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Improved coprolite identification in Patagonian archaeological contexts

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In the present work, we report a molecular approach that contributes to determine the zoological origin of unidentified coprolites from Patagonia. Three coprolites with morphological characters attributable to human or carnivorous, from Patagonian archaeological sites, were analyzed. Molecular analysis was conducted following the Authenticity Criteria to Determine Ancient DNA (aDNA) sequences. Two replicates of 398 and 521 coprolites were amplified. Specific fragments of *cytb* and *nadh5* genes were successfully sequenced. No PCR positives were reached to any aDNA replicates of coprolite 246. In the present work mammal coprolites aDNA between 2,700–5,100 years old were isolated. These results allowed us to establish coprolites identity as *Puma concolor*. This is the first genomic information produced in an Argentinian lab based on aDNA from Patagonian coprolites. In consequence, the present study would open the way to future paleogenomics studies in archaeological sites of the region.
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1. Introduction

Coprolites are dehydrated or mineralized feces from ancient fauna. These ancient feces have been preserved over time. In Holocenic archaeological contexts coprolites are dehydrated feces and they have mainly been found in caves, rock shelters and in open-air sites but with low frequency. Coprolites have been reported being part of caves or rock shelters floor but frequently there are other deposits associated to feces, as human latrines (e.g., Pike, 1968; Moore, 1981; Fernandes et al., 2005), rodent middens (Fugassa, 2014) and feces from mummified remains, denominated enteroliths. Palynological, botanical, zoological, entomological, molecular and biochemical studies have been performed to recover information about diet (e.g., Reinhard et al., 1992; 2007; Bon et al., 2012), sex (Sutton et al., 1996; Rhode, 2003), identification (Poinar et al., 2003), phylogeny (Bon et al., 2012), health and culture (e.g. Reinhard, 1988; Faulkner et al., 2000; Bouchet et al., 2003; Santoro et al., 2003) of its defecator.

In Argentina, coprolites have been analyzed in palynological (D'Antoni and Togo, 1974; Velázquez et al., 2010), botanical (Martínez and Yagueddú, 2012) and parasitological (e.g., Fugassa, 2006; Beltrame et al., 2010; Sardella and Fugassa, 2009; Taglioretti et al., 2015) studies. However, coprolite analysis based on ancient DNA (aDNA) has not yet been conducted in Argentina. Knowledge about coprolites zoological origins is critical to complement biological and cultural analysis obtained in Patagonian sites. Misdiagnosis could distort subsequent paleoecological and anthropological inferences.

Morphological identification of coprolites involves observation of macroscopic features, such as shape, size, weight, smell and color of the rehydrated solution (Chame, 2003; Bryant and Dean, 2006; Jouy-Avantin et al., 2003). However, characters as smell and color depend on the observer and shape, size and weight are subject to taphonomic processes that upset coprolites morphology. Diet content and specific parasites have contributed to the identification of the defector animal (Reinhard, 1992; Linseele et al., 2010, 2013).

The identification of feces is a problem in both wildlife and in archaeology studies. Coprolites have been associated with hunter-gatherers in archaeological contexts of Patagonia. In archaeological sites of *Cerro Casa de Piedra* (Santa Cruz Province, Argentina) a large number of coprolites were found. In some cases, coprolites zoological origin could not be defined. The difficulty to distinguish between hunter-gatherer and carnivore coprolites hindered the correct interpretation of the obtained parasitological evidences. In this sense, molecular analysis of coprolites is a useful tool. This method is based on identification of DNA from sloughed colonic

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epithelial cells (Albaugh et al., 1992). Mitochondrial DNA contains informative regions to allow discriminate between mammalian carnivores (e.g. Farrell et al., 2000; Roques et al., 2011; Naidu et al., 2012). In the last years, genomic information has contributed to identification of current and ancient feces. Ancient DNA (aDNA) sequencing advances have produced new information that complement morphometric features (e.g. Rasmussen et al., 2010; Bon et al., 2012; Caragiulo et al., 2014; Metcalf et al., 2016). In the present work, we report a molecular approach that helps to define the zoological origin of unidentified coprolites from archaeological sites of Patagonia.

2. Materials and methods

Three coprolites with morphological characters attributable to human or carnivorous, from *Cerro Casa de Piedra,* were analyzed. *Cerro Casa de Piedra* is a volcanic hill located in the National Park Perito Moreno to the west of Santa Cruz province, Argentina. Coprolite 398 was extracted from layer II of cave 5 (CCP5), dated in 2,740 ± 100 years (yr) before present (B.P). CCP5 site is a deep cave containing 7 levels and the human occupation takes places in three periods *ca*. 6,780-6,540 yr B.P., *ca*. 5,170-4,330 yr B.P. and *ca*. 2,740-2,550 yr B.P (Aschero, 1996). Coprolite 398 (Fig. 1A) showed a dark brown color and a large number of light brown short hairs and it exhibited an intense fecal odor after rehydration. According to morphological characters and its maximum width of 49.3 mm, coprolite 398 was attributable to *Puma concolor*, *Panthera onca* or human because its diameter greater than *Pseudalopex culpaeus* feces, the highest canid in the area (Cornejo Farfán and Jiménez Milón, 2001). Coprolites 521 and 246 came from cave 7 (CCP7), layer IV dated in 5,120 ± 90–3,970 ± 80 yr B.P and layer XVII/XVIII, dated in 9,100 ± 150–10,530 ± 620 yr B.P, respectively. CCP7 archaeological site displays a stratigraphic sequence of 19 levels showing human occupation between *ca.* 9,700 ± 100 and 3,600 ± 70 yr B.P (Aschero, 1996; Civalero and Aschero, 2003). Coprolite 521 (Fig. 1B) had four extremely calcareous fragments with similar aspect to felids previously found in CCP5 site (Fugassa et al., 2009). Smell of rehydrated fragments was compatible with fox and felids but diameter was higher, 34.3 mm, exceeding usual diameter of fox coprolites. Coprolite 246 (Fig. 1C) was dark brown, no recognizable odor after rehydration was detected. There were no identified macroscopic organic remains. Morphology and diameter of 27.8 mm suggested that this coprolite could be attributed to human.

Molecular analysis were conducted following the Authenticity Criteria to Determine Ancient DNA Sequences (Hofreiter et al., 2001; Willerslev and Cooper, 2005; Fulton, 2012). Special reagents for ancient molecular analysis were used. aDNA extraction, PCR, electrophoresis; PCR products purification and aDNA sequencing were carried out in separate places. Four replicates of 200–300 mg of each coprolite were analyzed. aDNA extraction was performed by using GENECLEAN FOR ANCIENT DNA™ KIT (MP Biomedicals). DeHybernation solution A (guanidine-based solution) and homogenization Matrix/Tubes were selected to lysis protocol following manufacturer instructions. aDNA was eluted with 50 μl of DNA-free elution solution. Finally, aDNA solutions were concentrated by Genomic DNA Clean & Concentrator™-10 (Zymo Research) in a final volume of 30 μl.

Molecular identification was first tested by PCR amplification using specific carnivores and felids primers to avoid misdiagnosis by contamination of aDNA with current human DNA from different sources reducing the risk of false positives.

In consequence, two fragments of mitochondrial aDNA, one of 146 bp of *cytochrome b* (*cytb*) gene from carnivores (among them humans) (Farrell et al., 2000) and other of 160 bp of *nicotinamide adenine dinucleotide dehydrogenase subunit 5* (*nadh5*) gene from felid species (primer pair NAD5C2-F; NAD5C2-R) (Roques et al., 2011) were selected to amplify aDNA from coprolites. PCR reactions were performed in a final volume of 12.5 μl containing 1 μl of 10-fold dilution of DNA sample, 200 μM of each dNTP (ThermoScientific), 0.4 μM of each primer and 1 units of AmpliTaq Gold® DNA Polymerase (Apply biosystems, Life Technologies) in 10X PCR Buffer II, 2 mM of $MgCl_2$ and 5% of DMSO (Sigma-Aldrich). The PCR conditions were as follows: an initial denaturation step (95 °C for 10 min), 40 cycles at 94 °C for 30 s (denaturation), 50 °C for 30 s (annealing), and 72 °C for 45s (extension), and 72 °C for 5 min (final extension). A negative control was included in all PCR experiments. Duplicates of the specific fragment were sequenced in forward and reverse sense and chromatograms were analyzed using BioEdit v7.2.0 (copyright © 1997–2013, Tomm Hall, Ibis Biosciences). The consensus sequences obtained were compared with the GenBank sequences by using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Center for Biotechnology Information (NCBI). Sequences were aligned using the multiple alignment tool: Clustal W2.0.9 (Larkin et al., 2007). Pairwise analysis was performed between sequences and identity percentage was determined by using LALIGN (Expasy Bioinformatics Research tools).

3. Results and discussion

Two replicates of 398 and 521 coprolites were amplified. Specific fragments of *cytb* and *nadh5* genes were successfully sequenced. No PCR positives were reached to any aDNA replicates of coprolite 246. This coprolite had been attributed to human according to morphometric characters, but *cytb* amplification could not be reached. However, assignment of this coprolite to human could not be ruled out, DNA could be damaged due to the age of the sample (9,100 ± 150–10,530 ± 620 yr B.P).

A consensus nucleotide sequence of 170 bp of *cytb* was obtained to coprolite 398. BLASTN analysis showed 98% of identity with Brazilian (Accession number: KC567527.1) and Northeast Argentinian (KC567478.1) sequences (Evalue 2e-58). A 97% of identity (Evalue 7e-68) with North American *Puma concolor cytb* sequence (AH014071.2). These results confirmed the zoological origin of coprolite complementing the morphometric study. On the other hand, BLASTN of 168 bp consensus of *cytb* sequence of coprolite 521 brought an identity of 99% (Evalue 2e-66) with sequences from Brazil (KC567527.1) and Argentina (KC567478.1). A 98% (Evalue 4e-70) with North American *Puma concolor* sequence (AH014071.2). Previous mitochondrial DNA analysis has separated pumas in three groups: North American, Central American and South American (Caragiulo et al., 2014).

Pairwise analysis of *cytb* sequences between 398 and 521 coprolites showed an identity of 99.4%. These coprolites were collected from different archaeological sites but both are just 400 m from each other. On the other hand, comparison of 398 and 521 sequences with *cytb* sequence from *Puma concolor* skin hairs from fur made by Selk'nam (Santa Cruz province, historical time) (Petrigh and Fugassa, 2015) showed a 98.8% of identity with 398 coprolite sequence and 98.2% with coprolite 521 sequence. *Cytochrome b* gene evolution has been widely studied, it is relatively constant among large terrestrial mammals fragment (Irwin et al., 1991) and this agree with results of *cytb* sequences of different ages. Nevertheless, *cytb* gene is informative and useful for species identification because it displays higher interspecies variation even studying short fragments (Tobe et al., 2009). In this sense, sequences of this work showed an 87% of identity with *cytb* sequence of *Panthera onca* (KP202264.1).

In addition, *nadh5* sequence allows to confirm *cytb* results, a 159

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