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Research paper

Mitochondrial DNA reveals species composition and phylogenetic relationships of hookworms in northeastern Brazil

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ABSTRACT

Hookworm infection persists focally in rural communities in Brazil. In this study, we analyze the mitochondrial nucleotide sequences obtained from hookworms infecting humans in order to characterize species composition and assess their genetic diversity and phylogenetic relationships. Field expeditions and cross-sectional surveys were carried out in three Brazilian municipalities from 2013 to 2017: Nossa Senhora de Nazaré (n = 605) and Teresina (n = 297), in the state of Piauí, and Russas (n = 213) in the State of Ceará. Parasitological methods were used to evaluate fecal samples. Hookworm-positive samples had a partial mtDNA *cox*1 amplified and sequenced. Maximum-likelihood and Bayesian analysis demonstrated two strongly-supported clades, including Group A, corresponding to *Necator americanus*, and Groups B and C, corresponding to *Necator* sp. Group A was divided into three main clusters: A1 grouped with Asian sequences, A2 grouped with African sequences, and A3 had only Asian sequences. Group B was closely related to *Necator* sp., showing a sequence similarity of 98%–99% with African samples circulating zoonotically among humans and non-human primates. Twenty three *N. americanus* haplotypes were identified. *N. americanus* Median-Joining network revealed three distinct groups, designated again as A1, A2, and A3. Group A1 presented a star-like shape, with one dominant haplotype. The molecular dating suggested that the two clades dividing *N. americanus* and *Necator* sp. began to diverge during the middle Pleistocene. The most recent common ancestor among *N. americanus* groups was dated to the late Pleistocene. Hookworms circulating in the studied communities are structured in well-defined subpopulations presenting both Asian and African genetic backgrounds. This reveals a double origin for hookworms in northeastern Brazil and opens up new possibilities in phylogeographic, evolutionary, and molecular epidemiological studies in regions where hookworms persists focally, despite control efforts. The presence of potentially zoonotic species and the specific identification of *Necator* sp. should be further investigated.

1. Introduction

Hookworms are soil-transmitted helminths (STH) that inhabit the small intestine of their hosts, sucking blood from the mucosa, which they adhere to through buccal parts. The blood loss they produce leads to varying degrees of iron-deficiency anemia, which is potentially severe (Casmo et al., 2014). Hookworm infection is one of the most widespread parasitic diseases in the world, affecting approximately 500 million people, mainly in developing countries (Bartsch et al., 2016).

Three major hookworm species infect humans: *Necator americanus*, *Ancylostoma duodenale*, and, less frequently, *Ancylostoma ceylanicum* (Loukas et al., 2016; Traub, 2013). Of the two main species, *N. americanus* can be considered more prevalent with having a global geographic distribution, while *A. duodenale* occurs mainly in the Mediterranean region, in northern China and India, and in northern Africa (Loukas et al., 2016).

In Brazil, the expansion of primary healthcare, improving sanitation, and the periodic administration of albendazole has led to a

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substantial decrease in the prevalence of hookworm infection and other STH (Chammartin et al., 2014). Nevertheless, enteric parasitic disease persists focally in rural areas, mainly in the north and northeast regions of the country (Bóia et al., 2006; Carvalho-Costa et al., 2007; Monteiro et al., 2018).

Concerning potentially zoonotic hookworm species, some previous work has reported the circulation of *Necator* sp. (presumably *Necator gorillae* by molecular and morphological methods) in Africa, among both humans and non-human primates (Hasegawa et al., 2014; Hasegawa et al., 2017; Kalousová et al., 2016). Human infections with other zoonotic parasites of the Strongylida order, such as *Trichostrongylus* sp. and *Oesophagostomum* sp. have also been described (Blotkamp et al., 1993; Gasser et al., 2006; Goldsmid, 1968; Lattes et al., 2011; Polderman and Blotkamp, 1995; Sato et al., 2011). Eggs from these parasites are not easily distinguished from hookworm eggs through light microscopy, and molecular approaches have demonstrated that misidentifications may be frequent (Verweij et al., 2001; Yong et al., 2007).

Mitochondrial (mt) DNA, such as cytochrome *c* oxidase subunit 1 encoding gene (*cox*1), has been used for specific identification, allowing for the genetic diversity to be evaluated and the genetic structure of hookworm parasites in some countries to be described (Gasser et al., 1998; Hawdon et al., 2001; Hu et al., 2002a; Hu et al., 2008; Kalousová et al., 2016).

Early molecular genetic studies with non-mitochondrial markers have pointed to substantial genetic differences at rDNA level (ITS-2), which suggests great population variation and the possibility of cryptic species when adult *N. americanus* from Africa and Malaysia are compared (Romstad et al., 1998). Later, the evaluation of complete mitochondrial DNA (12 genes) by nucleotide sequencing confirmed a large genetic divergence between *N. americanus* specimens from Africa and Asia (Hu et al., 2003). These genetic markers have not yet been applied for the species identification of hookworm in the American continent. In this study, we analyze mitochondrial nucleotide sequences obtained from hookworms infecting humans in northeastern Brazil – retrieved from GenBank – in order to characterize their species composition, to assess their genetic diversity and to perform phylogenetic inferences.

2. Methods

2.1. Study area, identification of hookworm-positive fecal samples, and ethics approval

Field expeditions and cross-sectional surveys were carried out in three Brazilian municipalities: Nossa Senhora de Nazaré (NSN, $n = 605$) and Teresina (TER, $n = 297$), in the state of Piauí, and Russas (RSS, $n = 213$) in the State of Ceará (Fig. 1), from 2013 to 2017. Parasitological methods (centrifugal-sedimentation with ethyl acetate and detergent, Kato-Katz thick smears, sucrose fluctuation, and Harada-Mori coprocultures) were used to evaluate fecal samples. The general abiotic conditions of each site, the prevalence rates of hookworm infection and the main population characteristics in each study area are presented in Table 1. A portion of the hookworm-positive fecal samples were cryopreserved, stored in microcentrifuge tubes and transported to the Laboratory of Epidemiology and Molecular Systematics/Oswaldo Cruz Foundation in Rio de Janeiro. 2 g of fecal samples or 1 mL of larvae-positive water from Harada-Mori technique were cryopreserved at −80 °C until the DNA extraction. This study was approved by the Research Ethics Committee (*Comitê de Ética em Pesquisa*, Instituto Oswaldo Cruz – Fiocruz, CAAE: 12125713.5.0000.5248).

2.2. DNA extraction and amplification, and the partial sequencing of cytochrome c oxidase subunit 1 gene

DNA was extracted from total fecal samples or larvae ($n = 109$) using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Partial mtDNA *cox*1 gene was amplified using Platinum Taq DNA Polymerase kit (Invitrogen, Waltham, USA). This set of primers was chosen to enable the DNA barcoding identification of other zoonotic parasites of the order Strongylida (Prosser et al., 2013). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were mixed with GelRed® Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), electrophoresed in a 1.5% agarose gel plate and detected using a UV illuminator (using the Camera Window Canon Power Shot S5 IS Software). After electrophoresis, DNA was extracted from the gel or directly purified with Illustra GFX PCR DNA and a Gel Band Purification kit (GE HealthCare, Pittsburgh, USA). PCR products were subjected to sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Foster City, USA) in both directions using M13F 5′ – TGT AAA ACG ACG GCC AGT – 3′ (forward) and M13R 5′ – CAG GAA ACA GCT ATG AC – 3′ (reverse) (Messing, 1993). Capillary electrophoresis was performed in an ABI3730 automated DNA sequencer (Applied Biosystems). In order to test for the presence of two or more haplotypes in the same individual, samples with overlapping peaks on electropherograms were cloned using pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA) using *Escherichia coli* DH5-alpha cells on Brain Heart Infusion Broth (Sigma-Aldrich, St. Louis, MO, USA) in disposable plates. Sequencing was performed as described above using the universal primers T7 5′ – TAA TAC GAC TCA CTA TAG G – 3′ (forward) and SP6 5′ – GAT TTA GGT GAC ACT ATA G – 3′ (reverse).

2.3. Data analysis

The nucleotide sequences were edited and analyzed with BioEdit v.7.0.9.0 and Molecular Evolutionary Genetics Analysis (MEGA) v.6.0 software (Hall, 1999; Tamura et al., 2013). The Basic Local Alignment Search Tool – BLAST (National Center for Biotechnology Information – NCBI, https://www.ncbi.nlm.nih.gov/) was used to verify nucleotide similarity with hookworm sequences from GenBank. Alignment problems, if any, were manually corrected. The whole mitochondrial genome of *N. americanus* (GenBank accession number AJ417719) was used as a reference sequence for the determination of variable positions. Orthologous sequences ($n = 33$) were retrieved from GenBank (Nucleotide NCBI – https://www.ncbi.nlm.nih.gov/nucleotide/) (S1 Table) (Hasegawa et al., 2017; Hawdon et al., 2001; Hu et al., 2002b; Hu et al., 2003; Shi et al., 2018). Bioedit v.7.0.9.0 software was used for fitting the sequences into equal fragments (Hall, 1999).

Initial phylogenetic inferences were performed by Maximum Likelihood (ML) method (465 bp) using MEGA v.6.0 software (Tamura et al., 2013). Evolutionary distances were computed using the Kimura's two-parameter (K2P) model. The clade stability of the branding topologies of *cox*1 sequences was evaluated using 1000 replicate bootstrap values. *Ancylostoma* spp. sequences were used as an outgroup (S1 Table). A dissimilarity matrix was calculated for determining the genetic distance among the sequences using MEGA v.6.0 software (Tamura et al., 2013).

A Median-Joining network based on distance criteria was constructed (483 bp) in order to establish the overall pattern of the intraspecific mitochondrial genetic variation of *N. americanus* using Network v.4.6.1.6 software (http://www.fluxus-engineering.com/) (Bandelt et al., 1999). The DNA *Sequence Polymorphism* (DNASP) v.5.10.01 software was used for editing the files (Librado and Rozas, 2009).

Bayesian inference (BI) was estimated for *cox*1 haplotypes using BEAST v.1.8.2, with 100 million generations (Drummond et al., 2012). The most appropriate substitution model of sequence evolution for the Bayesian tree was the Hasegawa-Kishino-Yano model (HKY) + I in jModelTest v.2.1.10 (Darriba et al., 2012). The approximate divergence time was estimated using an uncorrelated log-normal strict molecular-

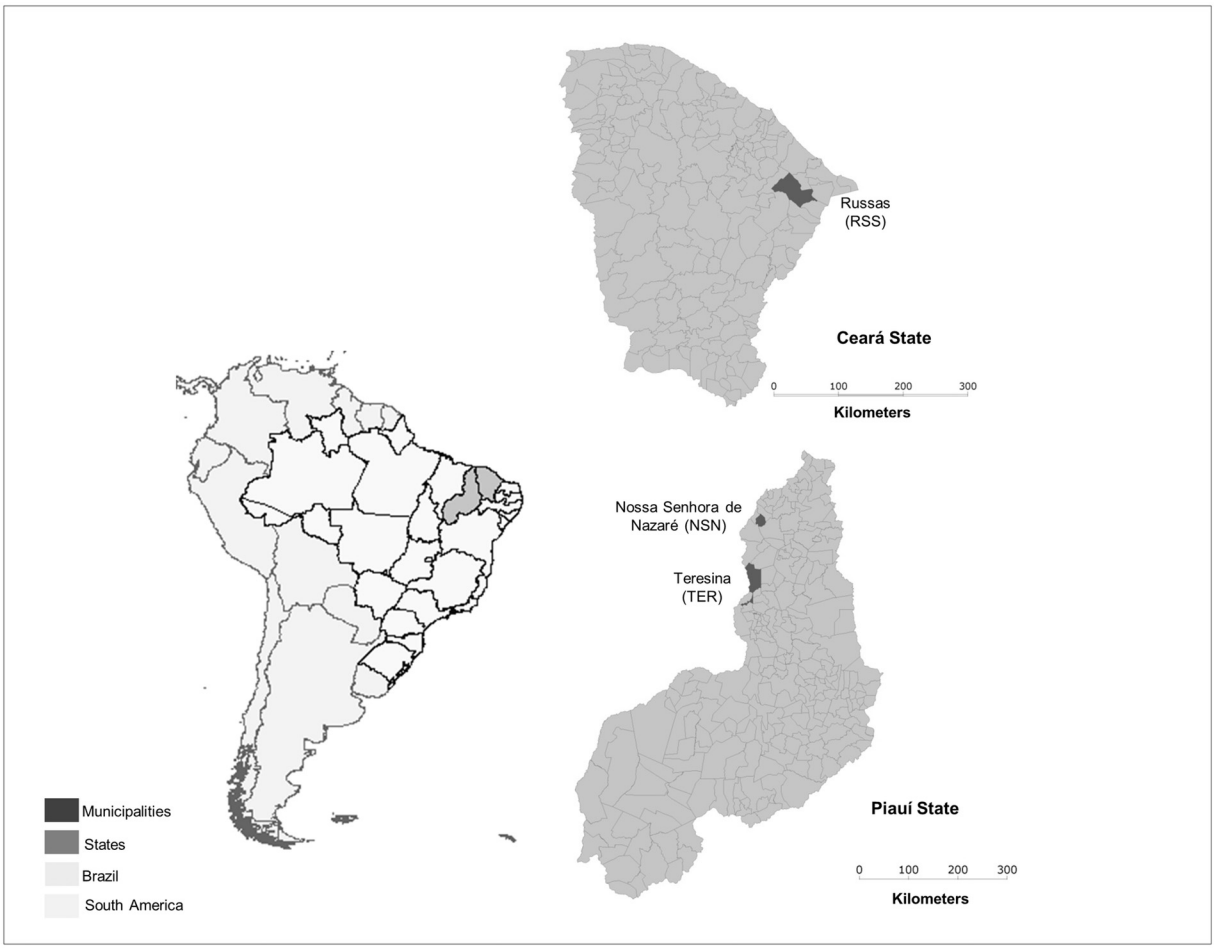

Fig. 1. Geographical location of the study in Piauí and Ceará state, northeast, Brazil.

clock model. The tree was generated using TreeAnnotator v.2.0.3 with 25% burn-in (Rambaut and Drummond, 2015). Branch-specific rates and lengths were visualized with FigTree v.1.4.2 software (Rambaut, 2014). A Bayesian skyline plot analysis (BSP) to estimate the change in population size over time for each *Necator* spp. haplotype was implemented in BEAST v.1.8.2 (Drummond et al., 2012). Plus, a piecewise-constant skyline model was selected. The molecular evolutionary rate of *cox*1 was fixed at 0.01 substitutions per site per million years ago (Mya) according to the substitution rate for nematode mtDNA (Zarlenga et al., 2006). Tracer v.1.5 software was then used to reconstruct the demographic history over time (Rambaut and Drummond, 2009).

3. Results

36 *cox*1 sequences were successfully amplified, bidirectionally sequenced, and used for molecular taxonomy and phylogenetic analysis. 24 (66.6%) samples were from NSN (Piauí state), 11 (30.6%) from TER (Piauí state), and 1 (2.8%) from RSS (Ceará state).

Maximum Likelihood reconstruction (Fig. 2) based on 465 bp *cox*1 sequences showed two strongly-supported clades belonging to *Necator* genus, including Group A, corresponding to sequences belonging to the *N. americanus* species, and Groups B and C, corresponding to *Necator* sp. *Necator americanus* was the predominant species and was characterized in 32 Brazilian sequences (NSN, $n = 19$; TER, $n = 12$; and RSS, n = 1). They presented 94–99% nucleotide similarity with each other and with reference sequences. Group B was composed of four Brazilian sequences

Table 1
Characteristics of the three studied localities.

Characteristic	Russas (Ceará)	Nossa Senhora de Nazaré (Piauí)	Teresina (Piauí)
Biome	Caatinga	Cerrado-Caatinga	Cerrado
Rainfall	775.6 mm/yearly	1.412 mm/yearly	1.400 mm/yearly
Temperature (minimum, maximum)	27–29 °C	26–34 °C	20–38
Population (no. of inhabitants)	76.475	4.786	850.198
Proportion of population performing open evacuation (%)	47	42.3	39.0
Main drinking water source	Harvested rain water stored in cisterns	Artesian wells	Artesian wells
Communities/districts included	2	27	2
Localization of districts	Rural	Rural and urban	Rural
Participants included	213	605	297
Year	2013	2014 and 2015	2017
Hookworm positive persons and prevalence (%)	8 (3.75)	75 (12.4)	26 (8.7)

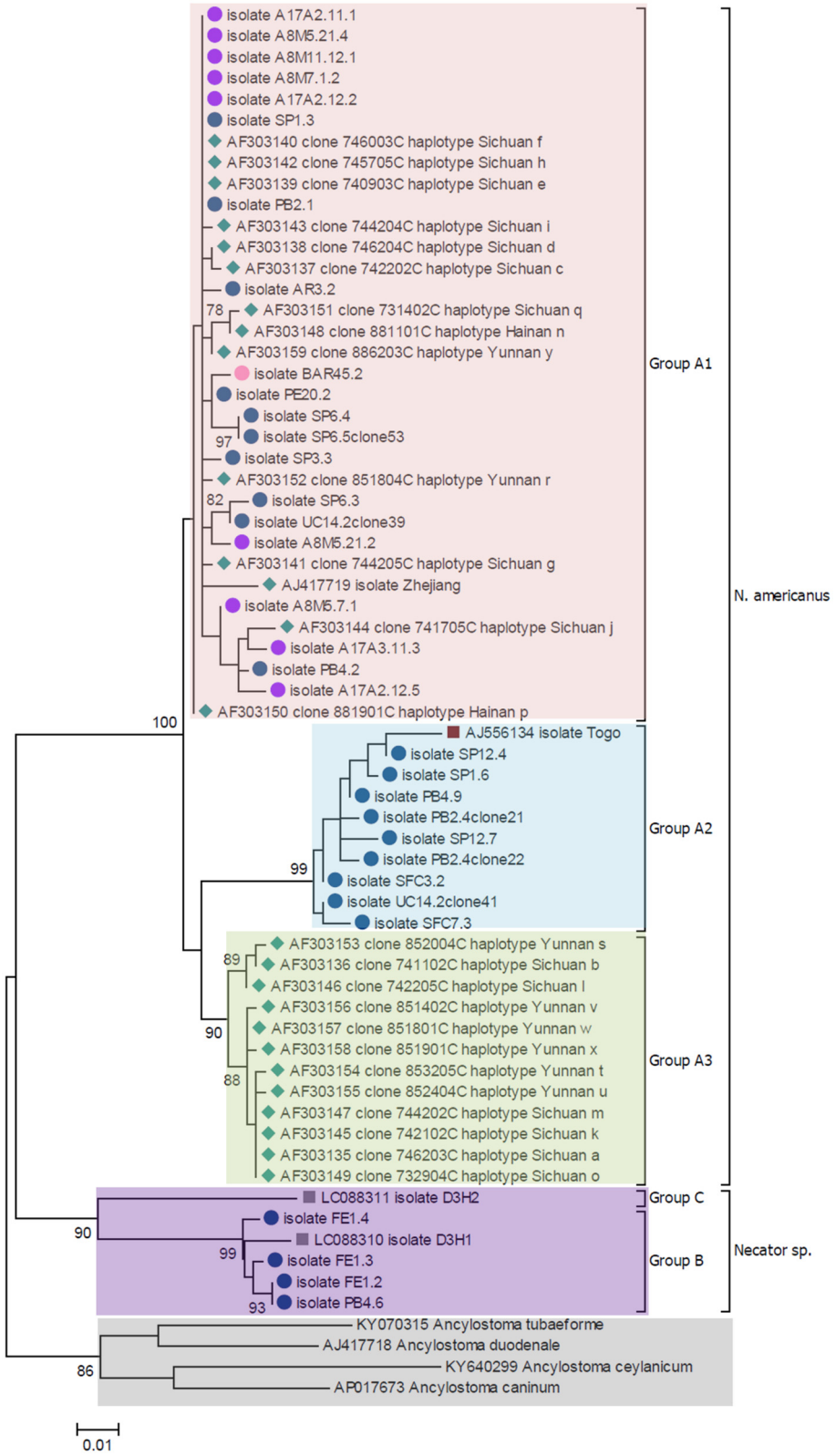

Fig. 2. Maximum Likelihood tree constructed using 465 bp *cox*1 sequences. Brazil (NSN): Blue Circle. Brazil (RSS): Pink Circle. Brazil (TER): Purple Circle. Africa: Red Square. China: Green Diamond. Africa: Gray Square. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(all from NSN), which presented a 98–99% nucleotide similarity with each other and with the reference sequences.

Group A (*N. americanus*) could be divided into three main clusters named A1, A2, and A3 (Fig. 2). The ML tree did not show a clear geographical division among the Brazilian samples. In Group A1, Brazilian sequences from all municipalities (NSN, TER, and RSS) were grouped with Asian sequences (four different sites in China). In Group A2, we observed Brazilian sequences only from NSN and one sequence

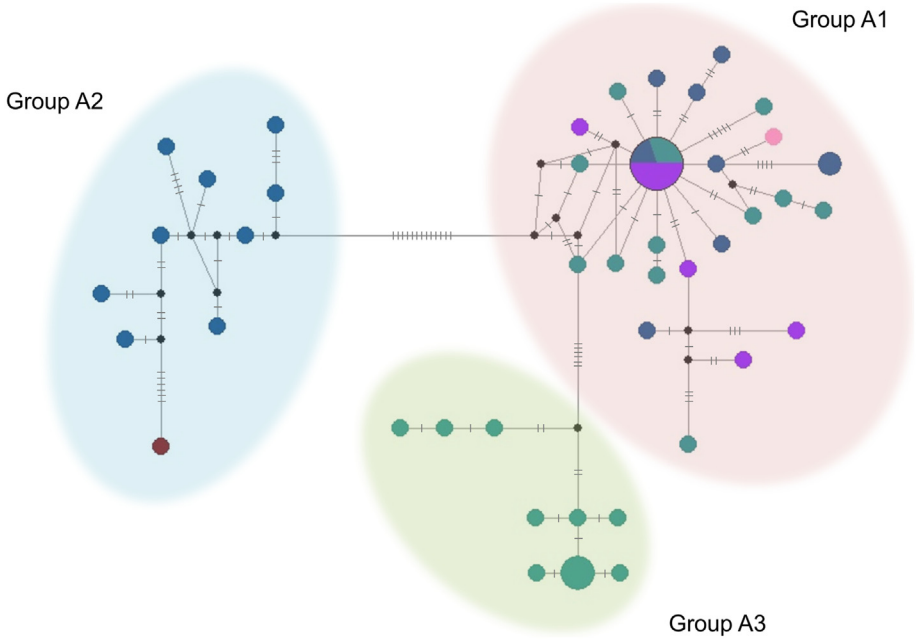

Fig. 3. The Median-Joining Network from 483 bp mtDNA *cox*1 sequences of *N. americanus*. Each haplotype is represented by a circle and the size of each circle is proportional to haplotype frequency. The median vector is indicated by a solid black circle. Brazil: (NSN) blue, (TER) purple, and (RSS) pink; Africa (Togo) red; Asia (China) green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from Africa (Togo); Group A3 included only Asian sequences (China). Interestingly, four Brazilian sequences were closely related to sequences identified as *Necator* sp. (Group B). These sequences presented a 98–99% nucleotide similarity with African (Gabon) samples and a 7–9% divergence when compared to *N. americanus* sequences. Three of these sequences of *Necator* sp. were identified in individuals of the same family (living in the same household).

23 haplotypes ($n = 29$ samples) were identified in 483 bp mtDNA *cox*1 sequences of *N. americanus*. When considering the three Brazilian municipalities included, we found 17, 5, and 1 distinct haplotypes in NSN, TER, and RSS, respectively. 22 haplotypes were unique and previously undescribed. Only one haplotype (two sequences from NSN and five from TER) was previously described in China and proved to be the most common haplotype. After the observation of overlapping peaks on electropherograms, the cloning of the mitochondrial *cox*1 segment confirmed the presence of co-infections with two distinct *N. americanus* haplotypes in two subjects.

A Median-Joining Network was created from *N. americanus cox*1 sequences obtained in the present study ($n = 29$) and Genbank reference sequences ($n = 27$) (Fig. 3). As in the ML tree, the pattern of the haplotype network revealed three distinct Groups designated again as A1, A2, and A3; and did not indicate any clear geographic pattern. Median-Joining Groups A1, A2, and A3 are connected to each other by long branches, indicating a large number of mutational steps (A1 vs. A2 = 13 mutational steps; and A1 vs. A3 = 6 mutational steps). Group A1 presents a star-like shape, with one dominant haplotype. No dominant haplotypes – or a star-like topology – were observed in Groups A2 and A3. In addition, we also observed the presence of many intermediate haplotypes (median vectors) indicating an absence of these haplotypes in the network.

Bayesian approaches generated a phylogenetic pattern similar to the ML tree (Fig. 4). The Bayesian tree also revealed three groups for *N. americanus* (A1, A2, and A3) and two groups for *Necator* sp. (B and C). Interestingly, the two groups of Chinese origin were grouped in the same clade. The molecular-dating analysis suggested that the two clades dividing *N. americanus* and *Necator* sp. began to diverge during the middle Pleistocene (300,000 years ago) (Fig. 4). The time of origin of *Necator* sp. clade is estimated to fall approximately in the middle Pleistocene (150,000 years ago). Moreover, the most recent common ancestor among *N. americanus* groups was in the late Pleistocene (approximately 60,000 years ago). Mismatch distribution analyses presented multi-modal frequency distributions for *Necator* spp. populations, rejecting possible population expansion (Fig 5A and B).

4. Discussion

In this study, most *cox*1 fragment sequences from hookworms infecting humans in northeastern Brazil were found to make possible the characterization of *N. americanus*. Previous studies carried out through morphologic taxonomy demonstrated the predominance of *N. americanus*, but did not discard the circulation of *A. duodenale* in Brazil (Kobayashi et al., 1995; Marzochi and Chieffi, 1978). The molecular characterization of hookworms with DNA restriction fragment length polymorphisms demonstrated the predominance of *N. americanus,* but the co-circulation of *A. duodenale* in Brazil (George et al., 2017). Nonetheless, species composition of hookworm populations is a poorly studied topic, and the proportion of infections attributable to *N. americanus* or *A. duodenale* in distinct endemic Brazilian regions is largely unknown.

The assessment of intraspecific genetic variability of *N. americanus* demonstrated the circulation of 23 distinct *cox*1 haplotypes of this parasite in the studied communities, including 22 haplotypes described by the first investigation. The study demonstrated that distinct haplotypes co-infected some individuals. The genetic variation of this species has already been observed in other populations and may be involved in differences in epidemiological and virulence characteristics, including distinct patterns of resistance to anthelmintic drugs (Hu et al., 2003). In this way, *N. americanus* populations present significant sub-structuring (Romstad et al., 1998).

The haplotype network, as well as the ML phylogenetic analysis, showed that some *N. americanus* haplotypes in Northeastern Brazil are closely related to Asian parasites. This part of the haplotype network (Group A1) presented a star-like shape pattern, suggesting that population expansion originating from an Asian haplotype. It is important to emphasize that there are few New World hookworm DNA sequences available for genetic studies and that the vast majority of data currently available refers to Asian and African isolates.

Our phylogenetic inferences reveal that, besides haplotypes of Asian origin, there is circulation in northeastern Brazil of haplotypes maternally related to African strains identified as *N. americanus* or *Necator* sp. This dichotomy of matrilineal inheritance is in agreement with the existing data on genetic variations of *N. americanus*, which point to the

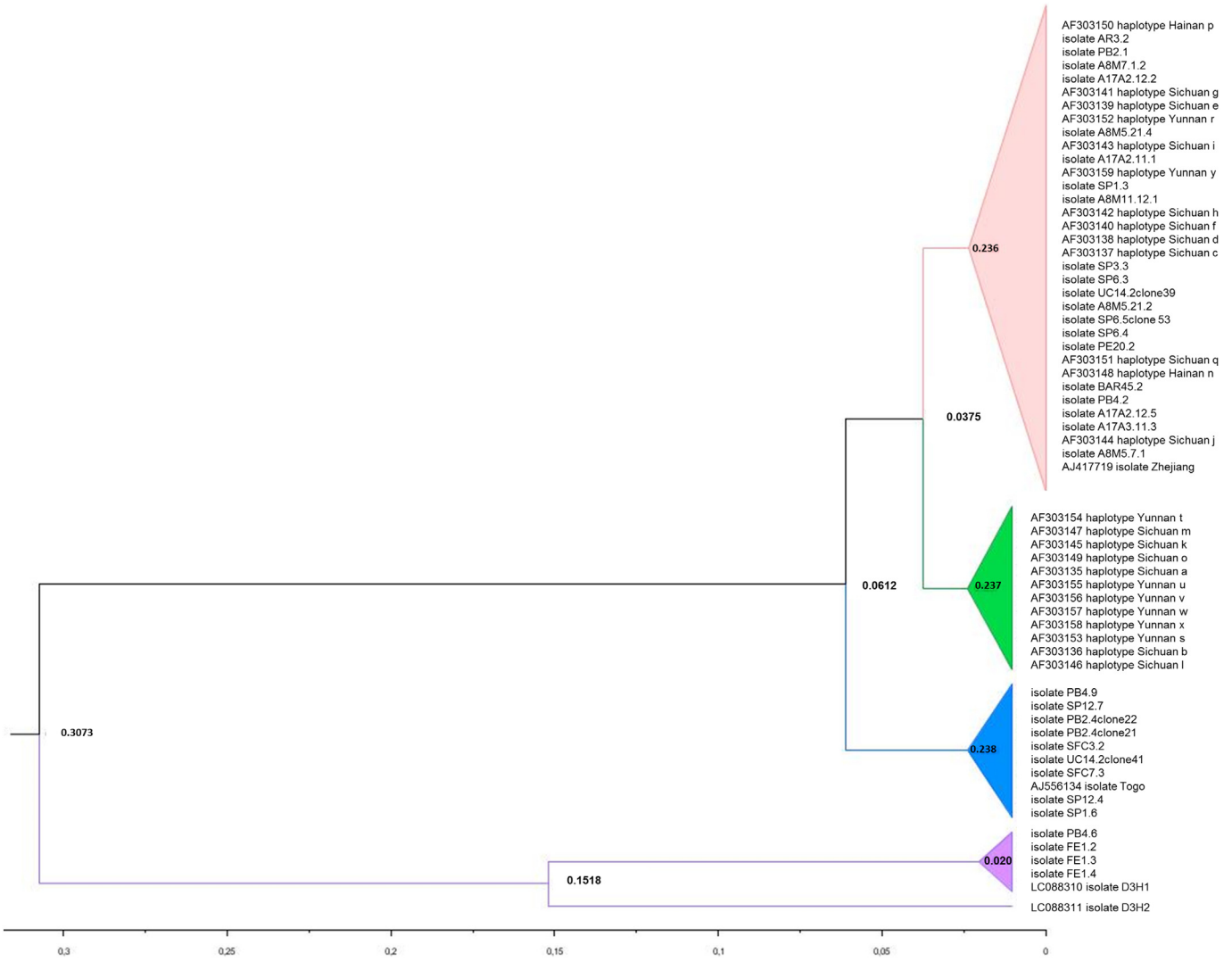

Fig. 4. Bayesian tree of *Necator* spp. sequences from Brazil, China, and Togo with divergence time estimation.

existence of two major lineages with ancestral divergence (Hu et al., 2003). Previous analyses demonstrated that Asian and African gene pools of *N. americanus* have been isolated for a long time and differ by 3–7% in 12 protein-coding genes and by 8–25% in non-coding regions, with 120 amino acid substitutions in 12 mitochondrial proteins (Hu et al., 2003).

Our analysis suggests that the divergence between *N. americanus* of Asian and African origin occurred over 60,000 years ago. This period finds a parallel in the interval of time elapsed in the migration of *Homo sapiens* from Africa towards Asia. The presence in the New World of *N. americanus* haplotypes of both Asian and African origin can be explained by the migratory phenomena responsible for the peopling of the American continent, with African slaves being introduced from the 17th century and integrated with native Amerindian peoples, which have Asian ancestry. In recent centuries, and until nowadays, Amerindians and African slaves' descendants have constituted the poorest social strata in Latin America, living in inadequate sanitation conditions, which has made hookworm infection an important public health problem since the early colonial times.

The presence of hookworms in pre-Columbian South America has been proved (Araújo et al., 2011). However, hookworm infection was also present in the European populations that colonized the continent, including Brazil. No genetic data from hookworms from Europe is currently available, and this infection has been successfully controlled in the continent through improvements in sanitation. It can also be hypothesized that the ancestry of hookworms potentially brought to Brazil by European colonizers is African, which would also explain the presence of *cox*1 sequences related to specimens from that continent. Nevertheless, we cannot rule out the emergence of new haplotypes along with the more recent migratory waves (recent decades) due to globalization. Our study did not use nuclear DNA genetic markers, so our phylogenetic inferences are limited to matrilinearity.

In this study, some samples could not be characterized at the species level, being identified as *Necator* sp. These *cox*1 sequences of parasites from the state of Piauí showed high similarity with sequences of specimens obtained from non-human primates circulating zoonotically among humans and apes in African forest reserves.

Hasegawa et al. (2014) sequenced *cox*1 and internal transcribed spacer (ITS) of hookworm larvae obtained from humans and apes living in the Dzanga Sangha Reserve in the Central African Republic, describing three phylogenetic clusters. The first presented homology with *N. americanus*, while the two others were not related to current available *N. americanus* sequences. This suggested that these clusters could be formed by other species within the genus *Necator*, and harbored by non-human primates, as the specimens were obtained from gorillas and chimpanzees, as well as the humans in the reserve. Kalousová et al. (2016) identified as *N. gorillae* adult worm specimens expelled by the researchers working in the Dzanga Sangha Reserve after treatment with

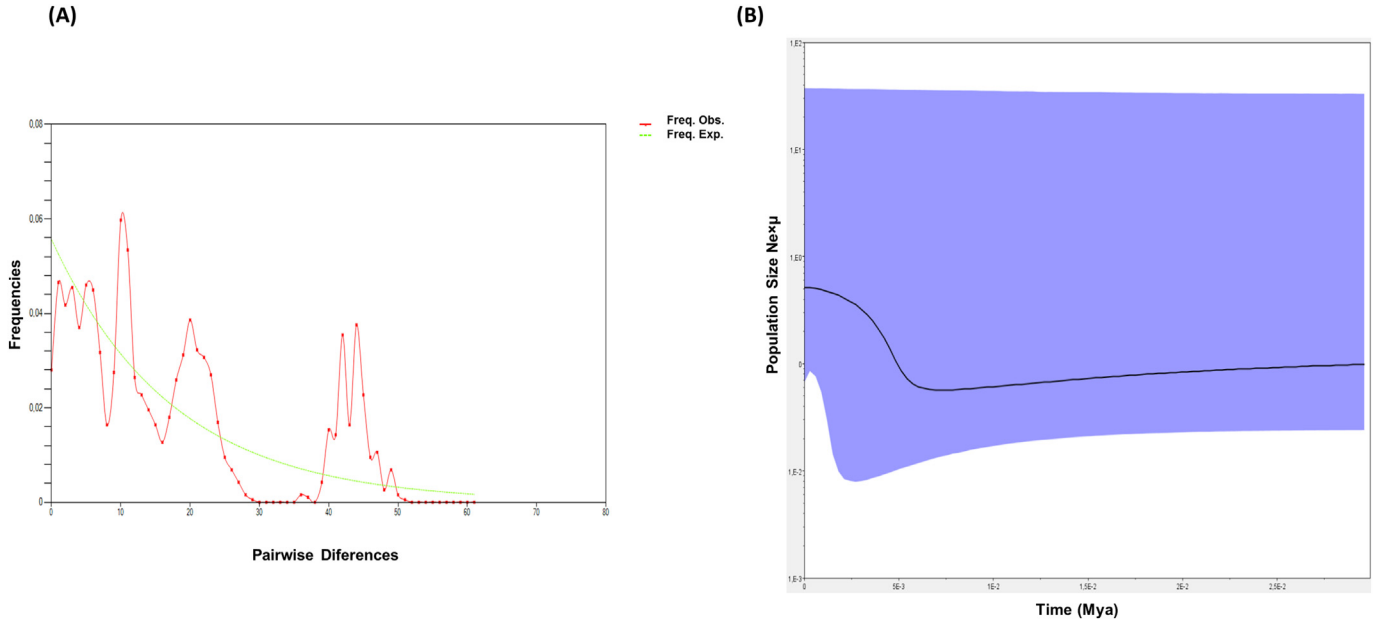

Fig. 5. (A) Mismatch distribution analysis for the *Necator* spp. population. The line charts represent the observed frequencies of pairwise differences among haplotypes. (B) Bayesian skyline plot calculates for the *Necator* spp. population. The X-axis is in units of per million years ago (Mya) and the Y-axis is Ne × μ (effective population size × mutation rate per site per generation). The purple area represents 95% highest posterior density (HPD) limits. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

albendazole, supporting a zoonotic cycle of *N. gorillae* in Africa. In addition, Hasegawa et al. (2017) analyzed mitochondrial and ITS nucleotide sequences of hookworm larvae obtained from gorillas and chimpanzees in Gabon and Uganda, grouping the specimens into three clusters, one of which had homology with *N. americanus*, and the other two being identified as *Necator* sp. The authors argue that the specimens identified as *Necator* sp. are, presumably, *N. gorillae*. Our study in northeastern Brazil identified *Necator* sp. related *cox*1 sequences, homologous to those observed in Africa in zoonotic cycles. Bayesian analyses suggest that the common ancestor of *N. americanus* and *Necator* sp. existed about 300,000 years ago. Thus, the divergence between these species can be traced back to the Middle Pleistocene. It is possible that, over time, humans and great apes became the preferential host of *N. americanus* and *N. gorillae*, respectively. However, these closely-related species of hookworms are able to cross the host barrier and circulate zoonotically.

Graber and Gevrey (1981) emphasized that parasites of the genus *Necator* obtained from non-human primates (and identified previously as *N. congolensis*) have a great morphological similarity with the swine parasite denominated *N. suillus*. *N. suillus* has been reported in domestic and wild swine in the Antilles and Madagascar. In the studied communities, there is intense pig raising in peridomestic environments, with great contamination of the environment with swine fecal matter. In this sense, the possibility of zoonotic transmission of hookworms between pigs and humans in NSN cannot be ruled out, and the specimens identified as *Necator* sp. could be, actually, related to *N. suillus*. Regarding the lifestyle of the houses where *Necator* sp. was identified, it is observed that families are living in poverty, practicing open evacuation and living in very close contact with pigs and their feces, in the domestic environment.

Human infections with *Trichostrongylus* sp. were not identified, despite the very close relationship of human populations with goats and sheep in the studied communities. The misidentification of hookworms in *Trichostrongylus* sp. infections in the Brazilian northeast was proposed in the state of Bahia and has been demonstrated in Asia and Europe (Buonfrate et al., 2017; Gholami et al., 2015; Sharifdini et al., 2017; Souza et al., 2013). Nevertheless, more extensive studies are needed in order to rule out the presence of zoonotic infections by Strongylida parasites in northeastern Brazil.

Data from this study opens up possibilities for phylogeographic, evolutionary, and molecular epidemiological studies in regions where hookworm infection persists focally, despite control efforts using MDA and improvements in sanitation. In addition, the assessment of genetic diversity can contribute to the development of hookworm vaccines and help to understand patterns of propagation of benzimidazole resistance. Studies with larger sample sizes, including other endemic areas in Brazil, are needed. STH control strategies should be improved by the characterization of the local parasite genetic profiles.

Conflict of interest statement

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.11.018.

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