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Clarifying prehistoric parasitism from a complementary morphological and molecular approach

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Abstract
This paper reports an approach to the identification of prehistoric parasitic infection, which integrates traditional morphological methods with molecular methods. The approach includes the strengths of each method while mitigating the limitations. Demonstrating the efficacy of this approach, we provide a case study from a 1400 year old desiccated fecal sample from La Cueva de los Muertos Chiquitos, archaeological site, near Rio Zape, Durango, Mexico. Traditionally prepared microscope slides were processed via microscopy and tentative ascarids were identified. Information regarding the parasites' developmental stage was recorded. DNA was then extracted directly from the slide material. From this DNA extract, a small segment of the 18S ribosomal RNA gene variant that is specific to *Ascaris*, and its phylogenetically close relatives, was targeted for PCR amplification and sequencing. Phylogenetic analysis of the DNA sequence best matched a member of physalopterids, rather than ascarids, with a single exception of a match to *Contracaecum spiculigerum*. Subsequent extractions, amplifications and sequencing of the original rehydrated coprolite material confirmed these results. The *C. spiculigerum* sequence represented a phylogenetic anomaly and subsequent analysis determined the sequence was an error in the BLAST database, likely attributable to misidentification of juvenile specimens prior to sequencing and submission. *Physaloptera* are a difficult genus to identify morphologically and can carry major health burdens. They may be underreported in humans, in part, because of morphological similarities to the more common human parasites belonging to ascarids. We conclude that integrating traditional morphological methods with molecular methods can help resolve this issue, in both contemporary and prehistoric populations.

Keywords: Ancient DNA, Archaeoparasitology, *Physaloptera,* Coprolites, *Ascaris, Contracaecum spiculigerum*

1. Introduction

Traditional parasitology methods, including archaeoparasitology (Reinhard, 1990), involve a microscopic examination of recovered parasite material to study diagnostic morphological characteristics of parasite life stages. Morphological identification is relatively straightforward and cost efficient. It requires little in the way of equipment or chemicals and can be completed in almost any setting, including a field lab. Morphological methods can provide information on the developmental stage of parasites. However, the resolution and precision of the taxonomic identification of parasites based on morphology is dependent on 1) whether the tissues observed are distinguishable between different taxa, 2) the level of preservation of parasite tissues. Egg morphology and homologous structures between larvae and adult parasites, including parasites from different taxonomic families, are frequently indistinguishable (Bott et al., 2009; Bryant and Dean, 2006; Reinhard and Bryant, 1992).

Molecular taxonomic identification can differentiate closely related genera bearing morphological similarities (Iniguez et al., 2003a, 2006; Oh et al., 2010) and may identify parasites within samples lacking intact diagnostic tissues. For these reasons, it has been suggested that the use of molecular methods can be applied directly to the coprolite material, limiting or replacing the need for traditional morphological approaches. (Iniguez et al., 2002, 2003a, 2003b, 2006; Leles et al., 2008; Oh et al., 2010). However, molecular approaches have their own limitations. In general, molecular identification is more time consuming and costly. It requires specialized acquisition and processing of samples, equipment, knowledge and skills. It also requires a specialized lab environment, especially when processing samples for ancient DNA analysis (Paabo et al., 2004).

Even with unlimited time and resources, molecular approaches require preserved DNA, and even when DNA preserves well, molecular data are unable to provide information regarding the developmental stage of the parasite.

Both approaches are constrained by available comparative data. The lesser known parasites have very few morphological references. Likewise, genetic sequences are biased toward the most commonly encountered parasites, and even these may be represented by only a single sequence.

Our protocol calls for a combined and modified methodology. This approach provides synergy, maximizing the benefits and minimizing the limitations of either method on its own. Our case study uses a coprolite sample from the archaeological site of La Cueva de los Muertos Chiquitos in Durango, Mexico to demonstrate benefits and future challenges of a combined methodology.

2. Methods

La Cueva de los Muertos Chiquitos is a rock shelter with excellent preservation, located approximately 50 feet above the Rio Zape in a cliff face (Brooks et al., 1962; Jiminez et al., 2012) (see Supplemental KML Map).

A trail connecting the Rio Zape site with other towns runs along the river and passes seven to eight feet below the cave entrance. The cave itself is accessed by finger and toe holds. The cave was first excavated in the 1960s and yielded a number of infant and adult burials, as well as well-preserved botanical and cultural material. Coprolites preserved by desiccation in the arid environment were also recovered. Adobe floors and walls provide evidence of human renovation of the cave interior for human habitation. Botanical and faunal evidence suggest that the inhabitants were an agricultural group, raising maize, beans and squash. They also gathered wild resources and hunted wild game for protein. Coprolites were excavated from a midden sealed beneath an adobe floor and in association with a number of human burials (Brooks et al., 1962). Archaeo-parasitological analysis of a number of these coprolites has recently been published (Jiminez et al., 2012). The coprolites have also been shown to have excellent DNA preservation for characterizing the ancient human gut (Tito et al., 2008, 2012).

Sample Zape 23, molecularly assigned to Native American Haplogroup B (Tito et al., 2012), was chosen for rehydration and morphological analysis. As part of the standard ancient DNA sample preparation protocol, the outer layer of bone and fecal material is generally removed to limit contamination. For example, studies of ancient human DNA from the coprolites could be confounded by modern human DNA on the sample's surface. However, some parasites such as *Enterobius vermicularis* may only be found on the exterior of the fecal bolus due to the nature of female egg-laying outside the rectum (Jiminez et al., 2012). Removal of the surface of the coprolite may remove evidence of this parasite. In an attempt to capture all potential parasites, we did not remove the outer layer of the bolus; instead, we reserved these subsamples "for parasite only" analyses. Approximately 1 g of coprolite material was removed from the original fecal bolus and clearly marked for use as a parasite only DNA extraction, to segregate them from other subsamples of the same coprolite.

2.1. Rehydration of "parasite only" subsamples

Homogenization and rehydration were completed in the University of Oklahoma's (OU) dedicated ancient DNA laboratory which includes positive pressure class 10,000 HEPA filtered ventilation. Researchers wore full sterile jumpsuits, goggles,

masks and double gloves. The lab was UVC irradiated prior to and after each work session. All workstations were bleached prior to and after the work session. Sterile scalpels were used to separate the subsamples.

The 1 g of dry fecal material was disaggregated using the sterile scalpel and mixed to homogenize the sample. For rehydration, we utilized Tris–EDTA pH 8 (TE) solution following the protocol used by Iniguez et al. (2003a). To each sample, 2 ml–5 ml of TE solution were added depending on the absorbency of the coprolite. The solution was then vortexed to further disaggregate and homogenize the sample. The samples were strapped to a slowly rotating orbiter and allowed to rehydrate for 72 h, samples were vortexed daily.

At the end of 72 h, 500 µl aliquots of both the aqueous and solid phases were transferred to 2 ml microcentrifuge tubes. The tubes were wrapped in plastic paraffin film and then sealed in double plastic bags for transport to the Veterinary Parasitology Laboratory at Oklahoma State University (OSU). The remaining rehydrated sample was then stored in the minus 20 °C freezer in the ancient laboratory

2.2. Morphological analysis

At the Veterinary Parasitology Laboratory, each aliquot was transferred to a 15 ml conical tube and Sheather's Sugar Solution was added until a reverse meniscus formed. A microscope slide cover slip was added to the top of each tube and the tubes were placed in a centrifuge. The samples were centrifuged for five minutes at 2500 rpm. The cover slips were lifted directly up at a 90° angle and immediately placed on a clean microscope slide. The slides were then transferred to a microscope and examined beneath 100× and 400× magnifications. Potential parasite eggs were noted. Additionally, insect fragments, pollen grains and plant materials were noted but were not analyzed for this study.

2.3. Extraction

The prepared microscope slides were transported back to the Molecular Anthropology Laboratories at OU and placed in the 4 °C refrigerator in the main laboratory. Using a buccal swab and molecular grade ddH20, each microscope slide was rinsed and swabbed to remove the fecal flotation material. The swab was then processed using the Mo Bio Ultra-Clean® Fecal DNA Isolation Kits according to the manufacturer's protocol with one minor modification: to facilitate lysis of durable parasite eggs we added a mechanical heat/freeze step to the Mo Bio extraction, by subjecting the samples to a cycle of heating and freezing (Leles et al., 2008). After 250 µl of sample were added to the Mo Bio bead tubes, the samples were heated for five minutes at 63 °C followed by five minutes in the minus 20 °C freezer and a final thawing step of five minutes at 63 °C.

Extraction of the original rehydrated samples were also performed on 25 µl aliquots using the Mo Bio Ultra Clean® Fecal DNA Isolation Kits in the dedicated ancient DNA lab in full protective gear and taking all routine ancient DNA precautions. An extraction blank was also processed in tandem with

the sample extraction; in the blank, water was substituted for sample material.

2.4. Amplification

A polymerase chain reaction (PCR) using *Ascaris* 18S primers (Table 1) was prepared with the following chemistry: 0.1 µl of 5U/µl Platinum Taq (Invitrogen 10966-018), 3 µl of 10X Platinum Taq buffer, 0.9 µl of 10 mM dNTP's, 1.5 µl of 50 mM $MgCl_2$, 1.8 µl of each 5 µM primer, 16.9 µl of molecular grade ddH20 and 4 µl DNA template. This PCR formula uses an increased amount of magnesium chloride following the published protocol of Loreille et al. (2001). We maintained this formula for all PCRs and all primer sets, previously published or designed as part of this study. Each PCR tube was individually capped and sealed prior to leaving the ancient lab for amplification. To provide a positive control by which to assess the success or failure of the PCR itself, 4 µl of modern *Ascaris* DNA, at 10 ng/µl, was then added to one tube only in the modern lab prior to being placed in the thermocycler. For the *Ascaris* primer pairs, the following thermocycler program was used: one cycle at 94 °C for 2:00 (initial denaturing), 60 cycles at 94 °C for 15 s (denaturing), 52 °C for 15 s (annealing), 72 °C for 15 s (extension) and one cycle of 72 °C for 5:00 (final extension). This PCR returned amplicons of the predicted size, ~99 bp and ~123bp, which were Sanger sequenced.

2.5. Sequence identification

The returned sequence data were trimmed and primers removed prior to inputting the samples in the US National Center for Biotechnology Information's Basic Local Alignment Tool (BLAST) using their BLASTN program on the whole NCBI refseq_genomic database. Scores were compared to *Ascaris* sequences for closeness of match. Additionally, scores with the highest coverage and identity were retained.

2.6. Cloning and sequencing

PCR product was then cloned following the TOPO TA protocol and using imMedia™ Kan Blue culture medium (Cat. No. 28236) and thirteen clones sent for sequencing. These data were trimmed, removing the M13 primers as well as the *Ascaris* primers, before submitting the sequences to the BLASTN program.

2.7. Replication

In order to test the replicability of our results, we performed two new extractions on the originally rehydrated material, submitting them to the same protocols as outlined above.

2.8. Additional primer design

We designed new primers to increase the length of our 18S sequence fragment. The same PCR chemistry was used with the exception of using 58 °C for the annealing temperature rather

Table 1. 18S gene primer sets. The initial PCRs were done using the *Ascaris* primers Asc6-9. The Physa18s primers were used in additional PCRs to expand the length of the sequence fragment.

Primer sets (18S Gene)	Primer sequence	Size	Initial or additional PCR	Annealing temp	Citation
Asc 6 F Asc7 R	CGAACGGCTCATTACAACAG TCTAATAGATGCGCTCGTC	~123bp	Initial	52 °C	Loreille et al., 2001
Asc 8 F Asc 9 R	ATACATGCACCAAAGCTCCG GCTATAGTTATTCAGAGTCACC	~99 bp	Initial	52 °C	Loreille et al., 2001
Physa18s243F Physa18s343R	TGAATAGCTCTGGCTGATCG CAACCATGGTAGGCACATAAAC	~100 bp	Additional	58 °C	This study

than the 52 °C. This resulted in a consensus sequence of ~190 bp for the 18S gene. There is a 28 bp gap, representing about 15% of the consensus sequence, which is attributed to difficulties in finding effective primers covering this region.

2.9. Phylogenetic tree construction

The Neighbor Joining algorithm (Saitou and Nei, 1987) was used to further assess the strength of the Rio Zape 23's match to physalopterids. We created a pool of published sequences for the 18S gene for *Ascaris, Contracaecum*, *Turgida and Physaloptera*. In Mega 5 (Tamura et al., 2011) we aligned the sequences and constructed a neighbor joining tree with 1000 bootstrap re-iterations. The evolutionary distances were estimated using the Maximum Composite Likelihood method (Tamura et al., 2004a; 2004b). To test the consistency of results, alternative tree building methods were constructed using Mega 5, specifically, Maximum Likelihood and Maximum Parsimony.

3. Results and discussion

3.1. Morphology

The microscopic examination identified potential ascarids as well as possible taenids in the fecal sample. Jiminez et al. (2012) identified *Dipylidium caninum* eggs in sample Rio Zape 23. Pollen, plant remains and insect remains were also noted in the flotation samples, but were not analyzed as part of this study. The results of the morphological analysis provided a guideline for the molecular analysis. Based on the finding of potential ascarids and possible taenids, we chose to pursue the ascarids with previously published protocols, for the initial PCR amplifications. Attempts to amplify taenids in the lab had been problematic due to the large size of previously published primers and difficulty in designing primers that worked well, for these reasons we chose not to pursue taenids in this study. This guided approach retains the valuable information provided by the morphological results, such as the parasite's developmental stage, while providing greater confidence in taxonomic identification, and even potentially impacting parasite taxonomy.

3.2. Extraction and PCR from the microscope slide

There is evidence to suggest that it is possible to obtain genetic results from a single worm, or a single egg (Carlsgart et al., 2009; Shayan et al., 2007). Therefore, the first extraction was performed on the flotation solution affixed to the microscope slide that contained the parasite remains. Because the parasites eggs were *Ascaris*-like, our PCR amplifation used primers that were specific for *Ascaris* and its close relatives.

The result of the initial extraction provided the first sequence identified to the *Physaloptera* genus rather than *Ascaris*. However, because the sample could have been contaminated during its preparation in the OSU Veterinary lab, we reserved consideration until more results were obtained directly from the rehydrated samples in OU ancient DNA lab.

This extraction step and the subsequent PCRs, clones, and DNA sequence data provided information for several considerations. First, it allows us to test the morphological identification directly on the organism identified on the slide. Second, it provides a baseline for comparison with subsequent DNA extractions and PCRs performed on the fecal samples that remained protected in the ancient DNA lab, which allows us to test for DNA contamination as well as consistency of our original identification. Third, it allows a more precise taxonomic identification when morphological identification is ambiguous.

3.3. Molecular analysis

The amplicons recovered from the PCRs and cloning of the original *Ascaris* primer set were consistent with the size expected. The clones were identical in sequence to the direct sequence from the PCR amplifications. The design of an additional *Physaloptera* specific primer set allowed us to increase the size of our sequence, with a gap of 28bp between the new and original primers sets. Because of the gap in our generated sequence data, the maximum possible coverage for any BLAST result would be 85%. With this level of coverage, there were several matches at 100% identity. The strongest matches were to data for *Physaloptera* sp. SAN-2007 and a *Contracaecum spiculigerum*, both having the highest bit scores of 191 and E-values of 2e-45. Additional results providing 85% coverage and 100% identity were to data for a *Physaloptera turgida,* with a bit score of 185 and an E-value of 8e-44 and a *Turgida torresi* with bit scores of 180 and E-values of 4e-42. *T. torresi* is a physalopterid and the only species in the genus *Turgida*; Ortlepp (1922) considers this genus and species to be synonymous with *Physaloptera torresi*. A *Physaloptera* identified as *Physaloptera* sp. SAN-2010 also had the same BLAST confidence as the *T. torresi*. A slightly less confident match was data for a *Physaloptera thalacomys*, which had the same bit score and e-values (180 and 4e-42, respectively) including 100% identity, but only 83% coverage (for discussion of bit scores and E-values see Madden (2002): http://www.ncbi.nlm.nih.gov/books/NBK21097/#A614).

The neighbor joining tree generated using the data from the BLAST matches as well as the available published data for ascarids is provided in Fig. 1. The resulting topology of the neighbor joining tree was consistent across alternative tree building methods, including Maximum Likelihood and the consensus tree from Maximum Parsimony. In the tree, *Ascaris* and *Physaloptera* form two distinct groups. The tree groups *T. torresi* with *Physaloptera* species, which is expected because this genera is a physalopterid. With one exception, the tree groups *Contracaecum* with *Ascaris* species, which is expected because *Contracaecum* is a genus of anisakid, in the ascarid family. The exception is data for a *C. spiculigerum* which presents an anomaly.

The *C. spiculigerum* sequence was a 100% match to *Physaloptera* sp. SAN-2007, but as demonstrated by the robust bootstrap values, it differed significantly from other *Contracaecum* and *Ascaris* sequences. The data for this *C. spiculigerum* is an exception to a tree that otherwise reflects a robust phylogenetic pattern of monophyletic groupings of the observed parasites. With this one exception, the results robustly separated the physalopterids (*Physaloptera* and *Turgida*) from the ascarids (*Ascaris* and *Contracaecum*) as monophyletic groups with bootstrap values of 95%. With this one exception, the tree further differentiates *Contracaecum* species from other parasites with a bootstrap value of 99%. For the physalopterids, the tree grouped the avian adapted species with bootstrap values of 90%, and, with this one exception, grouped mammalian adapted species with a bootstrap value of 86%.

The *C. spiculigerum* sequence is clearly a misidentified *Physaloptera*. The published study of this sequence (Sato and Suzuki, 2006) is a report on the genetic analysis of trematodes and does not report a genetic analysis of nematodes, like *Contracaecum*. The misidentified *Physaloptera* sequence was an unpublished direct submission to GenBank. This misidentified *Physaloptera* specimen further highlights the challenges associated with morphological identification of parasites. The error is likely attributed to the parasite's immature developmental stage; Sato and Suzuki (2006) noted that all studied individuals bearing *Contracaecum* also bore *Physaloptera* and that the *Contracaecum* specimens were either an immature female or one of four juveniles.

Figure 1. Neighbor-joining tree with 1000 bootstrap reiterations. The comparative taxa were chosen for their close relatedness to *Physaloptera* and to analyze the anomalous *Contracaecum spiculigerum* sequence. Green represent *Contracaecum*, blue represent *Ascaris*, orange represent *Physaloptera* infecting avian species and red represent *Physaloptera* infecting mammalian species. The Zape 23 consensus sequence falls securely among the mammalian *Physaloptera*, as does *Contracaecum* spiculigerum. This tree separates ascarids from physalopterids with a 95% confidence. It separates *Ascaris* and *Contracaecum* genera with a 99% confidence. It also separates the avian *Physaloptera* from the mammalian *Physaloptera* with 90% confidence for the avian branch and an 86% confidence for the mammalian branch.
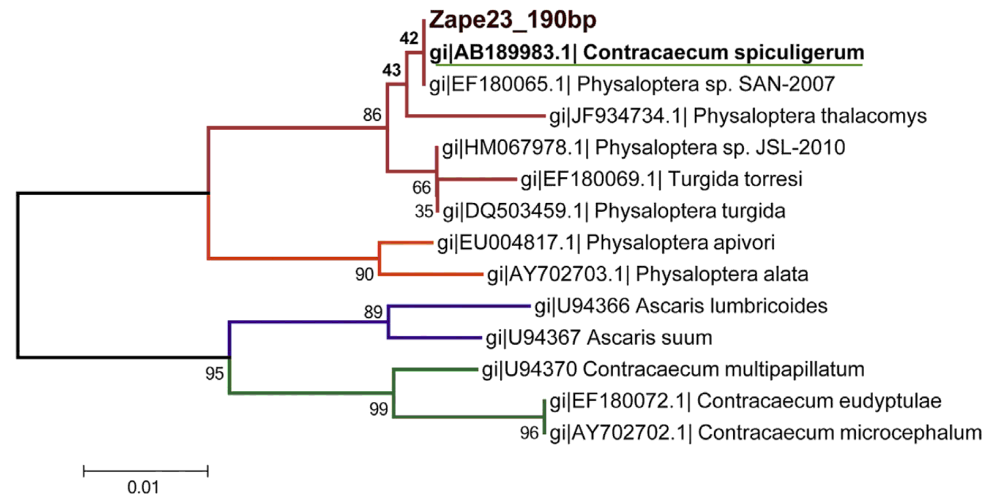
3.4. *Physaloptera and difficulty in diagnosis*

The recovery of an unexpected *Physaloptera* in the prehistoric sample highlights the importance of using a multi-pronged approach to parasite analysis in both modern and ancient samples. *Physaloptera* represent a model for the efficacy of combining methods in order to obtain more robust and informative results. This particular parasite is considered rare in humans, and as such is not a parasite that is routinely considered in parasite diagnosis. However, it is possible that this assumption is poorly supported by the available documentary evidence. *Physaloptera* are particularly difficult to identify in both veterinary and human samples and as a result may be underreported.

Physaloptera eggs are very similar to decorticated *Ascaris* eggs in appearance (Hira, 1978; Vandepitte et al., 1964). Several researchers note that this could be problematic in diagnosis and may have led to an underreporting of *Physaloptera* infections (Campbell and Graham, 1999; Gutierrez, 2000; Leiper, 1911; Vandepitte et al., 1964). *Ascaris* is a common parasite of humans both prehistorically and in modern populations (Leles et al., 2008; Loreille et al., 2001; Reinhard, 1990). A *Physaloptera* egg could very easily be misdiagnosed as a decorticated *Ascaris* egg. *Physaloptera* larvae are also often confused with *Ascaris* (Apt et al., 1965; Fain and Vandepitte, 1964; Flynn and Baker, 2007; Gutierrez, 2000; Hira, 1978; Leiper, 1911; Vandepitte et al., 1964). Eggs of *Physaloptera* are also few in numbers and relatively heavy, so they may not be captured in a flotation protocol, although a Sugar Solution Flotation has been recommended by veterinary parasitologists (David and Lindquist, 1982; Johnson-Delaney, 2009; Kazacos, 2010). Females may not produce a large number of eggs; there is little information on the number of eggs produced, unlike *Ascaris* which produce up to 200,000 eggs a day (Lee, 1955; Leles et al., 2008; Loreille et al., 2001; Olsen, 1986). It is suspected that adult *Physaloptera* are present in relatively small numbers, unlike *Ascaris* which can be present in rather large communities (Campbell and Graham, 1999; Johnson-Delaney, 2009; Kazacos, 2010; Naem et al., 2006; Nicolaides et al., 1977).

The genus *Physaloptera* is composed of between 92 and 126 identified species inhabiting a broad range of hosts, including mammals, birds, reptiles, amphibians and insects (Ortlepp,

1922; http://insects.tamu.edu/research/collection/hallan/Nematoda/Family/Physalopteridae.txt). Its broad range of host species suggests that this is a generalist parasite. From existing information, most species utilize insects such as crickets, cockroaches, and beetles as the intermediate host (Alicata, 1937; Cawthorn and Anderson, 1976; Fain and Vandepitte, 1964; Gray and Anderson, 1981; Guerrero et al., 2010; Gupta and Pande, 1970; Harrison and Hall, 1909; Hobmaier, 1941; Irwin-Smith, 1921; Lincoln and Anderson, 1972; Magnone et al., 2007; Naem et al., 2006; Petri, 1950; Schell, 1952). It is also possible that some species utilize snakes, frogs and possibly some rodents as paratenic hosts (Cawthorn and Anderson, 1976). Very little is known about the life cycle of most species of *Physaloptera*, but from information available, it suggests that this genus attaches itself to the mucosal lining of the gastrointestinal tract of its host by embedding its caudal end in the host tissue. Initially, the infective larvae attach in a spread out fashion individually, but later migrate to form small communities clumped together. They produce ulcers in the mucosal lining and result in bleeding, melena, vomiting and diarrhea and abdominal pain. The ulcers also provide an opening for opportunistic pathogens (Schell, 1952).

Third stage infective larvae are also capable of causing debilitating health consequences even in a non-definitive host (Nicolaides et al., 1977; Schell, 1952). Third stage larvae will attach themselves in the gastrointestinal tract in the same places they would in their definitive host and this complicates diagnosis because, while they will attach and cause the same symptoms, they will not develop to sexual maturity and therefore they will not produce eggs to be expelled in the feces (Alicata, 1937; Anderson, 1988; Basir, 1948; Cawthorn and Anderson, 1976; Schell, 1952). *Physaloptera* attach in the esophagus, stomach and small intestine. Its location is relative to whether or not vomiting or diarrhea is present as symptoms.

Physaloptera was first identified in humans from the Caucasus Mountains in Russia in 1902. It has also been identified in humans in Africa and South America. It is also known to infect baboons and chimpanzees as well as other Simian primates. *Physaloptera caucasica* and *Physaloptera mordens* have both been identified in humans; today they are considered synonymous (Irwin-Smith, 1921; Hahn et al., 2003; Hira, 1978; Lleras and Pan, 1955; Mbora and McPeek, 2009; Morgan, 1945; Murray et al., 2000; Mutani et al., 2003; Oliveira-Menezes et al., 2011; Ortlepp, 1922, 1926; Weyher et al., 2006). An infection has been

documented involving an 11 month old infant in 1977 which require surgical intervention to cure. The species infecting the infant was more typical of Australian bandicoots (Nicolaides et al., 1977); demonstrating the generalist nature and the ability of third stage larvae to negatively impact non-definitive hosts.

Possible *Physaloptera* have been identified in two other prehistoric coprolites, one considered to be a canid in origin and another human, both from Argentina (Fugassa et al., 2006, 2007). The current study represents the first molecularly confirmed infection of *Physaloptera* in a prehistoric human. Given the generalist nature and the difficulties of identifying *Physaloptera* infections, this parasitic infection may not be as rare as once believed—but additional testing of more samples is required to test this hypothesis.

4. Conclusion

The Zape 23 coprolite provided a sample for the testing of a combined and complimentary methodology for identifying the presence or absence of parasite remains in prehistoric samples. This methodology is applicable to modern samples as well. The approach resulted in the discovery of a parasite missed in previous traditional analyses of sample Rio Zape 23 (Jiminez et al., 2012). Jiminez and his colleagues were successful in recovering *D. caninum* eggs, but no other species.

Morphological analysis is often hampered by lack of intact physical remains or the similarity of morphological features between organisms at different developmental stages. This study demonstrates this difficulty by the initial identification of eggs as possible ascarids. By adding a step that extracts DNA from the microscope slide used for morphological analysis, we are able to isolate identified specimens for genetic analysis, as well as isolating ambiguous specimens. In this case, the initial genetic analysis on the microscope slide extraction returned an unexpected sequence related to a physalopterid rather than the anticipated ascarid. This highlights the difficulty in morphological certainty.

Molecular analysis can help to differentiate or confirm organisms, even in the absence of visible physical remains. This analysis highlights the efficacy of this approach, while also highlighting areas that are less efficient. For example, theoretically, researchers should be able to differentiate samples to the species level with molecular data. However, in this case study, we were able to identify the sequences recovered to a mammalian associated physalopterid, but not a specific species, because the database does not contain enough reference sequences. An additional issue highlighted by this study was the presence of a misidentified sequence—*C. spiculigerum,* in the national database.

It should be noted that absence of results either morphologically or molecularly do not necessarily mean that an organism is not present. Parasites can be differentially preserved depending on density and reproductive capacity, as well as, post-depositional environment. In this case, multiple lines of evidence support the presence of *Physaloptera* in this subsample.

Despite the limitations of each methodology by itself, by combining them, their complementarity provides a robust and informative methodology. We conclude that rather than being alternatives to one another, a combined morphological-molecular methodology for parasite identification is the most informative and most robust approach currently available to study both modern and prehistoric samples.

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