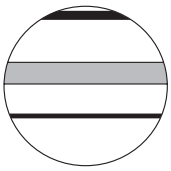

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Helminth infection and human mobility in *sambaquis*: Paleoparasitological, paleogenetic, and microremains investigations in Jabuticabeira II, Brazil (2890 ± 55 to 1805 ± 65 BP)

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Abstract

Sambaquis or shellmounds are archeological sites constructed by hunter-fisher-gatherers that inhabited the Brazilian coast about 10,000–2000 yrs BP. Jabuticabeira II (JABII: 2890 ± 55 to 1805 ± 65 BP) is one of dozens of contemporaneous *sambaquis* of the Santa Catarina state, South Brazil, and contains hundreds of neatly organized burials, indicating great population density. In order to gather information about the health, diet and way of life of people in JABII, a paleoparasitological, paleogenetic, and micro-human remains investigation was carried out. Pelvic region and environmental control samples from six individuals exhumed from JABII were submitted to microscopic and ancient DNA (aDNA) investigation. Paleoparasitological analyses based on light microscopy were negative. However, a variety of informative microremains were found. Diatoms, fish scales, and algae characterize the marine and estuarine environment. *Ipomoea batatas* and *Zea mays* starch grains suggested cultivated items as part of their diet in agreement with the literature. The finding of *Podocarpus* sp. pollen grain, characteristic of highlander vegetation, suggests human mobility of JABII individuals which were settlement in the coast. Paleogenetic analyses showed *Ascaris* sp. helminth infection based on *nad*1 gene fragment detected from an individual excavated at L3 FS7 burial (1826 ± 40 BP). This aDNA result places the antiquity of *Ascaris* sp. infection, and haplotypes that are circulating in humans and other animals nowadays, in Pre-Columbian South American times.

Keywords

ancient DNA, helminth, hookworm, paleogenetic, paleoparasitology, shellmounds

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Introduction

Sambaquis or shellmounds are archeological sites constructed by groups of hunter-fisher-gatherers that inhabited the Brazilian coast from about 10,000 to 2000 years Before Present (yrs BP). The Jabuticabeira II (JABII) *sambaqui* is located in the municipality of Jaguaruna, Santa Catarina state, Southern Brazil (28º35′25.061″S; 48º57′36.332″W) and is part of dozens of *sambaquis* that were constructed around Lake Camacho (Bianchini et al., 2011). This site was built between 2890 ± 55 and 1805 ± 65 BP (DeBlais et al., 2007; Fish et al., 2000). Some *sambaquis* are referred to as a habitational, working, and/or ritual places (Gaspar et al., 2008). JABII has about 8.4 hectares, 10 m high, and ending with 400 m on its largest axis and 250 m diagonally (Bianchini et al., 2011). It seems to have had strict ritual function, since there is no evidence of domestic use and elaborate as well as consecutive incremental funerary rituals explain the site construction history (DeBlais et al., 2007; Fish et al., 2000; Okumura and Eggers, 2005).

Paleoparasitology, the study of parasites found in human or other animal remains recovered from archeological or paleontological sites (Ferreira et al., 2011), have demonstrated intestinal infections in pre-Contact South American times. These infections caused by *Ascaris* sp., *Enterobius vermicularis*, *Trichuris trichiura*, *Trichostrongylus* spp., ancylostomids, cestodes, and acanthocephalans are related to the lifestyle, the increase in population, human mobility but also mutually influenced by process of agriculture and animal domestication (Bouchet et al., 1996; Gonçalves et al., 2003; Iñiguez et al., 2006; Leles et al., 2009; Vieira et al., 2018). Paleoparasitological studies in *sambaquis* recovered only very scant data of parasite evidence, despite a long investigative history (Bathurst, 2005; Camacho et al., 2016; Leles, 2010). This is mostly due to the intense biodegradation caused by humidity and rainfall. Some techniques have been tested in an attempt to recover parasites and plants from archeological remains. The study of parasites and diet in archeological material has recently produced combined information about the health and survival conditions of the ancient populations (Moore et al., 2020; Sawafuji et al., 2020; Søe et al., 2018; Wiscovitch-Russo et al., 2020).

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Table 1. Characteristics of burials, individuals and samples of JABII *sambaqui* from this study.

Burials	Sex/age	Burial characteristics	Samples	Sample characteristics
L3 FS7	Young adult ♂	15% bones preserved	JABII03	Pelvis sediment
			JABII04	Site control sediment
36A L2.05 E4	Young adult ♂	Primary; hiperflexed	JABII17	Pelvis sediment
10B L1.25 E1	N/A	N/A	JABII21	Sacral foramina sediment
17A L1.05 E4	Adult (31–50 years) ♂	Multiple; articulated; flexed	JABII26	Sacral foramina sediment
34 L2.05 E4	Young adult (21–35 years) ♀	Primary; simple; articulated; hiperflexed	JABII30	Sacral foramina sediment
			JABII32	Pelvis sediment
			JABII35	Site control sediment (Tibia)
25 L2 65 E3	Adult ♀	Simple; articulated; hiperflexed	JABII40	Pelvis sediment

♂: Male; ♀: Female; N/A: information not available.

The data achieved until now, however, could not provide direct information of helminth infection associated with burial in *sambaquis* archeological site. In this study, we aimed to contribute to the knowledge of health and lifestyle in *sambaquis* through paleoparasitological, microremain, and paleogenetic analyses of JABII individuals.

Materials and methods

Samples from pelvic region, sacral foramina and environmental controls were collected from six JABII individuals, exhumed about 20 years ago (Table 1). The team from the Paleogenetic Laboratory – Laboratório de Biologia de Tripanosomatídeos (LABTRIP), Instituto Oswaldo Cruz-Fundação Oswaldo Cruz (IOC/FIOCRUZ), Rio de Janeiro, collected the samples from the osteological collection housed at *Laboratório de Antropologia Biológica, Departamento de Genética e Biologia Evolutiva*, *Instituto de Biociencias*, University of São Paulo. Although in the process of curation bones were carefully cleaned off the original soil (no chemicals were used), it was possible to recover sediments associated to the sites of infection of intestinal parasites (Jaeger et al., 2013). In order to control environmental contamination, superficial sediments were first discarded and sediments from a second scraping were collected for this project, including controls.

Procedures were performed according to the paleogenetic collection protocol stablished by the Paleogenetic Lab (LABTRIP/IOC/FIORUZ), which requires the use of disposable tools, sterile instruments and containers and personal protective equipment, changed after each sample collection (Iñiguez, 2014). Standard measures to avoid aDNA degradation, contamination from modern DNA, and cross-contamination were applied as described elsewhere (Iñiguez, 2011). Samples were transported to the Paleogenetic Lab (LABTRIP/IOC/FIORUZ) and were frozen until paleoparasitological procedures (Iñiguez, 2021; Jaeger and Iñiguez, 2014).

Sediments of around 2–5 g were rehydrated using 0.5% aqueous solution of trisodium phosphate, during 72 h (Callen and Cameron, 1960). Later, the samples were submitted to the spontaneous sedimentation technique during 24 h (Lutz, 1919) at 4°C. Two aliquots of 200 µL of sediment were separated for each paleoparasitological and paleogenetic analysis. About of 20 slides per sample were prepared and fully screened for parasites and microremains detection. Slides were analyzed under a light microscope (ZEISS Primo Star) using 100× magnification and photographs were obtained at 400× magnification.

We performed the paleogenetic analysis to verify intestinal helminth infection by *Ascaris* sp. and *Trichuris trichiura*. For aDNA extraction we applied the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany), with modifications. First, sediments were submitted to liquid nitrogen, and then, to overnight proteinase K (Invitrogen) digestion (20 mg/µL) at 56ºC according to (Iñiguez et al., 2006). Two aDNA elution aliquots were done in a final volume of 80 µL. Primers and PCR conditions utilized for *Ascaris* sp. diagnosis were according to Loreille et al. (2001) for 18S ribosomal DNA (rDNA) and cytochrome b (*cyt*b); and to Botella et al. (2010) for NAD dehidrogenase (*nad*1) and cytochrome c oxidase subunit 1 (*cox*1) genes. For the diagnosis of *T. trichiura* were used primers and PCR conditions using 18S rDNA and 28S rDNA molecular targets as proposed by Oh et al. (2010) and Côté et al. (2016), respectively. Environmental (archeological site), aDNA extraction, and PCR negative controls were always included. Positive PCR controls were not carried out. PCR products were visualized on 3% agarose gel electrophoresis (Agarose NA, GE HealthCare Life Sciences, Vienna, Austria) and stained with GelRed nucleic acid stain (Biotium, Hayward, USA).

Sequencing reaction was performed using an ABI BigDye Terminator kit (Applied Biosystems, Foster City, CA) on an ABI 3730 (Applied Biosystems) automated sequencer. Lasergene Seqman v. 7.0.0 (DNASTAR, Madison, Wisconsin, USA) and Bio Edit v. 7.0.4 (Department of Microbiology, North Carolina State University, Raleigh, North Carolina) were used for editing and sequence analysis, respectively. BLAST searches were performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the obtained aDNA sequences.

Results

Paleoparasitological results by light microscopy allowed the detection of starch grains, phytoliths, diatoms, fish scales, algae, and fungi spores, including monolete spores, and a structure similar to an ascarid egg (Table 2 and Figure 1). The structure suggestive of the ascarid egg shows an oval to round shape and measures 64.7 × 57.2 µm. This size is compatible with *Ascaris* sp. (45–75 × 35–50 µm) (Roberts et al., 2013), although it looks a bit less elongated. Due to dirt on the surface, the outer mamilonated membrane can only be observed in some regions of the structure. The structure suggestive to *Ascaris* sp. eggs was found in sediment JABII35 associated to the tibia of the 34 L2.05 E4 individual buried in hyperflexed/crouched position, that could indicate true parasite infection. However, no parasite evidences were observed in samples JABII30 and JABII32 that are sediments collected from sacral foramina and pelvis of 34 L2.05 E4 individual, respectively. We resolve not to consider the structure suggestive ascarid egg conclusive of paleoparasitological positive result.

However, paleogenetic analyses demonstrated reproductible PCR amplifications using *Ascaris* sp. *nad*1 marker (Supplemental Material, available online), from JABII03 pelvic sediment sample of the L3 FS7 individual, yielding a nucleotide sequence of quality (151bp), and consequently a robust result of *Ascaris* sp. infection. BLAST comparison revealed 100% of both total coverage and identity only with *Ascaris lumbricoides* Linnaeus, 1758 (AJ968346) and *A. suum* Goeze, 1782 (HQ704901) sequences, as well as a 94–99% of identity with *A. lumbricoides*, *A. suum* and

Table 2. Paleoparasitological and micro-remain results of JABII *sambaqui* from this study.

Samples	Sample characteristics	Microscopic results
JABII03	Pelvis sediment	Diatom/*Actinoptychus* sp. Starch Fish scale Phytoliths/Poaceae (grass family) Fungus
JABII04	Site control sediment	Diatom/*Actinoptychus* sp. *Ipomoea batatas* starch *Zea mays* starch
JABII17	Pelvis sediment	Diatom/*Diploneis* sp. Starch/*Ipomoea batatas* starch *Zea mays* starch Phytolith Fungus Algae spore *Diporotheca* sp. spore Monolete
JABII21	Sacral foramina sediment	Diatom/*Grammatophora* sp. elongate grass phytolith
JABII26	Sacral foramina sediment	Starch/*Ipomoea batatas* starch
JABII30	Sacral foramina sediment	Diatom/*Actinoptychus* sp. and *Grammatophora* sp. Starch Phytolith/Poaceae (grass family)
JABII32	Pelvis sediment	Phytolith
JABII35	Site control sediment	Algae Foraminifera *Podocarpus* sp. pollen Structure suggestive of ascarid egg Pollen grain Mite
JABII40	Pelvis sediment	Fish scale Algae spore Phytolith Pollen grain

Ascaris sp. sequences. The *nad*1 primers were designed by us (Botella et al., 2010) exclusively for *Ascaris* sp. paleogenetic diagnosis. Until now no other nematodes were detected using this marker. The sequence alignment of *nad*1 sequence fragment analyzed demonstrated JABII03 haplotype is identical to H03CH, H14BR, H16BR, P10CH.BR, P15CH haplotypes, including the highly prevalent H12P17BR (Figure 2).

Discussion

Paleoparasitological examination by light microscopy did not yield robust results of positive parasite infection. The non-conclusive finding of suggestive *Ascaris* sp. egg in an JABII individual, refers to a control sediment collected from the surface of the tibia. However, regarding this issue, a reflection is needed. The 34 L2.05 E4 individual was buried in flexed position, and the permanent contact between the abdominal region and the inferior members, could allowed the deposition gastrointestinal vestiges. Taphonomic processes affect directly in the post-mortem body acting in the transport and dispersion of different elements present in the burial, enabling parasitic structures in the pelvic region to be found in control sample associated to the tibia (Ubelaker, 1997) The position of the body is evident when the paleogenetic collection is "in loco." However, in this study, samples were gathered from museological collection, when the original position was not perceived. The case calls for caution of appropriate collecting of environmental controls. We recommend to verify the body position in burials, before following standard local of collection, in order to rule out possible contact with sites of infection. This is especially critical when collecting samples in osteological collections, where bones are usually disarticulated, thus, the position of the body as the individual was placed in the grave must be carefully considered.

Regarding the microremains identified in the sediment samples by microscopic analysis, we believe that the low concentration of pollen grains, starch grains and phytoliths is due to conservation issues, and also, not specific protocols were following. Pollen grains are produced by flower angiosperms and have a tough layer consisting of axin. Starch grains are organic particles and, despite of being reported in sediment samples from different archeological sites, in some soil types they can be poorly preserved (Torrence and Barton, 2006). Concerning the phytoliths, since the solubility of these silica particles is higher in high pH environments, the high alkalinity that is expected in the deposit of shell matrix sites (Piperno, 1988), such as JABII, must play a role in their low concentration.

Despite the low concentration of microremains we can make some inferences on the taxa found. Fish scales denotes the fishing activity of hunter-fisher-gatherers from JABII. Diatoms characterize the estuarine environmental were JABII *sambaqui* was settled. *Actinoptychus* sp. is usually found in neritic collections, and *Diploneis* and *Grammatophora* are widespread marine genera with some of freshwater species. A study carried out in Conceição Lagoon in Santa Catarina with samples from different periods revealed a diatom flora characteristic of coastal and estuarines areas with the presence of several species, including *Actinoptychus senarius* Ehrenberg, 1843 and *Diploneis smith* (Brébisson) Cleve, 1894, being representative in about 80% of the samples (Souza-Mosimann et al., 2011). All three diatom records were already found in the JABII sediment (Villagrán, 2009), indicating the ecosystem location on the archeological site is inserted. In the same way, most foraminifera lives in a marine environment, some

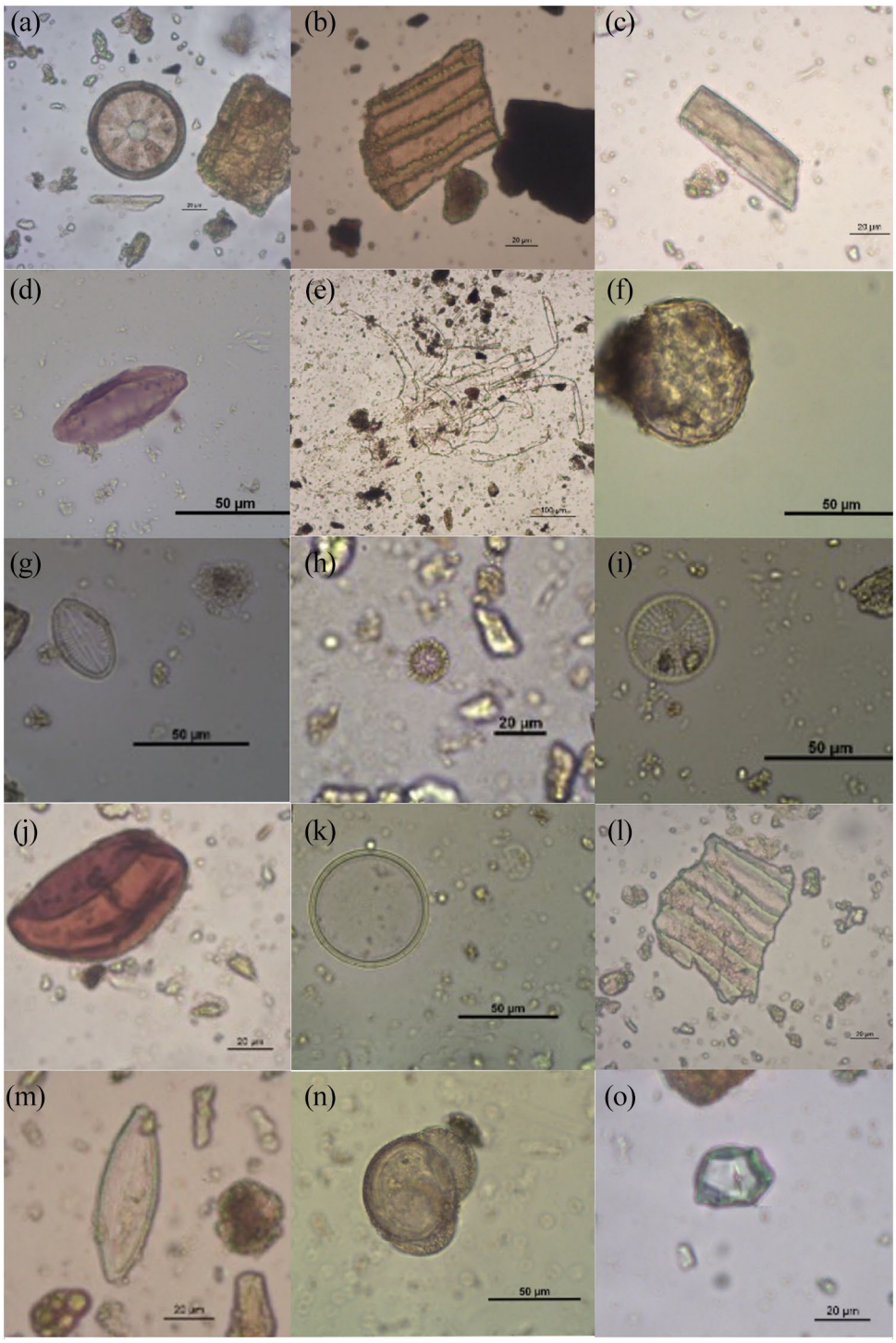

Figure 1. Micro remains findings based on light microscopy analysis. (a) Diatom/*Actinoptychus* sp. (b) Scale Fish. (c) Phytolith/Poaceae (Grass). (d) *Diporotheca* sp. spore. (e) Mite. (f) Structure suggestive of ascarid egg. (g) Diatom/*Diploneis* sp. (h) Algae spore. (i) Diatom/*Actinoptychus* sp. (j) Fungus spore. (k) Diatom. (l) Fish scale. (m) Algae. (n) *Podocarpus* sp. pollen grain. (o) Starch grain.

in a mixohaline environment, with rare freshwater species. *Diporotheca* sp. spore is widespread all over the world, as spores were not only found in paleoecological studies. The presence fungi is compatible with biodegradation (Boyadjian et al., 2016). Phytoliths of Poaceae (grass) indicate open spaces in the vegetation (Boyadjian et al., 2016). *Ipomoea batatas* (L.) Lam, 1793 and *Zea mays* (L.), suggested sweet potato and maize as sources of starch that the people of JABII consumed and/or cultivated, in agreement with the literature (Bianchini et al., 2011; Scheel-Ybert and Boyadjian, 2020). Archaeobotanical records of possibly domesticated (sweet potato) and domesticated (maize) items are consonant with an association of gathering and horticulture activities supporting the live of JABII under a mixed economy concept (Boyadjian et al., 2016; Wesolowski, 2007).

A *Podocarpus* sp. (evergreen shrubs or trees) identified from the sacral region of an JABII individual, is original from cold climate and mountainous region (Marinho et al., 2016). In the Mixed Ombrophilous Forest (FOM), also called Araucaria Forest, only two gymnosperms naturally occur, *Podocarpus lambertii* Klotzch ex Endl and *Araucaria angustifolia* (Bertol) Kuntze, 1898. *Podocarpus lambertii* could not occur in *sambaqui* environment, even if cultivated. Anthracological studies indicate that JABII was inserted in the *Restinga* forest (Scheel-Ybert, 2000), while typical species of wood debris found in the anthracological

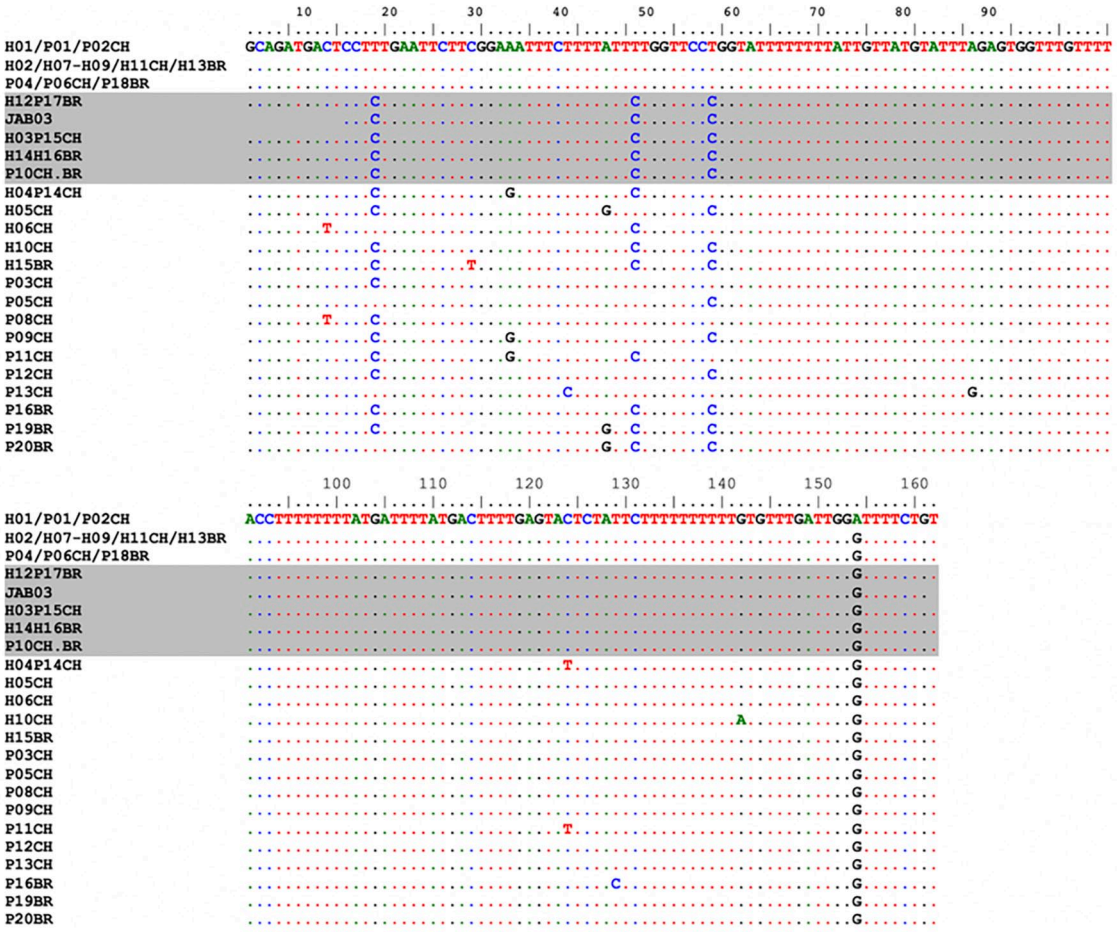

Figure 2. Alignment of the *Ascaris* sp. nad1 fragment sequence of JABII03 haplotype found in the individual L3 FS7 and *Ascaris* spp. reference sequences from GenBank. *Ascaris* sp. haplotypes that have identical polymorphisms to JABII03 haplotype are highlighted.

record of the Atlantic Forest (Bianchini et al., 2011), demonstrated contact and/or expansion for more coastal areas, since JABII was active at the time and was located 6 km from the seafront. An expansion of the Araucaria forest around 3000 years BP and its increase in the Northern highlands of Santa Catarina from 1000 years BP (Wesolowski, 2007) showed a new ecosystem explored a few miles from the coast. In the present study, the occurrence of *Podocarpus* sp. as an exotic plant record in a JABII, suggest a pattern of human mobility probably allowed contacts between coast and highlander populations.

We highlighted that even using not specific protocols on Palynology or starch proceedings, it is worthy the registration and examination of microremains by the specialists in order to explore in the maximum the archeological evidence.

It was estimated that thousands of people were buried over a period of more than 1000 years at JABII (Fish et al., 2000). Faunal and macroscopic plant remains indicated a rich surrounding estuarine environment, that could have been explored all-year round allowing permanent settlements, supporting sedentarism, crowding and complex socio-cultural organization (Okumura and Eggers, 2005; Scheel-Ybert et al., 2009). The high intake of terrestrial proteins in some JABII individuals, in contrast to the general subsistence pattern based on fish in *sambaquis*, was detected by the analysis of stable carbon and nitrogen isotopes, demonstrating that some JABII individuals had higher consumption of C3 protein (Colonese et al., 2014). The analysis of 49 native genomes present in ancient groups from Central America, South America, and North America, demonstrated through a population model that the strains found in South America are derived from one of the two ancient strains of North America generating important information to understand the origin of genetic variation in modern individuals in these regions (Posth et al., 2018). The distinct mtDNA haplotypes in JABII individuals, could indicate a population diversity, attributed to the presence of individual dietary restrictions or non-local individuals.

Paleogenetic analyses demonstrated helminth infection in one individual based on the detection of *Ascaris* sp. *nad*1 sequence from a pelvic region sample. Since molecular taxonomic studies using nuclear and mitochondrial genes demonstrated absence of *Ascaris* species discrimination (Leles, 2010; Peng et al., 2005; Zhou et al., 2011), *Ascaris* spp. were hypothesized to represent a single species (Iñiguez et al., 2012; Leles et al., 2012). A recent *Ascaris* spp. phylogenomic study suggested that *A. suum* and *A. lumbricoides* are a genetic complex infecting humans capable of interbreeding (Easton et al., 2020).

Human ascariasis is a major neglected tropical disease caused by helminth. Current molecular epidemiological studies showed a of *Ascaris* spp. panel of 16 and 20 *nad*1 haplotypes, described in humans (H) and pigs (P), respectively, distributed in worldwide (Iñiguez et al., 2012; Peng et al., 2005). When compared to *nad*1 panel, JABII03 haplotype t is identical to those found in both hosts and different countries, considering the gene fragment analyzed (Figure 2). The current epidemiology of *Ascaris* spp., based on *nad*1 gene, is driven by the globally distributed haplotype H12P17BR (Iñiguez et al., 2012; Peng et al., 2005). The JABII03 sequence retrieved from burial L3 FS7 (1826 ± 40 BP) is an irrefutable evidence of *Ascaris* sp. human infection. This is the first parasite aDNA sequence retrieved in an individual from a *sambaqui* burial. *Ascaris* sp. and *Trichuris* sp. were detected by aDNA hybridization in pelvic girdle sediments of individuals excavated

on Cubatão I *sambaqui* (3000 yrs BP to 2600–2500 yrs BP), also from Southern Brazil (Leles, 2010). The present finding not only point to the parasite infection, but also, the possible *Ascaris* sp. haplotype was verified, dating the circulation of modern *Ascaris* haplotypes since JABII times.

Paleoepidemiological data concerning parasitological information in *sambaqui* sites are extremely difficult due to scarce reports (Camacho et al., 2016; Leles, 2010). However, pondering that the humans who constructed JABII site lived in long-standing settlements (Okumura and Eggers, 2005), its opens the possibility of supposing that this scenario would be favorable to the circulation of *Ascaris* sp. Besides, other individuals were probably infected since ascariasis is a soil-transmitted helminthiasis and would not be sustained by just one individual. This would be the only way *Ascaris* sp. could have survived and spread through intra-community by fecal-oral transmission. Considering the taphonomic factors and biological characteristic of the parasite cycle, we can suggest that only a high parasite burden of individual could explain the positive record. The human remains associated with L3 FS7are incomplete and in a fairly bad state of conservation. Nevertheless, it is possible to distinguish fragments of an adult male individual who had a very severe and chronic infection on his right femur (Data not shown). The bad state of health may have left this individual more vulnerable to helminth infection and possibly, progression to a high burden.

Some studies have shown that JABII individuals presented paleopathological lesions such as treponematoses, which are conditions resulting from infections transmitted through person-to-person contact, also indirectly corroborate to the present finding. Finally, yet significantly, high frequency of paleopathological markers (Filippini et al., 2019) in the JABII inhabitants could have been caused by parasitic infections, probably triggered by poor sanitary conditions and considerable crowding of people.

As only this one from six individuals was found infected, and the few data on parasitology and paleogenetic recovery in *sambaquis*, we can consider that this result probably represents an underestimation of the true rate of infection. It is also possible that there was a greater diversity, at least of oral-fecal helminths infecting the JABII people. In addition, the curatorial process did not permit to achieve all the organic material from the abdominal region. The examination of abdominal samples collected during the excavation could have retrieved much more revealing results (Jaeger et al., 2013). We therefore stress the importance of a controlled curatorial process, with the conservation of organic material for future paleo-studies as mentioned before (Guedes et al., 2018, 2020; Jaeger et al., 2012).

Conclusion

The findings of the present study are an incontestable evidence of helminth infection in Pre-contact Southern Brazil, dating the *Ascaris* sp. human infection since the beginning of JABII times, circa 2000 years ago. Importantly, the paleogenetic result indicates that the haplotypes still present in human and other animal hosts worldwide afflicted people thousands of years ago. Furthermore, microremains and parasitological data corroborate with evidence of exploration of marine and estuarine environment, low-level of food production, gathering or/and exchanges of domesticated, and exotic plants, as recently stated by Scheel-Ybert and Boyadjian (2020), in addition to crowding and sedentary way of life of JABII people.

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Supplemental material

Supplemental material for this article is available online.

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