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ScienceDirect
International Journal for Parasitology 36 (2006) 1419–1425
www.elsevier.com/locate/ijpara

SL1 RNA gene recovery from *Enterobius vermicularis* ancient DNA in pre-Columbian human coprolites ☆

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Received 10 May 2006; received in revised form 14 July 2006; accepted 17 July 2006

Abstract

Enterobius vermicularis, pinworm, is one of the most common helminths worldwide, infecting nearly a billion people at all socio-economic levels. In prehistoric populations the paleoparasitological findings show a pinworm homogeneous distribution among hunter-gatherers in North America, intensified with the advent of agriculture. This same increase also occurred in the transition from nomad hunter-gatherers to sedentary farmers in South America, although *E. vermicularis* infection encompasses only the ancient Andean peoples, with no record among the pre-Colombian populations in the South American lowlands. However, the outline of pinworm paleoepidemiology has been supported by microscopic finding of eggs recovered from coprolites. Since molecular techniques are precise and sensitive in detecting pathogen ancient DNA (aDNA), and also could provide insights into the parasite evolutionary history, in this work we have performed a molecular paleoparasitological study of *E. vermicularis*. aDNA was recovered and pinworm 5S rRNA spacer sequences were determined from pre-Columbian coprolites (4110 BC–AD 900) from four different North and South American archaeological sites. The sequence analysis confirmed *E. vermicularis* identity and revealed a similarity among ancient and modern sequences. Moreover, polymorphisms were identified at the relative positions 160, 173 and 180, in independent coprolite samples from Tulán, San Pedro de Atacama, Chile (1080–950 BC). We also verified the presence of peculiarities (Splicing leader (SL1) RNA sequence, spliced donor site, the Sm antigen biding site, and RNA secondary structure) which characterise the SL1 RNA gene. The analysis shows that the SL1 RNA gene of contemporary pinworms was present in pre-Columbian *E. vermicularis* by 6110 years ago. We were successful in detecting *E. vermicularis* aDNA even in coprolites without direct microscopic evidence of the eggs, improving the diagnosis of helminth infections in the past and further pinworm paleoepidemiological studies.

Keywords: Ancient DNA; *Enterobius vermicularis*; Coprolites; 5S rRNA spacer; Splicing leader RNA gene; Paleoparasitology; Polymerase chain reaction

1. Introduction

For the past two decades molecular biology techniques have been used to study DNA recovered from archaeological remains or preserved biological material, establishing the research in ancient DNA (aDNA) (Higuchi et al., 1984; Brown and Brown, 1994; Herrmann and Hummel, 1994; Hofreiter et al., 2001a; Marota and Rollo, 2002; Pääbo et al., 2004). The aDNA research introduced one more possibility of studying human and infectious diseases within an evolutionary perspective. According to Drancourt and Raoult (2005) reliable diagnoses were done through aDNA analysis focusing on pathogens such as the bacterias *Mycobacterium tuberculosis* (Crubézy et al., 1998; Rothschild et al., 2001; Zink et al., 2003), *Mycobacterium leprae* (Haas et al., 2000; Montiel et al., 2003; Donoghue et al., 2001), *Treponema pallidum* (Kolman et al., 1999)

☆ Nucleotide sequences reported in this paper are available in the GenBank™ database under accession numbers: AY234771–AY234784.

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0020-7519/$30.00
doi:10.1016/j.ijpara.2006.07.005

and *Yersinia pestis* (Raoult et al., 2000; Drancourt et al., 2004); the parasitic protozoan *Plasmodium* sp. (Sallares and Gomzi, 2001) and more recently *Trypanosoma cruzi* (Guhl et al., 1999; Aufderheide et al., 2004); and the parasitic nematode *Enterobius vermicularis* (Iniguez et al., 2003a).

Enterobius vermicularis, pinworm, is one of the most common helminths worldwide, infecting nearly a billion people at all socio-economic levels and is known to have a major impact on the well-being of infants (Lukes et al., 2005). Parasite transmission has no environmental restrictions and the parasite can be transmitted from host to host without an obligatory stage in soil or intermediary hosts. It is considered that the human–*E. vermicularis* relationship originated in pre-hominid times, having evolved in Africa and dispersed to other continents through pre-historic human migrations (Ferreira et al., 1997; Hugot et al., 1999). Paleoparasitological findings and the parasite biological cycle suggest that pinworms crossed the Bering Land Bridge with human hosts during the first migratory movements into the Americas. However, transpacific routes have also been postulated (Ferreira et al., 1997).

The presence of pinworm eggs was shown in a 10,000-year-old human coprolite from Utah, USA, one of the oldest human coprolites found (Fry and Hall, 1969). Mummies and coprolites from several North American archaeological sites were positive for pinworm infection (Reinhard, 1990; Gonçalves et al., 2003), delineating a homogeneous distribution among hunter-gatherers in North America, intensifying with the advent of agriculture. This same increase also occurred in the transition from nomad hunter-gatherers to sedentary farmers in South America. *Enterobius vermicularis* infection encompasses the ancient Andean peoples, with no record among the pre-Colombian populations in the South American lowlands. Pinworm eggs were observed in coprolites from localities in Chile dating from 4100 BC (Before Christ) to 800 AD (Anno Domini) (Araujo et al., 1985; Ferreira et al., 1989); in Peru dating from 2277 ± 180 BP (Before Present) (Patruco et al., 1983) and in pre-Columbian human remains from Argentina (Zimmerman and Morilla, 1983). Old World data on this subject is curiously scarce (Bouchet et al., 2003). Herrmann (1985) found *E. vermicularis* eggs in Roman latrines and Horne (2002) recorded eggs in an Egyptian mummy.

Microscopic examination is useful in paleoparasitological diagnosis only when the recovered specimens are of good quality. Consequently, the prevalence of pinworm infection in ancient populations may have been underestimated (Reinhard, 1990; Araujo and Ferreira, 2000). Therefore, a molecular biology approach provides a specific and sensitive diagnostic tool and the opportunity to access a parasite's ancient genetic information. The 5S rRNA intergenic spacer was successfully used as a PCR target for *E. vermicularis* diagnosis in Amerindian coprolites (Iniguez et al., 2003a).

In order to verify the retrieval of *E. vermicularis* aDNA sequences directly from human coprolites and as well to investigate genetic diversity of pinworms and the relationship among geographically and temporally distinct populations of human pinworm, we have determined 5S rRNA spacer DNA sequences from 27 pre-Columbian coprolites that range in age from about 1100 to at least 6110 years and originate from four different North and South American archaeological sites. We have also characterised the presence of the SL1 RNA gene in the ancient pinworm populations.

2. Materials and methods

2.1. Coprolites and paleoparasitological analysis

Twenty-seven coprolites from Chilean and United States archaeological sites, previously examined microscopically for helminth eggs and larvae by microscopic techniques (Gonçalves et al., 2003) were used for aDNA extraction. Information about the coprolite samples is found in Table 1. The human origin of coprolites was suggested by archaeological context and confirmed by the finding of parasites specific to humans.

One sample from each of the 27 coprolites was rehydrated by immersion in a 0.5% aqueous solution of trisodium phosphate for 72 h, following the technique of Callen and Cameron (1960). The material was submitted to spontaneous sedimentation following the technique proposed by Lutz (1919). A portion of sediment was used for microscopic examination. The material was placed on a slide

Table 1
Coprolite locality, data, morphological and molecular paleoparasitological diagnosis results from *Enterobius vermicularis*

Archaeological site	Date	References	No.[a]	PA[b] (%)	PCR[c] (%)
Tiliviche, Iquique, Chile	4110–1950 BC	Araujo et al. (1985), Gonçalves et al. (2003)	2	50	50
Tulán, San Pedro de Atacama, Chile	1080–950 BC	Ferreira et al. (1989)	20	55	20
Caserones, Tarapaca Valley, Chile	400 BC–800 AD	Araujo et al. (1985)	2	50	50
Antelope House, Arizona, USA	900 AD	Reinhard (1990)	3	33.3	100
		Iniguez et al. (2003a)	27	51.8	33.3

BC: Before Christ; AD: Anno Domini.
[a] The total number of coprolites analysed.
[b] Paleoparasitological analysis. The percent of *Enterobius vermicularis* positive samples by microscopy assay.
[c] The percent of positive samples by PCR assay.

and examined for the presence of parasites. Twenty slides for each sample were examined at magnification of 100× and 400×.

2.2. Precautions in preventing contamination by modern DNA

Throughout the research, we implemented standard procedures for aDNA work in order to avoid contemporary DNA contamination and thus obtain authentic ancient sequences (Hofreiter et al., 2001a; Marota and Rollo, 2002; Drancourt and Raoult, 2005). Ancient DNA was manipulated in the Paleoparasitology Laboratory (ENSP/FIOCRUZ) and later reproduced at the Molecular Genetics Laboratory (IOC/FIOCRUZ), geographically distant from each other. Non-disposable tools and equipment was frequently pretreated with 2.5% NaOCl and work areas were irradiated by ultraviolet light for some hours. Handling of coprolite samples, aDNA extraction and purification were carried out in a physically environment separate from PCR and electrophoresis. DNA extractions were performed in a horizontal laminar flow equipped with ultraviolet germicidal lamps. Pipette filter tips and exclusive pipettes were used at each stage. All extraction reagents and primer solutions were separated into small aliquots. Negative controls, without coprolite samples or aDNA, were included in each step. Positive PCR controls were not included. All PCR-positive results were reproduced from the DNA extraction step in a second laboratory, and the aDNA sequences were determined by direct sequencing of both amplicons and clones.

2.3. DNA extraction

Coprolite samples were exposed to 254 nm of u.v. light at a distance of 50 cm for 10 min. Fine sheets of the surface of coprolites were eliminated and the core was ground. Samples of 0.5–2.0 g were hydrated at room temperature for 3 days in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 8.0) or 0.5% trisodium phosphate aqueous solution. Sediments of 150 μl were treated with 400 μl digestion buffer (NaCl 100 mM, Tris–HCl 50 mM, SDS 1%, EDTA 50 mM, pH 8.0), 20 μl dithiothreitol (DTT) 1 M, 60 μl proteinase K 10 mg/ml (Invitrogen) and 100 μl SDS 10% (Iniguez et al., 2003a). The reactions were incubated at 55–60 °C for 72 h with occasional homogenization. DNA extraction was performed by phenol/chloroform/isoamyl alcohol (25:24:1) and purified using a silica resin column Glass Max DNA Isolation Spring Cartridge System (Invitrogen).

2.4. PCR

PCR primers targeting a fragment of *E. vermicularis* 5S rRNA spacer region, which contained the Splicing Leader 1 RNA (SL1 RNA) gene were used. The protocol specificity was first tested in DNA extracted from modern human feces and *E. vermicularis* experimental coprolites (Iniguez et al., 2002, 2003a). *Enterobius vermicularis* amplification was performed by nested PCR targeting a 420 and 198 bp fragment, respectively. Alternatively, the DNA was submitted to reconstructive polymerization pretreatment before PCRs (Golenberg et al., 1996; Iniguez et al., 2003b). The final volume of the PCR was 25 μl and included 20 mM Tris–HCl, 50 mM KCl (Invitrogen 10 X Buffer), 2 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphates (dNTPs), 1 mg/ml BSA and 500 ng of each primer. The reaction mixture was exposed for 30 min to u.v. radiation before the addition of 2.5 U of *Taq* polymerase (Invitrogen) and 50–100 ng DNA extract or 3–5 μl of PCR product. The reactions were subjected to an initial cycle of 5 min at 94 °C, followed by 40 cycles of 94 °C for 1 min, 50–55 °C for 30 s and 72 °C for 30 s in a programmable thermal controller PTC100 60v (MJ Research). Extraction and PCR negative controls were included for each sample or reaction. PCR products were submitted to electrophoresis in 1.5% and 2% agarose for 420 and 198 bp products, respectively. Gels were stained with ethidium-bromide and observed over a u.v. transluminator. In order to verify the presence of PCR inhibitors in the aDNA extracted from coprolites, human mtDNA fragments were also amplified by PCR using two approaches: under conditions described by Pääbo (1990) for 92, 121 and 471 bp DNA fragments, and conditions described by Handt et al. (1996) targeting a 184 bp amplicon.

2.5. DNA cloning, sequencing and sequence analysis

PCR products were cloned into a pGEM-T Easy Vector System (Promega) and clones were purified with a QIA prep Spin Miniprep Kit (QUIAGEN). PCR products were purified using a Qia quick PCR Purification Kit (QUIAGEN) and utilised in direct sequencing reactions with an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems-Perkin Elmer). Samples were sequenced on both strands in an ABI PRISM 377 Automated DNA Sequencer (Applied Biosystems-Perkin Elmer) as described by suppliers. Chromas program version 1.45 (School of Health Science, Griffith University, Queensland, Australia), Bio Edit version 5.0.9 (Department of Microbiology, North Carolina State University, USA), and the package of programs GCG (Genetic Computer Group, version 9,1, Madison Incorporation, WI, the USA) were used for the sequence analysis. The *E. vermicularis* ancient sequences were aligned with the *E. vermicularis* sequences available in GenBank U65495/U65496 from the USA and from Brazil (AF493944–AF493944).

2.6. Identification of spliced leader SL1 RNA gene and SL1 RNA secondary structure analysis

The SL1 nematode motif was identified by BLAST command whereas the spliced donor and the Sm antigen binding sites were localised manually (Blaxter and Liu, 1996; Dassanayake et al., 2001). The secondary structure of SL RNA was inferred by the mfold program version 3.1 (Michael Zuker, Rensselaer Polytechnic Institute; Zuker,

2003) and previous conditions used to analyse *E. vermicularis* SL RNA secondary structure (Iniguez et al., 2002).

3. Results

3.1. Molecular paleoparasitological diagnosis of E. vermicularis from coprolites

Nested PCRs were performed using DNA from coprolites as a substrate. We were successful in obtaining the 198 bp DNA fragments in 9/27 coprolites. However, the 420 bp target was not amplified, even when reconstructive polymerization pretreatment was used. No DNA signal was observed in negative PCR and extraction controls. Three samples (706, 716 and 719) that were negative for microscopic analysis yielded an *E. vermicularis* diagnostic amplicon (Table 1). Dilutions of aDNA samples and/or new aDNA purifications were carried out to prevent *E. vermicularis* false negative PCR Results. Human mtDNA was also tested and mtDNA fragments were retrieved from all coprolites except samples 384 and 721.

3.2. Ancient DNA sequences and the putative secondary structure of the SL1 RNA gene

Fourteen *E. vermicularis* sequences were obtained and comparison with the GenBank database revealed their identity to *E. vermicularis* 5S rRNA spacer (Fig. 1). The presence of the 22-nucleotide motif, corresponding to the SL1 RNA sequence in nematodes, which is in the opposite direction (Liu et al., 1996) of transcription from the 5S rRNA gene, was verified. The alignment of contemporary and ancient *E. vermicularis* sequences showed a high degree of conservation within this segment (Fig. 1). Ten sequences from all archaeological sites were found to be identical to *E. vermicularis* contemporary sequences. Two *E. vermicularis* sequences from Tulán, San Pedro de Atacama, Chile, had three nucleotide substitutions at the same positions. Sequences from coprolites collected in Caserones, Tarapaca Valley, Chile, were the same as the modern consensus, except for two clones that displayed one difference at a distinct position (Fig. 1).

In addition to the SL1 RNA motif, the canonical dinucleotide spliced donor and the *Sm* antigen binding sites, were identified in all aDNA sequences, which gives evidence to the presence of the SL1 RNA gene (Stratford and Shields, 1994; Blaxter and Liu, 1996; Favia et al., 2000; Dassanayake et al., 2001). The nucleotide substitutions observed in aDNA *E. vermicularis* sequences were three T → C transitions and two T/C → G/C transversions (Fig. 1). All these substitutions were found outside the conserved regions of the SL1 RNA gene.

The sequence 385B displays a substitution 28 nucleotides downstream from the SL1 RNA sequence and the

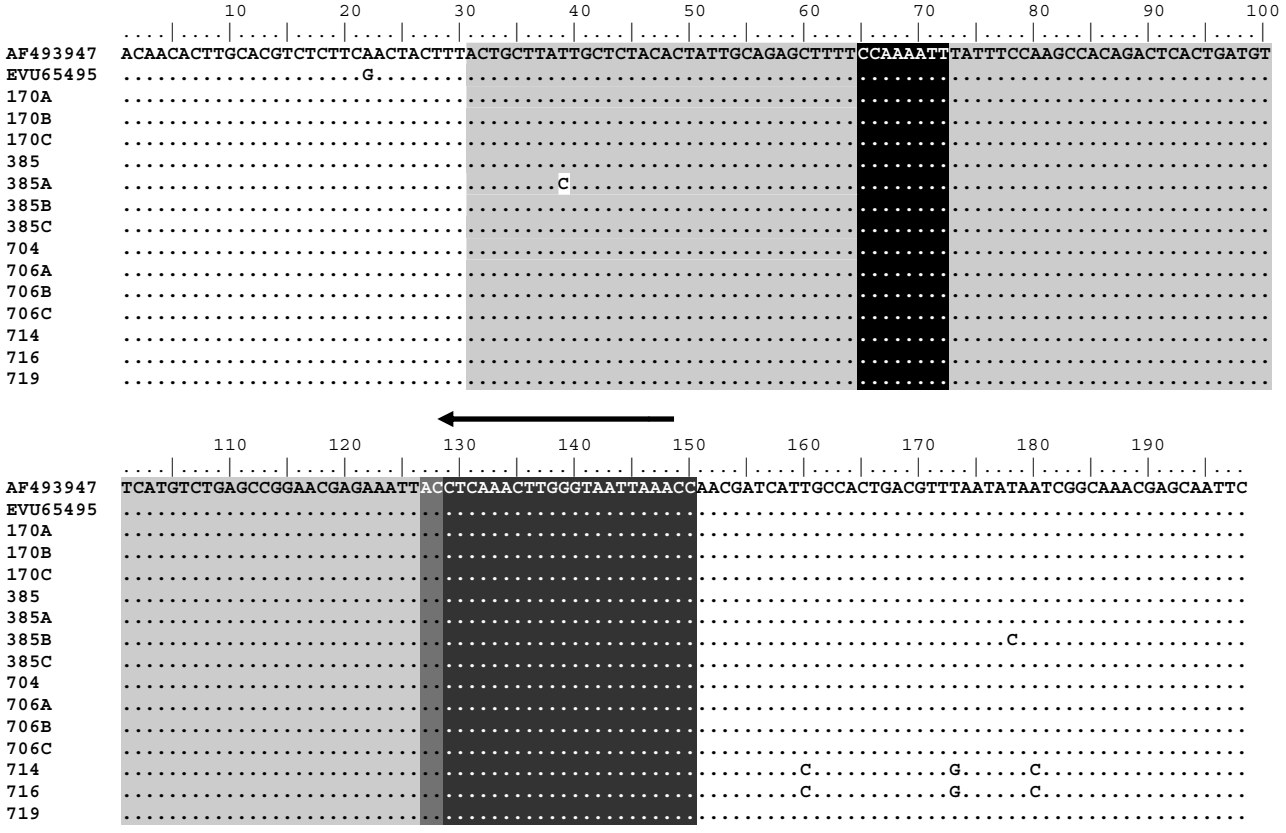

Fig. 1. Ancient DNA sequence alignment from *Enterobius vermicularis* intergenic 5S rRNA region. Areas shaded represent: light gray = putative primary sequence of *Enterobius vermicularis* spliced leader (SL) RNA gene; dark gray = the spliced donor site; black = the Sm antigen-binding site and the SL1 RNA sequence. The orientation of the SL1 RNA sequence is indicated by the arrow.

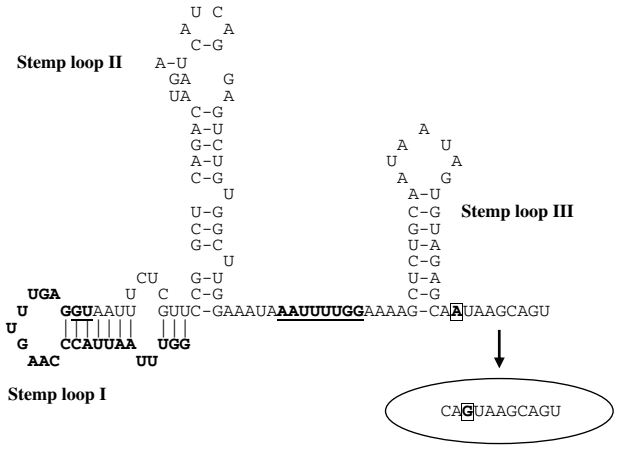

Fig. 2. The putative secondary structure of *Enterobius vermicularis* spliced leader 1 (SL1) RNA. The three stem–loops are indicated. The SL1 RNA sequence in stem–loop I is bolded. The spliced donor and the Sm antigen-binding sites are bolded and underline. The detail from the putative secondary structure of *Enterobius vermicularis* SL1 RNA from 385A ancient DNA sequence. The nucleotide changes are in boxes.

first substitution in sequences 714 and 716 occur 10 nucleotide downstream. The SL1 RNA secondary structure was determined for sequence 385, which has one substitution 26 nucleotides upstream the consensus sequence of *Sm* antigen binding site (Fig. 2). The remaining SL1 RNA gene sequences were identical to the modern *E. vermicularis* sequence and therefore their SL1 RNA gene secondary structures are maintained (Iniguez et al., 2002; Fig. 2). The potential SL1 RNA secondary structure from the 385A sequence was shown to be similar to the SL1 RNA gene three stem–loops previously described in modern *E. vermicularis*, other nematodes and trypanosomatids (Evans et al., 1997; Sturm et al., 1999; Evans and Blumenthal, 2000; Iniguez et al., 2002). The unique nucleotide substitution in sequence 385A does not interfere in the structure of the stem–loops since it is found in the linear strand downstream of the third stem–loop.

The remaining structure of both sequences was shown to be the same: the 22 nucleotides SL1 RNA exon molding the first stem–loop, the *Sm* antigen binding-site situated in the intron moiety, and the stem–loops II and III localised upstream and downstream of the *Sm* binding-site, respectively (Evans et al., 1997; Vandenberghe et al., 2001; Fig. 2).

4. Discussion

The 5S rRNA spacer region has been used for systematic, diagnostic and phylogenetic inferences in nematodes due to its variability in size and sequence (Stucki et al., 1993; Xie et al., 1994; Liu et al., 1995; Favia et al., 2000; Iniguez et al., 2002; Veronico et al., 2004; van der Giessen et al., 2005). In contrast with the variability of ribosomal spacers in other species, *E. vermicularis* display at most 1% difference in the nucleotide composition between contemporary North and South American isolates (Liu et al., 1995; Iniguez et al., 2002). An SL1 RNA gene was identified in the 5S rRNA spacer region from *E. vermicularis* aDNA. The sequences from all archaeological sites were identical to the consensus 5S rRNA spacer modern sequence. Interestingly, all nucleotide substitutions in the four *E. vermicularis* sequences from the Caserones and Tulán archaeological sites were outside the SL1 RNA gene. The high conservation of the 5S RNA spacer region in geographically and temporally distant samples revealed in this work is probably a consequence of selective pressure due to the presence of an essential gene in this region. aDNA studies have previously shown that 18S rRNA gene sequence recovered from Middle-Age *Ascaris* eggs is identical to moderns *Ascaris* sequences (Loreille et al., 2001). The DNA analysis of bacteria from pre-Columbian mummy coprolite (1170–980 AD) using the 16S rDNA gene, has also revealed a total or high similarity between modern and ancient sequences (Luciani et al., 2006).

The SL RNA secondary structure has a functional role in the *trans*-splicing process (Sturm et al., 1999; Xu et al., 2000; Nilsen, 2001). Analyses of structure–function of SL1 RNA in nematodes showed that stem–loop II and the Sm binding-site are crucial for *trans*-splicing (Denker et al., 1996; Evans and Blumenthal, 2000). Of the 22 nucleotides of the SL1 sequence that are totally conserved in all nematodes studied, the first 16 nucleotides are apparently fundamental for transcription and *trans*-splicing (Xie and Hirsh, 1998; Ferguson and Rothman, 1999; Evans and Blumenthal, 2000). The recovery of an *E. vermicularis* 5S RNA spacer from coprolites allowed the inference of the secondary structure for pinworm SL1 RNA ancient sequences. The parasite sequences retrieved from coprolites had the same SL1 RNA gene sequence and conformation as modern pinworms.

Damage in aDNA may cause the addition of an incorrect base during the PCR, but these misincorporations are unlikely to arise at the same point in independent samples (Poinar et al., 1998; Hofreiter et al., 2001b). Therefore, the polymorphism in samples 714 and 716 from Tulán, San Pedro de Atacama, Chile are authentic of Tulán *E. vermicularis.* The territory currently known as the San Pedro de Atacama region was an important pre-Columbian trade route from the Pacific coast to the Andes Mountains (Ferreira et al., 2000). If the substitutions identified in ~3000-year-old *E. vermicularis* are present in current pinworm populations from San Pedro de Atacama, Chile, these polymorphisms would be evidence of a different parasite lineage. On the other hand, the conservation and length of 5S rRNA spacer target sequences, did not allow any evolutionary inferences.

We have demonstrated the recovery of *E. vermicularis* aDNA sequences, directly from coprolites, opening new possibilities for studying helminth infections in the past. Moreover since human aDNA has been successfully retrieved from coprolites, our results provide one more approach to investigate the host–parasite relationship through time.

Acknowledgements

We thank the San Pedro de Atacama Archaeological Museum (Chile) for provided the archaeological samples. We also thank Koko Otsuki (FIOCRUZ/Brazil) technical assistance. This study was supported by grants from FIOCRUZ (Brazil), FAPERJ/FIOCRUZ (Brazil), CNPq (Brazil), CAPES (Brazil), and Fullbright Commission (USA).

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