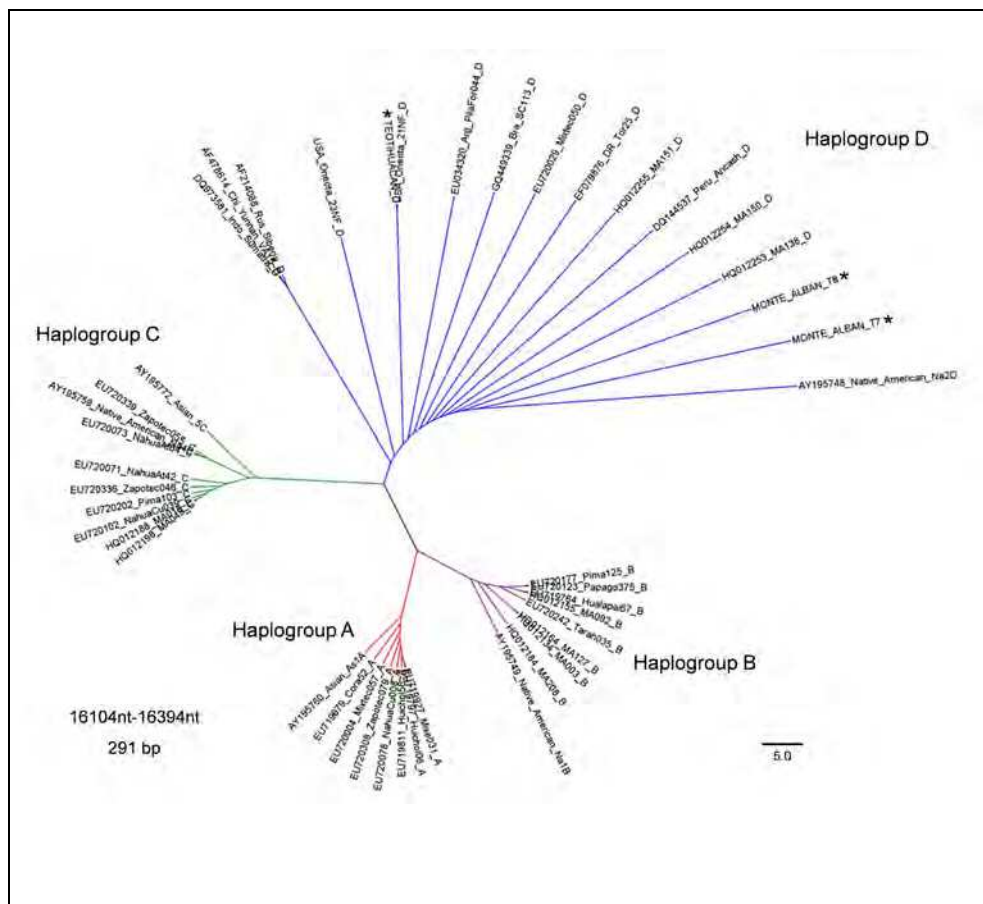

Fig. 12. Phylogenetic analyses of American native populations including five sequences from samples of pre-Hispanic populations. Tree of Native American and ancient pre-Hispanic Amerindian, constructed with the Jukes-Cantor method, and the distances were obtained from a neighbour joining algorithm and optimised for maximum likelihood using Hy-Phy software (Kosakovsky-Pond et al., 2005). The lanes in different colours indicate the haplogroup designation of lineages. Sequences of this study are marked with an asterisk.
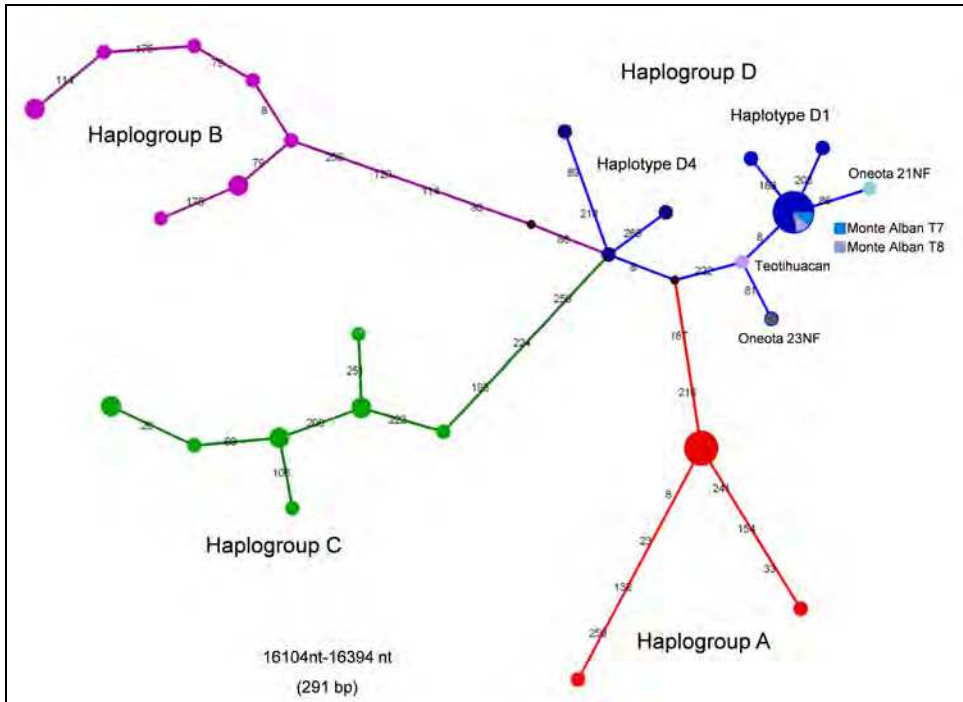
Fig. 13. Haplotype network of American native populations including five sequences from samples of pre-Hispanic populations. Each haplotype is represented by a circle in which the square radius (surface) is proportional to its population frequency. Circle colours show the site location as represented by the indicated colours. Dark circles without number in the network indicate mutational steps between haplotypes (theoretically extinct or unrepresented in the sample). Numbers between haplotypes represent mutational steps.

5. Conclusion

This review offers a direct overview of the different methods of aDNA extraction, including all special conditions needed in the laboratory to avoid contamination by contemporary DNA. It reveals the complexity involved in demonstrating the authenticity of human aDNA because the risk of contamination is very high. However, exogenous DNA contamination can be avoided if the necessary care is taken. In our experience and the experience of other laboratories, obtaining the ancient sample with coat, gloves and mask, and maintaining it in sterile conditions without human contact reduces the chances of sample contamination. It is also very important to test all reagents to verify that they are free of contemporary DNA. In addition, we also recommend performing negative control PCR experiments with at least 45 cycles to convincingly demonstrate the absence of contemporary DNA contamination. In our experience the best method to purified aDNA is phenol-chloroform-isoamyl alcohol with concentration using Amicon® Ultra-0.5 30 kDa columns (Millipore, Billerica, USA) or the Silica gel method using the QIAquick (Qiagen) columns. We also prefer to include the EDTA in the extraction buffer to optimise the aDNA extraction. This is supported by recent

publications that have demonstrated that some DNA may be lost during decalcification (Campos et al., 2011). It is also important to keep DNA at -70°C in aliquots to maintain its integrity. Maintaining bone tissue samples at -70°C during aDNA extraction is useful to avoid additional DNA degradation. In our point of view, the best method will be that containing the least sample manipulation because this will avoid DNA contamination. Finally, there will be always risk of contamination by contemporary human DNA; however, next generation sequencing methods do provide a greatly improved means of measuring the degree of contamination in a sample.

Sequencing of the PCR products from aDNA as well as phylogenetic and network analyses of remains from America would allow testing of the hypotheses concerning single versus multiple waves of migration to the New World. This analysis will also reveal new haplotypes that were lost through time because not all migrations were successful in terms of leaving descendants among contemporary populations. Furthermore, the development of next generation sequencing is revolutionising aDNA research. The examples presented in Figure 13 and 14 display the relationship between the Oneota sample and that from Teotihuacán showing different haplotypes. There were also two ancient samples from Monte Albán that were grouped with the more frequent haplotype in the D1 haplogroup. Further analysis of more pre-Hispanic human samples will give us more detailed information about the history of these populations.

6. Acknowledgment

7. References

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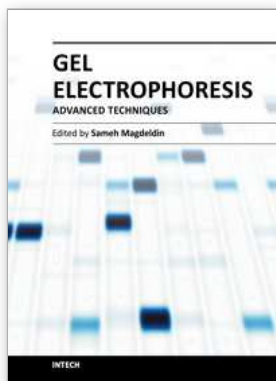
Gel Electrophoresis - Advanced Techniques

Edited by Dr. Sameh Magdeldin

As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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