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Victor Hugo Borba Nunes

Taxonomia Integrativa, Filogenia e Paleodistribuição de Capilarídeos

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2019

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Microbiologia, da Universidade do Estado do Rio de Janeiro. Área de concentração: Microbiologia Médica Humana.

Orientador: Prof. Dr. José Roberto Machado Silva

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Coorientadora: Profª. Dra. Alena Mayo Iñiguez
Fundação Oswaldo Cruz

Banca Examinadora: _____
Prof. Dr. José Roberto Machado e Silva (Orientador)
Faculdade de Ciências Médicas - UERJ

_____
Prof. Dr. Eduardo José Lopes Torres
Faculdade de Ciências Médicas - UERJ

_____
Profª Dra. Simone Cohen
Fundação Oswaldo Cruz

_____
Profª Dra. Claudia Maria Antunes Uchôa Souto Maior
Universidade Federal Fluminense

_____
Prof. Dr. Luis Muniz
Fundação Oswaldo Cruz

Rio de Janeiro
2019

DEDICATÓRIA

A minha mãe, sem ela nada seria possível.

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Isn't it enough to see that a garden is beautiful without having to believe that there are fairies at the bottom of it too?

Douglas Adams

RESUMO

NUNES, Victor Hugo Borba. *Taxonomia integrativa, Filogenia e Paleodistribuicao de capilarídeos.* 2019. 165 f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2019.

Existem mais de 300 espécies de capilarídeos em vários grupos de vertebrados (peixes, anfíbios, répteis, aves e mamíferos) em todo o mundo. A dificuldade de identificação, pelas poucas estruturas taxonomicamente informativas e seu tamanho reduzido, fazem a tarefa laboriosa e a definição controversa. Assim, sua taxonomia é uma das mais complexas entre os Nematoda. Achados de ovos de capilarídeos são relatados em material arqueológico tanto no Velho como no Novo Mundo, principalmente na Europa e América do Sul. Apesar de numerosos achados, existe pouco refinamento no diagnóstico das espécies devido aos poucos trabalhos que caracterizam a morfologia dos ovos, principal estrutura de identificação em material arqueológico. A principal proposta deste projeto foi fornecer informações básicas da taxonomia integrativa de capilarídeos, baseada na caracterização morfológica e morfométrica dos ovos, assim como desenvolvimento de uma metodologia para identificação de espécies com a abordagem de Inteligencia Artificial aplicada em espécies referencias de coleções biológicas, para construir um modelo de discriminação dos morfotipos encontrados nos sítios arqueológicos. Para atingir os objetivos, ovos de espécies referências de duas coleções institucionais, Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC) e *Collection helminthologique du Muséum National d'Histoire Naturelle de Paris* (MNHN), foram examinados por microscopia de luz e eletrônica de varredura, análise estatística dos dados e aplicação de metodologia de Inteligência Artificial. A tese é apresentada no formato de "coletânea", composta por 4 artigos. O primeiro artigo foi realizado uma revisão sistemática do cenário paleoepidemiológico mundial das espécies de capilarídos, além de demonstrar a atual taxonomia dos gêneros por análise filogenética, fazendo alusão as dificuldades encontradas em identificar as espécies em material antigo e classificar nos gêneros. O segundo artigo faz a caracterização morfológica por microscopia de luz dos espécimes das duas Coleçõoes biológicas institucionais, sendo no total de 28 espécies distribuídas em 8 generos, além de desenvolver uma metodologia de Inteligência Artificial. As semelhanças e diferenças das espécies e gêneros são expostas e discutidas com análise estatística e é criada uma árvore de decisão para identificação de espécies. O terceiro artigo caracteriza os ovos depositados na CHIOC por microscopia eletrônica de varredura, o que permite evidenciar as diferenças observadas pela microscopia de luz. É uma ferramenta que discrimina melhor os morfotipos dos ovos e ajuda a entender sua ultraestrutura, porém não ainda não substitui o microscópio de luz na prática de rotina. No quarto artigo, são analisados sítios do Novo e Velho mundo onde são identificados ovos de capilarídeos. Assim foi feito uma análise dos ovos para identificação. Os resultados fornecem as bases para estabelecer a relação dos capilarídeos com seus hospedeiros humano e animal no passado, estabelecendo um cenário da paleodistribuição da infecção. Como conclusão os resultados do estudo poderão subsidiar futuras pesquisas na identificação de capilarídeos tanto modernos como em cenários arqueológicos.

Palavras-chave: Paleoparasitologia. Filogenia. Inteligência Artificial. MEV.

ABSTRACT

NUNES, Victor Hugo Borba. *Integrative taxonomy, phylogeny and capillariid paleodistribution.* 2019. 165 f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2019.

There are more than 300 species of capillaries in various vertebrate groups (fish, amphibians, reptiles, birds and mammals) all over the world. The difficulty of identification, by the few taxonomically informative structures and their small size, make the task laborious and the definition controversial. Thus, its taxonomy is one of the most complex among the Nematoda. Findings of capillary eggs are reported in archaeological material both in the Old and New World, mainly in Europe and South America. Despite numerous findings, there is little refinement in the diagnosis of the species due to the few works that characterize the main egg morphology identification structure in archaeological material. The main proposal of this project is to provide basic information on the integrative taxonomy of capillaries, based on the morphological and morphometric characterization of the eggs, as well as the development of an Artificial Intelligence methodology applied to reference species from biological collections, to construct a discrimination model of the morphotypes found in archaeological sites. To achieve the objectives, eggs of reference species from two institutional collections, Helminthological Collection of the Oswaldo Cruz Institute (CHIOC) and Collection helminthologique du Muséum National d'Histoire Naturelle de Paris (MNHN), were examined by light microscopy and scanning electron microscopy, statistical analysis data and application of Artificial Intelligence methodology. The thesis is presented in the format of "collection", composed of 4 articles. The first article was a sitematic review of the global paleoepidemiological scenario of capillary species, as well as showing the current taxonomy of genus by phylogenetic analysis, alluding to the difficulties encountered in identifying species in ancient material and classifying them in genera. The second article makes the morphological characterization by light microscopy of the specimens of the two institutional biological Collections, being a total of 28 species distributed in 8 genera, in addition to developing an Artificial Intelligence methodology. The similarities and differences of the species and genera are exposed and discussed with statistical analysis and a decision tree is created for species identification. In the third article the CHIOC eggs were characterized by scanning electron microscopy, which shows the observed differences between light microscopy. It is a tool that better discriminates the egg morphotypes and helps to understand its ultrastructure but does not yet replace the light microscope in routine practice. In the fourth article, New World and Old World sites are analyzed where capillary eggs are identified. Thus, an analysis of the eggs for identification is made. The results provide the basis for establishing the relationship of capillaries with their human and animal hosts in the past, establishing a scenario of paleontribution of infection. As conclusion, the study may support future research on the identification of both modern and archaeological capillaries.

Keywords: Paleoparasitology. Phylogeny. Artificial Intelligence. SEM.

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LISTA DE ABREVIATURAS E SIGLAS

18S rDNA	18S DNA ribosomal
AD	*Anno Domini*
aDNA	DNA antigo (*ancient* DNA)
AP	Antes do presente
CDC	Centers for Disease Control and Prevention
CHIOC	Coleção Helmintologica do Instituto Oswaldo Cruz
*cox*1	Citocromo oxidase subunidade 1
DNA	Ácido desoxirribonucléico
FCM/UERJ	Faculdade de Ciências Médicas da Universidade do Estado do Rio de Janeiro
FIOCRUZ	Fundação Oswaldo Cruz
GL	Geographical Location / Localização Geográfica
H	Hospedeiro
IAB	Instituto de Arqueologia Brasileiro
IOC	Instituto Oswaldo Cruz
LABTRIP	Laboratório de Biologia de Tripanosomatídeos
MM	Mofológia e Morfometria
MNHN	Museu Nacional de História Natural de Paris
ML/AI	Machine Learning/Artificial Inteligence
N	Número
SSCP	Polimorfismo de conformação de fita simples
IA	Inteligência Artificial
MA	Milhões de anos
AM	Aprendizado de Máquinas

LISTA DE SÍMBOLOS

%	Porcentagem
±	Mais ou menos
μm	Micrometro
μl	Microlitro
ml	Mililitro

SUMÁRIO

INTRODUÇÃO

A Paleoparasitologia é um ramo da Paleopatologia, que se caracteriza pela busca de vestígios parasitológicos em material arqueológico ou paleontológico (FERREIRA; ARAÚJO; CONFALONIERI, 1979). Tem como principais objetos de estudo os coprólitos (fezes dessecadas ou mineralizadas), porém também são estudados parasitos em tecidos mumificados, enterramentos, latrinas, urnas funerárias e outros restos preservados que podem ser encontrados em camadas arqueológicas ou paleontológicas (BOUCHET et al., 2003).

Foi definida assim, como campo da ciência capaz de contribuir com estudos para a antropologia, arqueologia, zoologia, ecologia, entre outros, mas sempre com profundas raízes na parasitologia (DITTMAR, 2009). Entre os resultados mais relevantes publicados estão o uso de parasitos como marcadores biológicos de migrações humanas pré-históricas, com evidências de novas rotas para a colonização das Américas (ARAÚJO et al., 1988; REINHARD et al., 2001; ARAUJO et al., 2008). Também são significativos os achados de parasitos de animais em coprólitos humanos caracterizando, em alguns casos, zoonoses em populações pré-históricas do nordeste brasileiro, na região arqueológica da Serra da Capivara, sudeste do estado do Piauí (SIANTO et al., 2009). Mesmo com toda a contribuição para o entendimento das parasitoses no passado a ausência de determinados parasitos em material arqueológico ainda não foi esclarecida.

Achados de ovos de *Capillaria sensu lato* têm sido relatados em material arqueológico, principalmente na Europa e América do Sul. No Velho Mundo, Bouchet (BOUCHET, 1997) analisou 23 coprólitos humanos, na França, datados de 5150-4930 antes do presente (AP), e encontrou 21 positivos para ovos de *Capillaria* sp. No sítio arqueológico localizado na Bélgica, na “Place d’Armes” foram encontrados ovos de *Capillaria* sp. em 3 contextos arqueológicos, no Período Gallo-Romano (século II e III Anno Domini (AD)), no Período Carolíngea (século IX – XI AD) e entre os séculos XII e XIII (DA ROCHA et al., 2006). Na Alemanha, pela análise de sedimento retirado da região pélvica do esqueleto datado de 4500 anos, foram revelados ovos de *Capillaria* sp. (DITTMAR; TEEGEN, 2003). No Velho Mundo, ainda tem relatos de ovos desse parasito em sítios arqueológicos das regiões que hoje são a Itália (BOSI et al., 2011), Rússia (SAVINETSKY; KHRUSTALEV, 2013) e República Tcheca (MYŠKOVÁ et al., 2014).

No Novo Mundo, a maioria dos achados se encontra na região da Patagônia, Argentina. Fugassa e Guichón (FUGASSA; GUICHÓN, 2005) encontraram ovos de *Capillaria* sp. em coprólito humano de 6540±110 anos AP. Foram encontrados, também em sedimentos associados a ossos da região pélvica provenientes de sítio arqueológico, ao sul da província de Santa Cruz, Argentina (FUGASSA; BARBERENA, 2006). Em sedimentos de *pellets* do sítio arqueológico Cerro Casa de Piedra, também de Santa Cruz, foram recuperados numerosos ovos identificados primeiramente como *Capillaria* sp. e mais tarde como *C. hepatica* (Bancroft, 1893) (FUGASSA; SARDELLA; DENEGRI, 2007). No entanto, apesar de numerosos achados de ovos de *Capillaria* sp. na América do Sul, no Brasil existem apenas dois relatos deste parasito (CONFALONIERI, 1985; SIANTO *et al.*, 2014). A escassez de achados de ovos de *Capillaria sensu lato* em material paleoparasitológico não indica necessariamente a ausência deste parasito no ambiente ou mesmo de infecções (SIANTO et al., 2009). Quando a conservação do material arqueológico está muito comprometida e consequentemente a morfologia dos ovos, há dificuldade na distinção entre ovos de tricurídeos e capilarídeos por microscopia de campo claro. Segundo Le Bailly (LE BAILLY, 2005) a aplicação da microscopia eletrônica de varredura tem permitido a identificação precisa de ovos de *Capillaria sensu lato* discriminando de ovos de tricurídeos.

Esses achados representam a circulação de diversas espécies de capilarídeos em hospedeiros, tanto humanos como animais no passado. A relação zoonótica desse grupo de parasito pode não ser clara na análise coprológica, porém indica o contato humano tanto no Novo e Velho Mundo.

O grupo dos capilarídeos é um dos que possuem a taxonomia mais complexa, além de existirem descritas mais de 300 espécies em diversos hospedeiros. Portanto, apesar dos numerosos achados arqueológicos, tanto no Novo e no Velho Mundo, a identificação dessas espécies através dos ovos é muitas das vezes imprecisa, resultando em um diagnóstico genérico de *Capillaria sensu lato*.

Portanto, abordagens diversas para caracterizar e identificar esses ovos passam a ser necessárias. Dentre os métodos de microscopia já utilizados para tal caracterização, é possível apontar a microscopia de luz (ML), utilizada de rotina em laboratórios, e utilizada em todos os trabalhos supracitados. Os ovos possuem características na superfície das cascas, como ornamentações, já apontadas por alguns autores (CONBOY, 2009; TRAVERSA et al., 2011; ZAJAC; CONBOY, 2012). A microscopia eletrônica de varredura (MEV), nos permite observar e descrever em detalhes das ornamentações, porém ainda pouco utilizada e com poucas espécies de capilarideos descritos (SUKONTASON et al., 2006; TRAVERSA et al.,

2011; MACCHIONI et al., 2013). Um trabalho desenvolvido por Romashov em 1985 (ROMASHOV, 1985), tentar classificar as espécies de capilarídeos pela ornamentação presente nos ovos, os categorizando em 6 tipos, ao utilizar ambas as microscopias, ML e MEV. Porém não propõe uma metodologia de identificação de espécies.

A abordagem de Aprendizado de Máquinas é uma ferramenta da Inteligência Artificial para realizar funções “inteligentes”, como aprendizado, tomada de decisão, adaptação, controle e percepção. Possui aplicabilidade em diversas áreas como na computação e robótica (TSAI et al., 2009; CHEN et al., 2019), assim como na biologia principalmente na área de detecçao de novos grupos de doenças (PEROU et al., 1999; ALIZADEH et al., 2000; ROSS et al., 2000). Porém não existem trabalhos para a identificação identificaçao de espécies de parasitos em qualquer nível taxonomico.

1. REVISÃO DA LITERATURA

1.1 Paleoparasitologia e Paleogenética

A Paleoparasitologia é uma ciência multidisciplinar que tem profundas raízes na Parasitologia. Utiliza-se da arqueologia e paleontologia para investigar questões referentes a interação parasito-hospedeiro-ambiente e a origem e evolução das doenças parasitárias. Através de coprólitos (fezes preservadas por dessecação ou mineralização) (SOUTO, 2008), esqueletos, múmias, latrinas e urnas funerárias, recuperadas de camadas arqueológicas e paleontológicas, é possível encontrar evidências deixadas pelas infecções parasitárias, tais como ovos e larvas de helmintos, cistos de protozoários, fragmentos de material genético e mesmo antígenos (ARAÚJO; FERREIRA, 2000).

Os coprólitos são o "padrão ouro" para pesquisas relacionadas a parasitos intestinais devido à melhor preservação dos ovos nesse tipo de material. Porém em alguns casos de ovos mais delicados, devido a ações de fatores tafonômicos, como temperatura e umidade, degradam o material havendo a destruição das evidências evolutivas ali presentes (MORROW et al., 2016). Temos como exemplo os ovos de *Enterobius vermicularis* (Linnaeus, 1758), leves e de estrutura delicada, são muito escassos em sítios europeus e locais de clima tropical (ARAÚJO et al., 1985), com exceção de lugares com ótima preservação (EUA e algumas regiões da América do Sul). No caso de esqueletos, a obtenção de ovos também é favorecida pela região do sacro que serve como depósito do material fecal em um corpo em decomposição, mesmo em posição vertical (REINHARD et al., 1992; BERG, 2002).

A identificação desses coprólitos deve-se a estudos em fezes recentes, que através de sua morfologia é possível identificar a origem zoológica (CHAME, 1988, 2003). Quando não é possível a identificação precisa das fezes apenas com a morfologia, a análise de macro e microvestígios é essencial, determinando assim a dieta. A presença de determinados ovos de parasitos específicos de alguns hospedeiros também pode solucionar essas questões, como o *Enterobius vermicularis*, parasito específico da espécie humana.

Essa linha de pesquisa, a Paleoparasitologia, foi nomeada e estruturada no Brasil, pelo Dr. Luiz Fernando Ferreira em 1979, na Fundação Oswaldo Cruz. Porém existem alguns trabalhos estudos anteriores. Sir Marc Armand Ruffer (RUFFER, 1910), o pai da

paleopatologia, foi o pioneiro na área, identificando ovos de *Schistosoma haematobium* (Fisher, 1934) em rins de múmias egípcias datadas de 3.200 anos antes de presente (AP). E Szidat (SZIDAT, 1944) foi um dos primeiros a estudar coprólitos, de corpos preservados em um pântano na Prússia, datados de 1.500 anos AP, onde encontrou ovos de *Trichuris trichiura* (Linnaeus 1758) e *Ascaris lumbricoides* (Linné, 175).

A Paleogenética é um ramo da paleoparasitologia que consiste na recuperação de material genético de vestígios biológicos preservados de sítios arqueológicos ou paleontológicos, denominado de DNA antigo (aDNA, *ancient* DNA). Surgiu com o propósito de responder indagações que as análises tradicionais não conseguiam, além de conseguir confirmar os resultados obtidos desses estudos. Tem aplicações nas áreas de antropologia, arqueologia, paleontologia, entre outras (IÑIGUEZ, 2011, 2014).

A princípio, esses estudos eram realizados com materiais extremamente preservados, pois a degradação do material genético começa a partir do momento da morte celular, quando os processos de reparo do DNA cessam. Com isso surgem as dificuldades de extração de DNA antigo, muitas vezes impossibilitando-o. Porém, existem condições de preservação que retardam ou interrompem esse processo, o que ocorre em mumificações por exemplo.

Com o surgimento de técnicas de PCR (Reação em Cadeia da Polimerase), com a possibilidade de amplificação de uma única fita, as dificuldades para trabalhar com aDNA foram minimizadas. Então, pesquisas com materiais de diversas origens, humana, animal e vegetal, assim como vestígios de bactérias, vírus e fungos, aumentaram. As datações também chegaram a tempos bem antigos, com trabalhos em âmbar e *permafrost* (um exemplo de condição de ótima preservação do DNA, devido as baixíssimas temperaturas), os mais antigos com datações de 135 milhões de anos (MA) e 3 MA respectivamente.

Uma das aplicações da Paleogenética está no estudo das doenças infecto-parasitárias nas populações do passado, que pode ser chamada também de Paleoparasitologia molecular. Esse interesse pelas doenças que acometiam o ser humano no passado, e que não era possível a recuperação pelas técnicas clássicas da Paleoparasitologia, culminou em diversos trabalhos apontando infecção como por exemplo *Mycobacterium tuberculosis* (causador da tuberculose) (JAEGER et al., 2013), *Yersinia pestis* (causador da peste negra) (HAENSCH et al., 2010), *Trypanosoma cruzi* (causador da doença de Chagas) (FERNANDES et al., 2008), dentre vários outros microrganismos como os helmintos e protozoários causadores das infecções intestinais (IÑIGUEZ et al., 2003).

Trabalhos com conteúdo intestinal e coprólitos tiveram grandes resultados também, apesar das dificuldades metodológicas devido a diversos fatores que interferem na

amplificação do aDNA. A exemplo deles, trabalhos com aDNA de *Escherichia coli*, recuperado de múmias datadas de 2250 AP por Fricker, Spilgelman e Fricker (FRICKER; SPIGELMAN; FRICKER, 1997), assim como um estudo que mostrou a possibilidade de acesso ao aDNA sem o isolamento de parasitos, feito por Iñiguez e colaboradores (IÑIGUEZ et al., 2003, 2006) em coprólitos de povos pré-colombianos. Esses estudos demonstraram que a análise molecular evidencia a presença de parasitos quando análises tradicionais não identificaram.

1.2 Capilarídeos

1.2.1 Classificação Taxonômica

O presente trabalho segue como base para as discussões a classificação taxonômica proposta por Moravec (2001), descrita abaixo.

Filo: Nematoda Rudolphi, 1808
Classe: Enoplea Inglis, 1983
Subclasse: Dorylaimia Inglis, 1983
Ordem: Trichocephalida Spasski, 1954
Superfamília: Trichinelloidea Ward, 1907 (Hall, 1916)
Família: Capillariidae Railliet, 1915
Subfamília: Capillariinae Railliet, 1915

1.2.2 História Taxonômica

Existem descritas atualmente mais de 300 espécies de capilarídeos em diversos hospedeiros vertebrados (peixes, anfíbios, répteis, aves e mamíferos) (Tabela 1) por todo o mundo. A sua sistemática é uma das mais complexas dentre os Nematoda. A dificuldade de

identificação primariamente se da devido ao pequeno tamanho das espécies (MORAVEC, 1982) e as poucas estruturas morfológicas taxonomicamente informativas para a identificação, torna a tarefa laboriosa e controversa. Outro fator que dificulta a sua classificação é a plasticidade fenotípica causada pelos diferentes hospedeiros, sítios de infecção e localidades (SPRATT, 2006). Além disso, muitas espécies têm sido descritas de forma inadequada ou errônea (MORAVEK, 2001). O diagnostico de espécie é feita principalmente pelas estruturas da região posterior dos machos, já que as fêmeas de algumas espécies são indistinguíveis.

A classificação formal dos capillarídeos começou em 1800 com Zeder e a definição do gênero *Capillaria*, sendo descritos como nematoides pequenos com aparecia de cabelo. Posteriormente Rudolphi em 1819 criou o gênero *Trichosoma* onde descreveu seis espécies novas e mencionou outras 16 como sinonimos do gênero *Capillaria*, que só deixaria de ser usado em 1911 por Ransom. Quatro gêneros foram estabelecidos por Dujardin (1845), *Calodium*, *Eucoleus*, *Liniscus* e *Thominx*, e dividido por Diesing (1851) em dois grupos, Trichotrachelida: Gymnothecae (*Calodium*, *Liniscus*, *Trichosoma*) e Echinothecae (*Eucoleus*, *Thominx*), com a adição do gênero Eucoleus no último grupo.

Ransom em 1911 utiliza o gênero *Capillaria* e o aloca na subfamília Trichurinae na família Trichinellidae. Esse gênero também é utilizado de maneira genérica por Travassos em 1913, utilizando *Capillaria* em detrimento dos gêneros *Calodium*, *Eucoleus*, *Liniscus*, *Thominx* e *Trichosoma*, descritos até então, que eram divididos em dois subgêneros, *Capillaria* e *Thomynx*.

Hall em 1916 redescreve a então espécie *Trichocephalus hepaticus* para o gênero criado, *Hepaticula*, que posteriormente seria realocada para Capillaria e Calodium por último. Além de estabelecer uma nova superfamília Trichinelloidea. Já em 1926, Yorke e Maplestone criaram a subfamília Capillariinae com o reconhecimento apenas dos gêneros *Capillaria*, *Hepaticola* e *Eucoleus*. A subfamilia foi descrita na família Trichuridae e superfamília Trichuroidea que apresentava outras famílias como Trichosomoididae e Trichinellidae.

López-Neyra, em 1947, revisou o grupo de capilarídeos e aceitou a existência dos gêneros *Capillaria*, *Capillostrongyloides*, *Eucoleus* e *Trichosomoides*, além de criar o gênero *Aonchotheca*.

Em 1959, Freitas adicionou novos gêneros e dividiu o grupo, totalizando 10 gêneros: *Aonchotheca*, *Capillaria*, *Capillistrongyloides*, *Hepaticola*, *Skrjabinocapillaria*, *Gessyella*, *Pseudocapillaria*, *Pterothominx* e *Ritaklossia*.

Além de outros autores que continuaram usando o único gênero *Capillaria* (Roman, 1960, 1965; Yamaguti, 1961), Anderson e Bain (1982) classificaram taxonomicamente os

capilarídeos na subfamília Capillariinae Railliet, 1915, pertencente à família Trichuridae (Nematoda: Trichinelloidea), baseado na descrição feita por Roman em 1965. Foi reconhecido pelos autores apenas um gênero, *Capillaria* Zeder, 1800, e todos os demais como sinônimos.

A classificação de Moravec (1982) foi proposta como um novo sistema para os capilarídeos para servir de alicerce para futuros estudos. Assim, é mantida a família Capillaridae Neveu-Lemaire, 1936 (Nematoda: Trichinelloidea), que devido à diversidade na morfologia dos espécimes adultos, à variedade de sítios de infecção e de hospedeiros definitivos de suas espécies, o autor propõe a necessidade de separação em diversos gêneros. A distinção taxonômica dos gêneros foi baseada principalmente nas características morfológicas da extremidade posterior dos machos. Assim, foi sugerido a divisão dos capilarídeos em 16 gêneros, onde 12 foram redefinidos, 2 resgatados e 2 criados.

Posteriormente foram adicionados outros gêneros a família, totalizando 22 gêneros (HUFFMAN; MORAVEC, 1988). Lomakin e Romashov (LOMAKIN; ROMASHOV, 1987) (*apud* (GIBBONS, 2010) dividem a família Capillariidae em 3 subfamílias, Capillariinae (5 gêneros), Baruscapillariinae Lomakin et Romashov, 1987 (10 gêneros), Skrjabinocapillariinae Lomakin et Romashov, 1987 (1 gênero). Dentro dessas subfamílias são distribuídos os 16 gêneros descritos por Moravec em 1982, e os outros gêneros descritos a partir de então. Vicente e colaboradores (VICENTE et al., 1990, 1993, 1995, 1997; VICENTE; PINTO, 1999) consideram os gêneros de capilarídeos como pertencentes da família Trichuridae, subfamília Capillarinae. Os autores descrevem os capilarídeos presentes no Brasil segundo os gêneros descritos por Moravec (MORAVEC, 1982). Recentemente, Gibbons (2010) expande a classificação propondo outros gêneros na subfamília Capillarinae. Apesar de escassos, alguns estudos moleculares foram feitos para embasar e refinar a classificação sistemática do grupo, corroborando com a classificação pelos gêneros proposta por Moravec (1982).

Tabela 1. Gêneros da família Capillariidae relacionados às classes de hospedeiros vertebrados

Gênero	**Peixes**	**Anfíbios**	**Répteis**	**Aves**	**Mamíferos**
Paracapillaria	+	+	+	+	+
Capillaria	+	+	-	+	+
Pseudocapillaria	+	+	-	+	+
Aoncotheca	-	+	-	+	+
Amphibiocapillaria	-	+	+	-	-
Baruscapillaria	-	-	-	+	+
Echinocoleus	-	-	-	+	+
Eucoleus	-	-	-	+	+
Pterothominx	-	-	-	+	+
Freitascapillaria	+	-	-	-	-
Capillostrongyloides	+	-	-	-	-
Schulmanela	+	-	-	-	-
Piscicapillaria	+	-	-	-	-
Paracapillaroides	+	-	-	-	-
Pseudocapillaroides	-	+	-	-	-
Paratrichosoma	-	-	+	-	-
Crocodylocapillaria	-	-	+	-	-
Tridentocapillaria	-	-	-	+	-
Brevithominx	-	-	-	+	-
Liniscus	-	-	-	-	+
Pearsonema	-	-	-	-	+
Calodium	-	-	-	-	+
Skrjabinocapillaria	-	-	-	-	+
Tenoranema	-	-	-	-	+

Legenda: + Hospedeiro; - Não é hospedeiro.

Fonte: (MORAVEC, 1982).

1.2.3 Biologia de capilarídeos

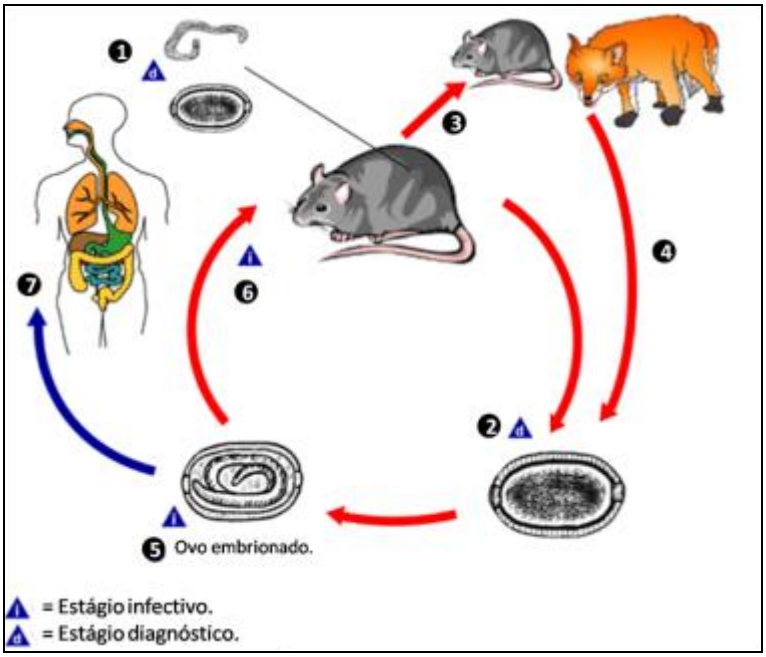
Por ser um grupo muito diverso e com variados tipos de hospedeiros (Tabela 1), de difícil diagnóstico e classificação, além de ser pouco estudado, muito pouco foi descrito sobre a biologia dos capilarídeos. Nesse item será apresentada a biologia dos gêneros dos capilarídeos descritos, restrito aos identificados em humanos.

Os ciclos biológicos da maioria das espécies não são bem conhecidos, com exceção das identificadas na infecção no homem, como *Calodium hepaticum* (Bancroft, 1893), *Paracapillaria philippinensis* (Chitwood, Velasquez, and Salazar, 1968) e *Eucoleus aerophilus* (Creplin, 1839). As capilaríases humanas são infecções raras causadas por parasitos zoonóticos, com distribuição global (LALOŠEVIĆ et al., 2008; FUEHRER; IGEL; AUER, 2011). Apesar da sua baixa frequência no homem, podem ocasionar doenças severas resultando no óbito (MORAVEC, 2001).

Uma das espécies que infecta o homem pertence ao gênero *Calodium*. A espécie *C. hepaticum* (syn. *Capillaria hepatica*, *Hepaticola hepatica*) tem como hospedeiros os roedores (REDROBE; PATTERSON-KANE, 2005), sinantrópicos tais como *Rattus rattus* (MOREIRA et al., 2013) e *Rattus norvegicus* (SIMÕES et al., 2014), e silvestres como *Agouti paca* (ALMEIDA et al., 2013). Embora os roedores sejam os principais hospedeiros, podem ocasionalmente infectar outros mamíferos (SOARES et al., 2011; ROCHA et al., 2015). Alguns estudos indicam que os ratos domésticos podem desempenhar uma função primordial na transmissão dessa zoonose (RESENDES et al., 2009). A infecção ocorre com a ingestão de ovos embrionados no solo, podendo ser ingeridos através da água ou alimentos contaminados. As larvas eclodem no intestino e migram pelo sistema porta até o fígado. Os vermes adultos ficam retidos no tecido hepático onde liberam os ovos. Para serem embrionados, e se tornarem infectantes para um novo hospedeiro, os ovos devem ser liberados ao ambiente, que ocorre quando o hospedeiro morre e se decompõe no solo, ou quando sofre canibalismo ou é predado (Figura 1).

Casos do aparecimento dos ovos nas fezes de animais e humanos são comuns devido ao consumo do hospedeiro. Assim os ovos passam pelo sistema gastrointestinal sem causar infecção no hospedeiro. Isso ocorre com frequência em populações indígenas no Brasil, devido ao hábito alimentar de consumir fígado cru de roedores (CARVALHO-COSTA et al., 2009). Também acontece com animais carnívoros como felideos e canídeos que se alimentam de pequenos roedores.

Figura 1 - Ciclo biológico de *Calodium hepaticum*.



Legenda: (1) Vermes adultos e os ovos ficam retidos no fígado; (2) ovos não embrionados são liberados no ambiente com a morte e decomposição do hospedeiro; (3) ou liberados nas fezes após canibalismo ou predação (4); (5) os ovos são embrionados no ambiente; (6-7) e ingeridos pelo hospedeiro definitivo.
Fonte: modificado de CDC. http://www.cdc.gov/dpdx/

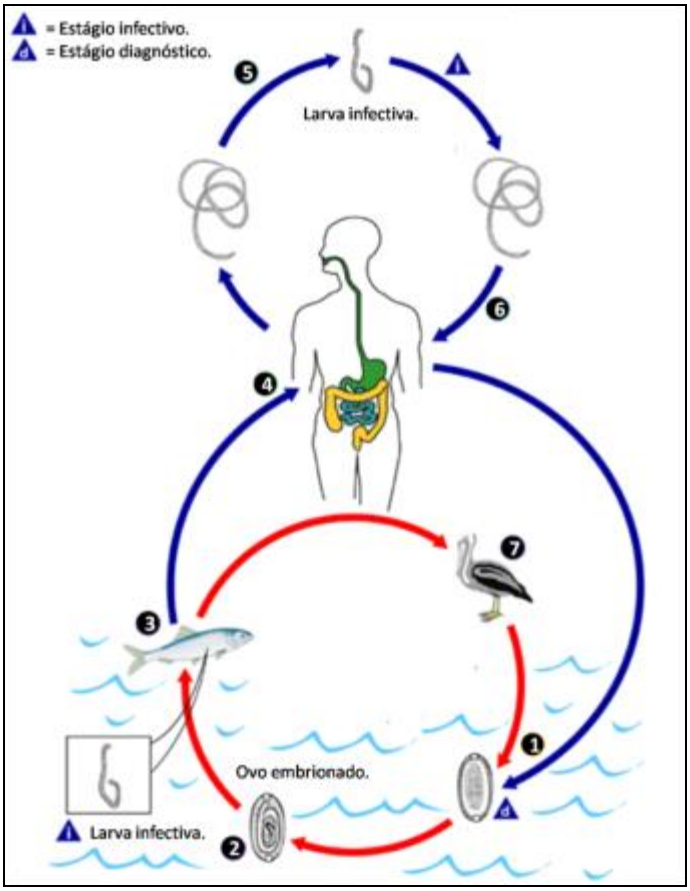
Descrito em pequenos mamíferos *C. soricicola* (Nishigori, 1926) também infecta o fígado. Porém outras espécies do mesmo gênero podem ter outros sítios de infecção (MURAI; MÉSZÁROS; SEY, 1992). *C. splenaecum* (Dujardin, 1843), por exemplo, infecta o baço principalmente de pequenos mamíferos como os mussaranhos, e acredita-se que necessita da morte do hospedeiro para a liberação dos ovos no ambiente, assim como *C. hepaticum* (MILLÁN et al., 2014).

As espécies do gênero *Paracapillaria* infectam em sua maioria esôfago e estomago de peixes, anfíbios e répteis, com exceção de *P. philippinensis* (syn. *Capillaria philippinensis*, *Aonchotheca philippinensis*) que parasita o intestino delgado, principalmente, de aves que se alimentam de peixes (estes infectados com larvas) e eventualmente o homem. Outra particularidade é que além da liberação de ovos, também há a liberação de larvas diretamente das fêmeas do parasito ocasionando reinfecções no hospedeiro, o que não ocorre com nenhuma outra espécie de capilarídeo (Figura 2) (SUKONTASON et al., 2006). Outro caso

peculiar é *P. rhamdiae* (Moravec, González–Solís e Vargas–Vázquez, 1995), cujas fêmeas liberam ovos larvados (MORAVEC, 2001).

Casos de infecção humana por *P. philippinensis* já foram relatados nas Filipinas, Japão, Coréia, Taiwan, China, Indonésia, Iran e Egito (SAICHUA; NITHIKATHKUL; KAEWPITOON, 2008). É considerada uma das infecções gastrointestinais mais virulentas, causando o óbito em casos não tratados (CROSS, 1992).

Figura 2. Ciclo biológico de *Paracapillaria philippinensis*.



Legenda: (1) Ovos não embrionados são liberados pelas fezes; (2) embrionam no ambiente; (3) são ingeridos por peixes, e liberam as larvas que penetra no intestino e migram para os tecidos; (4, 7) a ingestão de peixe cru ou mal cozido infectam humanos, aves ou mamíferos marinhos; (5) no intestino, as fêmeas podem liberar larvas infectantes que penetram na mucosa intestinal e reinfectam o hospedeiro (6).
Fonte: modificado de CDC. http://www.cdc.gov/dpdx/

Dentre as espécies do gênero *Eucoleus*, *E. aerophilus* (syn. *Capillaria aerophila*, *Thominx aerophilus*) é zoonótico. É um parasito que infecta as vias respiratórias, encontrados

na mucosa da traquéia, brônquios e bronquíolos. Infecção comum em canídeos e felideos, tanto selvagens quanto domésticos (TRAVERSA et al., 2009; DI CESARE et al., 2012, 2014), que eventualmente podem causar infecção em humanos. Ao contrário das outras espécies que infectam humanos, ainda não tem um ciclo biológico bem elucidado, porém acredita-se que ocorra com a ingestão dos ovos larvados. Esses eclodem no intestino e as larvas migram para as vias respiratórias pelo sistema sanguíneo. Os ovos são liberados junto com a secreção através da tosse, que são expelidas pela boca ou deglutidos e liberados junto às fezes. O embrionamento ocorre no solo e tornam-se infectante para um novo hospedeiro (BOWMAN et al., 2008).

Infecções causadas por parasitos do gênero *Eucoleus* podem ocorrer nas vias respiratórias, mucosa do esôfago e no estômago de mamíferos e aves (MORAVEC; PROKOPIC; SHLIKAS, 1987). Além de *E. aerophilus*, *Eucoleus bohemi* (Supperer, 1953) (syn. *Capillaria bohemi*) também infecta as vias respiratórias de mamíferos. Ambos possuem morfologia semelhante dos ovos encontrados nas fezes, o que dificulta o diagnóstico laboratorial (MAGI et al., 2012). Já *E. anullatus*, *E. perforans* e *E. contortus*, por exemplo, foram descritos no esôfago e estômago de aves (PINTO et al., 2008).

Infecções causadas por parasitos desse gênero podem ocorrer nas vias respiratórias, mucosa do esôfago e no estômago de mamíferos e aves (MORAVEC; PROKOPIC; SHLIKAS, 1987). Além do *E. aerophilus*, *Eucoleus bohemi* (syn. *Capillaria bohemi*) também infecta as vias respiratória de mamíferos. Ambos possuem morfologia semelhante dos ovos encontrados nas fezes, o que dificulta o diagnóstico laboratorial (MAGI et al., 2012). Já *E. anullatus*, *E. perforans* e *E. contortus*, por exemplo, foram descritos no esôfago e estômago de aves (PINTO et al., 2008).

As infecções na bexiga urinária de carnívoros domésticos e selvagens são provocadas pelo gênero *Pearsonema*, tais como, *P. plica* (Rudolphi, 1819) (syn. *Capillaria plica*) e *P. feliscati* (Diesing, 1851) (syn. *Capillaria feliscati*) (CALLEGARI *et al.*, 2010; BELDOMENICO *et al.*, 2002). O ciclo desse grupo de capilarídeo é pouco conhecido. Após os ovos serem eliminados na urina, maturam no solo e são ingeridos por minhocas, que seriam os hospedeiros intermediários. As larvas eclodem e se desenvolvem no celoma da minhoca. Após serem ingeridas pelo hospedeiro definitivo, as larvas penetram na mucosa intestinal e pelo sistema circulatório chegam na bexiga urinária. Sugere-se que os gatos sejam infectados após se alimentarem do hospedeiro paratênico, as aves, já outros sugerem que seja um ciclo direto (BOWMAN et al., 2008).

O gênero *Aonchoteca* inclui parasitos do trato gastrintestinal frequentemente de mamíferos, assim como em aves e anfíbios. *Aonchotheca putorii* (Rudolphi, 1819) (syn. *Capillaria putorii*), dentre outras, são encontradas principalmente no estômago de felinos (IBBA et al., 2013), canídeos (MAGI et al., 2015) e mustelídeos (CERBO et al., 2008). O ciclo também é pouco conhecido, podendo se direto ou indireto, com minhocas como hospedeiro intermediário. Na porção do intestino delgado, a espécie *Aonchotheca annulosa* (Dujardin, 1845) infecta principalmente roedores, além de outros mamíferos como macacos (UMUR et al., 2012). Em aves, *A. bursata* (Freitas e Almeira, 1934) e *A. caudinflata* (Molin, 1858) são encontrados no intestino delgado (MORAVEC; PROKOPIC; SHLIKAS, 1987).

O gênero *Capillaria* é bem heterogêneo, provoca infecções intestinais em peixes, anfíbios, aves e mamíferos, com exceção de *C. cyprinodonticola* (Huffman et Bullock, 1973) que infecta fígado de peixes (MORAVEC, 1982). Sabe-se um pouco sobre os ciclos biológicos de algumas espécies, algumas apresentam ciclo direto, *C. pterophylli*, e outras com ciclo indireto, *C. anatis* (Schrank, 1790) (syn. *Thominx anatis*) e *C. phasianina* (Kotlan, 1940) (syn. Thominx phasianina), necessitando do hospedeiro intermediário, a minhoca, para a infecção do hospedeiro definitivo, as aves (MORAVEC; PROKOPIC; SHLIKAS, 1987).

As espécies do gênero *Baruscapillaria* infectam o intestino delgado e estomago de aves e mamíferos. O pouco que se conhece de alguns ciclos, não há participação de hospedeiros intermediários. *B. obsignata* (Madsen, 1945) e *B. ovopunctata* (Linstow, 1873) parasitam o intestino delgado de aves, onde os ovos não larvados são postos e liberados no ambiente junto às fezes. Em aves marinhas foi descrito a espécie *Ornithocapillaria appendiculata* (Freitas, 1933) (syn. *Baruscapillaria appendiculata*, *Capillaria appendiculata*) que também provoca infecção intestinal, porém possui o peixe de água doce como hospedeiro intermediário (MORAVEC; SALGADO-MALDONADO; OSORIO-SARABIA, 2000).

Os gêneros *Echinocoleus* e *Pterothominx* têm como hospedeiros aves e mamíferos. Esses gêneros parasitam o sistema gastrintestinal, a exemplo da espécie *E. hydrochoeri* (Travassos, 1916), que infecta o intestino delgado de capivaras (ROBLES et al., 2013) e *P. angrensis* e *P. brevidelphis* (Freitas e Mendonça, 1960) que parasita morcegos (SANTOS; GIBSON, 2015).

Também com espécies que parasitam o intestino e estômago dos hospedeiros, a *Pseudocapillaria* pode infectar mamíferos, aves, anfíbios e peixes. *Pseudocapillaria tomentosa* (Dujardin, 1843) e *P. salvelini* (Polyansky, 1952), são duas das espécies que parasitam o intestino de peixes, que apresentam ciclo direto ou indireto com a presença de oligoquetas como hospedeiros paratênicos. Já *P. moraveci* (Iglesias, Centeno, García y

García-Estévez, 2013) infecta estômago de peixes (IGLESIAS et al., 2013). Em aves *P. corvorum* pode ter minhocas como hospedeiros intermediários no seu ciclo.

Além dos gêneros que parasitam mamíferos já mencionados, *Liniscus*, *Skrjabinocapillaria* e *Tenoranema*, parasitam apenas mamíferos, totalizando 13 gêneros para esse grupo de hospedeiros. Os sítios de infecção variam de bexiga urinária e rins em *Liniscus* (ROBLES; CARBALLO; NAVONE, 2008), e sistema gastrintestinal em *Tenoranema* (MAS-COMA; ESTEBAN, 1985). Já dentre os que parasitam somente aves, temos *Brevithominx* e *Tridentocapillaria* (BARUS; SERGEEVA, 1990).

Pseudocapillaroides é o único gênero que parasita exclusivamente anfíbios. A espécie *P. xenopi* (Moravec et Cosgrov, 1982) localiza-se na pele de anfíbios, porém seu ciclo é pouco conhecido. Sabe-se que a infecção ocorre com a ingestão de ovos larvados em um ciclo direto. *Amphibiocapillaria* parasita anfíbios, porém a espécie *A. freitasilenti* (Araujo and Gandra, 1941) infecta estômagos de lagartos. Este parasito produz ovos larvados possibilitando a reinfecção do hospedeiro (BURSEY; GOLDBERG; PARMELEE, 2005).

Dentre os gêneros que parasitam répteis, *Paratrichosoma* e *Crocodylocapillaria* ocorrem apenas nesse grupo de animais, descritos em crocodilos. As espécies *P. recurvum* (Solger, 1877) (syn. *Trichosoma recurvum*, *Capillaria recurvum*) e *P. crocodylus*, únicas espécies do gênero, infectam a epiderme. O ciclo não é conhecido por completo, mas sugere-se que as larvas são ingeridas e maturadas no estômago. As fêmeas se localizam na epiderme, onde colocam os ovos, que são eliminados para o ambiente (TELLEZ; PAQUET-DURAND, 2011). Já a espécie do outro gênero, *C. longiovata* (Moravec and Spratt, 1998), infecta o estômago desses animais (MORAVEC; SPRATT, 1998).

Existem vários gêneros que infectam exclusivamente peixes, *Gessyella*, *Freitascapillaria*, *Capillostrongyloides*, *Piscicapillaria*, *Paracapillaroides*, *Schulmanela*. A espécie *S. petruschewskii* (Schulman, 1948) (syn. *Capillaria petruschewskii*) é a única no gênero e possui baixo grau de especificidade ao hospedeiro infectando peixes de várias famílias e ordens. Também necessita de um hospedeiro intermediário para o desenvolvimento larvar, para então se estabelecer no fígado do hospedeiro definitivo (PEKMEZCI; UMUR, 2010). Espécies dos outros gêneros possuem como principal sítio de infecção o sistema gastrointestinal (CANTATORE et al., 2009; MORAVEC; MUZZALL, 2009; ROSSIN; TIMI, 2009a, 2009b).

Alguns trabalhos relatam a presença de infecção por capilarídeos, porém sem o diagnóstico da espécie (RATAJ et al., 2011; TAKEUCHI-STORM et al., 2015). O que ocorre provavelmente pela dificuldade de diagnóstico a esse nível.

1.2.4 Dados moleculares

Poucos estudos de caracterização genética de capilarídeos foram feitos até hoje, porém existem para algumas espécies, com a utilização de diversos alvos moleculares (Tabela 2).

Tabela 2 - Espécies da família Capillariidae com sequências depositadas no GenBank (acesso 08.2016).

Espécies*	Acessos no GenBank	Alvos moleculares	Referências
Aonchotheca musimon	1	18S	-
Aonchotheca putorii	24	18S / *cox*1	Tamaru et al. 2015; Guardone et al. 2013
Aonchotheca riukiuensis	2	18S	-
Baruscapillaria obsignata	2	18S	Tamaru et al. 2015
Capillaria aerophila	87	18S / *cox*1 / 28S	Di Cesare et al. 2014; Guardone et al. 2013
Capillaria anatis	2	18S	Tamaru et al. 2015
Capillaria boehmi	9	*cox*1 / 18S	Di Cesare et al. 2014; Guardone et al. 2013
Capillaria gastrica	3	*cox*1	Zhu et al. 2000
Capillaria hepatica	9	18S / *cox*1	Guardone et al. 2013
Capillaria madseni	5	18S	Tamaru et al. 2015
Capillaria navoneae	3	18S / *cox*1	-
Capillaria plica	15	18S / *cox*1 / 5.8S / 28S / ITS 1 / ITS 2	Guardone et al. 2013 -
Capillaria pudendoteca	6	18S	Tamaru et al. 2015
Capillaria suis	2	18S	-
Capillaria tenuissima	1	18S	Honisch et al. 2008
Capillaria xenopi	1	18S	-
Paracapillaria philippinensis	1	18S	El-Dib et al. 2015
Aonchotheca sp.	9	18S	
Capillaria sp.	72	*cox*1 / 18S / 28S	
Eucoleus sp.	6	18S	
Pearsonema sp.	5	18S	

Legenda: - Sequências que não foram publicadas em artigos científicos;

Nos estudos moleculares, se destaca o uso do DNA mitocondrial (mtDNA), em especial o gene citocromo c oxidase subunidade 1 (*cox*1), para discriminar espécies de *Capillaria* sp. Com este alvo molecular, um estudo determinou a diversidade genética de diferentes espécies de *Capillaria sensu lato* de diversos hospedeiros e diferentes sítios de infecção. Para a caracterização, foram aplicadas as técnicas de PCR e de polimorfismo de conformação de fita simples (SSCP) seguido de seqüenciamento nucleotídico. Os resultados mostraram que apesar da alta variabilidade das morfoespécies dentro do hospedeiro, assim como em relação ao sítio de infecção, existe uma grande especificidade a nível de gênero-hospedeiro (ZHU et al., 2000).

A detecção molecular de *Capillaria aerophila* em canídeos e felinos na Itália foi alcançada com o mesmo alvo *cox*1. A aplicação da técnica de *seminested*-PCR mostrou uma sensibilidade de 97 a 100% e uma especificidade de 100%, mesmo na presença de nematóides filogeneticamente próximos como *Trichuris vulpis* e *C. bohemi* (DI CESARE et al., 2012).

O mesmo grupo de pesquisa caracterizou com o alvo mitocondrial espécies de *Eucoleus bohmi* comum em canídeos. Por ser um parasito que infecta o epitélio nasal que resulta na eliminação dos ovos nas fezes, foram utilizadas amostras fecais e vermes adultos de várias localidades na Europa. A sensibilidade da técnica foi de 85,14% e uma especificidade de 100% em amostras fecais, inclusive de animais coinfectados com parasitos filogeneticamente próximos, como *E. aerophilus* (DI CESARE et al., 2015).

Outro trabalho investigou a variabilidade genética entre hospedeiros silvestres e domésticos de países diferentes na Europa de *E. aerophilus*. A análise do *cox*1 identificou 15 diferentes haplotipos, sendo 5 deles compartilhados por hospedeiros domésticos e silvestres, e os outros restritos a determinados hospedeiros ou países (DI CESARE et al., 2014).

Guardone e colaboradores (GUARDONE et al., 2013) analisaram tricurídeos e capilarídeos de pequenos mamíferos carnívoros pelos alvos ribossomal e mitocondrial, 18S rDNA e *cox*1 respectivamente. A análise filogenética com base no 18S rDNA (Figura 3) corrobora com a classificação atual dos capilarídeos, descritos na família Capillaridae, e a maioria das espécies anteriormente pertencentes ao gênero *Capillaria*, foram reclassificadas e divididas em diversos gêneros em relação aos diferentes sítios de infecção. Foi visto uma grande distinção entre os ramos, atualmente considerados de Trichuridae e Capillaridae. Já o alvo *cox*1 demonstrou diferenciar espécies muito próximas, e apresentou mais informação genética de diagnóstico.

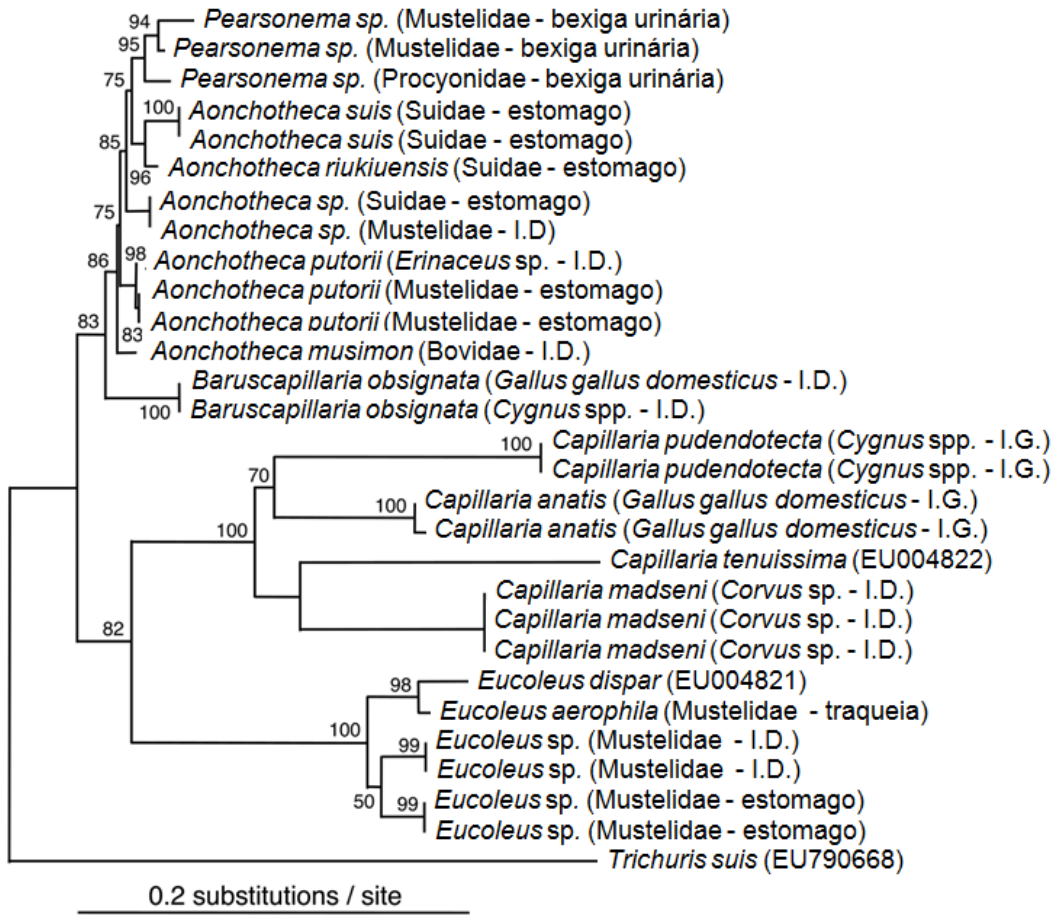
As relações filogenéticas entre parasitos de aves de rapina da Alemanha foram investigadas com base no gene 18S rDNA. Apesar do foco na infraordem Spiruromorpha, foram posicionadas filogeneticamente duas espécies de capilarídeos encontrados no trato digestivo desses animais, *Capillaria tenuissima* (Travassos, 1915) e *Eucoleus dispar*, porém não foi discutida a fundo a relação com outras espécies. Além disso, pelos espécimes analisados, *E. dispar* apresentou o mais amplo espectro de hospedeiro, infectando 12 das 15 espécies de aves do estudo (HONISCH; KRONE, 2008).

Para estudar a posição filogenética de *Pseudocapillaroides xenopi*, parasito de anfíbio (*Xenopus laevis*), foram analisados sequência de 18S rDNA de 22 parasitos da ordem Trichocephalida. Por meio da análise Bayesiana e Máxima Verossimilhança foi observado uma maior proximidade filogenética da espécie com a família Capillaridae. Nesse trabalho também foi aplicada PCR quantitativa por *TaqMan*, para a detecção de *P. xenopi* no sedimento de aquários. Dos 12 sedimentos analisados, dois foram positivos. Porém os autores retratam que essa análise não garante que outros Capillaridae de espécies aquáticas não sejam detectados também, gerando um falso positivo para *P. xenopi* (FELDMAN; RAMIREZ, 2014).

Um estudo conduzido no Egito demonstrou a identificação por PCR de *Capillaria philippinensis* em amostras fecais. Justificado pela emergência da infecção causada pelo parasito no país, o estudo com base no alvo 18S rDNA demonstrou sensibilidade e especificidade de diagnóstico, inclusive em amostras com outros parasitos (EL-DIB et al., 2015).

Na tentativa de solucionar o problema de identificação precisa de capilarídeos por grupos não especialistas, Tamaru e colaboradoes (TAMARU et al., 2015) fizeram a caracterização morfológica e genética de várias espécies de parasitos de aves. Pelas características morfométricas machos e fêmeas apresentaram muitas semelhanças, consequentemente uma difícil discriminação. Porém a análise filogenética por Máxima Verossimilhança demonstrou concordância com a última classificação do grupo por Moravec (1982) (Figura 3). E ainda foi possível observar uma relação dos clusters aos hospedeiros e aos sítios de infecção.

Figura 3. Árvore filogenética baseada nas sequências parciais do alvo 18S rRNA de Capillaridae.



Legenda: (I.D.) Intestino delgado; (I.G.) Intestino grosso.
Nota: *Trichuris suis* como grupo externo.
Fonte: modificado de (TAMARU et al., 2015)

Os trabalhos de caracterização genética e filogenia de capilarídeos corroboraram entre si. Em geral, os resultados mostram que há um agrupamento de gêneros de capilariídeos isolado em relação aos outros Tricocephalida, além de agrupamentos gênero-específicos.

1.3 Inteligencia Artificial

Inteligência artificial (IA) foi criada por John McCarthy (1963) e denominado como a ação de uma máquina de realizar uma função considerada inteligente se desempenhada por um ser humano. Essas ações abrangem o aprendizado, tomada de decisão, adaptação, controle e percepção (DE MELLO; DE SOUZA, 2019).

Desde que essa ciência foi criada o modo como é usada está em constante progresso, com o avanço da tecnologia, em inovações na informática e computação. Nos anos 90 a IA era usada como Representação do Conhecimento (RC), onde um conjunto de informações associado a especificações de símbolos e regras de interpretação, possibilitam a descrição de um dado alvo, a exemplo da descrição das posições e movimentos em um jogo de xadrez (BENCH-CAPON, 1990). Atualmente o foco está no Aprendizado de Máquinas (AM) e algoritimos estatísticos, que é a habilidade do programa desenvolver melhores cenários a partir do conhecimento adquirido com os dados fornecidos, ou seja, se adaptar a novas circunstâncias e detectar padrões (RUSSELL; NORVIG, 1995).

O uso do AM é amplo, abrange áreas mais tecnológicas, como o desenvolvimento de sistemas de detecção de intrusão na internet (TSAI et al., 2009), também vale destacar os sistemas multiagentes que podem ser usados para construir sistemas complexos, como na robótica (STONE; VELOSO, 2000), ou mesmo sistemas de wireless que simulam redes neurais artificiais tentando modelar o comportamento neural real para ampliar a conectividade (CHEN et al., 2019).

O uso do AM na biologia já tem uma relação longa, os trabalhos pioneiros utilizaram algoritmos de perceptron para distinguir sítios de iniciaçao da tradução com a análise de sequências em *Escherichia coli* (STORMO et al., 1982). Nos anos seguintes, com a melhoria da estrutura matemática dos algoritimos a flexibilidade das técnicas de aprendizado de máquina cresceu junto assim como sua confiabilidade. Dados de expressão gênica foram usados com sucesso para classificar os pacientes em diferentes grupos clínicos e para identificar tipos distintintos de linfócitos B em linfomas (ALIZADEH et al., 2000), padrões de células mamarias em câncer de mama (PEROU et al., 1999), assim como a variação sistemática nos padrões de expressão gênica em linhas celulares de câncer humano (ROSS et al., 2000). A predição de variáveis contínuas com algoritmos de aprendizado de máquina foi usada para estimar o viés em dados de microarray de cDNA.

Apesar de toda a vasta aplicação da AM, não se tem nenhuma utilização da técnica na identificação de espécies biológicas em qualquer nível taxonômico.

2. OBJETIVOS

2.1 Objetivo geral

Identificar e caracterizar morfologicamente e morfometricamente ovos de capilarídeos como modelo taxonômico de definição de espécies em morfotipos encontrados em sítios arqueológicos do Novo e Velho Mundo, e assim propor uma paleoepidemiologia e relacionar com o status filogenético molecular atual de Capillaridae.

2.2 Objetivos específicos

- Estabelecer a Paleoepidemiologia de Capillaridae por meio de revisão sistemática de achados no Novo e Velho Mundo;
- Estabelecer o status atual da filogenia molecular de Capillaridae;
- Caracterizar morfológicamente e morfométricamente ovos de espécies de capilarídeos parasitos de humanos e animais disponíveis em coleções biológicas institucionais utilizando diferentes microscopias;
- Desenvolver uma abordagem de identificação de espécies de capilarídeos com uso de tecnologia de Inteligência Artificial/Machine Learning ;
- Caracterizar morfologicamente e morfometricamente os morfotipos de ovos de capilarídeos encontrados em sítios arqueológicos do Velho e Novo Mundo;
- Identificar/Sugerir as espécies de capilarídeos de ovos recuperados de sítios arqueológicos do Novo e Velho Mundo, com base na tecnologia de IA/ML proposta.

3. JUSTIFICATIVA

A pesquisa a cerca de identificação de helmintos é utilizada em diversas áreas: taxonomia clássica e molecular, análise de rotina para o diagnóstico de infecções humanas e de outros animais, levantamento ecológico da fauna de helmintos, e pesquisas em material arqueológico e paleontológico. Destacando a Paleoparasitologia, crescem a importância dos estudos da sobre evidências de infecções parasitárias em vestígios antigos. A identificação de parasitos, nesse contexto, em material antigo abre perspectivas de estudos evolutivos que podem contribuir com novos dados sobre mudanças no comportamento das infecções parasitárias em seus hospedeiros, ao longo do tempo.

Alem da paleoparasitologia, a maioria das áreas supracitadas utilizam principalmente material coprológico como fonte de trabalho. Nesse material são recuperadas algumas formas evolutivas como ovos, assim a identificação de capillarideos é prejudicada devido a falta de literatura com caracterização de ovos e definição de suas estruturas. Portanto, é importante dados morfológicos de diferentes espécies, utilizando diferentes tipos microscopias, além da aplicação de ferramentas de IA / *Machine Learning* para auxiliar na identificação.

Atualmente, os capilarídeos são amplamente distribuídos pelo mundo. Suas espécies têm tanto importância médica como veterinária, que podem desenvolver quadros clínicos graves, tanto com infecções no fígado, como intestinais e pulmonares, levando ao óbito.

No passado, a distribuição dos capilariídeos também era bem ampla, com achados de infecção e circulação em muitos países, tanto no Novo e no Velho Mundo. Embora sejam publicados vários achados de capilarídeos, tanto no Velho como no Novo Mundo, há uma incerteza sobre as espécies encontradas, devido à escassez de trabalhos com uma detalhada caracterização da morfologia dos ovos das diversas espécies, a carência de trabalhos com identificação molecular para esse grupo e a complexa taxonomia do grupo. Mesmo com a diversidade de gêneros, e a dificuldade na diferenciação dos mesmos, o diagnóstico muita das vezes é realizado. Outras vezes, quando identificada a espécie, esta é apenas sugerida.

Portanto a justificativa central desse estudo foi a necessidade de fornecer informação taxonômica básica baseado na identificação e caracterização morfológica e morfométrica dos ovos das diferentes espécies de capilarídeos identificados de Coleções Biológicas Institucionais. Além de desenvolver uma metodologia de identificação de ovos utilizando a abordagem de Machine Mearning. Essas estratégias possibilitarão a discriminação de

morfotipos encontrados em amostras arqueológicas do Velho e Novo Mundo. Consequentemente, o estudo fornecerá a base para estabelecer a real relação dos capilarídeos com seus hospedeiros humanos e animais do passado, estabelecer um autêntico cenário da paleodistribuição da infecção, e ainda dando subsídio para futuras pesquisas de identificação de capilarídeos tanto em material moderno como arqueológico.

4. MATERIAIS E MÉTODOS

A tese foi escrita no formato “coletânea”, composta de 4 artigos científicos: Worldwide paleodistribution of capillariid parasites: Paleoparasitology, current status of phylogeny and taxonomic perspectives”; Taxonomic characterization of capillaridae eggs from species deposited in Institutional Hlminthological Collections: morphological, morphometric and statistical”; Eggshell of Capillariid species: an ultrastructural perspective; Capillariid diversity in ancient samples. Neste tópico é descrito detalhadamente a metodologia e/ou as abordagens teórico-metodológicas utilizadas para cada uma das produções científicas.

4.1 Worldwide paleodistribution of capillariid parasites: Paleoparasitology, current status of phylogeny and taxonomic perspectives

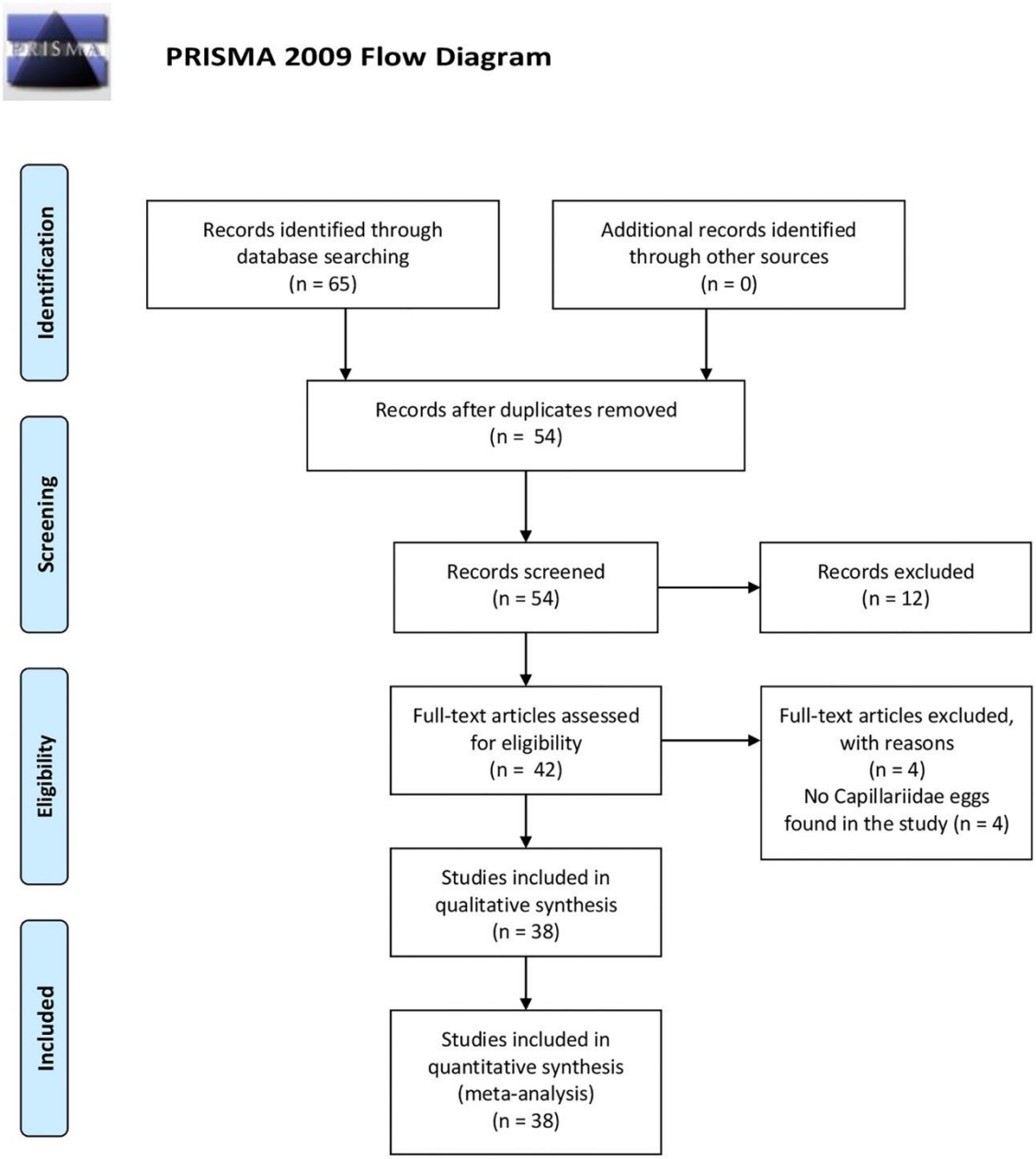
Nesta revisão sistemática seguimos uma diretriz definida pelo PRISMA (MOHER et al., 2009). Esse processo gerou um fluxograma do PRISMA ilustrado na Figura 4. Basicamente, a literatura foi identificada como possíveis artigos, depois selecionados e acessos para elegibilidade e incluídos na revisão.

4.1.1 Compilação da literatura paleoparasitológica

A revisão da literatura objetivou identificar e sintetizar os achados dos ovos capilares em amostras arqueológicas em qualquer contexto, e mostrar sua paleodistribuição. Um banco de dados foi criado por artigos coletados no PubMed e no ScienceDirect usando palavras-chave como (“capillariid” OU “Capillariidae” OU “Capillariinae” OU “capillaria” ou “Trichuridae”) E (“paleoparasitologia” OU “arqueoparasitologia” OU “coprolita” OU “amostras antigas”).

Apesar da *Capillaria* sp. ser o diagnóstico em alguns estudos, nesta revisão, revimos particularmente os esforços realizados por alguns autores para alcançar uma identificação taxonômica do gênero Capillaria. Caso contrário utilizamos a nomenclatura genérica “capillariids”, que envolve todos os gêneros, incluindo *Capillaria* sp.

Figura 4. Fluxograma PRISMA.



Legenda: Fluxograma da revisão bibliográfica.
Fonte: Borba et al., 2019.

4.1.2 Análise filogenética de capillarídeos

Análises filogenéticas foram conduzidas para estabelecer as relações genéticas atuais entre as espécies para elucidar sua complexa taxonomia e arranjo filogenético para corroborar com a classificação taxonômica de Moravec, 1982.

Todas as sequências disponíveis no GenBank (08/2018) dos genes rDNA 18S e mtDNA cox1 foram utilizadas para a análise filogenética. Sequências com tamanho de sequência reduzido não foram utilizadas, como o DNAr 18S de *Paracapillaria philippinensis* (235 pb). O alinhamento e a visualização da edição sequencial foram realizados no Bioedit v.7.0.5 e no MUSCLE (CHOJNACKI et al., 2017). Matrizes de distância genética para cada gênero / grupo de espécies foram construídas para determinar as distâncias inter e intraespecíficas entre gêneros e espécies usando o parâmetro Kimura-2 com distribuição gama (K2P + G) em MEGA v. 7.0.26 (TAMURA et al., 2013). No gene 18S rDNA, uma vez que a maioria das sequências estava localizada nas extremidades 5 'ou 3', o alinhamento foi separado em dois conjuntos de dados para usar o maior número de sequências de gênero / espécie disponíveis. Os nomes das espécies foram mantidos como depositados no GenBank, mas os nomes dos gêneros foram especificados de acordo com a classificação taxonômica proposta por Moravec (MORAVEC, 1982). Seqüências idênticas ou com distâncias genéticas menores que 0,020 foram excluídas.

O conjunto de dados I incluiu 105 sequências (1090 pb) de 16 espécies pertencentes a 7 gêneros de capilarídeos: *Pearsonema, Aonchoteca, Baruscapillaria, Pseudocapillaria, Paratrichosoma, Eucoleus* e *Capillaria*. Além disso, seqüências de gêneros indefinidos foram incluídas. As seqüências das espécies *Trichuris* e *Trichinella* foram utilizadas como grupo externo. O conjunto de dados II foi de 40 seqüências (678 pb) de 18 espécies pertencentes a 8 gêneros: *Pearsonema*, *Aonchoteca*, *Baruscapillaria*, *Pseudocapillaria*, *Pseudocapilaroides*, *Eucoleus*, *Capillaria* e *Calodium*. O grupo externo foi o mesmo da análise anterior.

Para a construção da árvore filogenética, dois métodos foram aplicados em MEGA (TAMURA et al., 2013). Modelo Neighbor-Joining (NJ) e K2P + G seguindo o protocolo para identificação molecular de espécies (protocolo DNA barcoding CBOL) (http://www.barcodeoflife.org/content/resources/ Standards-and-guidelines), e Máxima Verossimilhança (ML), também usando o modelo K2P + G, conforme determinado pelo modelo de melhor ajuste do comando de substituição de DNA usando os critérios de informação Bayesiana no MEGA. Em ambos, o suporte estatístico dos ramos foi gerado por 500 réplicas de bootstrap.

Um conjunto de dados para o gene cox1 consistiu em 30 sequências do GenBank (255 pb) de 7 espécies pertencentes a 4 gêneros: *Pearsonema*, *Aonchoteca*, *Eucoleus* e *Calodium*. As seqüências das espécies *Trichuris* e *Trichinella* foram utilizadas como grupo externo. O

método NJ foi baseado no modelo K2P + G como defendido pela análise de código de barras e o método ML foi baseado no modelo de 3 parâmetros de Tamura. Ambos com distribuição Gamma e suportados por 500 réplicas de bootstrap.

4.2 Taxonomic species discrimination based on helminthological collections data and machine learning technology

4.2.1 Análises morfológicas e morfométricas

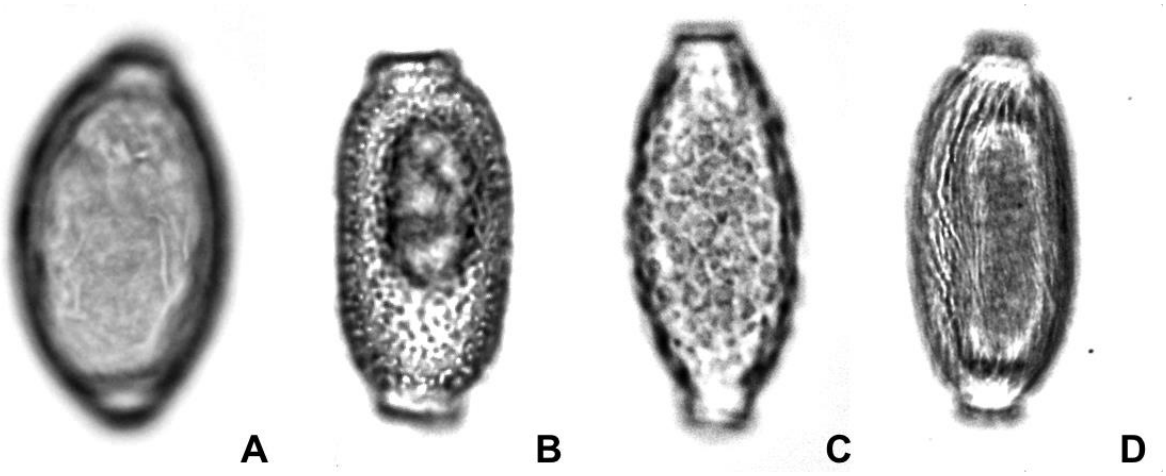
Os espécimes foram analisados em duas Colecções Helmintológicas institucionais: no Brasil, na Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC), Fundação Oswaldo Cruz (FIOCRUZ), 14 espécies (20 espécimes) e na França, na coleção do Muséum National d'Histoire Naturelle de Paris (MNHN), 16 espécies (17 espécimes).

Os espécimes tiveram seus ovos separados para análise morfológica e morfométrica. Quando presentes, foram coletadas fêmeas contendo ovos para a separação dos ovos ou fragmentos contendo os ovos, quando não é possível extraí-los manualmente do interior das fêmeas. Para uma visualização clara da morfometria do ovo, as amostras são submetidas ao banho ultrassônico (Cristófoli®) por 60 segundos na freqüência de 42Khz. O processo é feito para limpar a sujeira e os fragmentos de fêmeas, resultando apenas na presença de ovos com a casca de quitina formada.

Os ovos tiveram sua morfologia e morfometria caracterizadas por um microscópio óptico (Nikon Eclipse E200) na ampliação de 400X, com o uso de software de análise de imagens (IMAGE PRO PLUS - MEDIA CYBERNETICS, EUA). As medidas consideradas foram: diâmetro total (largura) e comprimento dos ovos, valor médio da largura e altura dos dois plugs e espessura da casca (Fig. 6). Também foi feita uma qualificação dos ornamentos apresentados na casca externa dos ovos capilariídeos. O parâmetro de ornamentação do ovo foi dividido em categorias: 1) liso, que não possui ornamentos na casca, como descrito por Conboy em ovos de *Trichuris trichiura* (CONBOY, 2009); 2) pontuado, que tem pontos como uma superfície sem caroço, como descrito em *Eucoleus bohemi* (CONBOY, 2009; TRAVERSA et al., 2011); 3) reticulado tipo I, que se apresenta como uma rede como um

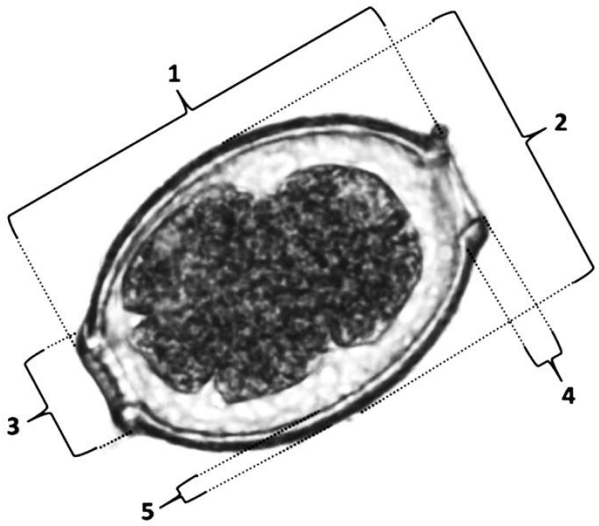
cordão interconectado descrito em *Eucoleus aerophilus* por Conboy (CONBOY, 2009); e 4) tipo reticulado ll, que é apresentado como uma rede mas com uma orientação de sulcos longitudinais profundos como descrito em *Aonchotheca putorii* (ZAJAC; CONBOY, 2012) (Fig. 5A-D).

Figura 5. Representação de cada padrão de ornamentação considerado neste estudo com base nos ovos de capillariidae das espécies depositadas na CHIOC.



Legenda: A: Lisa de *Aonchotheca pulchra* voucher CHIOC9804; B: Pontuado do comprovante *Capillaria brasiliana* CHIOC7046; C: Reticulado Tipo I de *Pearsonema plica* voucher MNHN373c; D: Reticulado Tipo II de voucher de *Baruscapillaria resecta* MNHN1073.

Figura 6 - Representação das medidas de ovos capilares consideradas neste estudo com base no comprovante *Bariuscapillaria obsignata* CHIOC26715.



Legenda: (1) comprimento total; (2) largura total; (3) base da largura do plugue polar; (4) base da altura do plugue polar; (5) espessura da casca.

4.2.2 Análises discriminantes e abordagens de inteligência artificial / aprendizado de máquina

Um conjunto de dados de espécies de capillariídeos das coleções Helmintológicas CHIOC e MNHN foi construído com os parâmetros morfológicos e morfométricos (MM) gerados. Além disso, foram incluídos os parâmetros ecológicos como informação sobre o hospedeiro (H) e localização geográfica (GL) dos espécimes (Tabela S1).

Análises discriminantes foram realizadas usando o software Past 3.16 para separar os grupos de espécies. Primeiramente, plotando-se o comprimento total e a largura dos ovos de todas as espécies e, em seguida, a análise da função discriminante foi gerada por cada ornamentação de casca do ovo: tipo reticulado pontual I e tipo II reticulado. A exceção foi a ornamentação suave com apenas uma espécie identificada.

Para análises de ML / IA, a ornamentação e os parâmetros ecológicos foram transformados em variáveis numéricas. Os parâmetros ecológicos foram definidos como hospedeiros incluindo peixes, anfíbios, répteis, aves, mamíferos e como localização geográfica, compreendendo, América do Sul, América Central, América do Norte, Europa, África, Ásia, Oceania. A variável resposta foi 1 = sim ou presença; 0 = não ou ausência; e -1 = sem informação. Os parâmetros MM foram testados isoladamente e em combinação com parâmetros ecológicos como: MM + H, MM + GL e MM + H + GL.

Como não há literatura de algoritmos ML para definição de espécies taxonômicas, o objetivo deste estudo foi a realização de um teste exaustivo de algoritmos disponíveis no software Weka 3.8.3 (EIBE; HALL; WITTEN, 2016). As árvores de decisão produzidas pelo sistema ML / IA são semelhantes às chaves taxonômicas propostas / usadas pela sistemática para discriminar espécies biológicas. Portanto, nos concentramos em algoritmos que retornaram representações de árvores de decisão como: J48, Random Tree, REPTree. Apesar disso, as árvores de modelo logístico (LMT) também foram testadas.

Para análise estatística, o teste de proporção de comparação foi utilizado para verificar a hipótese nula para proporções iguais entre as taxas de algoritmos (J48, Árvore Aleatória, Árvore REP e Árvores de Modelo Logístico) e entre os parâmetros (MM + H + GL, MM + H, MM + GL e MM + H + GL). Para se chegar a uma conclusão sobre a hipótese com 95% de confiança, o valor de p da estatística do teste Qui-Quadrado deve ser menor que 0,05 indicando que a diferença é significativa. Em seguida, o procedimento Marascuilo foi aplicado para verificar quais proporções são diferentes entre os algoritmos e a combinação entre os parâmetros aplicados. As análises de dados foram realizadas usando o software RStudio versão 3.5.1 (2018-07-02).

4.3 Eggshell struture of Capillariid species: a sem perspective

As espécies de capillariídeos foram obtidas da Coleção Helmintológica do Instituto Oswaldo Cruz. Os espécimes foram preservados em solução de álcool a 70% e lavados com solução salina tamponada com solução salina tamponada com fosfato (PBS). A seção final do útero das fêmeas foi coletada e submetida a um ultrassom ultrassônico (Cristófoli®) por 60 segundos na frequência de 42Khz, para romper o verme e extrair os óvulos.

Para ML, as amostras foram preparadas entre lâminas de vidro com uma gota de glicerol. As imagens foram capturadas com um microscópio Nikon Eclipse E200, na ampliação de 400X, com o software Image Pro Plus - Media Cybernetics, EUA.

A preparação para MEV foi realizada em lâmina de vidro. Os ovos foram aderidos na lâmina com uma solução de gelatina, e então fixados por desidratação com uma série de etanol (20%, 30%, 50%, 70%, 80%, 90% e absoluto) por 10min em cada concentração e então seco com secador de ponto crítico. As amostras foram revestidas com ouro em uma camada de 20-25 nm de espessura. Foi observado em um microscopio eletrônico de varredura JEOL.

Tabela 3. Informações de espécies de Capillariidae caracterizadas neste estudo

Espécies	**Coleção voucher**	**Espécie do Hospedeiro**	**Classe do Hospedeiro**	**Sítio de Infecção**
Aonchotheca pulchra	CHIOC 18215	*Tadarida laticaudata*	Mamífero	Mucosa do estômago
Baruscapillaria obsignata	CHIOC 26715	*Gallus gallus domesticus*	Aves	Intestino delgado
Baruscapillaria rudolphii	CHIOC 7770	*Tinamus solitarius*	Aves	Intestino delgado
Baruscapillaria spiculata	CHIOC 2863	*Carbo vigua*	Aves	Intestino delgado
Capillaria venusta	CHIOC 23408	*Ramphasto toco*	Aves	Intestino delgado
Capillaria collaris	CHIOC 18904	*Gallus gallus domesticus*	Aves	Ceco
Capillaria brasiliana	CHIOC 7046	*Nycticorax naevius*	Aves	Intestino delgado
Echinocholeus hydrochoeri	CHIOC 11214	*Hydrochoerus capybara*	Mamífero	Intestino delgado
Echinocholeus auritae	CHIOC 7786	*Metachirops opossum*	Mamífero	Intestino delgado
Eucoleus perforans	CHIOC 9898	*Numida meleagris*	Aves	Mucosa do Esofago
Eucoleus contortus	CHIOC 6307	*Sterna maxima*	Aves	Mucosa do Esofago
Eucoleus dubius	CHIOC 7004	*Attila cinereus*	Aves	Mucosa do Esofago

CHIOC: "*Coleção Helmintológica do Instituto Oswaldo Cruz*"

4.4 Capillariid diversity in archaeological material from the new and old world: clustering and artificial inteligence aproaches

4.4.1 Área de estudo

4.4.1.1 Gruta do Gentio II (GGII)

O local está localizado em Unaí, estado de Minas Gerais, Brasil, datado de 12000 - 3500 BP, com dois períodos culturais, caçador-coletor (12000-7295 +- 150 AP) e horticultor (3490 +- 120 - 410 +- 60 AP). A caverna tinha uma área interna de 200m2 associada a uma parede calcária. Restos humanos, animais e vegetais foram observados no interior. Além disso, 80 coprólitos de diferentes formas foram coletados, e seu produtor foi identificado por análise de DNA Barcoding (GURJÃO, 2019).

4.4.1.2 La Rochelle Augustin (LRA)

Ele está localizado no oeste da França, perto do Golfo da Biscaia e é datado do século XVII-XVIII.

4.4.1.3 Calais ZAC des Turqueries (CAL)

Ele está localizado no norte da França. O site é datado do século 8-10 e está localizado em um antigo pântano. Tem evidências de atividades agro-pastoris, focadas em gado e processamento. Foi descoberto restos de fauna, mas não evidência de habitação, mas apenas lugares com poços.

4.4.1.4 Bourges Avaricum (AVA)

Está localizado na França, no centro da região de Val de Loire. É datado do século XIII-XVII.

4.4.2 Análise paleoparasitológica

Para a recuperação dos ovos do parasito, foram realizadas duas técnicas. Amostras brasileiras foram previamente identificadas, como GGII-01, Panthera onca; GGII-15, Didelphis albiventris; GGII-33 e GGII-51, Bos taurus. Eles foram reidratados como sugerido por Callen e Cameron (CALLEN; CAMERON, 1960), com uma solução de fosfato trissódico a 0,5% (Na3PO4.H2O) por 72h a 4 ° C. Em seguida, foi homogeneizado e sedimentado por 24 horas com uma gaze tripla como proposto por Lutz (LUTZ, 1919). Dez lâminas foram analisadas para cada amostra. Todo o processo foi realizado no Laboratório de Paleogenetc / LABTRIP / IOC na Fundação Oswaldo Cruz, Brasil.

As amostras européias também foram reidratadas com solução de fosfato trissódico a 0,5%, por 7 dias, e com uma gota de solução de formalina. Após a homogeneização, as amostras foram submetidas a um tratamento com ultra-som (50/60 Hz) por 1 minuto, e a amostra é submetida a tensão nas malhas de 315 μm, 160 μm, 50 μm e 25 μm. Seis lâminas foram analisadas para cada amostra. As amostras foram processadas em um Laboratório de Paleoparasitologia da Universidade Franche-Comté, França.

Todas as amostras foram analisadas sob um microscópio de luz na ampliação de 100 e 400X.

4.4.3 Análises morfológicas e morfométricas

As mensurações dos ovos foram feitas conforme proposto por Borba e colaboradores (BORBA et al.,), considerando a largura, comprimento, comprimento da base do *plug*, altura da base do *plug* e espessura da casca.

Os morfotipos de superfície de casca de ovo foram separados em quatro categorias: liso (S), pontuado (P), reticulado tipo I (RTI) e I reticulado tipo I (RTII), seguindo no prelo, e REFS. O tipo S, denominado liso, não possui ornamentação na superfície do ovo (ZAJAC; CONBOY, 2012). Tipo pontuado apresenta ornamentos como buracos em casca de ovo, como pouca perfuração em todo o ovo (CONBOY, 2009; ZAJAC; CONBOY, 2012). Reticulado tipo I, forma uma rede sem orientação como uma grade (CONBOY, 2009), diferente do tipo II reticulado que tem uma orientação longitudinal, similar a um monte de pequenos raios de um plugue polar para outro.

4.4.4 Análise estatística

As espécies depositadas em duas coleções institucionais, a Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC) / Brasil e a Coleção de Amelitologia do Museu Nacional de História Natural de Paris / França, foram utilizadas como referências na análise estatística com ovos em amostras arqueológicas. A análise estatística teve o objetivo de mostrar as medidas mais próximas dentro do banco de dados. Foi feita uma análise discriminante para construir um modelo preditivo e gerar uma função discriminante para prever a melhor variável para discriminar os ovos. Como um agrupamento hierárquico usando a distância de Gower e a variância mínima de Ward para encontrar proximidades nas medidas em uma análise multivariada, usando um software PAST 3.16.

4.4.5 Inteligência Artificial / Abordagens de Aprendizagem de Máquina

Conforme proposto por Borba e colaboradores (BORBA et al.,), uma abordagem de Inteligência Artificial / aprendizado de máquina foi aplicada neste estudo para identificar os espécimes encontrados. Selecionou-se uma árvore de decisão que apenas informava a morfologia e morfometria do ovo para ampliar o alcance e maximizar as possibilidades de identificação de espécies.

5. RESULTADOS

Os resultados serão apresentados no formato de "coletânea", composto por 4 artigos científicos publicados ou submetidos a periódicos.

Artigo 1: "Worldwide paleodistribution of capillariid parasites: Paleoparasitology, current status of phylogeny and taxonomic perspectives"
Periódico: PlosOne
Status: publicado. PLoS ONE 14(4): e0216150. DOI: 10.1371/journal.pone.0216150

Artigo 2: "Taxonomic species discrimination based on helminthological collections data and machine learning technology"
Periódico: PlosOne
Status: submetido

Artigo 3: "Eggshell structure of capillariid species: a SEM perspective"
Periódico: Parasite
Status: submetido

Artigo 4: "Capillariid diversity in archaeological material from the New and Old World: clustering and artificial intelligence approaches"
Periódico: Parasitology International
Status: manuscrito

5.1 Artigo 1: Worldwide paleodistribution of capillariid parasites: Paleoparasitology, current status of phylogeny and taxonomic perspectives

RESEARCH ARTICLE

Worldwide paleodistribution of capillariid parasites: Paleoparasitology, current status of phylogeny and taxonomic perspectives

Victor Hugo Borba1,2, Jose´ Roberto Machado-Silva1, Matthieu Le Bailly3, Alena Mayo Iñiguez 2*

1 Laborato´rio de Helmintologia Romero Lascasas Porto, Faculdade de Ciências Me´dicas, Universidade do Estado do Rio de Janeiro (FCM/UERJ), Rio de Janeiro, Rio de Janeiro, Brazil, 2 Laborato´rio de Biologia de Tripanosomat´ıdeos, Instituto Oswaldo Cruz, Fundac¸ão Oswaldo Cruz (IOC/FIOCRUZ), Rio de Janeiro, Rio de Janeiro, Brazil, 3 University of Bourgogne Franche-Comte, CNRS UMR 6249 Chrono-environment, Besanc¸on, France

* alenainiguez@gmail.com

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Abstract

Introduction

Paleoparasitology, the study of parasites in the past, brings the knowledge of where and when they occurred in preterit populations. Some groups of parasites, as capillariids, have a complex and controversial systematic, hindering the paleoparasitological diagnosis. In this article, we synthesized the occurrence of capillariids in both the New and the Old World in ancient times, and discussed the difficulty of the diagnosis of species and the strategies for identification. The present review also shows the current status of the phylogeny in capillariids and indicates the necessity to try new approaches for a better understanding of capillariid paleodistribution.

Methods

For the systematic review, a predefined guideline defined by PRISMA was used. The articles collected were identified, screened, and included in the review following criteria for eligibility. The current status of the phylogeny of capillariids was accessed using MUSCLE, Bioedit v.7.0.5 and MEGA v. 7.0.21 programs.

Results

The review discussed 38 articles that presented information about capillariids in past populations. Most of capillariid eggs found in the New and Old World were not identified. However, Calodium hepaticum eggs were the most identified, as some from Eucoleus genus. It

was observed that sites from the New World had a better chance for capillariid egg identification, due to previous knowledge of its host, when compared to the Old World. In the

18S rDNA phylogenetic analyses, two datasets were constructed, one including sequences from 7 Moravec's genera, where 3 genus-specific clusters, with high bootstrap values, could be observed for Capillaria (ML = 99%, NJ = 96%), Eucoleus (ML / NJ = 100%) and Paratrichosoma (ML / NJ = 100%). A fourth cluster of 18S rDNA dataset I revealed lack of definition of Pearsonema and Aonchotheca genera. The 18S rDNA dataset II comprised 8 Moravec's genera and defined 3 clusters, 2 genus-specific for Eucoleus (ML = 99%, NJ = 100%) and Capillaria (ML / NJ = 98%). The third 18S rDNA dataset II cluster included 6 gen- era and exhibited, once again, Pearsonema and Aonchotheca poor discrimination. The cox1 gene data consist of 4 Moravec's genera, and in spite of grouping some species-specific clusters, did not show genera-specific definition.

Conclusions

Despite the numerous archaeological findings, both in the New and the Old Worlds, the identification of capillariid species based on the morphology and morphometry of eggs remains imprecise, often resulting in a generic diagnosis of a group or morphotype of capillariid. Capillariid is one of the most diverse group of helminths recovered in archaeological sites. The phylogenetic trees produced in this study showed limited genetic information available, unresolved genera and incongruence with the classical taxonomy. The elucida- tion of the paleodistribution of capillariids can give insights of the ancient host-parasite associations but also in modern sceneries.

Introduction

Over 300 species of capillariids have been described throughout the world in different vertebrate hosts, as fishes, amphibians, reptiles, birds or mammals [1]. The difficulty of identification, due to the small size of the specimens and the few morphological structures that are characteristics for species identification, make their systematic and taxonomy one of the most complex among the phylum Nematoda. Another factor that hinders their classification is the phenotypic plasticity caused by different hosts, infection sites and locations [1]. In 1982, Mora- vec changed the classification of the group when proposed 16 genus of capillariids [2]. After that, a number of new genera, synonyms and reclassifications have been proposed.

Currently, it is know that capillariids are spread throughout the world, with a slight knowledge of the genus or species distribution within the different hosts and localities. However, the scenario of the paleodistribution of capillariid species in the past is very uncertain. There are a few findings, both in the New and in the Old World, in a sort of archeological material, such as coprolites, sediment associated to skeleton, latrines, pits and cesspits, with uncertain diagnoses of species. Most of taxonomic classification in the papers is Capillaria sp., which

does not nec- essary means the species identification of Capillaria genus, as proposed by Moravec (1982). In other publications, the capillariid genus is specified.

There are scarce molecular and phylogenetic studies regarding capillariids, most of them focus on 18S rDNA or cox1 targets, considering just a few of the current known genera. Some authors showed an integrative study, which gives a robust molecular result of species discrimi- nation. Despite the little information, it was possible to discriminate some genera as Eucoleus or Capillaria [3].

In this study, we present and discuss the paleoparasitological findings and paleodistribution of capillariids worldwide. In addition, we present the current status of the phylogeny and suggests new taxonomic perspectives for the understanding of the capillariid paleodistribution.

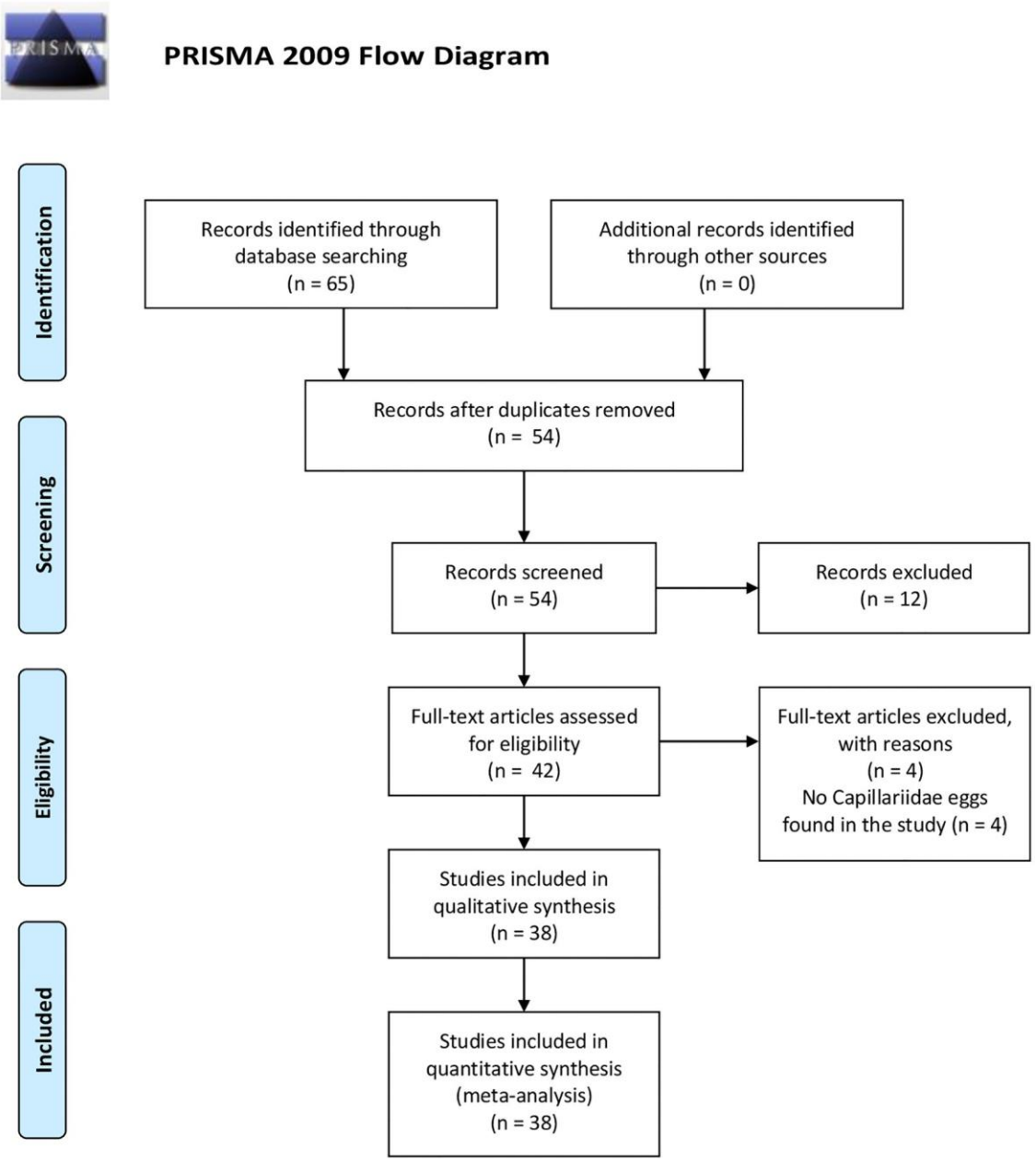

Fig 1. PRISMA flow diagram. From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/joumal. pmed1000097 For more information, visit www.prisma-statement.org.

https://doi.org/10.1371/journal.pone.0216150.g001

Materials and methods

In this systematic review we followed a guideline defined by PRISMA [4]. This process gener- ated a PRISMA flow chart illustrated in Fig 1. Basically, the literature was identified as possible articles, then screened and accesses for eligibility and included in the review.

Paleoparasitological literature compilation

The literature review aimed to identify and summarize the findings of capillariid eggs on archaeological samples in any context, and to show its paleodistribution. A database was made by articles collected on PubMed and ScienceDirect using keywords such as ("capillariid" OR "Capillariidae" OR "Capillariinae" OR "capillaria" or "Trichuridae") AND ("paleoparasitol- ogy" OR "archaeoparasitology" OR "coprolite" OR "ancient samples").

In spite of the Capillaria sp. diagnosis in some studies, in this review, we particularly revised the efforts conducted by some authors to reach a taxonomic identification of Capillaria genus, otherwise we used the generic nomenclature "capillariids", which involves all genera including Capillaria sp.

Capillariid phylogenetic analysis

Phylogenetic analyses were conducted to establish the current genetic relationships among the species to elucidate its complex taxonomy and phylogenetic arrangement to corroborate with the taxonomic classification of Moravec [2].

All sequences available in GenBank (08/2018) of the 18S rDNA and cox1 mtDNA genes were used for the phylogenetic analysis. Sequences with reduced sequence size were not used, as 18S rDNA from Paracapillaria philippinensis (235 bp). Sequence edition alignment and visualization were performed in Bioedit v.7.0.5 and MUSCLE [5]. Genetic distance matrixes for each genus / species group were constructed to determine the inter and intraspecific distances between genus and species using the Kimura-2-parameter with Gamma distribution (K2P + G) on MEGA v. 7.0.26 [3]. In the 18S rDNA gene, since most of sequences were located at the 5' or the 3'extremities, the alignment was separated in two datasets to use the largest number of genus / species sequences available. The names of the species were maintained as was deposited in GenBank, but the genus names were specified according to the taxonomic classification proposed by Moravec [2]. Identical sequences or with genetic distances less than 0.020 were excluded.

Dataset I included 105 sequences (1090 bp) of 16 species belonging to 7 genera of capillariids: Pearsonema, Aonchoteca, Baruscapillaria, Pseudocapillaria, Paratrichosoma, Eucoleus, and Capillaria. In addition, sequences of undefined genus were included. Trichuris and Trichinella species sequences were used as outgroup. Dataset II was of 40 sequences (678 bp) of 18 species belonging to 8 genera: Pearsonema, Aonchoteca, Baruscapillaria, Pseudocapillaria, Pseudocapil- laroides, Eucoleus, Capillaria, and Calodium. The outgroup was the same as in the previous analysis.

For the phylogenetic tree construction, two methods were applied in MEGA [3]. Neighbor-Joining (NJ) and K2P + G model following the protocol for the molecular identification of spe- cies (DNA barcoding CBOL protocol) (http://www.barcodeoflife.org/content/resources/Standards-and-guidelines), and Maximum Likelihood (ML), also using the K2P + G model, as determined by the best-fit model of DNA substitution command using the Bayesian

informa- tion criteria in MEGA. In both, the statistical support of the branches was generated by 500 bootstrap replicas.

A Dataset for the cox1 gene consisted of 30 GenBank sequences (255 bp) of 7 species belonging to 4 genera: Pearsonema, Aonchoteca, Eucoleus, and Calodium. Trichuris and Trichi- nella species sequences were used as outgroup. The NJ method was based on the K2P + G model as advocated by the barcoding analysis and the ML method was based on the Tamura 3-parameter model. Both with Gamma distribution and supported by 500 bootstrap replicas.

Results

Paleodistribution of capillariids

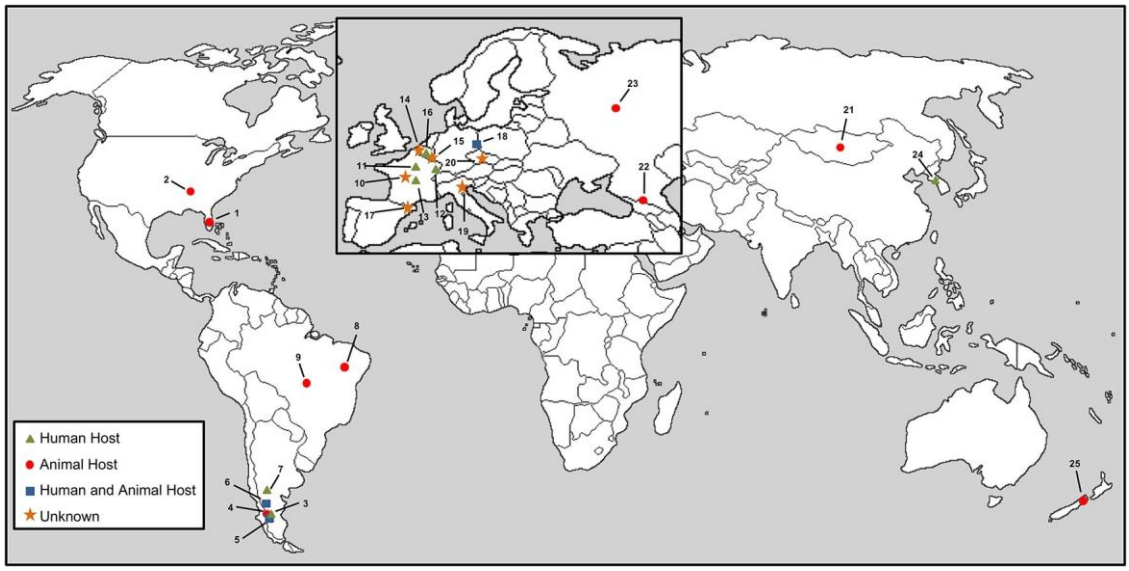

Fig 2. Worldwide paleodistribution of capillariids. The symbols represent the locals where the parasites were found in the archeological material. Relative data see map numbers in Tables 1 and 2. The host of capillariids is indicated whenever is known.

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Capillariids in the New World. Capillariid findings in the New World are not much numerous than in the Old World (Fig 2). In North America, only two studies have identified capillariid eggs. Reinhard and coauthors (1986) were the first to find capillariid in archaeolog- ical material, i.e. samples of crocodile coprolites dated about 6000 years before present (BP), collected in the United States, in the Florida state. It was possible to observe the presence of two species of capillariid in which despite the good preservation of the eggs, the larval material had been degraded [6]. McConnell and Zavada (2013) found capillariids in sediments associ- ated with skeleton of an extinct tapir (Tapirus polkensis), which lived in the Miocene-Pliocene period (4.5–7 million years BP). The authors revealed very poorly preserved

structures that have similar characteristics and measures to capillariid eggs [7]. This structure may represent an evidence of a longstanding host-parasite interaction of capillariid with this group of mammals.

In South America, the research on archaeological material, mostly coprolites, have revealed numerous findings of capillariid eggs. It is noteworthy the findings in Patagonia region, Argentina. There are a number of studies with the identification of different morphotypes of capillariid eggs in a range of hosts, such as canids, rodents, camelids, felids, birds and even humans.

The first publication, by Fugassa and Guicho´n, (2005), showing capillariid parasites in archaeological material from Patagonia, was in human coprolites [8]. In the same region, sediments taken from skeletons and graves dating from 3720–3978 BP were also analyzed. In this material, capillariid eggs were identified, but the authors suggested the presence of more than one species, since a variety of measures and ornaments on the eggshells was found [9]. Two studies examined parasites in sediment associated with sacral region. The first study was conducted on skeletons recovered in three archaeological sites (Nombre de Jesu´s, Las Mand´ıbulas, Caleta Falsa) from the Patagonia region, and stored in museum collections in Argentina.

Although the cleanse of the skeletal remains, as part of museological curation process, the recovery of helminth eggs was possible with the identification of two capillariid morphotypes, one of them attributed to Calodium hepaticum [10]. In the second study, an egg of capillariid was found in a rodent coprolite found in sediment associated with a body dating from the Hispanic post-contact period (212 ± 35 years BP) [11].

Some coprolites of uncertain zoological origin, with characteristics of both human and canid feces, were also analyzed in the Patagonia region. Coprolites dated of 3480 and 2740 years BP, showed eggs of Calodium sp. (n = 98), and another unidentified capillariid species (n = 4) [12]. In coprolites with older dating (9730 ± 100 to 8920 ± 200 years BP), correspond- ing to the transition from the Pleistocene to Holocene, the presence of Calodium sp. eggs suggested the consumption by hunter-gatherer of infected rodents, possibly by C. hepaticum [13]. The taxonomic identification was performed based on eggshell ornaments, which have particular striations that permit the genus discrimination. A canid coprolite (6540 ± 110 years BP), probably from Pseudalopex culpaeus, revealed numerous eggs (n = 171) with different measures, that were identified as capillariid [14].

Feline coprolites were also analyzed in Patagonia [15]. A number of Calodium sp. eggs (n = 563) were found and identified as C. hepaticum based on the morphometric data and the similarity of eggshell ornaments. Taking into consideration the life cycle of the parasite, the finding could represent an environment contaminated by C. hepaticum, which could have affected humans who shared the same location. In the same study, another capillariid, similar to Eucoleus sp. was also found, but in lower frequency (n = 4) [15]. A carnivore coprolite from Epulla´n Chica site, in Argentina, revealed one egg of Eucoleus sp., probably a Eucoleus aerophi- lus [16].

Although the morphological characteristics of C. hepaticum egg are very singular (small average size and typical ornamentation), the possibility of other species with similar egg morphology cannot be ruled out. The analysis of a coprolite of camelid from Patagonian region, dating 8300 ± 130 to 7920 ± 115 BP [17], revealed eggs similar to C. hepaticum. However, the infection is not supported by the parasite life cycle and their herbivore host diet. Therefore, it was suggested that other species with eggs similar to C. hepaticum circulated in that environ- ment. Coprolites of camelid collected from the Andean region, dated to 9640 ± 190 to 3920 ± 80, revealed three capillariid morphotypes. One morphotype was compatible with Calo- dium sp., which usually infects the gastrointestinal tract of a variety of host, and has been found in coprolites of humans, canids, felids and rodents. Other morphotype was comparable to Eucoleus sp. and the third morphotype had not matching with any capillariid described in camelids until that time [18]. An study done in this region showed once more the same mor- photype compatible with Calodium sp. and one other morphotype never seen before in cam- elid coprolites, increasing the number of species found in this host [19].

In coprolites of rodents, dating from 7920 ± 130 years BP, the presence of capillariids identified as Eucoleus sp. (n = 3), and other helminth eggs were described. Although the genus identified represent parasites that usually infect the respiratory tract, the presence in coprolites could be a result of swallowing of expelled secretion with eggs [20]. Another analysis in rodent coprolites showed a high number (n = 239) and variety of capillariid morphotypes (n = 4). The eggs were identified as suggestive of genera Calodium, Eucoleus, Echinocoleus and an unidenti- fied capillariid species [21].

Besides coprolites, ancient pellets were analyzed, which are structures resulting of the regurgitation process of prey birds. Some studies have performed microscopic analysis on this material and showed capillariid eggs with radially ornamented eggshell, and morphometry compatible with C. hepaticum. Because rodent bones, often preys of raptors, were recovered in the pellets, Fugassa and collaborators (2007) attributed the infection to rodents [22]. In Beltrame and coauthors (2011), despite the identification of C. hepaticum eggs in raptor pellets, it was pondered the presence of others capillariid species attributed to the genera Aonchotheca and Eucoleus, which include some species that parasitize bird esophagi [23]. The archaeological site has several caves and rock shelters with evidence of human occupation, suggesting a probable exposure of humans to zoonotic parasites of rodents and, perhaps, of birds [23].

In Brazil, only two findings of capillariids are known in coprolites. Sianto and coauthors [24] recovered Calodium sp. eggs (N = 2) in feline coprolite (2840 ± 100 years BP), collected in Serra da Capivara, Northeast Brazil. Morphologically identified as Calodium cf. hepaticum, the finding could represent a case of false parasitism by consuming the parasite's final host, and consequently, the eggs appeared in feces without acquiring the infection [24]. Confalonieri [25] identified Echinocoleus hydrochoery (syn. Capillaria hydrochoery) in coprolites of capy- bara (Hydrochoerus hydrochoeris) from Lapa da Ange´lica archaeological site, Goia´s state, cen- tral west region. Currently, the parasite affects capybaras in the region. However, the coprolite eggs are slightly larger (Table 1) than those found nowadays [25].

The identification of eggs in all studies is defined as capillariids, although it could include all genera, or be restricted to Capillaria genus. The species identification is infrequent, due to morphological variety of capillariid eggs, an important lack of knowledge on egg structures, and/or by the action of taphonomic agents that would have altered the structure of eggs and therefore, made the taxonomic identification more difficult. It was possible to suggest the capillariid species in few cases (Table 1).

Capillariids in the Old World. As in the New World, capillariid findings in the Old World are quite frequent. However, unlike the New World, the findings are more common in sediments, latrine, or burial, mainly in Western Europe where most of the analyses were con- ducted (Table 2).

In France, Bouchet [26] accomplished the first recovery of capillariid egg in the Old World. Sediments of latrines and pits from a medieval site in Beauvais (13th to 17th century) revealed capillariid eggs [26]. Later, during the investigations on the lakeside archaeological site of Cha- lain (Jura, France), human coprolites dated to the Neolithic period (5216–4996 years BP) tested positive for two different morphotypes of capillariids. The first presenting reticulated outer eggshell, and the second presenting punctuated outer eggshell [27]. The same two mor- photypes were recovered during the analysis of several Neolithic sites from Germany and Swit- zerland in Lakes Federsee and Bodensee [28].

In the scenario of World War I, Le Bailly et al. (2014) analyzed samples from German soldiers [29]. The samples were collected in the abdominal cavity of three bodies, recovered in a military gallery collapsed in March 1918. Two individuals showed capillariid eggs, which were identified using morphometric analysis as Eucoleus gastricus, a parasite of rodents. The authors explained the finding by the historical data recording the presence and the circulation of rats inside the galleries, as well as, the close contact and interaction between soldiers and these rodents.

An unusual capillariid finding was made in a body dating from the Roman period in France [30]. X-ray and cross section analysis identified two hydatid cysts, probably developed by the larval stage of Echinococcus sp. tapeworm. Analysis of the residue allowed to visualize Calodium hepaticum eggs, showing a coinfection by these two parasites [30].

In Belgium, in the Raversijde archaeological site (16th century) mainly inhabited by fishermen, few eggs of capillariids were observed in organic materials [31]. Based on the findings of eggs of Trichuris trichiura and Ascaris lumbricoides, it was possible to suggest the human origin of the material. In the Place d'Armes site in Namur, which had seven stratigraphic layers repre- senting different historical ages, organic materials were collected from latrines, pits and cess- pits. Capillariid eggs were found in layers dated to the Roman period (2nd-3rd centuries), the Carolinean period (9th-11th centuries), and a medieval period from12th to 13th centuries [32]. In addition, during the analysis performed in the historical center of Nivelles, dated to the medieval period, human samples from pelvic region were analyzed. One individual revealed a high abundance of capillariid eggs (332 eggs per gram), but the species could not be identified [33].

Table 1. Capillariids on New World.

Locality/Country	Period (BP)	Sample	Host	Capillariid identification	Measures (µm)	Map number	References
Florida / USA	6000–7000	Coprolite	Alligator	Capillariid morph1	-	1	[6]
				Capillariid morph2	-		
Tennessee / USA	4.5–7 m.a.	Sediments	*Tapirus polkensis*	Capillariid	54 x 30	2	[7]
CCP5 / Santa Cruz / Argentina	6540±110	Coprolite	Human	Capillariid	-	3	[8]
CCP/ Santa Cruz / Argentina	6540±110	Coprolite	Canid	Capillariid	27.5–85 x 20–47.5 (n = 174)	4	[14]
CCP/ Santa Cruz / Argentina	2740	Coprolite	Human or Canid	*Calodium* sp.	47.5–77 x 30–42.5 (n = 87)	5	[12]
				Capillariid	55–70 x 28.8–43 (n = 4)		
	3480			*Calodium* sp.	53–75 x 33.5–42 (n = 11)		
CCP5 / Santa Cruz / Argentina	6540±110	Coprolite	Feline	*Calodium hepaticum*	57.5–75 x 35–45 (n = 563)	4	[15]
				Eucoleus sp.	67.5 x 35.7 (n = 4)		
CCP7 / Santa Cruz / Argentina	7920±130	Coprolite	Rodent	*Eucoleus* sp.	60–62.5 x 37.5–40 (n = 3)	4	[20]
CCP/ Santa Cruz / Argentina	3440±70–6700 ±70	Coprolite	Rodent	*Calodium* sp.	60–70 x 33.7–47.5 (n = 153)	4	[21]
				Eucoleus sp.	50–55 x 22.5–35 (n = 56)		
				Echinocoleus sp.	65 x 31.5 (n = 1)		
				Capillarid morph	55–67.5 x 32.5–42.5 (n = 29)		
CCP7 / Santa Cruz / Argentina	9730±100–8920 ±200	Coprolite	Human or Canid	*Calodium* sp.	60–71.25 x 28.7–42.5 (n = 48)	5	[13]
CCP7 / Santa Cruz / Argentina	9640±190–3920 ±80	Coprolite	Camelid	*Calodium hepaticum*	55–70 x 32.5–45 (n = 47)	4	[18]
				Eucoleus sp.	80–87.5 x 45–52.5 (n = 5)		
				Morphotype	80–87.5 x 45–57.5 (n = 7)		
Patagonia / Argentina	Historic	Sediments[♮]	Human	*Calodium hepaticum*	- (120–360 eggs/g)	3	[10]
	850			Capillarid morph	60 x 30 (n = 1)		
CCP5 / Santa Cruz / Argentina	6540±110	Pellet	Rodent	*Calodium hepaticum*	37.5–42.5 x 63.7–68.7 (n = 4)	4	[22]
CCP / Santa Cruz / Argentina	3990±80–2740 ±100	Pellet	Rodent	*Calodium* sp.	35–45 x 62.5–75 (n = 60)	4	[23]
OB1 / Santa Cruz/ Argentina	3575–3931	Sediment[♮]	Human	Capillariid morph1	56–65 x 25–32.5	3	[9]
	3720–3978			Capillariid morph2	55.5–62.5 x 36.2–37.5		
				Capillariid morph3	62.5–72 x 35		
CCP7 / Santa Cruz / Argentina	8300±130–7920 ±115	Coprolite	Camelid	*Calodium hepaticum*	63.7–70 x 35–37.5	4	[17]
ADG / Santa Cruz / Argentina	3440±70–4900 ±70	Coprolite	Camelid	*Calodium sp*.	57.5–77.5 x 32.5–50 (n = 116)	4	[19]
				Capillariid	61.25–67.5 x 37.5–47.5 (n = 10)		
Rio Mayo / Chabut / Argentina	212±35	Coprolite	Rodent	Capillariid	65 x 35 (n = 1)	6	[11]
Epullán Chica / Patagonia / Argentina	2220±50	Coprolite	Carnivores	*Eucoleus aerophilus*	62.5 x 27.5 (n = 1)	7	[16]
Serra da Capivara / Piau´ı / Brazil	2840±100	Coprolite	Feline	*Calodium* cf. *hepaticum*	52.2–54 x 31.1–33 (n = 2)	8	[24]
Lapa da Angélica / Goiás / Brazil	-	Coprolite	Capybara	*Echinocoleus hydrochoery*	49–41 x 24–19	9	[25]

Sediment extracted from skeletal remains

BP, before present; CCP, Cerro Casa de Piedra; m.a., millions years ago; cf., confer; eggs/g, eggs per gram of feces; morph, morphotype; -, no information available in the paper.

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Table 2. Capillariids on Old World.

Locality/Country	Period (BP)	Sample	Host	Capillariid identification	Measures (μm)	Map Number	References
Beauvais / France	Cen. 13 to 17	Latrine	-	Capillariid	-	10	[26]
Jura / France	3200–2980	Coprolite	Human	Capillariid morph1	70 x 31.5	11	[27]
				Capillariid morph2	62 x 35		
Carspach / France	1915/16 (First World War)	Sediment	Human	*Eucoleus gastricus*	65.5±1.6 x 28.6±0.4 (n = 6)	12	[28]
Amiens / France	Cen. 3 to 4 (Roman)	Burry	Human	*Calodium hepaticum*	46.7–25	13	[29]
Raversijde / Belgian	Cen. 16	Sediment	-	Capillariid	-	14	[30]
Place d'Armes / Namur / Belgian	Cen. 2 and 3 (Gallo-Roman)	Latrines and Pits	-	Capillariid	-	15	[31]
	Cen. 9–11 (Carolinian)			Capillariid	24 x 50		
	Cen. 12 and 13			Capillariid	-		
Nivelles / Belgian	Cen. 10–13 (Medieval)	Sediment	Human	Capillariid	- (n = 332)	16	[32]
La Draga / Lake Banyoles / Spain	7270–6930	Sediment	Soil	Capillariid morph1		17	[33]
				Capillariid morph2			
Saale-Unstrut Valley / Germany	4500	Sediment	Human and Cattle	Capillariid	-	18	[34]
EmiliaRomagna / Italy	Cen. 10–11 (Medieval)	Pits	-	Capillariid	- (n = 1913)	19	[35,36]
Prague / CzechRepublic	Cen. 18 and 19	Pits and Cesspits	-	Capillariid	33 x 15	20	[37]
Shahr-e Sukhteh / Iran	5150–3750	Coprolite	Sheep	*Aonchotheca bovis*	47.5–59.8 x 27.5–35.5 (n = 3)		[38]
Mongolia	1440	Coprolite	Rodent	Capillariid	-	21	[39]
North Ossetia	700	Coprolite	Goat	Capillariid	-	22	[40]
Moscow / Russia	Neolithic and Mesolithic	Coprolite	Canid	Capillariid	-	23	[40]
Korea	Cen. 17 (JoseonDynasty)	Mummy	Human	*Paracapillaria philippinensis*	34–35 x 17–20	24	[41]
New Zealand	<3000 and 6268±31	Coprolite	Moa	Capillariid	52–60 x 30–35 (n = 1423)	25	[42]

BP, before present; Cen., century or centuries; morph, morphotype; -, no information described in the paper.

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In Spain, a Neolithic site La Draga, dated of 7270–6930 BP, showed the presence of two capillariid morphotypes with different ornamentations. Considering the characteristics of the eggs, especially the ornamentation, the authors suggested that the reticulated-type egg was from a Bovidae infection and the punctuated-type was probably related with fish-eating habits or the presence of rodents [34].

In Germany, Dittmar and Teegen (2003) found capillariid eggs in sediment associated with pelvic region of human and cattle, dated of 4500 years BP. The authors considered the finding as a probably contamination by feces of rodents [35].

In Italy, an study analyzed pits from both Roman and Medieval period. These pits, particularly in the medieval period, were used as garbage, and contained organic materials, wood, charcoal, pottery and various artifacts. Capillariid eggs (n = 1913) were found in two samples. Because of the nature of the samples it was not possible to associated the parasite to a host [36,37]. Finally in the Czech Republic, it was also possible to recover capillariid eggs from a well and a pit belong to 18thand 19th centuries, but here again no species discrimination could be made [38].

In Asia, some findings of capillariid were recorded in different hosts and contexts. In coprolites of rodents (Alticola sp.) from the eastern Mongolia, dating from at least 1440 years BP [39]; in coprolites of goats (Capra cylindricornis) from the North Ossetia republic, Russian Federation, dating from 700 years BP; and in canid coprolites from sites on northern Moscow, recovered from layers dating from the Neolithic and Mesolithic periods. Regarding the findings in goats, the authors discussed that the defeat of tribes in the region by Mongolian troops, followed the establishment of settlements with breeding of herds of these animals nearby, and consequently, the increased frequency of these parasites [40]. In Iran two morphotypes of capillariids were found in sheep coprolites, and assumed as Aonchotheca bovis due the host [41]. In Korea, capillariid eggs with morphometry comparable to Paracapillaria philippinensis, were identified in a human burial from the Joseon Dynasty (17th century). However, the authors affirmed that further investigations should be conducted to defined the diagnosis because of the low number of cases of intestinal capillariasis is known in the country [42].

In Oceania, New Zealand, some studies with coprolites of extinct birds known as Moa was performed [43,44]. Coprolites dated of <3000 and 6368 ± 31 years BP of four moa species from different regions were analyzed. Capillariid eggs were found in two moa species from dif- ferent regions, but the parasite species could not be identified [43].

Despite the lack of findings in Africa, that could be explained by less paleoparasitological material studied in the region, capillariids are distributed worldwide (Fig 2). Both, animal and human capillariids were found in the New and the Old World since several millenniums, which show a presence and a circulation of zoonotic capillariids, as C. hepaticum, in ecosystems that were inhabited by humans and animals. Besides, humans as other carnivores can act as paratenic hosts, carrying and dispersing the parasite in the environment.

Morphological differentiation and taxonomic identification strategies. In attempt to identify capillariid eggs and understand the dynamics of the paleodistribution of different capillariid morphotypes, some works have performed biostatistical analysis.

First, Fugassa and collaborators (2008) based on data of findings in Patagonian archaeological material, conducted a statistical analysis to evaluate morphometric and morphological differences, considering the size of eggs, ornament patterns in eggshell and size of polar plugs.

The results showed a significant discrimination between four egg morphotypes using the linear variables, length and width, with greater importance for width parameter. Thus, to the authors correlated egg morphotypes with some well-known species, such as C. hepaticum, Aonchotheca putorii and E. aerophilus [45].

The biometric analysis conducted by Taglioretti and coauthors (2014), evaluated length and width variables and investigated if eggs that had similar morphotype in different scenarios belong to the same species. In the analysis, the eggs were selected randomly from different archaeological samples and Permutation Multivariate Analysis of Variance (PERMANOVA) approach was applied. The result showed that hosts, diet, archaeological sites, or dating, had no significant effect in morphometry of capillariid eggs. Thus, the authors concluded that capillariid eggs with the same morphotype in different scenarios were, in fact, from the same species [46].

In samples from German soldiers of World War I, Le Bailly and collaborators (2014) applied a statistical approach for capillariid identification. The study used Gower Algorithm followed by a Multivariate Regression Tree on a data set on various capillariid eggs including measurements and ornamentation patterns. The statistical test suggested species definition ofthe egg as E. gastricus, and proving a complementary tool to conventional diagnostic techniques for taxonomic identification [29].

These studies showed that with a mathematical approach it might be possible to achieve the differentiation and identification of capillariid eggs found in archaeological material. This approach can supplement paleoparasitological data obtained by microscopy, molecular and immunological techniques.

Current phylogenies of capillariids

The phylogenetic trees generated for dataset I showed four clusters, three of them genus-specific with high statistical support: Capillaria (ML = 99%, NJ = 96%), Eucoleus (ML / NJ = 100%) and Paratrichosoma (ML / NJ = 100%). The fourth cluster included sequences from at least 4 genera and presented short branches with medium to high support (ML = 94%,

NJ = 100%), but no genus-specific subclusters are observed. A lack of monophyletic definition is clear among species from Pearsonema and Aonchotheca (Fig 3). This is also confirmed by the low distance values between Aonchotheca and Pearsonema (0.037 ± 0.004) in the genetic matrix (S2 Table). In the distance matrix, a great distance was observed in the genus Capillaria (0.098 ± 0.008). The 18S rDNA gene does not appear to be informative in order to discriminate these genera, at least for the segment of this Dataset I.

In the trees generated with 18S rDNA dataset II (Fig 4), the strongly supported monophy- lies of the genera Eucoleus (ML = 99%, NJ = 100%) and Capillaria (ML /NJ = 98%) are also observed. A third cluster, with medium support (ML = 73%, NJ = 86%), grouping 6 genera, including 12 species, but not with monophyletic subclusters. Calodium spp. appear paraphyletic. Calodium splenecum species was basal, and Calodium hepaticum grouped with Aonch-

otheca spp. sequences. Once more Pearsonema spp. sequences clustered with Aonchotheca species despite their morphological differences.

In relation to the genetic analyses of Dataset II, the genus Aonchotheca presented one of the highest distances (0.027 ± 0.007) with Pseudocapillaroides and the shortest with Aonchotheca and Pearsonema (0.007 ± 0.002) within the cluster showing a paraphyletic origin. Aonchotheca and Pearsonema genera revealed an evolutionary distance (0.007 ± 0.002) smaller than between genera with similar posterior and spicule regions, such as Baruscapillaria and Pearso- nema (0.015 ± 0.005), or Aonchotheca and Calodium (0.012 ± 0.004) (S3 Table).

The morphological characteristics of the posterior region in the adult male are taxonomic informative for the discrimination of capillariid genera [2]. The close proximity observed in our results could not be explained by Moravec taxonomic key based the hypothetical evolution of capillariids [2]. The author proposes that genera Pearsonema is evolutionary close related to Baruscapillaria, while Aonchotheca is close related to Calodium.

Unlike the 18S rDNA phylogeny, no genus-specific cluster was found in the cox1 phylogenetic trees generated (Fig 5). However, species-specific clusters were observed as noted before by Guardone et al. [47]. The genus Pearsonema, although represented by a single sequence, grouped with Aonchotheca for both models analyzed (ML = 58%, NJ = 97%). Eucoleus genus was paraphyletic, which was reflected by greater intraspecific distance (0.080 ± 0.012), and the smaller interspecific distance with the genus Calodium (0.161 ± 0.029). Aonchotheca and Pear- sonema genera demonstrated a low interspecific distance (0.159 ± 0.031) (S4 Table), as also observed in the 18S rDNA analyses.

Remarks on worldwide paleoparasitological findings of capillariids

Despite the numerous archaeological findings, both in the New and the Old Worlds, the identification of capillariid species based on the morphology and morphometry of eggs remains

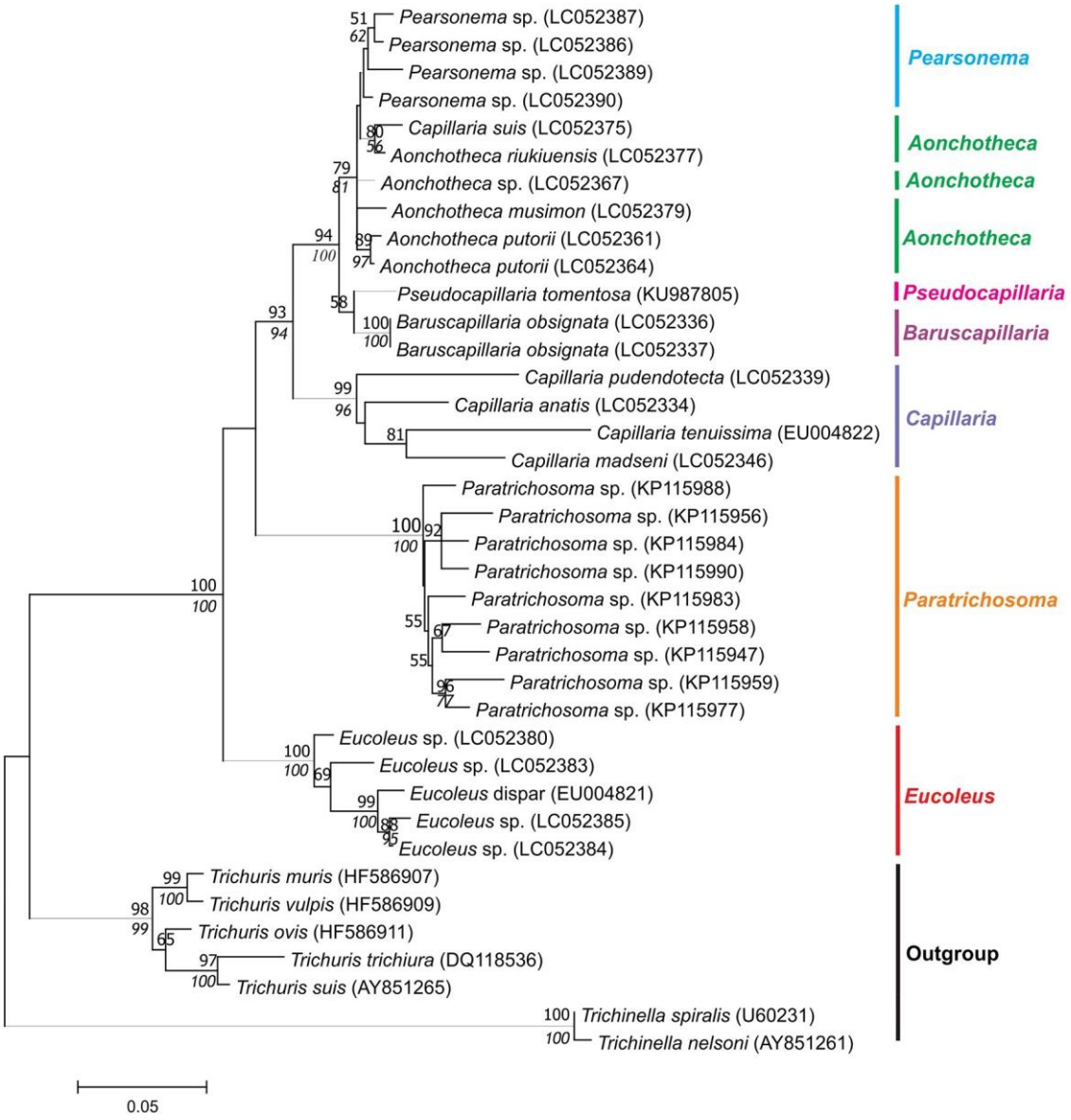

Fig 3. Dataset I phylogenetic tree based on 18s rDNA of capillariids inferred by MEGA v. 7.0.21 using Maximum Likelihood (ML) method, Kimura 2-parameter (K2P) model, and 500 replicates of bootstrap. Only bootstrap values 50% are shown.

Neighbor Joining values are on italic.

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imprecise, often resulting in a generic diagnosis of a group of capillariid. The challenge in working with the characterization of egg morphology and morphometry is clearly due to the diversity of species and the similarity of eggs from different species. A coupled of statistic treat- ment have showed interesting results in the identification of taxon. However, this approach is limited by the poor knowledge on the egg morphology, mainly considering

animal capillariids. Paleogenetic studies have been shown as promissory approach for identifying a variety of para- sitic species in archeological materials [48–51]. Molecular techniques have not been employed in ancient capillariids and require an important development in the genetic characterization of the capillariid group.

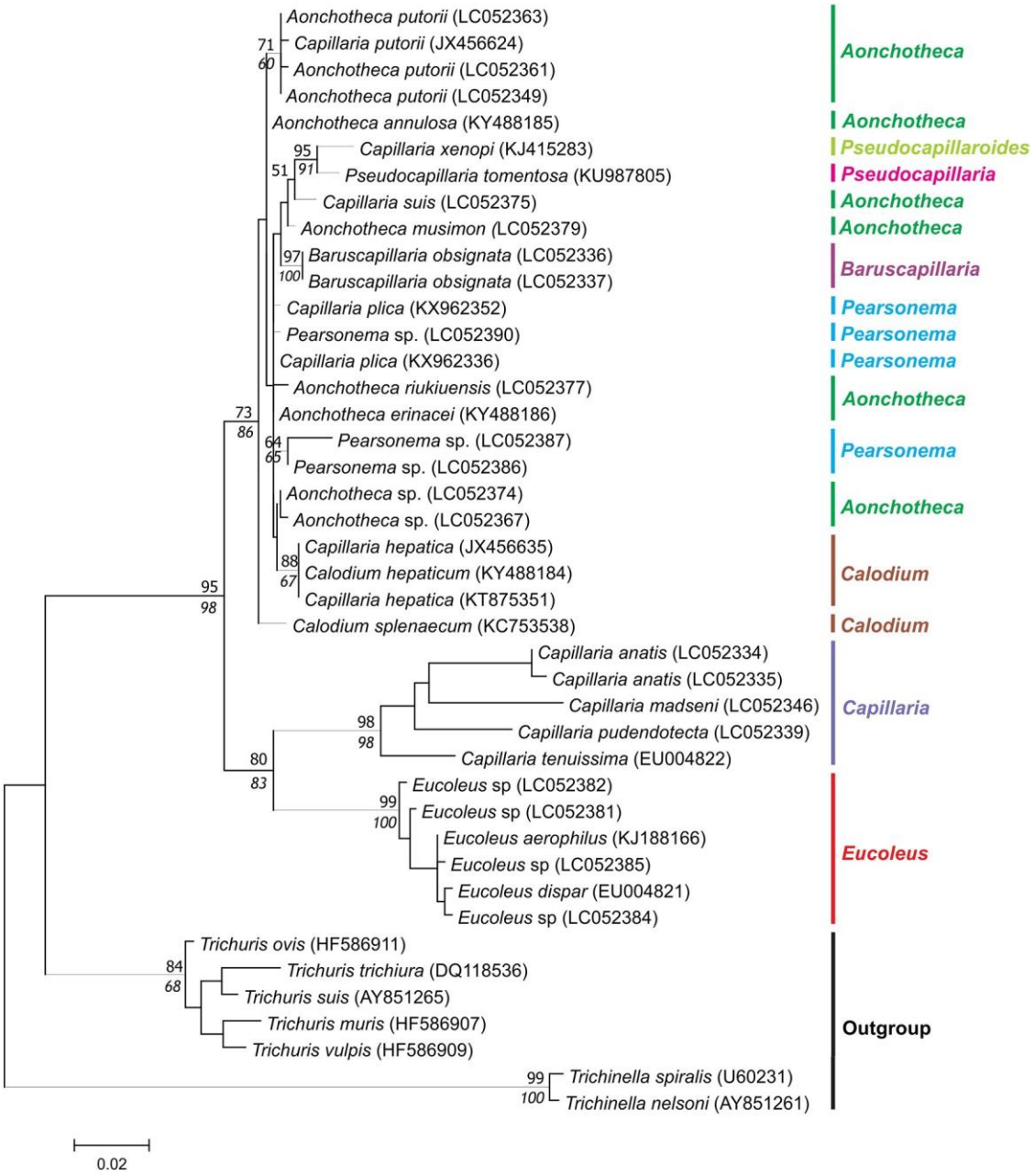

Fig 4. Dataset II phylogenetic tree based on 18s rDNA of capillariids inferred by MEGA v. 7.0.21 using Maximum Likelihood (ML) method, Kimura 2-parameter (K2P) model, and 500 replicates of bootstrap. Only bootstrap values 50% are shown.

Neighbor Joining values are on italic.

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In this study, it was observed that most of samples were coprolites 21/37 (57%) (Fig 6A), which is an expected result since they are the main source of paleoparasitological studies. Characteristics of coprolites, including morphology, morphometry and vestiges, are informative of their origin. The review showed that all Capillaridae findings from coprolites had their host defined (21/21), therefore it was the type of sample with higher number of species and genera identified (14/21) when compared to other materials (6/16) (Fig 6B).

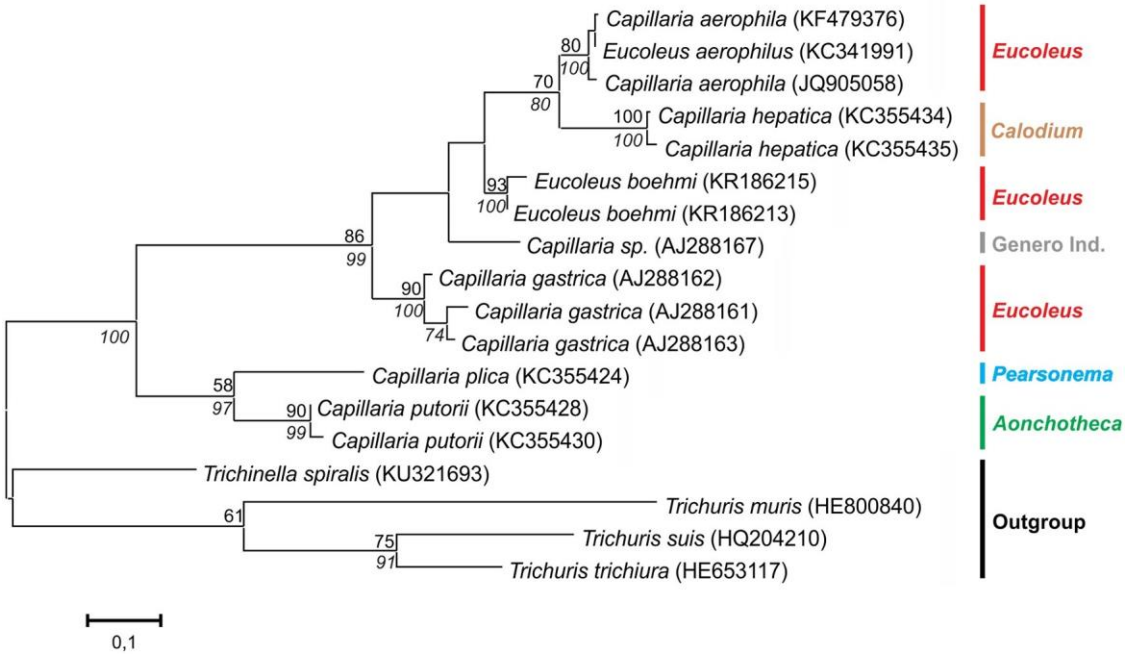

Fig 5. Phylogenetic tree based on cox1 gene of capillariids inferred by MEGA v7.0 using ML method, Tamura 3-parameter model, and 500 replicates of bootstrap. Only bootstrap values ≥ 50% are shown. Neighbor Joining values are on italic.

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We summarized that from 54 egg morphotypes found in archaeological samples only 11 (20.37%) were identified at species level. Seven of them were recognized as C. hepaticum, the species with most singular morphological peculiarities. In other 6 morphotypes indicated as Calodium sp., it is possible to observe clear characteristics of C. hepaticum, but the species defi- nition was not mentioned, which limited the biological, ecological and archaeological interpre- tations of results. From the ecological point of view, C. hepaticum eggs appear in

human feces when liver and related viscera of the animal host are consumed. Consequently, this scenario does not represent a true infection, but show that the parasite, and their animal hosts, are cir- culating in the environment. In addition, the specific parasite identification could be under- stood as indirect archaeological evidence of lifestyle, diet and/or practices that involve a close relationship with a variety of hosts or a particular contact with site of infection.

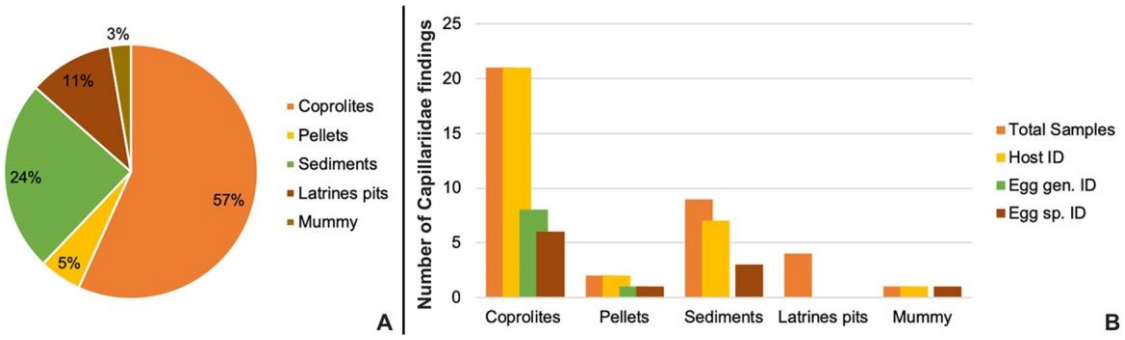

Fig 6. Graphical distributions of Capillaridae findings (n = 37) included in the present systematic review. (A) Distribution by types of samples; (B) Distribution by host and taxonomic egg identifications.

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In Le Bailly et al [29], the precise identification of E. gastricus, parasite of rodents, in WWI human samples, gives a possibility of a deeper interpretation of the host-parasite relationship, indicating a false parasitism related to soldier environments and practices. Eggs found in a Korean human mummy [42] could be identified as P. philipinensis, due to the associating of egg morphology and host origin. Regarding the parasite life cycle, the finding permits us to suggest a diet of the individual based on fish since it is the unique final host of

P. philipinensis. In the same way, the identification of C. hepaticum eggs in feline coprolite by Sianto et al [24] would allow to suggest the animals involved in the network of transmission by integration of the data of parasite's final host and the prey of felines that had habited Northeast Brazil.

Four morphotypes were identified at species level. In two, E. aerophilus, and E. hydrochoery, the information of the host made the parasite identification possible. The first is a carnivore parasite and the second a parasite specific of capybaras, respectively.

At genus level only 5 morphotypes were identified (Fig 6B), 4 as Eucoleus and 1 as Echinocholeus, both genera parasitize birds and mammals. The Eucoleus sp. morphotypes were found in feline and camelid, and both genera in rodents. The other 32 morphotypes were classified as family taxa, as the authors could not found taxonomic characters for a more

specific definition, although 14 capillariids hosts were identified (Fig 6B). We remark that host data is the most value information for capillariid taxonomic identification (Fig 6B), after, obviously, the mor- phological and morphometric data. However, some capillariids have a large range of hosts, so little could be restricted from the broad species spectrum in the parasite definition.

As all samples studied from the New World had information about the parasite host, the egg identification was probably easier than the Old World parasites, since the host information is not available in most of samples. The importance of host origin data for the taxonomic discrimination results is ratified.

Despite the limited number of publications that conduct any statistical analysis of morphometric information for egg identification, the author effort applied with this objective, could be a bias. All studies that applied statistical evaluation of egg measures and structures reached an identification on genus or even species level [29,45,46].

The ornamentation of the wall of eggs is a very important character for taxonomic identification of capillariids, and we could notice that most of the studies did not described it properly, possible because the lack of specific pattern of nomenclature. Researches focusing the detailed differentiation of these eggs structure is crucial for a robust classification in future findings.

Molecular techniques have shown promising results in parasite diagnosis in archeological material [47,50], and new methodologies are rising facilitating the recovery of parasite aDNA [48,49]. The insertion and/or comparison of aDNA sequences with modern capillariid phylogeny could open discussions on taxonomy, host-parasite association or even on parasite evolution. Since the paleoepidemiology of capillariids is not truly known as not the properly species identification, studies involving molecular and morphological characterization of eggs, of as many capillariid species as possible, would help to show a most clear and complex picture of their distribution in the past and present. The phylogenetic trees produced in this study showed limited genetic information available, unresolved genera and incongruence with the classical taxonomy. It is evident the necessity of more genetic studies, manly of integrative tax- onomy, in order to solve taxonomic conflicts, and to complement the systematic in Capillarii- dae, that, in addition, would permit the design of paleogenetic approaches. The elucidation of the paleodistribution of capillariids can give insights of the ancient host-parasite associations but also in modern sceneries.

Supporting information

S1 Table. PRISMA checklist.

(DOCX)

S2 Table. 18S rDNA– Dataset I. K2P Distance Matrix with estimates of evolutionary divergence over sequence pairs between groups. Bold numbers are those of evolutionary divergence within groups.

(DOCX)

S3 Table. 18S rDNA– Dataset II. K2P Distance Matrix with estimates of evolutionary divergence over sequence pairs between groups. Bold numbers are those of evolutionary divergence within groups.

(DOCX)

S4 Table. cox1 gene. K2P Distance Matrix with estimates of evolutionary divergence over sequence pairs between groups. Bold numbers are those of evolutionary divergence within groups.

(DOCX)

Author Contributions

Conceptualization: Alena Mayo Iñiguez.

Data curation: Victor Hugo Borba, Alena Mayo Iñiguez. Formal analysis: Victor Hugo Borba, Alena Mayo Iñiguez. Funding acquisition: Alena Mayo Iñiguez.

Investigation: Victor Hugo Borba, Alena Mayo Iñiguez. Methodology: Victor Hugo Borba, Alena Mayo Iñiguez. Project administration: Alena Mayo Iñiguez.

Resources: Matthieu Le Bailly, Alena Mayo Iñiguez.

Software: Alena Mayo Iñiguez.

Supervision: Jose´ Roberto Machado-Silva, Matthieu Le Bailly, Alena Mayo Iñiguez.

Validation: Victor Hugo Borba, Jose´ Roberto Machado-Silva, Matthieu Le Bailly, Alena Mayo Iñiguez.

Visualization: Alena Mayo Iñiguez.

Writing – original draft: Victor Hugo Borba, Alena Mayo Iñiguez.

Writing – review & editing: Victor Hugo Borba, Jose´ Roberto Machado-Silva, Matthieu Le Bailly, Alena Mayo Iñiguez.

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5.2 Artigo 2: Taxonomic species discrimination based on helminthological collections data and machine learning technology

Taxonomic species discrimination based on helminthological collections data and machine learning/artificial intelligence technology

Victor Borba[1,2], Coralie Martin[3], José Roberto Machado-Silva[1], Samanta C. C. Xavier[2], Flávio Mello[4], Alena Iñiguez[2*]

[1]Laboratório de Helmintologia Romero Lascasas Porto, Faculdade de Ciências Médicas, UERJ, Rio de Janeiro, RJ, Brazil.

[2]Laboratório de Biologia de Tripanosomatídeos-LABTRIP, Instituto Oswaldo Cruz, IOC-FIOCRUZ, Rio de Janeiro, RJ, Brazil.

[3]Unité Molécules de Communication et Adaptation des Microorganismes (MCAM, UMR 7245), Sorbonne Universités, Muséum National d'Histoire Naturelle, CNRS, CP52, Paris, France

[4]Departamento de Engenheira Eletrônica e Computação. Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

*Corresponding author

E-mail: alenainiguez@gmail.com

Abstract

There are more than 300 species of capillariids that parasitize various vertebrate groups over the world. There is a difficulty in genera identification due to few taxonomically informative structures that make the task laborious and genus definition controversial. Thus, its taxonomy is one of the most complex among Nematoda, which makes more difficult the identification of species or genus only by the egg structures. Eggs are the parasitic structures most viewed in coprological analysis both in modern and ancient samples, and consequently, their presence is an indicative of positive diagnosis for infection. Institutional Biological Collections are taxonomic depositories of specimens described and strictly identified to specific level for systematics. In this regard, this work aims to characterize eggs of capillariid species deposited in institutional helminthological collections, and process the morphological, morphometric and ecological data using Machine Learning (ML) as a new taxonomic approach for a more precise species identification. Specimens from *Coleção Helmintológica do Instituto Oswaldo Cruz* (IOC/FIOCRUZ/Brazil) e *Collection du Muséum National d'Histoire Naturelle de Paris* (MNHN/France), with a total of 42 species and 9 genera, were examined by light. In the morphometric analysis (MM) of the eggs, the total length and width, as well as plugs and shell thickness were considered. In addition, eggshell ornamentations categorized in 4 groups and ecological parameters of geographical location (GL) and host (H) were included. ML approaches showed that the algorithm J48 produced the most reliable decision tree for species identification (93.072). As statistical evaluation of dataset showed that a statistical significance between trees with GL+H+MM and using only MM. The study will support future research on taxonomic identification and diagnosis of both modern and archaeological capillariids. The research revealed a novel procedure to taxonomic species identification integrating data from centenary biological collections and the logic of ML techniques.

Keywords

Taxonomy, Artificial Intelligence, Species identification, Capillaridae, Parasite eggs.

Introduction

There are more than 300 species of capillariids that parasitize various vertebrate groups over the world (fish, amphibians, reptiles, avian and mammals). There is a difficulty in species identification due to few taxonomically informative structures that make the task laborious and the genus or species definition controversial. Thus, its taxonomy is one of the most complex among Nematoda, which makes difficult the identification of species or genus.

Moravec (1982) proposed a classification of a new taxonomy classification for capillariids to serve as a foundation for future studies. Thus, raised the genera to family Capillaridae Neveu-Lemaire, 1936 (Nematoda: Trichocephalida), due to the difference in adult morphologies the variety of infection sites and their definitive hosts. The taxonomy of the genera was based mainly on morphological characteristics of the posterior termination of males. Thus, it was suggested the division of capillariids into 16 genera, 12 redefined, 2 rescued and 2 created [1].

The suggested genera were: *Schulmanela* Ivashkin, 1964, *Paracapillaria* Mendonça, 1963, *Capillostrongyloides* Freitas et Lent, 1935; *Pseudocapillaria* Freitas, 1959; *Liniscus* Dujardin, 1845; *Pearsonema* Freitas et Mendonça, 1960; *Echinocoleus* López-Neyra, 1947; *Capillaria* Zeder, 1800; *Eucoleus* Dujardin, 1845; *Pterothominx* Freitas, 1959; *Aonchotheca* López-Neyra, 1947; *Calodium* Dujardin, 1845; *Gessyella* Freitas, 1959; *Skrjabinokillaria* Skarbilovich, 1946; besides the description of 2 new genera, *Freitascapillaria* gen. n .; *Baruscapillaria* gen. n. [1]. Subsequently other genera were added to the family, totaling 22 genera. These are: *Pseudocapillaroides* Moravec et Cosgrove, 1982; *Piscicapillaria* Moravec, 1982; *Amphibiocapillaria* Moravec, 1982; *Tenoranema* Mas-Coma et Esteban, 1985; *Paratrichosoma* Ashford and Muller, 1978 [2].

Recently, Gibbons (2010) expands the classification proposing other genera in the subfamily Capillarinae. Some of the genera that were classified in this subfamily are: *Tridentocapillaria* Barus et Sergeeva, 1990, *Brevithominx* Teixeira de Freitas and Machado de Mendonça, 1964, *Paracapillaroides* Moravec, Salgado-Maldonado and Caspeta-Mandujano, 1999, *Crocodylocapillaria* Moravec and Spratt, 1998 [3].

Although scarce, some molecular studies were done as a base for refining the systematic classification of the group, corroborating with the classification of the genera proposed by Moravec (1982) [4–6].

Eggs are the parasitic structures most viewed in coprological analysis both in modern, as in a public health or ecological surveys, and ancient samples, in researches as paleoparasitology. Most of the eggs found in ancient sample are not identified in species or even in genus level, and in modern samples when just eggs are found, the identification is impaired [6]. Despite species and genera of capillariids are identified primarily with the structure of the posterior end of male adults, but it was pointed out that the structure of the egg, could play a role in genera or species differentiation [1,7].

Artificial Intelligence (AI) was primary described as the ability of a machine to perform “intelligent” functions, as learning, decision-making, adaptation, control, and perception [8]. In order to execute such functions, a classification process must be triggered so that scenarios can be identified, grouped and properly treated. Machine Learning (ML) is a useful AI approach when this classification process depends on features extracted from several samples. For instance, ML has been used for epidemiological research [9], diagnosis [10] and discriminate pathogens [11]. In this regard, we propose that the complexity of Capillariidae in the species definition, based on egg structures, could be clarified with the use of AI tools. A taxonomic dataset including morphological and morphometrical characteristics of parasite eggs, and ecological information was constructed based on specimens from Institutional Helminthological Collections. Institutional Biological Collections are taxonomic depositories of specimens described and strictly identified to specific level for experienced systematics. The research revealed a novel procedure to taxonomic species identification integrating data from centenary biological collections and the logic of Artificial Intelligence approaches.

Material and methods

Morphological and morphometric analyses

The specimens were collected in two Institutional helminthological collection, in Brazil at Fundação Oswaldo Cruz (FIOCRUZ) in *Coleção Helmintológica do Instituto Oswaldo Cruz* (CHIOC), 14 species (20 specimens); and in France at the collection of Muséum National d'Histoire Naturelle de Paris (MNHN), 16 species (17 specimens).

The specimens had their eggs separated for morphological and morphometric analysis. When present, females containing eggs were collected for the separation of the eggs or fragments containing the eggs, when it is not possible to manually extract them from the inside of females. For clear visualization of egg morphometry, samples are subjected to ultrasonic bath (Cristófoli®) for 60 seconds at the frequency of 42Khz. The process is done to clean the dirt and the fragments of females, resulting only in the presence of eggs with the chitin shell formed.

The eggs had their morphology and morphometry characterized by an optical microscope (Nikon Eclipse E200) in the 400X magnification, with the use of image analysis software (IMAGE PRO PLUS - MEDIA CYBERNETICS, USA). The measures considered were: total diameter (width) and length of eggs, the mean value of width and height of the two plugs and the thickness of the shell (Fig 1A). It was also done a qualification of the ornaments presented in the outer bark of capillariid eggs. The parameter of egg ornamentation was divided in categories: 1) smooth, that has no ornaments on the shell; 2) punctuated, that has a lot of dots; 3) reticulated type I, that is present like a network; and 4) reticulated type ll, that is presented like a network but with an orientation (Fig 1A-D).

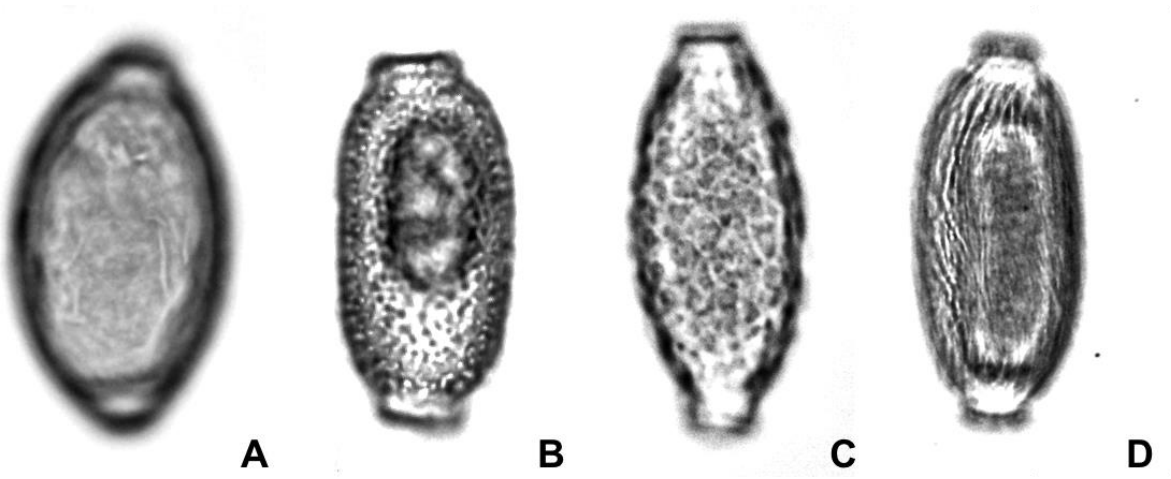

Fig 1. **Representation of each ornamentation pattern considered in this study based on capillariid eggs from CHIOC species**. A: Smooth from *Aonchotheca pulchra* voucher CHIOC9804; B: punctuated from *Capillaria brasiliana* voucher CHIOC7046; C: reticulated type I from *Pearsonema plica* voucher MNHN373c; D: reticulated type II from *Baruscapillaria resecta* voucher MNHN1073.

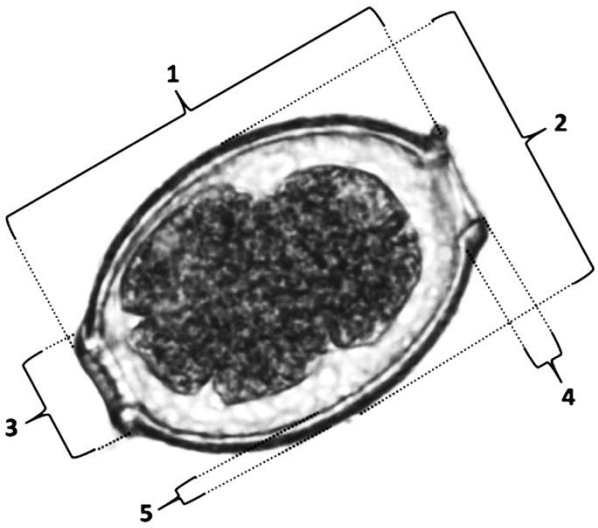

Fig 2. **Representation of capillariid egg measurements considered in this study based on *Baruscapillaria obsignata* voucher CHIOC26715.** (1) Total length; (2) total width; (3) base of polar plug width; (4) base of polar plug height; (5) shell thickness.

Discriminant analyses and artificial intelligence/machine learning approaches

A dataset of capillariid species from CHIOC and MNHN Helminthological collections was constructed with the morphological and morphometric (MM) parameters generated. In addition, ecological paraments as information about host (H) and geographical location (GL) of specimens were included (S1 Table).

Discriminant analyses were performed using Past 3.16 software in order to separate species groups. Firstly, plotting total length and width of eggs from all species and then, the discriminant function analysis was generated by each eggshell ornamentation: punctual, reticulated type I and reticulated type II. The exception was smooth ornamentation with only 1 species identified.

For ML/AI analyses, ornamentation and ecological parameters were transformed into numerical variables. Ecological parameters were defined as host includes fish, amphibian, reptile, avian, mammal, and as geographical location comprising, South America, Central America, North America, Europe, Africa, Asia, Oceania. Response variable were 1 = yes or presence; 0= no or absence; and -1 = no information. MM parameter were tested alone, and in combination with ecological parameters as: MM+H, MM+GL, and MM+H+GL.

Since, there is no literature of ML algorithms used to taxonomic species definition, the goal of this study, it was conducted an exhaustive test of algorithms available using Weka 3.8.3 software [12]. The decision trees yielded by the ML/AI system are similar to the taxonomic keys proposed/used by systematics in order to discriminate biological species. Therefore, we focused in algorithms that returned representations of decision trees such as: J48, Random Tree, REPTree. Despite, Logistic Model Trees (LMT), were also tested.

For a statistical analysis, the comparison proportion test was used for checking the null hypothesis for equal proportions among the algorithms rates (J48, Random Tree, REP Tree and Logistic Model Trees) and among parameters (MM+H+GL, MM+H, MM+GL and MM+H+GL). To make a conclusion about the

hypothesis with 95% confidence, *p*-value of the Chi-Square statistic should be less than 0.05 indicating that the difference is significant. Then the Marascuilo procedure was applied to check what proportions are different among the algorithms and the among combination of parameters applied. Data analyses were performed using RStudio version 3.5.1 (2018-07-02) software.

Results

Morphological and morphometric analyses

The species of Capillariidae studied herein have, in general, a barrel shape, varying between round and elongated, with polar plugs, and the egg shell usually has ornamentation, as described in the literature [13]. A total of 28 species of capillariids, distributed in 9 genera were characterized. Regarding egg shell ornaments, they were classified in smooth (n=1), punctuated 10, reticulated type l 7 and reticulated type ll 10 (Fig 3 - 5, Table 1).

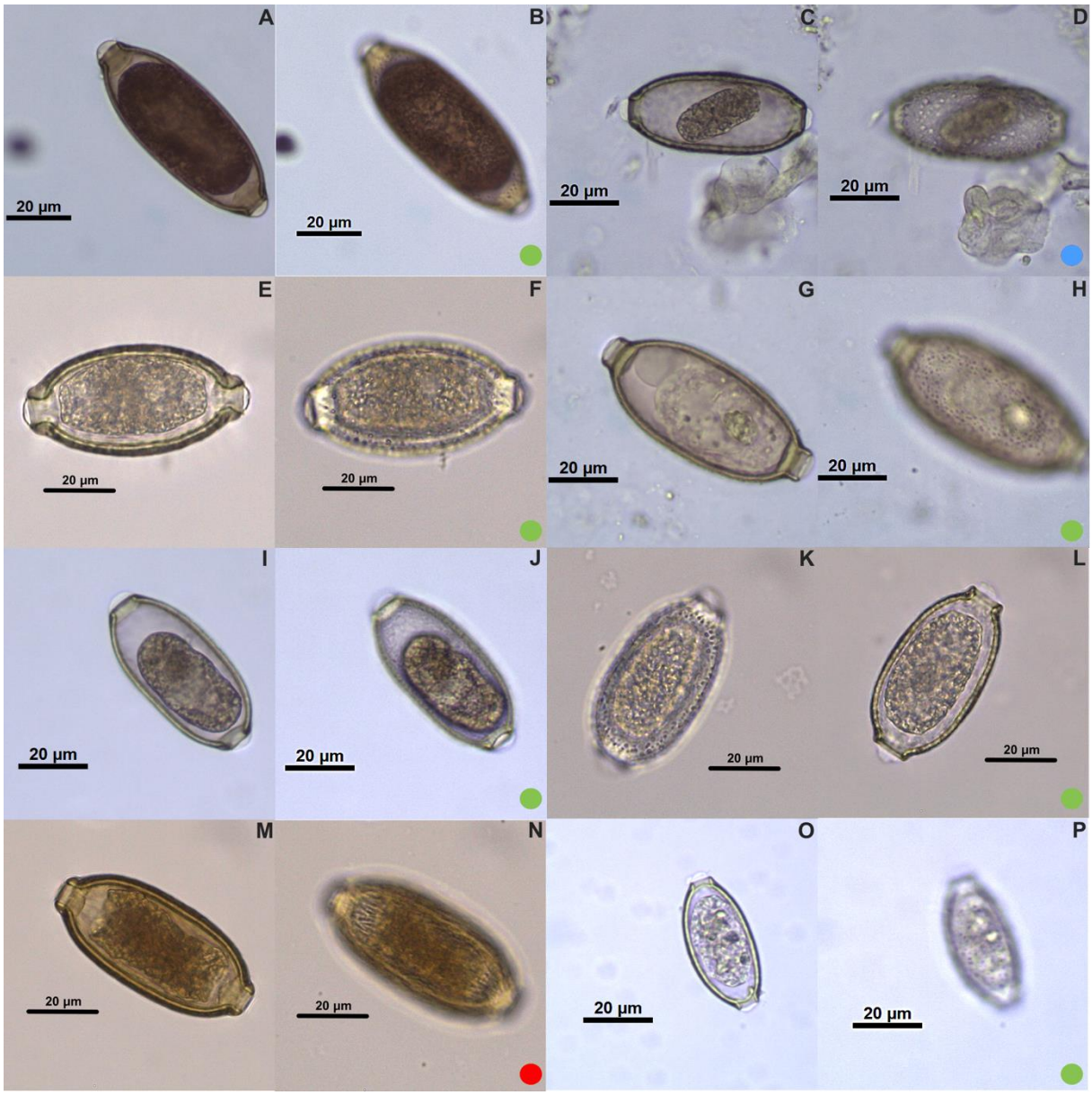

Fig 3. Micrographies of *Eucoleus* genus eggs. *Eucoleus anullatus* (A, B) *E. dubius* (C, D) *E. bacilatus* (E, F) *E. eberthi* (G, H) *E. contortus* (I, J) *E. madjerdae* (K, L) *E. dispar* (M, N) *E. perforans* (O, P). Each color dot represents an ornamentation pattern: Green dot - punctuated; blue dot - reticulated type I; red dot - reticulated type II.

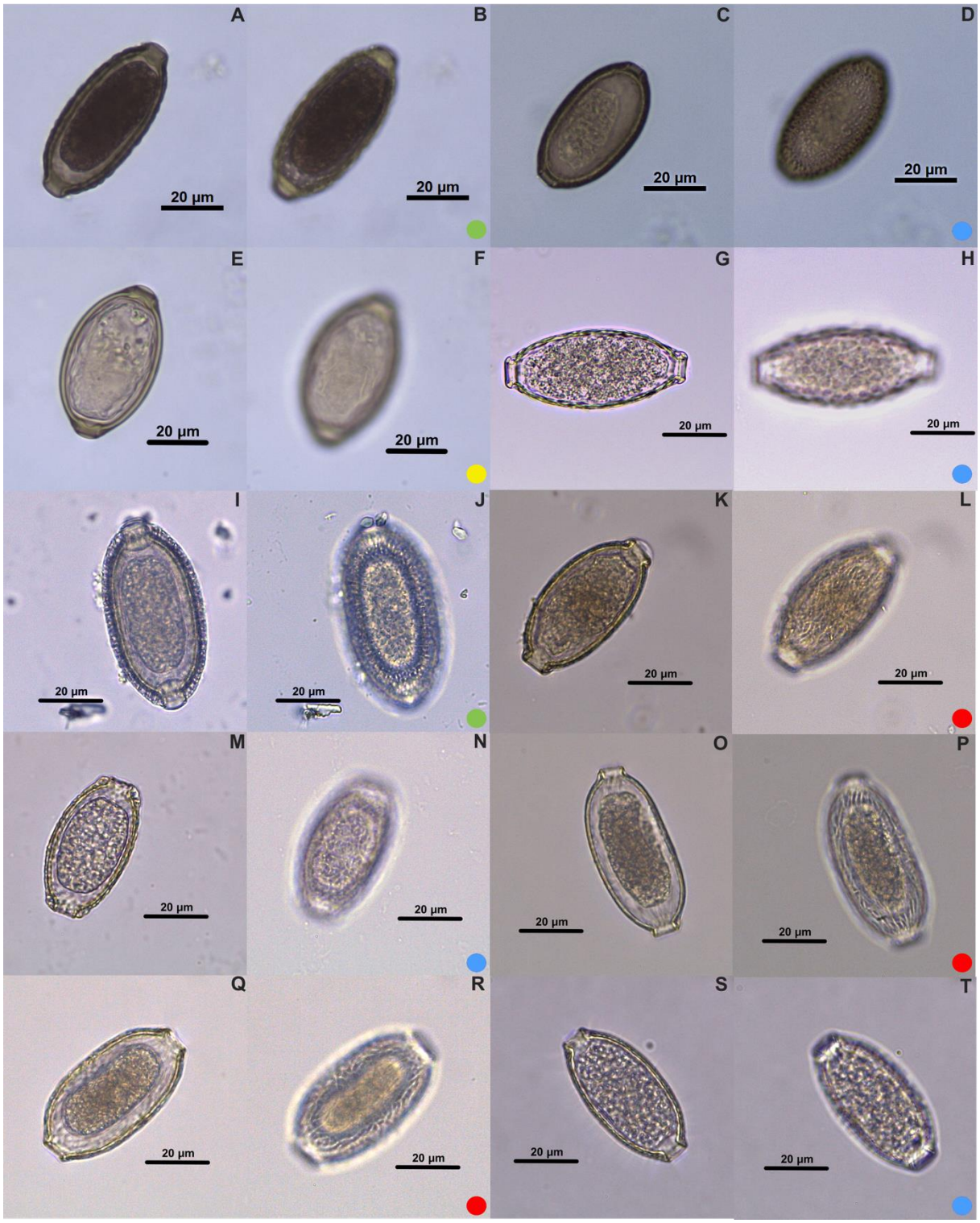

Fig 4. Micrographies of eggs belonged to *Echinocoleus, Pterothominx, Pearsonema, Calodium* and *Aonchotheca* genera. *Echinocoleus auritae* (A, B); *E. hydrocoeri* (C, D); *P. pulchra* (E, F); *P. plica* (G, H); *C. hepaticum* (I, J); *A. annulosa* (K, L); *A. baylisi* (M, N); *A. myoxinitelae* (O, P); *A. erinacei* (Q, R), *A. murissylvatici*

(S, T). Each color dot represents an ornamentation pattern: Yellow dot - smooth; Green dot - punctuated; blue dot - reticulated type I; red dot - reticulated type II.

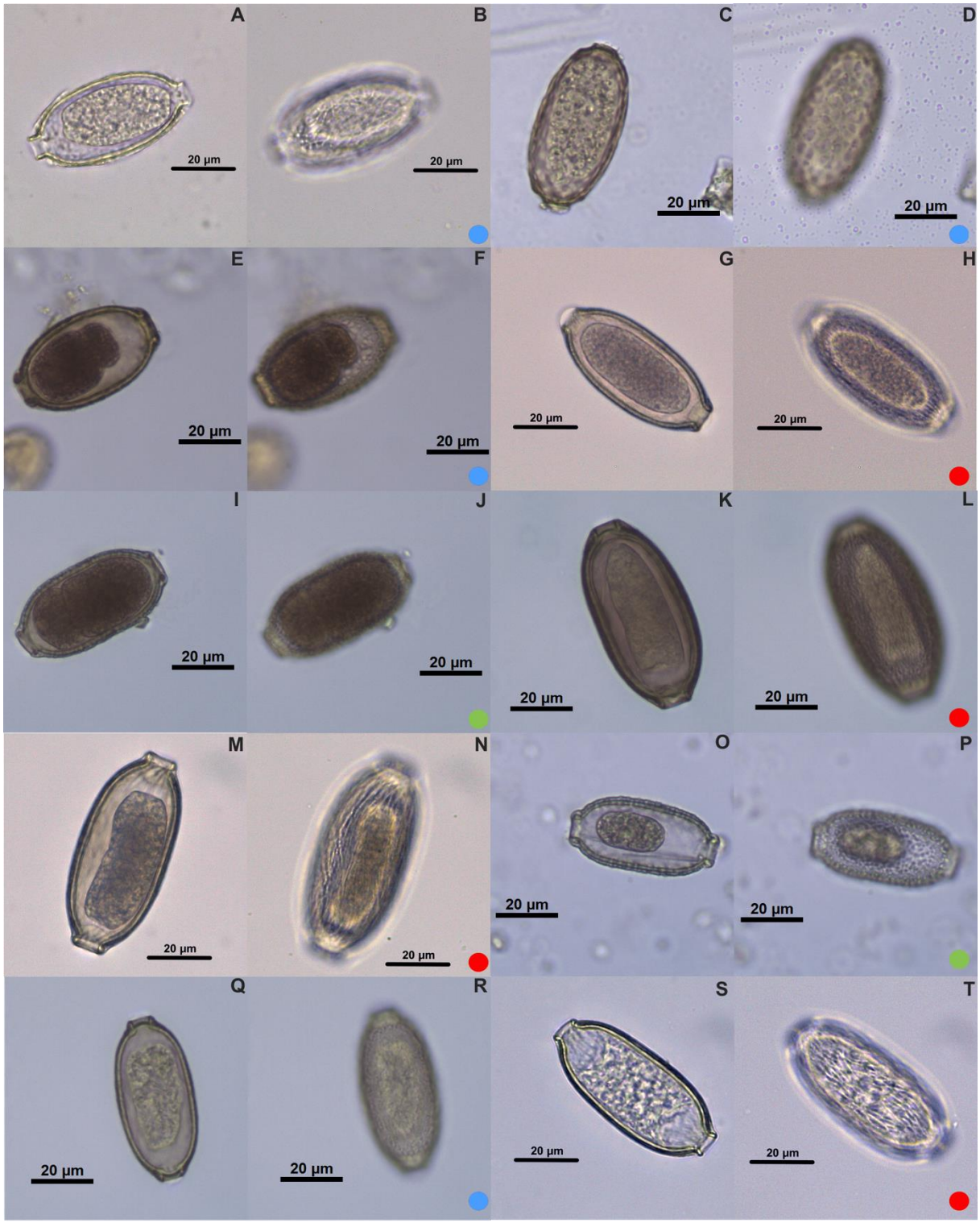

Fig 5. Micrographies of eggs belonged to *Baruscapillaria, Capillaria* and *Tridentocapillaria* genera. *Baruscapillaria falconis* (A, B); *Capillaria collaris* (C, D); *B. obsignata* (E, F); *C. exigua* (G, H); *B. spiculata* (I, J); *C. venusta* (K, L); *B. resecta*

(M, N); *C. brasiliana* (O, P); *B. rudolphi* (Q, R); *Tridentocapillaria tridens* (S, T). Each color dot represents an ornamentation pattern: Green dot punctuated; blue dot reticulated type I; red dot reticulated type II.

In all genera with more than one species to compare a high heterogeneity in measurements was seen. An amplitude of 37.06 - 70.39μm for length, 18.15 – 34.40μm for width, 5.43 – 12.95μm for plug base width, 1.09 – 5.68μm for plug base height and 0.78 – 5.57μm for eggshell thickness (Table 1).

Genus *Aonchotheca*

Five species were collected for this genus at helminthological collection of MNHN. The host of all the species were register as mammals: *A. annulosa* in *Apodemus sylvaticus*; *A. baylisi* in *Lophuromys sikapusi*; *A. erinaceid* in *Erinaceus europaeus*; *A. murissylvatici* in *Evotomys glareolus*; *A. myoxinitelae* in *Eliomys quercinus; A. pulchra* in *Tadarida laticaudata* and *Nyctinomus brasiliensis*. In general, the egg morphologies are very similar, the plug bases in mostly prominent, except in *A. baylisi* which has a thickening in the eggshell in plug base region, masking it. The egg ornamentation most presented is reticulated type ll with 4 species (Fig 5L, 5N, 5P, 5R). One punctuated type was present (Fig 5T). *A. pulchra* only species in this study, that does not have ornamentation on the eggshell surface (Fig 5F). *A. baylisi* is the smallest egg in the genus (44.75 – 50.14 x 24.92 – 28.97μm), in contrast with *A. myoxinitelae* (55.44 – 61.57 x 24.77 – 26.76μm).

Genus *Baruscapillaria*

Five species were collected in both helminthological collections, MNHN and CHIOC. The host of all the species were register as avian: *B. obsignata* in *Gallus*

gallus domesticus; *B. rudolphi* in *Tinamus solitarius*; *B. spiculata* in *Carbo vigua*; *B. falconis* in *Tyto alba*; *B. resecta* in *Garrulus glandariu*. All the egg ornamentation classifications are present in this genus. Reticulated type I (Fig 5F, 5R) and II (Fig 5B, 5N) was observed in two species each type, and one punctuated (Fig 5J). The eggs are very similar within the genus in shape and in plug base morphology. The species with the biggest egg measurement registers of all species of capillariids is *B. resecta* (65.47 – 70.39 x 29.58 – 31.81μm).

Genus *Capillaria*

Four species were collected at both MNHN and CHIOC helminthological collections. The host was register as avian and mammal: *C. venusta* in *Ramphasto toco*; *C. collaris* in *Gallus gallus domesticus*; *C. brasiliana* in *Nycticorax naevius*; *C. exigua* in *Erinaceus europaeus*. The morphologies of eggs are very different in shape. The genus showed the 3 different types of ornamentations.

Genus *Calodium*

Only one species was collected at helminthological collection of MNHN. The host was recorded in mammal: *C. hepaticum* in *Meriones persicus* and *Rattus rattus.* This species has a very peculiar morphology. The ornamentation is punctuated, and in a transversal view, a radial ornamentation is seen in eggshell. The thickest egg shell is seen in this species (5.54μm).

Genus *Echinocoleus*

Two species were collected at helminthological collection of CHIOC. The hosts registered were mammals: *E. hydrochoeris* in *Hydrochoerus capybara*; *Ec. auritae* in *Metachirops opossum.* The ornaments identified were punctuated (Fig 4D) and reticulated type I (Fig 4B), respectively. *E. auritae* has a particular eggshell ornament, with a prominent reticulated on a transversal view. Both have a very thick eggshell (2.1 – 3.51μm and 1.59 – 3.63μm, respectively).

Genus *Eucoleus*

Four species were collected at both helminthological collections. The hosts indicated were avian and mammal: *E. perforans* in *Numida meleagris*; *E. annulatus* in *Gallus gallus domesticus*; *E. contortus* in *Sterna maxima* and *Ajaja ajaja*; *E. dubius* in *Attila cinereus*; *E. eberthi* in *Metachirops opossum*; *E. bacilatus* in *Apodemus sylvaticus*; *E. madjerdae* in *Mus musculus*; *E. dispar* in *Atlapetes semirufus*. The majority of eggs observed in genus *Eucoleus* has the punctuated egg ornamentation (Fig 3B, 3F, 3H, 3J, 3L, 3P), but two species have each reticulated type I and II. *Eucoleus* genus showed the most different measurements of length (37.06 – 68.82μm) and width (18.15 – 33.65μm) among the species of the genus. The same was seen on plug base measurements, plug base length and plug base width, and also on egg shell thickness. The smallest of all capillariid species is *E. perforans* (37.06 x 18.91μm) and the thinnest is *E. annulatus* (0,78μm).

Genus *Pearsonema*

Only one species was collected at helminthological collection of MNHN. The host was register in mammal: *P. pulchra* in *Vulpes vulpes*. This species has a very elongated morphology, with a prominent reticulated type I eggshell.

Genus *Tridentocapillaria*

Only one species was collected at helminthological collection of MNHN. The host was register in avian: *Tridentocapillaria tridens* in *Cyanolanius madagascarinus*. The species *T. tridens* has reticulated type II ornamentation.

Discriminant analyses and artificial intelligence / machine learning approaches

The graphic XY of length and width measures for all species revealed a strong superposition of data with a more discriminant distribution in length than in width parameter (Fig 6A). The graphics of discriminant analysis by eggshell ornamentation showed the same pattern of species overlapping, with only 1-3 species group showed the adequacy of parameter for capillariid identification (Fig 6B - D), with the discrimination of *E. perforans*, *E. annulatus*, *E. eberthi* (punctuated) (Fig 6B), *P. plica* (reticulated type II) (Fig 6C) and *A. baylisi* (reticulated type II) (Fig 6D).

Table 1. Morphometry of Capillariidae species with measurements of length, width, plug width, plug thickness and shell thickness. Shell ornamentations 1, smooth; 2, punctuated; 3, reticulated type I; 4, reticulated type II.

Species	Lenght (μm)		Width (μm)		Plug base W (μm)		Plug base H (μm)		Shell (μm)		Shell Ornamentation
	Mean	Amplitude	Mean	Amplitude	Mean	Amplitude	Mean	Amplitude	Mean	Amplitude	
Aonchotheca annulosa	53.11	49.28 – 55.96	28.04	26.03 – 32.60	8.91	7.23 – 10.28	3.50	2.52 – 4.58	2.66	1.98 – 3.36	4
Aonchotheca baylisi	46.56	44.75 – 50.14	26.50	24.92 – 28.97	7.75	6.40 – 9.28	3.85	2.69 – 5.27	2.66	1.78 – 3.48	4
Aonchotheca erinacei	54.81	52.67 – 57.37	30.98	27.99 – 33.70	9.66	7.99 – 12.26	3.00	1.83 – 4.04	2.50	1.64 – 3.09	4
Aonchotheca murissylvatici	53.10	50.75 – 55.70	26.17	24.91 – 27.82	8.43	7.33 – 9.74	3.74	2.75 – 4.83	2.80	2.29 – 3.42	2
Aonchotheca myoxinitelae	57.74	55.44 – 61.57	25.83	24.77 – 26.76	8.25	6.83 – 9.24	3.21	2.47 – 4.06	1.68	1.34 – 2.11	4
Aonchotheca pulchra	49.52	46.15 – 52.96	30.89	28.15 – 34.40	8.52	7.42 – 9.38	4.09	2.77 – 5.68	1.82	1.41 – 2.32	1
Baruscapillaria obsignata	47.46	42.17 – 51.78	27.94	24.76 – 33.45	9.97	7.13 – 12.94	2.84	1.09 – 4.62	1.77	1.18 – 2.52	3
Baruscapillaria rudolphi	54.34	52.57 – 57.56	24.84	22.88 – 26.84	8.33	6.74 – 9.37	4.01	3.34 – 4.85	2.18	1.78 – 2.61	3
Baruscapillaria spiculata	53.17	51.48 – 55.69	27.02	24.11 – 30.47	1.85	9.17 – 12.01	2.67	1.98 – 3.61	2.38	1.88 – 2.79	2
Baruscapillaria falconis	54.30	52.54 – 55.85	26.23	25.28 – 28.19	7.95	6.92 – 8.68	3.32	2.51 – 4.06	1.51	1.00 – 2.54	4
Baruscapilaria resecta	68.12	65.47 – 70.39	30.53	29.58 – 31.81	9.56	8.37 – 10.79	3.88	2.87 – 5.07	2.73	1.85 – 3.43	4
Capillaria venusta	60.21	54.01 – 63.29	30.00	21.95 – 32.27	9.95	8.56 – 11.65	3.60	2.54 – 4.60	2.83	1.45 – 3.98	4
Capillaria colaris	52.33	46.91 – 56.82	26.46	23.81 – 30.03	8.32	7.24 – 9.04	3.36	2.20 – 4.64	1.46	0.84 – 2.22	3
Capillaria brasiliana	46.45	43.10 – 50.24	21.41	19.06 – 22.88	8.80	7.23 – 10.66	2.67	1.66 – 3.6	2.05	1.63 – 2.86	2
Capillaria exigua	55.10	52.73 – 56.73	26.33	25.18 – 27.12	8.06	7.13 – 9.08	2.84	1.94 – 3.59	1.61	1.31 – 1.98	4
Calodium hepaticum	55.44	50.07 – 62.02	30.42	27.38 – 33.84	8.08	7.07 – 9.86	4.24	3.16 – 5.18	4.34	3.26 – 5.57	2
Echinocholeus hydrochoeri	49.34	46.18 – 51.74	25.14	22.43 – 27.62	6.72	5.92 – 7.91	3.78	2.58 – 5.00	2.65	1.59 – 3.63	2
Echinocholeus auritae	57.69	56.13 – 59.84	26.13	24.71 – 27.82	7.74	6.85 – 8.80	4.84	3.99 – 6.15	2.72	2.1 – 3.51	3

Eucoleus perforans	39.86	37.06 – 42.81	20.41	18.15 – 23.94	6.37	5.43 – 7.45	2.50	2.05 – 3.38	1.57	1.08 – 2.24	2
Eucoleus annulatus	65.36	61.45 – 68.78	26.75	24.87 – 27.71	8.56	6.24 – 10.22	3.33	2.47 – 5.33	1.13	0.78 – 1.63	2
Eucoleus contortus	51.07	46.70 – 54.07	26.24	24.49 – 28.21	8.03	6.71 – 9.62	2.36	1.59 – 3.77	1.42	1.08 – 1.82	2
Eucoleus dubius	52.14	47.07 – 55.25	23.62	22.40 – 25.57	8.82	7.73 – 9.80	3.43	2.44 – 4.24	2.28	1.76 – 2.73	3
Eucoleus eberthi	65.89	63.15 – 69.82	29.27	28.55 – 29.86	9.59	8.53 – 10.55	5.30	4.23 – 7.01	1.59	1.25 – 1.97	2
Eucoleus bacilatus	63.09	60.53 – 68.29	32.78	32.04 – 33.65	11.24	9.70 – 12.95	4.78	3.86 – 6.05	3.97	3.17 – 4.63	2
Eucoleus madjerdae	53.41	51.81 – 55.10	29.23	28.41 – 29.99	10.38	8.82 – 11.19	3.29	2.16 – 4.53	2.13	1.53 – 2.53	2
Eucoleus dispar	63.42	60.34 – 68.39	29.65	28.19 – 32.81	8.89	7.63 – 10.93	3.94	2.79 – 5.40	2.90	2.34 – 3.68	4
Pearsonema plica	62.62	60.30 – 65.32	27.47	26.35 – 28.76	9.62	8.48 – 10.87	4.47	3.40 – 5.28	2.24	1.71 – 2.55	3
Tridentocapillaria tridens	60.60	57.39 – 63.33	27.54	25.84 – 29.81	8.34	7.04 – 9.23	2.84	1.99 – 4.22	2.86	2.49 – 3.13	4

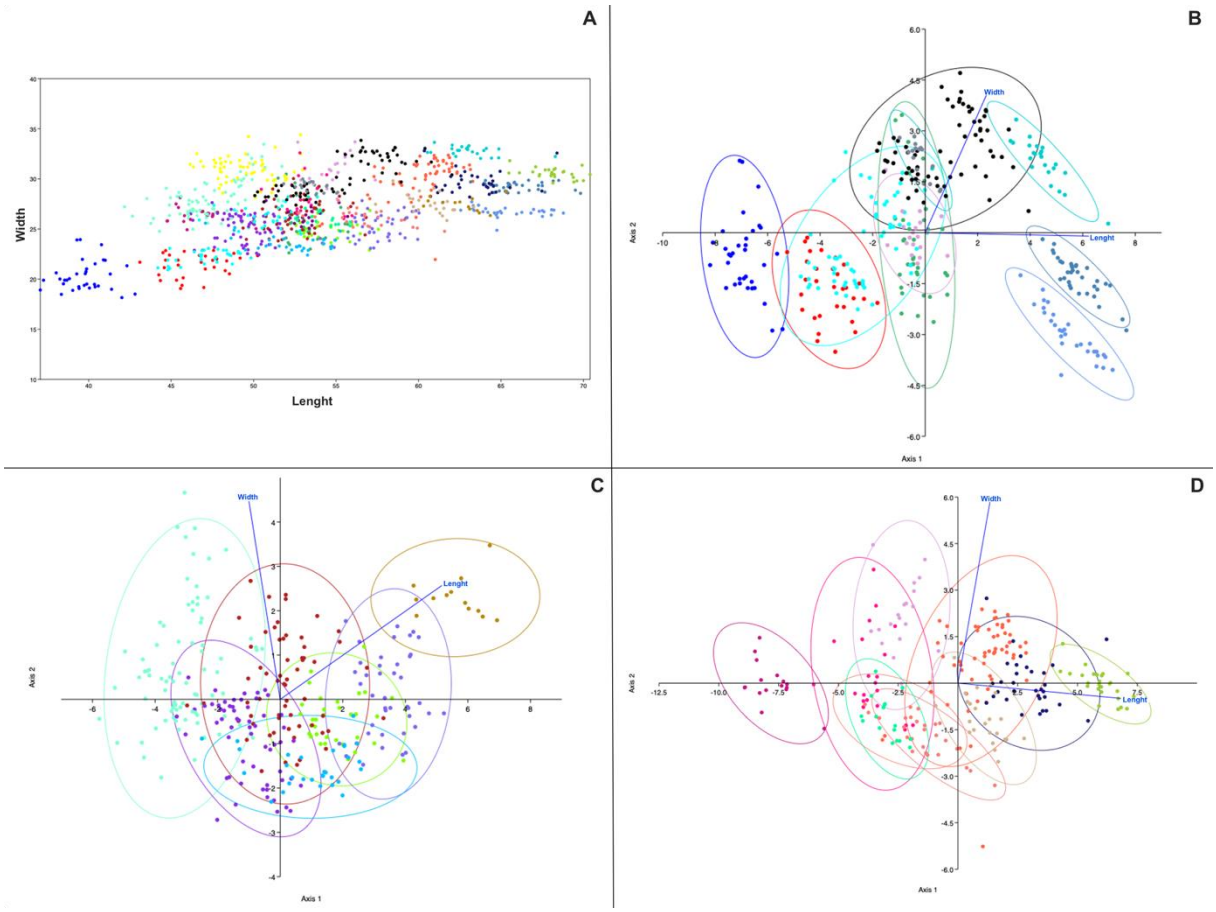

Fig 6. **Discriminant analyses considering measures of length and width of capillariids eggs.** All species of this study (A). According to the classification of ornamentation, punctual (B), reticulated type I (C) and reticulated type II (D). Each color represents one species.

In the AI/ML analyses, graphics of distribution of each attribute exhibited, in general, that there are most entries, and overlapping, in the average measures, except in the shell thickness distribution that have most entries in thinner shells (Figure 7).

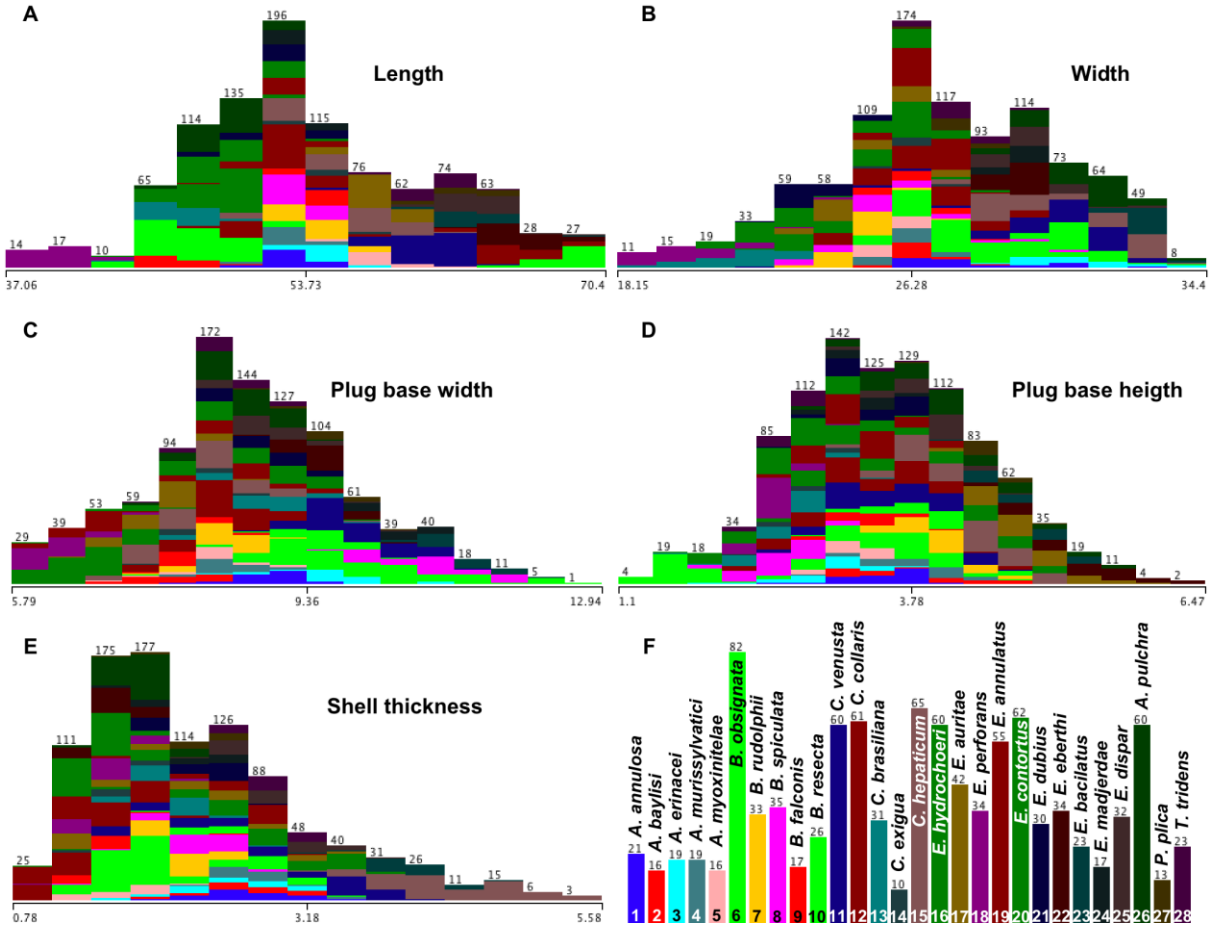

Fig 7. **Graphics of distribution of egg measures by attribute.** A: length; B: width; C: plug base width; D: plug base height; E: shell thickness. Numbers in the X-axes are maximum, minimum and average values of each parameter. F: Representation of entries by each species. Numbers in the columns represent the total of entries. Each color represents one species, as indicated. Generated by Weka 3.8.3 software.

The results of cross-validated and corrected classified instances showed higher values when LMT algorithm was used, with the highest efficiency (97.289%) in the combination of morphological and morphometric with all ecological parameters (Table 2). However, LMT algorithm does not return representations of decision trees. From all the algorithms that produce decision trees, J48 algorithm showed higher values, with the highest efficiency (93.072%) when all parameters were used (Table 2).

The decision trees generated by J48 algorithm, applying all ecological parameters and morphological and morphometric data (Fig 8). In the supporting information are available the decision trees constructed using morphological and morphometric data plus only host (S1 Fig), plus only geographical location (S2 Fig), and only morphological and morphometric data (S3 Fig), considering 3 different ornamentation type, punctuated (Fig 1B), reticulated type I (Fig 1C) and reticulated type II (Fig 1D).

Table 2. **Algorithms and parameters considered in the ML/IA analysis.** Corrected classified instances cross-validated in percentages per algorithms and per parameters. Generated by Weka 3.8.3 software.

Algorithmes	MM+GL+H (%)	MM+H (%)	MM+GL (%)	MM (%)
J48	93.072	90.060	91.566	87.550
Random tree	90.361	87.249	89.558	84.236
REPTree	90.361	87.349	88.654	84.739
LMT	97.289	95.381	95.582	92.871

MM, morphological and morphometric data; GL, geographical location; H, host.

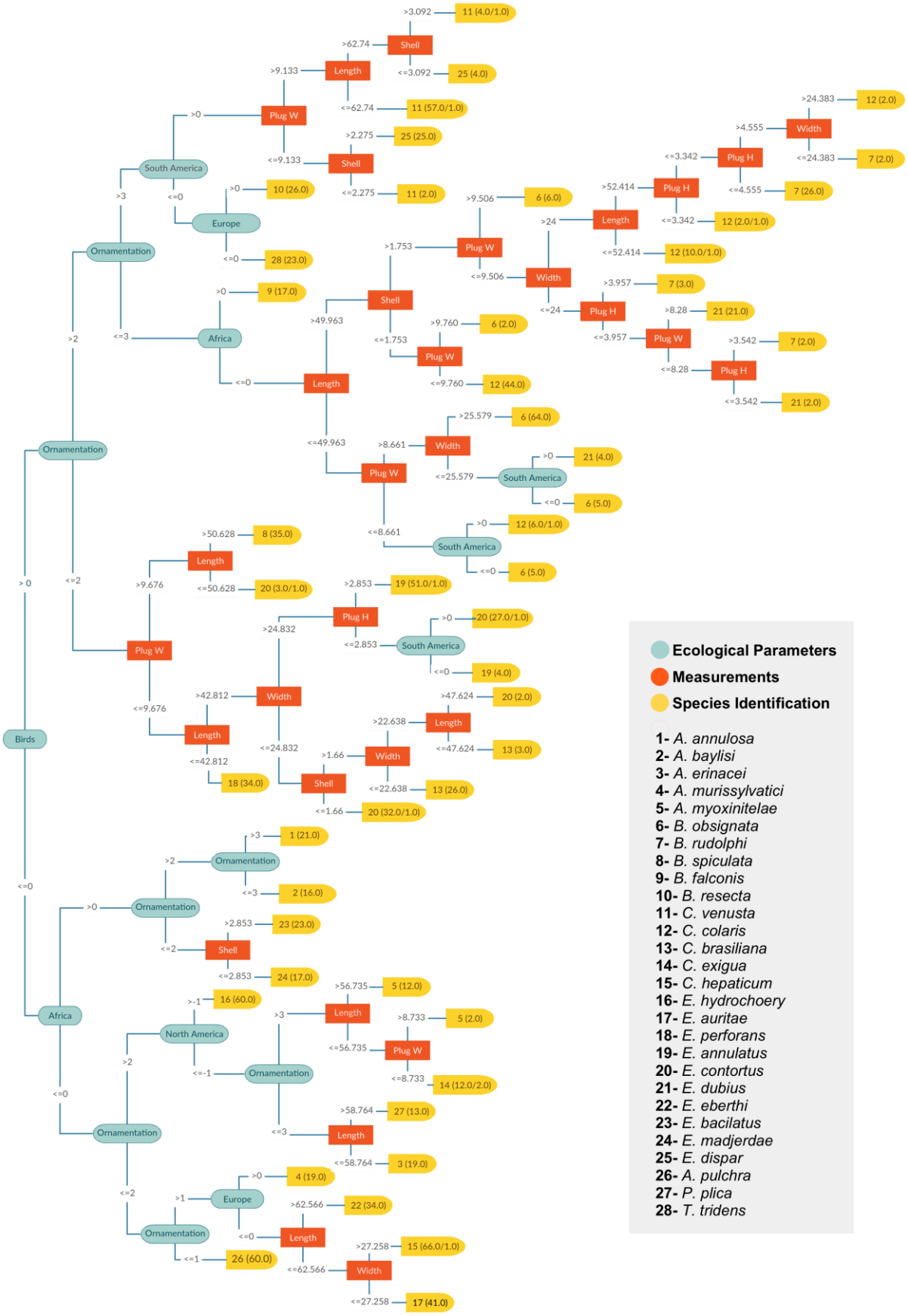
Birds
> 0
<=0
Ornamentation
>2
<=2
Africa
Plug W
Ornamentation
>0
<=3
<=9.676
>9.676
Length
>50.628
8 (35.0)
<=50.628
20 (3.0/1.0)
Africa
Ornamentation
>3
<=0
South America
>0
10 (26.0)
<=0
Europe
<=0
28 (23.0)
Plug W
>0
>9.133
Length
>3.092
11 (4.0/1.0)
>62.74
Shell
<=3.092
25 (4.0)
<=62.74
11 (57.0/1.0)
<=9.133
>2.275
25 (25.0)
Shell
<=2.275
11 (2.0)
Ornamentation
>2
<=3
Africa
>0
9 (17.0)
<=0
Length
>49.963
Shell
>1.753
Plug W
<=1.753
>9.760
6 (2.0)
<=9.760
Plug W
12 (44.0)
<=49.963
Ornamentation
>0
<=2
Plug W
>8.661
Width
>25.579
6 (64.0)
<=25.579
Plug W
South America
>0
21 (4.0)
<=0
6 (5.0)
<=8.661
South America
>0
12 (6.0/1.0)
<=0
6 (5.0)
Plug W
>24.832
Plug H
>2.853
19 (51.0/1.0)
<=2.853
South America
>0
20 (27.0/1.0)
<=0
19 (4.0)
<=24.832
Width
>42.812
Length
<=42.812
18 (34.0)
Shell
>1.66
Width
>22.638
Length
>47.624
20 (2.0)
<=47.624
13 (3.0)
<=22.638
13 (26.0)
<=1.66
20 (32.0/1.0)
Ornamentation
>3
1 (21.0)
>2
Ornamentation
<=3
2 (16.0)
Ornamentation
>0
<=2
Shell
>2.853
23 (23.0)
<=2.853
24 (17.0)
Africa
<=0
Ornamentation
<=2
Ornamentation
>1
Europe
<=0
Length
>62.566
22 (34.0)
<=62.566
Width
>27.258
15 (66.0/1.0)
<=27.258
17 (41.0)
<=1
26 (60.0)
North America
>-1
16 (60.0)
<=-1
Ornamentation
>2
<=0
Ornamentation
>3
Length
>56.735
5 (12.0)
<=56.735
Plug W
>8.733
5 (2.0)
<=8.733
14 (12.0/2.0)
<=3
Length
>58.764
27 (13.0)
<=58.764
3 (19.0)
>0
4 (19.0)
Plug W
>9.506
6 (6.0)
<=9.506
Width
>24
Length
>52.414
Plug H
<=3.342
12 (2.0/1.0)
<=52.414
12 (10.0/1.0)
<=24
Plug H
>3.957
7 (3.0)
<=3.957
Plug W
>8.28
21 (21.0)
<=8.28
Plug H
>3.542
7 (2.0)
<=3.542
21 (2.0)
Width
>24.383
12 (2.0)
>4.555
Plug H
<=24.383
7 (2.0)
<=4.555
7 (26.0)
Ecological Parameters
Measurements
Species Identification
1- A. annulosa
2- A. baylisi
3- A. erinacei
4- A. murissylvatici
5- A. myoxinitelae
6- B. obsignata
7- B. rudolphi
8- B. spiculata
9- B. falconis
10- B. resecta
11- C. venusta
12- C. colaris
13- C. brasiliana
14- C. exigua
15- C. hepaticum
16- E. hydrochoery
17- E. auritae
18- E. perforans
19- E. annulatus
20- E. contortus
21- E. dubius
22- E. eberthi
23- E. bacilatus
24- E. madjerdae
25- E. dispar
26- A. pulchra
27- P. plica
28- T. tridens

Fig 8. **Decision tree for Capillaridae species discrimination using J48 algorithm with MM+GL+H parameters.** MM: include the attributes: length, width, plug base width, plug base height, shell thickness, and ornamentation type. GL: geographic location includes North America, Central America, South America, Europe, Africa, Asia and Oceania. H: include fish, amphibian, reptile, avian, and mammal. Ornamentations 1: Smooth; 2: Punctuated; 3: Reticulated Tipo I; 4: Reticulated Tipo II. Numbers in yellow are the capillariid species and between parentheses are correct/incorrect entries by the program. Generated by Weka 3.8.3 software.

The proportions comparison test showed a statistically significant difference between among the algorithms (p-value < 0.001) and the ecological parameters (p-value < 0.001) thus, rejecting the null hypothesis. The Marascuilo results indicate that, with algorithm J48, no significant difference between the parameters employed was found, except between using all the parameters and without ecological parameters, MM+GL+H vs MM (p-value < 0.001). In addition to MM+GL+H vs MM statistical difference, was also observed between MM+ H vs MM) (p-value < 0.001). for Random tree, REPTree and LMT algorithms. The Marascuilo procedure also demonstrated significant difference between algorithms for each combination of parameters (p-value < 0.001).

Discussion

The adult specimens were preserved in the institutional collection in alcohol in 70% solution. And in this study the eggs were recovered from the interior of females. Although there was a concern of get the eggs from the final portion of uterus, to guarantee the final stages of development, eggs once liberated can change in measure. So, a slighted difference could be seen in the data presented here to the eggs found in coprological surveys.

A lot of species of capillariids are non-species specific for host, and even non class specific, as *Paracapillaria phillipinensis* that is the only one known to parasitized two class of vertebrates, described in mammals and birds [14]. That difference in natural hosts could imply in variability in shape and/or size of eggs, as phenotypical plasticity. That phenomenon occurs when the same species infecting different hosts present different morphologies [15], that has been already seen in adult trematodes as *Schistosoma mansoni*. As observed in adult worms, it is possible that can occur in others development stages, such as eggs.

In the first attempts to classify genera using eggs of capillariids, Romashov (1985) divided the eggs into six groups, considering only the eggshell surface ornaments and the site of parasite infection. All capillariids analyzed were from mammals, and the author concluded that a relation between those variables is enough to determine the genus, relatively unmistakably [7]. However, in coprological surveys and paleoparasitological studies it is impossible to define the site of infection, as the only data recorded is the egg itself, sometimes also the host.

In the present study, there is a dominance of punctuated ornament (6 species presented) in the genus *Eucoleus*. Although *E. dispar* has a different ornamentation from the other 7 species, it is similar to *E. aerophilus* seen in the literature [16], which is supported by molecular phylogenetic analyses, showed a close relation between them [4,6]. However, there was not molecular information of other known species of the genus. *Eucoleus dubius* is also in another category, with type I reticulated ornamentation.

In the other hand, genus *Aonchotheca* has dominance of type ll reticulated ornamentation. Although these ornaments are seen in other genera, the frequency is not too high, it appears in *Baruscapillaria*, *Capillaria*, *Eucoleus* and *Tridentocapillaria*. The genus *Echinocoleus* has reticulated type l and punctuated ornamentation, but as it has only two species studied, it cannot be assumed that has any pattern. *Pearsonema plica* also presented reticulated type l, with a very similar characteristics of *C. collaris*, although the egg morphology is different, one narrowed on the extremity (Fig. 4G) and the other rounder (Fig. 5C), respectively.

Regarding the statistical analysis of egg measures, no relation between genera was seen. The same to discriminate species, even though length showed

more relevance than width, great part of measures is overlapped, and it is impossible to discriminate among most of them. Despite it was not a good method for discrimination, 5 species (*E. perforans*, *E. annulatus*, *E. eberthi* , *P. plica* and *A. baylisi*) among 28, could be identified when applied a discriminant analysis separated by ornamentations. For this reason, the necessity of application of another tool, more robust, that can integrate more variables for species identification.

The ML/AI analysis showed that when parameters of geographical location and host were included the reliability of the decision tree is higher with all algorithms used (Table 1). The LMT algorithm showed a more reliable results, however it doesn't produce a decision tree, which it wasn't functional for the revision of the results in the biological sense and is not useful for an application for future taxonomic species identifications, as mentioned before.

Regarding ecological parameters, the H parameter may be more robust since, except for two genera (*Capillaria* and *Eucoleus*), it was possible to employ the taxonomic level of class and use one H entrance, avoiding decision errors. Regarding geographical location, firstly, for a more complete dataset, the parameter was defined by all the continents where the species was recorder, based on an extensive literature revision of capillariid identifications. However, species with worldwide distribution, as *C. hepaticum*, presents multiples reentrances and consequently decision errors were observed. When used two different entrance for the same specimens, both for H and GL, the program tends to choose whose differentiates more between species, which could be erroneous because it does not consider the second entrance as a possible variable. For this reason, the GL parameter was then expressed as the local where specimens were collected, based on CHIOC and MNHN files. Therefore, there is a restriction of information used for geographical location.

Although the12 decisions trees produced, as showed in Table 2, the trees generated by J48 algorithm showed the most reliable values. The tree with all ecological variables (MM+GL+H) is the most complete (Fig 8), with a higher reliable value (93.072) than MM (87.550%) and showed significant difference when no ecological parameter was used. These results showed the relevance of ecological characteristic of specimens in the species discrimination. However, no significant

difference was shown when only one of ecological parameter was present (MM+H or MM+GL), that means that one could compensated the absence of any those data.

The present study has some limitations. Relating to the dataset constructed, this contains 28 species and 8 genera of capillariids, a portion of more than 300 and 25 described, respectively. Therefore, it does not give the real scenario of the biological diversity and/or similarity in capillariids. In addition, some species are represented by one specimen, that could be a restriction in the possible intraspecific and ecological variations. Multiple host or geographical origin in the same species could be interpreted by the system as a discrepant character, and consequently the learning is wrongly addressed. However, it is known that capillariid species in general are not so restricted. The solution that we found was both a generalization and constriction of host and location of species information, respectively. The addition of new curated information from other biological helminthological collections will allow the construction of a stronger support dataset, and consequently a better species definition by means of ML/AI. To our knowledge, this is the first study that applied artificial intelligence techniques to the taxonomic definition of biological species, opens an opportunity of application in other important taxa for health, biodiversity and technology purposes.

Conclusions

This work is a start in the Machine Learning/Artificial Intelligence approach applied to taxonomic species definition using capillariids as model. It supplies new data in the characterization of nematode eggs, a field that has a lack of knowledge in parasite morphological description that compromises studies such as ecological and health surveys, as well as paleoparasitological research. Therefore, it gives support for the identification of capillariids with the characterization of 28 species and 8 genera of the family and generates a catalog for future references.

The study makes available a solid representation of capillariids deposited in two large and important institutional collections, CHIOC and MNHN. Starting from this, others collection can be accessed to increase the species and families described and applied the same ML/AI methodologies proposed.

The Machine Learning/Artificial Intelligence approach showed herein is an initial methodology presentation for identification of parasite species. It showed a concise result that can assist on the species identification of Capillariidae family and we believe and hope that can be also extrapolated for other parasites.

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Supporting Information

S1 Fig. Decision tree generated by Weka software using J48 algorithm with only MM plus only geographical location parameters that include the attributes; for MM: length, width, plug base width, plug base height, shell thickness; and for geographical location: South America, Central America, North America, Europe, Africa, Asia, Oceania. Generated by Weka 3.8.3 software.

S2 Fig. Decision tree generated by Weka software using J48 algorithm with MM plus only Host parameters that include the attributes; for MM: length, width, plug base width, plug base height, shell thickness; and for Host: fish, amphibian, reptile, avian and mammals. Generated by Weka 3.8.3 software.

S3 Fig. Decision tree generated by Weka software using J48 algorithm with only MM parameters that include the attributes: length, width, plug base width, plug base height, shell thickness, for punctuated ornamentation. Generated by Weka 3.8.3 software.

S4 Fig. Decision tree generated by Weka software using J48 algorithm with only MM parameters that include the attributes: length, width, plug base width,

plug base height, shell thickness, for reticulated type I ornamentation. Generated by Weka 3.8.3 software.

S5 Fig. Decision tree generated by Weka software using J48 algorithm with only MM parameters that include the attributes: length, width, plug base width, plug base height, shell thickness, for reticulated type II ornamentation. Generated by Weka 3.8.3 software.

S1 Table. **Morphometry: measurements of length, width, plug width, plug thickness and shell thickness; morphology: shell ornamentations, 1, smooth; 2, punctuated; 3, reticulated type I; 4, reticulated type II; host: fish, amphibian, reptile, avian, mammals; geographical location: South America, Central America, North America, Europe, Africa, Asia, Oceania.** Response variable were 1 = yes or presence, 0= no or absence, and -1 = no information.

5.3 Artigo 3: Eggshell struture of Capillariid species: a SEM perspective

Eggshell structure of capillariid species by a SEM perspective

Victor Borba[1,2], Makoto Enoki[1], Eduardo José Lopes-Torres[1], José Roberto Machado-Silva[1], Alena Iñiguez[2*].

[1]Laboratório de Helmintologia Romero Lascasas Porto, Faculdade de Ciências Médicas, UERJ, Rio de Janeiro, RJ, Brazil.

[2]LABTRIP, Instituto Oswaldo Cruz, IOC-FIOCRUZ, Rio de Janeiro, RJ, Brazil.

*Corresponding author

E-mail: alenainiguez@gmail.com

ABSTRACT

Capillariidae is group of nematode parasites of vertebrates with one of most difficult and complex taxonomy. The structure of eggshell, which was indicated as the most important characteristic for identification of genus or species by means of eggs, is very diverse among genera. This work aimed to present and characterized the eggshell structure of Capillariidae species, parasites of mammals and avian, deposited in helminthological collections using Scanning Electron Microscope (SEM). Institutional Biological Collections are taxonomic depositories of specimens

described and strictly identified to specific level for systematics. SEM eggshell imagens were obtained from 12 species belonged to 5 genera (*Eucoleus*, *Aonchotheca*, *Capillaria*, *Echinocoleus*, *Baruscapillaria*) and compared to their respective light microscope (LM) micrographs. Six eggshell patterns by SEM were observed and associated according to 4 categories of eggshell ornamentation by LM: Smooth, Punctuated, Reticulated Type I and Reticulated Type II. The data indicate that eggshell categories are not in agreement with capillariid genera or sites of infection, since all Types, except Types 1 and 6 are described for more than one genus. However, the study provides previously unknown SEM eggshell information, from curated species, which contributes with a specific and supplementary taxonomic feature at the species level of Capillariidae.

Keywords: Scanning electron microscope, Capillariidae, Taxonomy, Eggshell Ornamentation

Résumé

Les Capillariidae sont un groupe de parasites nématodes de vertébrés présentant une taxonomie des plus difficiles et complexes. La structure de la coquille, indiquée comme caractéristique la plus importante pour l'identification du genre ou de l'espèce au moyen d'œufs, est très diverse parmi les genres. Ce travail visait à présenter et à caractériser la structure de la coquille d'œuf des espèces de Capillariidae, parasites de mammifères et d'oiseaux, déposée dans des collections helminthologiques à l'aide d'un microscope électronique à balayage (MEB). Les collections biologiques institutionnelles sont des dépôts taxonomiques de spécimens décrits et strictement identifiés à un niveau spécifique pour la systématique. Les images de coquille d'oeuf MEB ont été obtenues à partir de 12 espèces appartenant à 5 genres (*Eucoleus*, *Aonchotheca*, *Capillaria*, *Echinocoleus*, *Baruscapillaria*) et comparées à leurs micrographies respectives au microscope photonique (MP). Six motifs de coquille d'œuf par MEB ont été observés et associés selon 4 catégories d'ornementation de

coquille d'œuf par MP : lisse, ponctuée, réticulée Type I et réticulée Type II. Les données indiquent que les catégories de coquilles d'œufs ne sont pas en accord avec les genres ou les sites d'infection des capillaires, car tous les Types, à l'exception des Types 1 et 6, sont décrits pour plus d'un genre. Cependant, l'étude fournit des informations sur la coquille d'oeuf inconnues dans MEB, provenant d'espèces sélectionnées, qui contribuent à une caractéristique taxonomique supplémentaire et supplémentaire au niveau de l'espèce Capillariidae.

Mots-clés: Microscope électronique à balayage, Capillariidae, Taxonomie, Ornementation de coquille d'oeuf

Introduction

Nematodes of the family Capillariidae Railliet, 1915, parasites of vertebrates, have a difficult and controversial taxonomy, mostly because the lack and/or misinformation of morphology of many species. The main feature for identification of genera is based on the morphology of the posterior end of adult males. Other features used are the specular sheath, spicule, structure of stichosome, bacillary bands, vulvar appendage, and among other characteristics the structure of eggs [1,2]. The structure of the outer surface of the eggshell are very diverse among genera and have been recognized as the most important characteristic for identification of capillariid species by means of eggs.

Researches characterizing eggs are scarce, especially in capillariids. However, among all the structures used to identify species, the egg is the evolutionary form that appears in most of studies, as coprological surveys on medical, ecological and paleoparasitological fields. The most studied species are of public health or veterinary concern, such as *Calodium hepaticum* [3], *Paracapillaria philipinensis* [4], *Eucoleus aerophilus* [5]. Romashov (1985) was a pioneer in the categorization of Capillariidae species by the ornaments of the eggshell. He proposed 6 groups according to the similarities of the ornamentations relating to the

site of infection by light microscopy (LM), as well, scanning electron microscopy (SEM) [6]. Recently, we proposed Types of eggshell ornamentations after evaluation of 9XX morphotypes of 26 capillariid species deposited on institutional helminthological collections using LM. The study allows to develop a new methodology for capillariid species identification based on artificial intelligence technology [7]. Paleoparasitological studies, which investigate parasites in archaeological and paleontological samples, also have used eggshell ornamentations for supporting species discrimination [8,9]. Coprological and paleoparasitological studies, are usually conducted by LM, and do not have a high support of SEM assays, which are scarce with few species characterized [3–6]. Although helminth eggs have little taxonomic characters for species identification, they are the only structure available for parasite diagnosis, as mentioned.

This work aimed to characterize Capillariidae eggshells from species deposited in an Institutional Helminthological Collection using SEM in order to supply additional information in the identification of parasite species by means of eggs.

Materials and methods

Capillariid species were obtained from the Institutional Collection "*Coleção Helmintológica do Instituto Oswaldo Cruz*" (CHIOC). The specimens were preserved in 70°GL ethanol, and were washed with a saline solution, phosphate buffered-saline (PBS). The final portion of the uterus of females were sectioned in order to collect fully developed eggs. Worms were broken and their eggs extracted using an ultrasonic sonicator (Cristófoli®) for 60 seconds at frequency of 42Khz.

For LM procedures, the samples were fixed in temporary slides with glycerol, examined and analyzed using a Nikon Eclipse E200 microscope. Thirty eggs of each species were measured in the 400X magnification, with the software Image Pro Plus - Media Cybernetics, USA.

The preparation for SEM was performed with the samples adhered in microscope slide cover glass (22 x 22 mm) previously prepared with porcine gelatin

solution 1%. Then, samples were dehydrated in a graded ethanol series (30°-absolute), critical point dried in CO2, mounted on stubs, coated with gold (20–25 nm) and examined using the SEM Jeol JSM-6390LV, under 15kV acceleration voltage [10].

Table 1. Information of capillariid species characterized in this study

Species	Collection voucher	Host Species	Host Class	Infection Site
Aonchotheca pulchra	CHIOC 18215	*Tadarida laticaudata*	Mammal	Stomach mucosa
Baruscapillaria obsignata	CHIOC 26715	*Gallus gallus domesticus*	Avian	Small intestine
Baruscapillaria rudolphii	CHIOC 7770	*Tinamus solitarius*	Avian	Small intestine
Baruscapillaria spiculata	CHIOC 2863	*Carbo vigua*	Avian	Small intestine
Capillaria venusta	CHIOC 23408	*Ramphasto toco*	Avian	Small intestine
Capillaria collaris	CHIOC 18904	*Gallus gallus domesticus*	Avian	Caecum
Capillaria brasiliana	CHIOC 7046	*Nycticorax naevius*	Avian	Small intestine
Echinocholeus hydrochoeri	CHIOC 11214	*Hydrochoerus capybara*	Mammal	Small intestine
Echinocholeus auritae	CHIOC 7786	*Metachirops opossum*	Mammal	Small intestine
Eucoleus perforans	CHIOC 9898	*Numida meleagris*	Avian	Eosophagus mucosa
Eucoleus contortus	CHIOC 6307	*Sterna maxima*	Avian	Eosophagus mucosa
Eucoleus dubius	CHIOC 7004	*Attila cinereus*	Avian	Eosophagus mucosa

CHIOC: Helminthological Collection of Instituto Oswaldo Cruz - "*Coleção Helmintológica do Instituto Oswaldo Cruz*"

Results

The capillariid eggs showed the characteristic barrel-shaped morphology with polar plugs, with measurements described in Table 2. Egg shell surface were discriminated by LM into 4 groups: Smooth, Punctuated, Reticulated Type I and

Reticulated Type II, based on Borba *et al*. (in press) [7]: 1) Smooth, that has no ornaments on the shell, as described by Conboy in *Trichuris trichiura* eggs, Trichuridae; Ransom, 1911 [11]; 2) Punctuated, that has dots like a pitted surface, as described in *Eucoleus boehmi* by Conboy (2009) and Traversa et al (2011) [5,11]; 3) Reticulated Type I, that is presented like a network as an interconnected ridges described in *Eucoleus aerophilu*s by Conboy [11]; and 4) Reticulated Type ll, that is presented like a network but with an orientation of deep longitudinal ridges, as described in *Aonchotheca putorii* by Zajac and Conboy (2012) [12].

For SEM eggshell patterns, it was considered the arrangement of the ornamentation unit over its appearance on egg surface. It was possible to characterize 6 Types of eggshell surface ornamentations using the SEM method (Fig. 1-4). SEM ornamentation Types 1 and 6 corresponding to Smooth and Reticulated Type II LM patterns, respectively (Table 2). While Punctuated and Reticulated Type I were associated to 2 different eggshell ornamentation patterns each by SEM (Table 2). The SEM eggshell patterns were characterized and classified as follows:

Type 1: The shell is overall smooth, with discreet longitudinal rays but does not present an ornamentation (Fig. 1A-1C). Only one species was identified with these characteristics, *Aonchotheca pulchra*. When LM was used, no visible ornamentation is seen (Fig. 1C), but in SEM images the mild rays appeared, specially near polar plugs (Fig. 1A).

Type 2: The outer surface has holes when look in detail as the entire egg itself, this appearance is seen both in SEM (Fig. 1D-1E, 1G-1H) and LM images (Fig. 1F e 1I). Two species presented these characteristics, *Eucoleus perforans* (Fig. 1D-1F) and *Echinocoleus auritae* (Fig 1G-1I), with more thin and rough shell respectively.

Type 3: The ornamentation has a Reticulated Type, as a network that creates spaces as holes. Three species show this pattern. *Baruscapillaria spiculata* (Fig. 2A-2C) and *Capillaria brasiliana*, are very similar, with a slightly difference in the network connection. *Capillaria brasiliana* (Fig. 2D-2F) has a tumid connection, whereas the third species, *Eucoleus contortus* (Fig. 2G-2I) has a tangle network, with no tumid connections.

Type 4: The egg surface has a very thin tangle appearance. In *Baruscapillaria rudolphi* (Fig. 3D-3F), undulation is observed as a wrinkle, formed with the tangle, while *Echinocoleus hydrochoeri* (Fig. 3A-3C), has flaws in the surface forming holes.

Type 5: The egg ornament has different depths of depression, like a shallow disk with different shapes/sizes and close to each other, which gives an appearance of punctuated Type in SEM images, but as reticulated Type I in LM. Three species fit in this description, *Baruscapillaria obsignata* (Fig. 4A-4C) that has a smoother disk holes, *Capillaria collaris* (Fig. 4G-4I) that has a very particular ornament, as the disk holes looks like ball of wool, and *Eucoleus dubius* (Fig. 4D-4F) which have sparse depressions.

Type 6: *Capillaria venusta* (Fig. 3G-3I) presented the same tangle pattern as Type 4 when observed in detail, but it seems to have a layer on the tangle surface, that produce other Type of ornament seen farther, which is seen in LM micrography.

Table 2. Results of capillariid eggshell ornamentation characterized in this study

Species	LM analysis	SEM analysis	Amplitude Measures (LxW) (μm)
Aonchotheca pulchra	Smooth	Type 1	46.35-52.96 x 28.15-34.40
Baruscapillaria obsignata	Reticulated type I	Type 5	47.52-51.78 x 26.67-33.45
Baruscapillaria rudolphii	Reticulated type I	Type 4	52.57-57.56 x 22.88-26.84
Baruscapillaria spiculata	Punctuated	Type 3	51.48-55.69 x 24.11-30.47
Capillaria venusta	Reticulated type II	Type 6	54.01-61.54 x 25.29-32.27
Capillaria collaris	Reticulated type I	Type 5	46.91-56.03 x 25.80-30.03
Capillaria brasiliana	Punctuated	Type 3	43.10-50.24 x 19.06-22.88
Echinocholeus hydrochoeri	Reticulated type I	Type 5	46.18-51.58 x 22.88-26.95
Echinocholeus auritae	Punctuated	Type 2	56.13-59.84 x 24.71-27.82
Eucoleus perforans	Punctuated	Type 2	37.06-42.81 x 18.15-23.94
Eucoleus contortus	Punctuated	Type 3	46.70-54.07 x 24.49-28.21
Eucoleus dubius	Reticulated type I	Type 4	47.07-55.25 x 22.40-25.57

LxW - length x width

Discussion

Researches regarding capillariid shell ornamentation by LM [6–9] categorized the species by the shell surface, which is cited as the most important characteristic of egg, for species identification. In reason of the resolution limitation of the LM method, details of the eggshell surface ultrastructure with minor differences can be not identified. The categories of ornamentation can be improved using the SEM method due to the resolution and image formation process, allowing a detailed characterization by high magnification and add new features of the surface eggshell. While some similarities can be recognized among some species, no pattern are seen within genus.

In a structural point of view, there are more differences between species by SEM that are seen by LM method. The SEM patterns observed in the outer shell are not genus specific. Some SEM patterns repeat LM categories, as Type 1, with a smooth surface; and Type 2, with holes characteristic of a punctuated LM Type ornament. On the other hand, some LM Types have different patterns in SEM. As seen in Type 3, that a tangle structure produces a punctuated Type when observed in LM. Another example is Type 5, that has a punctuated appearance in SEM images, but when seen in by LM method looks like a grid on the surface, characteristic of a reticulated Type I. Type 6 diverges from all observed, with a different eggshell pattern under higher magnification.

Although there is a difference in categorization by LM and SEM methods, the objectives are different. As a routine method, the LM offers a rapid preparation and diagnosis, in addition to use a cheaper and accessible equipment [13–15]. On the other hand, SEM is not a routine methodology in most laboratories, mostly used as a tool for description and characterization of species [3,5]. Given that, it is plausible some differences in categorization by two different approaches. As can be seen in Table 2, most of the categories can be related, but with some overlapping. We must emphasize Type 5, which produces two different ornamentation in SEM and LM, punctuated and reticulated Type I. In contrast, Type 2 represents a precise punctuated pattern in both of microscopies applied.

In that matter, the great difference observed between the two microscopies, was the reticulated Type I. When visualized by SEM method, it can appear as holes in the outer shell, similar to Punctuated Type. The species *E. dubius* and *E. hydrochoeri*, are both examples of Reticulated Type I that has a punctuated like Type based on SEM visualization.

The species *C. venusta* shows a completely different disposition on LM and SEM. It is possible to see a striate pattern from one pole to another, although in a detailed view, it cannot be seen. Unfortunately, it was not possible to register another species with the same pattern using SEM. However, we believe that the sixth Type is needed, which allocate the Reticulated Type II category. Otherwise, it can be a particularity of the species, as it has been already described in *P. philipinensis*, that showed two Types of eggshell [4].

No relation between genus and ornamentation was verified, as well as site infection or host class. The results presented here disagree with the previous study that showed that eggshell ornamentation was related to the site of infection in capillariids of mammals [6]. This could be explained by the different solutions used to fixed the materials, 3% formalin in the preceding work by Romashov, and 70°GL ethanol in the present work [16,17].

Conclusion

The SEM method is a powerful tool for eggshell characterization due to the higher magnification of ornamentation. Although egg is not the main structure for species identification, as well SEM is not a technique used in routine surveys, the results give auxiliary information of capillariids not previously studied by this method. In the present study, we established Type categories of eggshell ornamentation in order to verify a relationship between the methods, and particularly, to capillariid genera, or site of infection. Nevertheless, the results revealed no association, corroborating with the taxonomic complexity of capillariid group. More specimens must be analyzed with the purpose of fill information about all genera described.

The study provides SEM eggshell information from curated species that contributes with specific and supplementary taxonomic feature at the species level of Capillariidae.

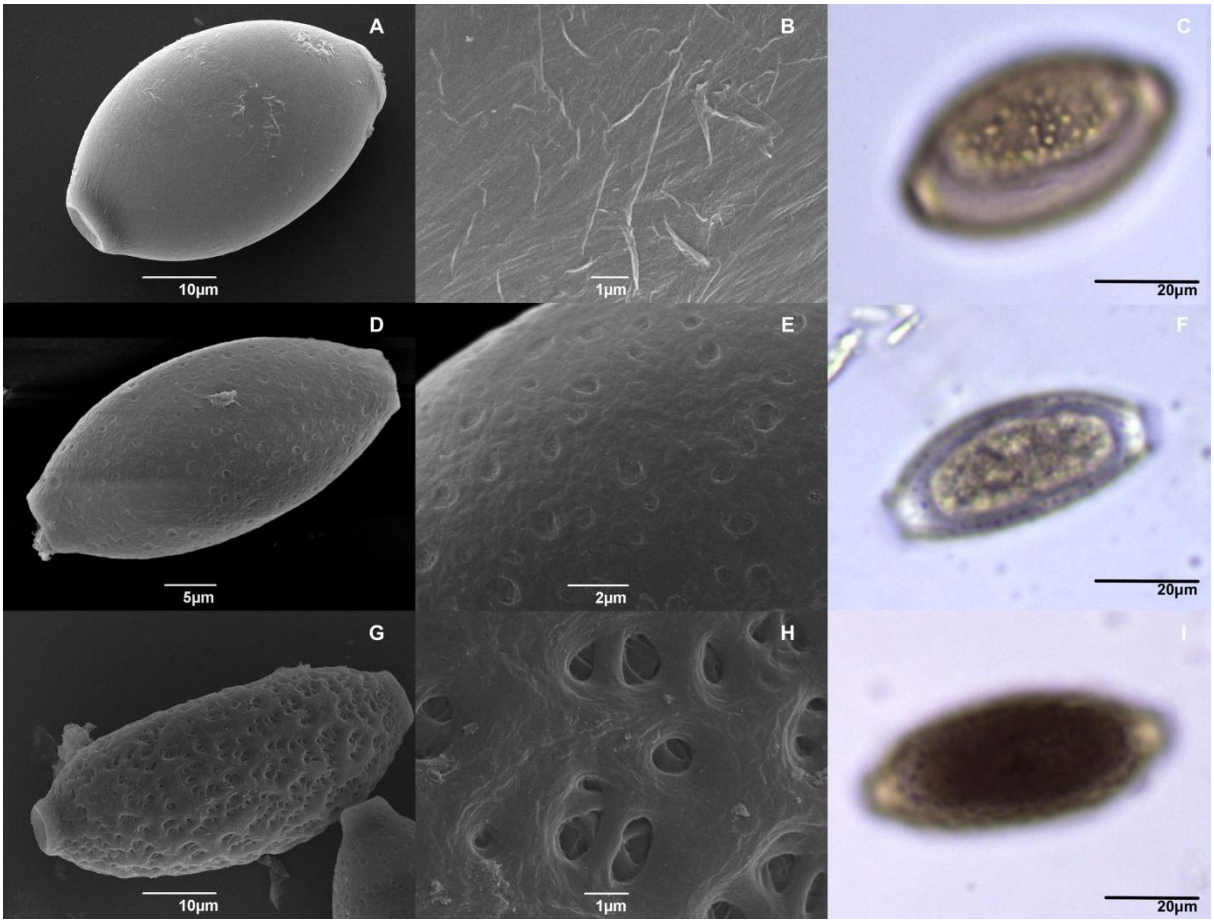

Figure 1. SEM overview, SEM detail and LM micrographies of eggs surface of capillariids from Helminthological Collection of Oswaldo Cruz Institute (CHIOC). Type 1 (A-C) and Type 2 (D-I). A-C: *Aonchotheca pulchra*; D-F: *Eucoleus perforans*, G-I: *Echinocoleus auritae*, showing the egg and its eggshell.

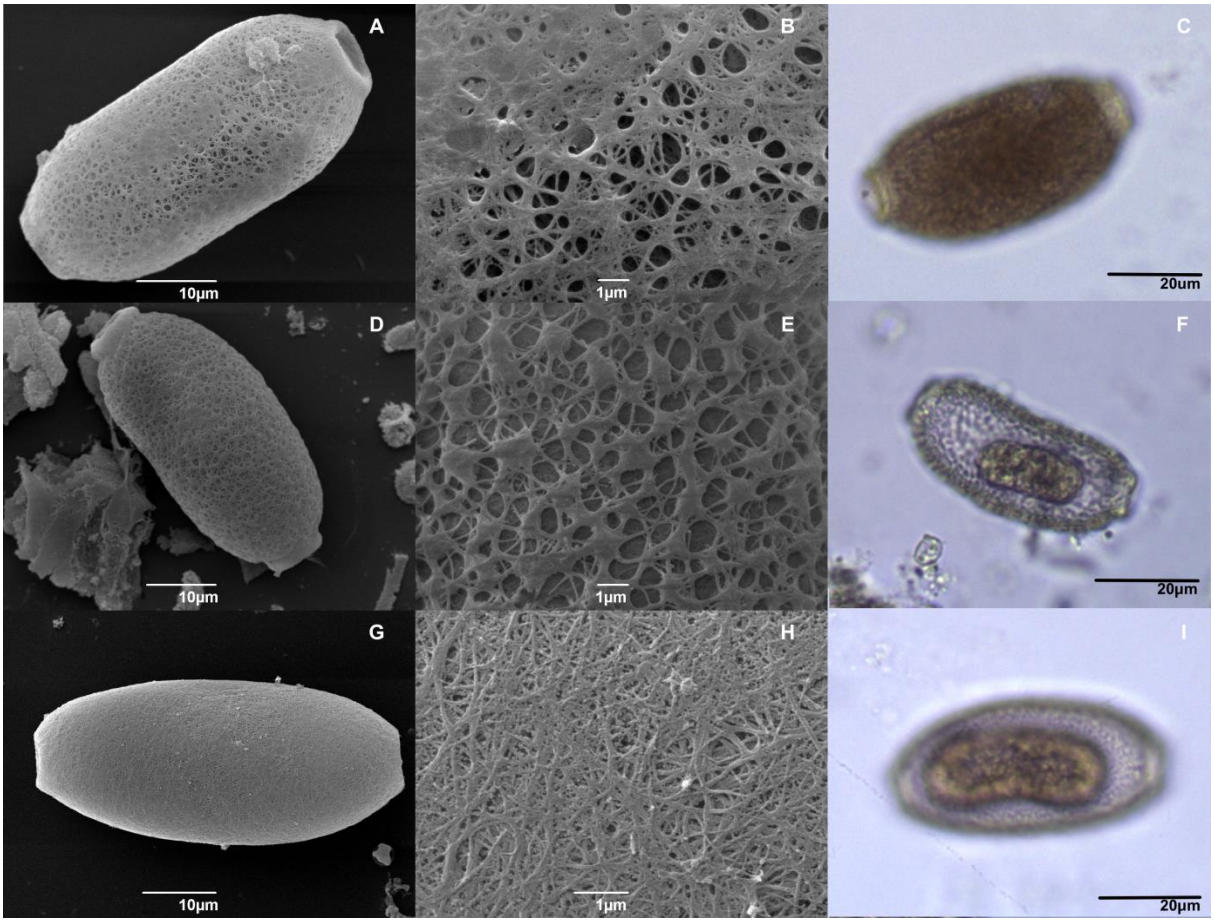

Figure 2. SEM overview, SEM detail and LM images of eggs surface of capillariids from Helminthological Collection of Oswaldo Cruz Institute (CHIOC)., Type 3. A-C: *Baruscapillaria spiculata*; D-F: *Capillaria brasiliana*, G-I: *Eucoleus contortus*, showing the egg and its eggshell.

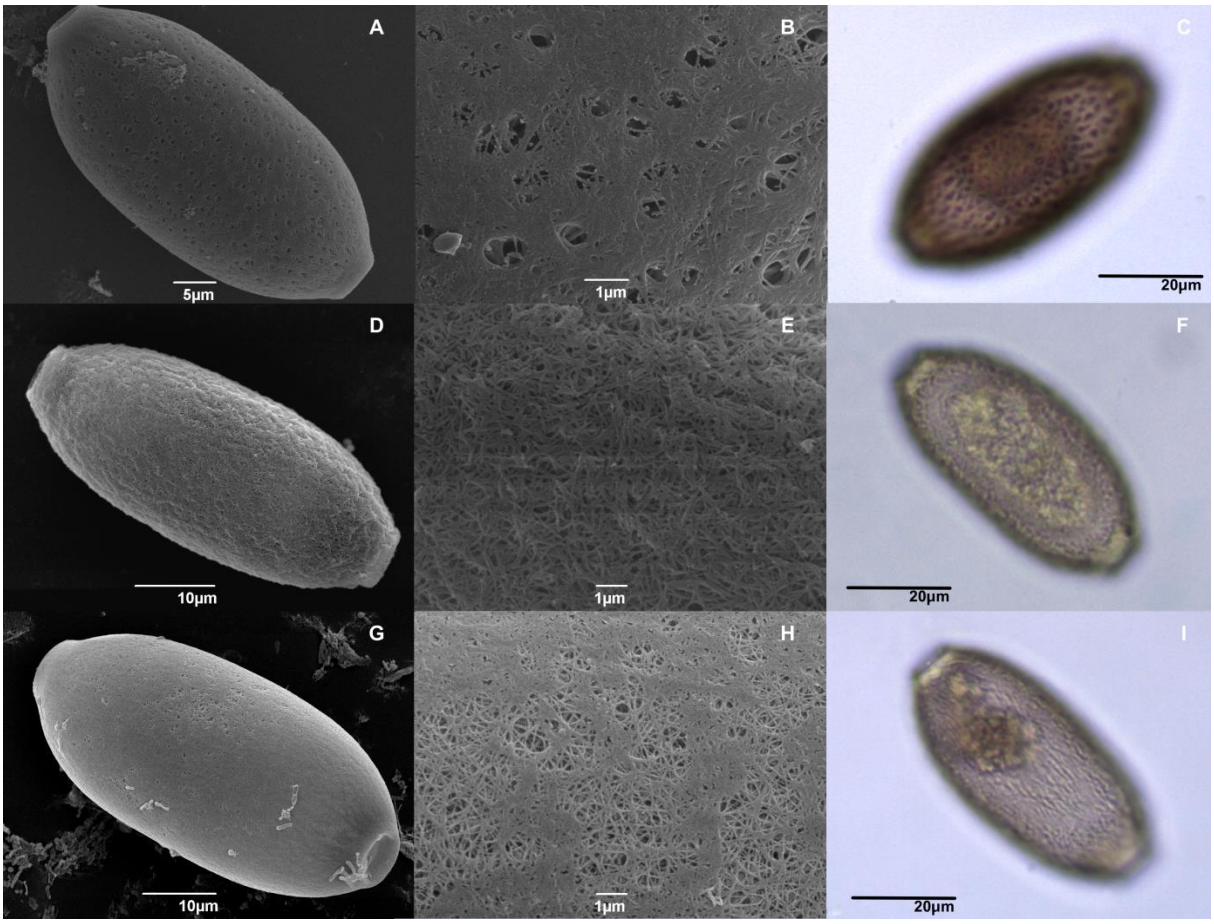

Figure 3. SEM overview, SEM detail and LM images of eggs surface of capillariids from Helminthological Collection of Oswaldo Cruz Institute (CHIOC), Type 4 (A-F) and Type 6 (G-I). A-C: *Echinocoleus hydrochoeri*; D-F: *Baruscapillaria rudolphii*, G-I: *Capillaria venusta*, showing the egg and its eggshell.

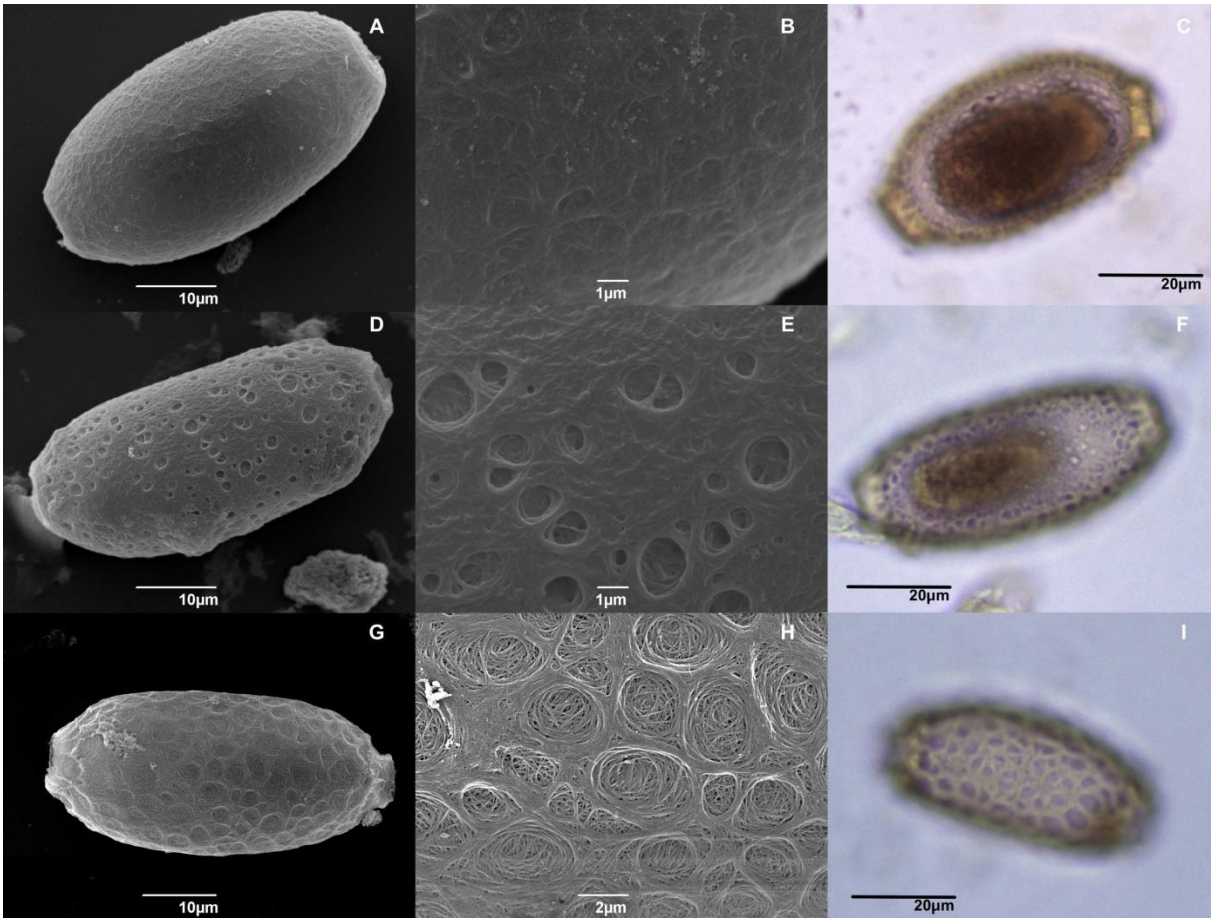

Figure 4. SEM overview, SEM detail and LM images of eggs surface of capillariids from Helminthological Collection of Oswaldo Cruz Institute (CHIOC), Type 5. A-C: *Baruscapillaria obsignata*; D-F: *Eucoleus dubius*, G-I: *Capillaria collaris*, showing the egg and its eggshell.

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5.4 Artigo 4: Capillariid diversity in archaeological material from the New and Old world: clustering and artificial intelligence approaches

Capillariid diversity in archaeological material from the New and Old World: clustering and artificial intelligence approaches

Victor Borba[1,2], Ludmila Gurjão[1], Coralie Martin[3], Benjamin Dufour[3], Matthieu Le Bailly[3], Alena Iñiguez[1*]

[1]LABTRIP, Instituto Oswaldo Cruz, IOC-FIOCRUZ, Rio de Janeiro, RJ, Brazil.

[2]Laboratório de Helmintologia Romero Lascasas Porto, Faculdade de Ciências Médicas, UERJ, Rio de Janeiro, RJ, Brazil.

[3]Unité Molécules de Communication et Adaptation des Microorganismes (MCAM, UMR 7245), Sorbonne Universités, Muséum National d'Histoire Naturelle, CNRS, CP52, Paris, France

[3]University of Bourgogne Franche-Comté, CNRS UMR 6249 Chrono-environment, Besançon, France.

*Corresponding author

E-mail: alenainiguez@gmail.com

Abstract

Capillariid eggs are reported in archaeological material both in the New and Old World, mainly in Europe and South America. It can be found in all types of samples, as coprolites, sediments from latrines, pits or burial. More than 300 species of capillariids have been described in all vertebrate taxa, including humans, which makes it a very diversified group. The main proposal of this work is to characterize and identify capillariid eggs found in archaeological sites from Europe and Brazil. A total of 39 samples of archeological sites from Europe, deposited in the Paleoparasitological collection of the University of Bourgogne Franche-Comté, Besançon, France were analyzed. In addition, coprolites from the pre-Columbian archaeological site Gruta do Gentio II, Brazil, deposited in the Paleogenetic Laboratory of Instituto Oswaldo Cruz (IOC/FIOCRUZ) were evaluated. Samples were treated following the protocols according to each laboratory and then analyzed under light microscopy. Capillariid eggs found were characterized by length, width, plugs and eggshell sizes, and statistical analysis of morphometric dataset was performed. As results, 10 samples from Europe and 4 from Brazil were positive for capillariids, showing 13 different morphotypes. Using a dataset of specimen's provided by two institutional collections, with 3 approaches for identify the species, discriminant analysis, hierarchical clustering and artificial intelligence/machine learning. As European samples were mainly collected from latrines and pits, parasite host information was absent, and consequently, species identification was impaired. In contrast, the definition of possible capillariid species was less complicated with the information of host of Brazilian coprolites. The possibility of a pseudo-parasitism makes the capillariid findings harder to interpret. The results suggested the presence of *Capillaria exigua* (Dujardin, 1845) in feline coprolite, *Baruscapillaria resecta* (Dujardin, 1845) in opossum, *Aonchotheca bovis* (Schnyder, 1906) in bovid, in Brazilian site, while in European sites, *Capillaria venusta* (Freitas e Mendonca, 1958), *Aonchotheca myoxinitelae* (Diesing, 1851), *Eucoleus madierdae* (Bernard, 1964), *Baruscapillaria spiculata* (Freitas, 1933) were suggested. The study provides new data for capillariid identification and for a better understanding of the relationship parasite and human or animal hosts in the past.

Keywords: Paleoparasitology, Capillariidae, Archaeology, Parasites, Taxonomy.

Introduction

Paleoparasitology, which is the study of parasites in archaeological and paleontological material, tries to explain the host-parasite-environment interaction in the past, elucidating parasites origin and evolution. The main structure that is recovered from ancient samples is parasite egg, due to the chitinous eggshell, that enables egg preservation through time. The species identification of parasites in the paleoparasitological record is one of the difficulties in the field, as it is challenging to do based only on egg structures. In some cases, it is impossible to identify at species, or even at genus level, using the accessible scientific literature.

The family Capillariidae (Railliet, 1915) has a very complex and controversial taxonomy, with more than 20 genera described [1,2]. Capillariids parasite all vertebrate classes, including fish, amphibian, reptile, birds, and mammals, in a various infection sites. Because of this diversity, the identification of parasites in ancient samples is impaired.

In the Old World, most of paleoparasitological findings have no host definition [Borba et al., 2019 Paleodistribution]. Bouchet [3] analyzed 23 human coprolites in France, dating to 5150-4930 before present (BP), and found 21 positive for capillariids eggs. In the archaeological site located in the Place d'Armes, Belgium, capillariids eggs were found, in the Gallo-Roman Period (2nd and 3rd century AD), in the Carolingian Period (IX-XI AD) and between the 12th and 13th centuries [4]. In Germany, eggs of capillariids were also found by the analysis of sediment removed from the pelvic region of an skeleton dating to 4500 years[5]. In the Old World, there are still reports of capillariid eggs in archaeological sites of regions that today belong to Italy [6], Russia [7] and Czech Republic [8] countries.

In the New World, most of the findings are in the region of Patagonia, Argentina [9]. Fugassa and Guichón [10] found capillariids eggs in human coprolite of

6540 ± 110 years BP. They were also found in sediments associated with pelvic bone from an archaeological site from the south of the province of Santa Cruz, Argentina [11]. In pellets from the Cerro Casa de Piedra archaeological site, also in Santa Cruz, numerous eggs were identified as *Capillaria* sp. and later as *Calodium hepaticum* (Bancroft, 1893) [12]. However, in spite of numerous findings of capillariids in South America, there are only two reports of parasite in Brazil [13,14]. The scarcity of Capillariidae egg findings in paleoparasitological material does not necessarily indicate the absence of this parasite in the environment or even of infections [15]. When the preservation of archaeological material is very damaged and consequently the morphology of eggs, it is difficult to distinguish between trichurid and capillariid eggs by light microscopy [14].

The main proposal of this study is to characterize Capillariidae eggs from archaeological sites from Europe and Brazil and to provide taxonomic information of capillariid eggs diversity.

Material and methods

Study sites

Gruta do Gentio II (GGII)

The site is located in Unaí, Minas Gerais state, Brazil, dated of 12000 – 3500 BP, with two cultural periods, hunter-gatherer (12000-7295 ± 150 BP) and horticulturist (3490 ± 120 – 410 ± 60 BP). The cave had an internal area of 200m^2 associated with a calcareous wall. Human, animal and plant remains were observed inside. In addition, 80 coprolites of different shapes were collected, and their producer identified by DNA Barcoding analysis [16].

La Rochelle Augustin (LRA)

It is located in western France near Bay of Biscay and is dated of 17th-18th century.

Calais ZAC des Turqueries (CAL)

It is located in North France. The site is dated of 8th-10th century and it is located on an old marsh. It has evidence of an agro-pastoral activities, focused on livestock and processing. It was discovered fauna remains but no evidencing of housing, but only places with pits.

Bourges Avaricum (AVA)

It is located in France, in the centre Val de Loire region. It is dated of 13th-17th century.

Paleoparasitological analysis

For the recovery of parasite eggs two techniques were performed. Brazilian samples were previously identified, as GGII-01, *Panthera onca*; GGII-15, *Didelphis albiventris*; GGII-33 and GGII-51, *Bos taurus* [13]. They were rehydrated as suggested by Callen and Cameron [17], with a 0.5% trisodium phosphate solution (Na3PO4.H2O) for 72h at 4°C. Then it was homogenized and sedimented for 24 hours with a triple-folded gauze as proposed by Lutz [18]. Two hundred microliters of sediment equivalent to about twenty slides were analyzed for each sample. All the process was made at Paleogenetc Laboratory/LABTRIP/IOC at Oswaldo Cruz Foundation, Brazil.

European samples were also rehydrated with a 0.5% trisodium phosphate solution, for 7 days, and with a drop of formalin solution as well. After the homogenization, the samples are submitted to an ultrasound treatment (50/60 Hz) for 1 minute, and then the sample is strained through 315 μm, 160 μm, 50 μm and 25 μm meshes [19]. Six slides were analyzed for each sample. The samples were processed at a Paleoparasitology Laboratory at Franche-Comté University, France.

All the samples were analyzed under a light microscope at the magnification of 100 and 400X.

Morphological and Morphometric analyses

The egg measurements were made as proposed by Borba et al (in press), considering length width, plug base length, plug base height and shell thickness.

Eggshell surface morphotypes were separated based on Borba et al (in press), in four categories: Smooth (S), Punctuated (P), Reticulated Type I (RTI) and Reticulated Type II (RTII) as follow: S type has no ornamentation on the egg surface as described by Conboy in *Trichuris trichiura* (Linnaeus 1758) eggs [20]; P type present ornaments like holes on eggshell, as little perforation all over the egg as described in *Eucoleus boehmi* (Supperer, 1953) by Conboy (2009) and Traversa et al (2011) [20,21]; RTI forms a network without an orientation as a grid described in *Eucoleus aerophilu*s (Creplin, 1839) by Conboy [21], different from RTII that has a longitudinal orientation seems like a lot of small rays from one polar plug to another as described in *Aonchotheca putorii* (Rudolphi, 1819) by Zajac and Conboy (2012) [20].

Statistical analysis

The species deposited on two institutional collection, *Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC)*/Brazil and *Collection d'Helminthologie du Museum National d'Histoire Naturelle de Paris*/France, were used as references on the statistical analysis with eggs in archaeological samples. The statistical analysis had the objective of show the closest measures within the database. It was made a discriminant analysis to build a predictive model and generate a discriminant function to predict the best variable to discriminate the species [22]. As a hierarchical clustering using Gower distance and Ward minimum variance [23] to found proximities in measures in a multivariate analysis, using a PAST 3.16 software [24].

Artificial Intelligence/Machine Learning Approaches

An Artificial Intelligence/Machine Learning approach was applied in this study to propose/identify the species of specimens found, following Borba et al. (in press). The data base applied was from two institutional collection, Oswaldo Cruz Institute Helminthological Collection (CHIOC/FIOCRUZ) and Helminthological Collection of National Museum of Natural History of Paris (MNHN), with 26 species characterized. It was selected a decision tree generated by Borba et al. (in press) with the information only of egg morphology and morphometry (MM) to extend the reach and maximize the possibilities of species.

Results

A total of 27 capillariid eggs were identified in archaeological sites from the New World (Fig. 1) and the Old World (Fig. 2). Four samples were positive in GGII, two in LRA, one in CAL and seven in AVA (Table 1, Fig. 1-2).

The discriminant analysis showed eggs by its length and width plotted in a XY axis (Fig. 3). In addition, hierarchical clustering trees using Gower distance and Ward minimum variance were generated based on the length and width measures of eggs per 2 eggshell ornamentation type, reticulated type II (Fig.4A) and punctuated (Fig. 4B).

Table 1. Sample information and results of morphometric morphological analyses.

Samples	N° eggs	Length	Width	Plug L	Plug H	Eggshell	Morphotypes	Sample type
GGII-01	1	51.672	25.032	6.444	2.348	1.776	RTII	Coprolite
GGII-15	1	76.923	30.312	9.739	7.192		RTII	Coprolite
GGII-15	5	63.556–56.485	43.221-41.027	12.253-7.832			S	Coprolite
GGII-33	1	48.83	21.103		6.396		RTII	Coprolite
GGII-51	1	49.779	26.073	6.808	6.714		RTII	Coprolite
LRA201-03	1	47.771	24.917	7.985	3.287	1.832	RTI	Latrine
LRA201-05	1	46.265	26.484	5.811	2.106	3.476	P	Latrine
CAL-P530	2	57.073-58.404	31.19-31.971	8.648-9.299	3.088-3.156	1.14-1.542	P	Pits
AVA10-P17	1	50.744	28.275	8.071	4.558	3.926	RTI	Pits
AVA10-P28	1	58.39	29.687	8.665	3.676	0.938	P	Pits
AVA10-P37	2	56.282-55.491	34.497-34.771	9.197-9.367	1.935-2.037	0.938-1.606	P	Pits
AVA10-P73	1	57.79	28.858	9.952	3.861	1.832	RTII	Pits
AVA10-P72	2	56.9-58.963	31.292-30.941	9.726-9.538	3.294-2.653	2.661-1.192	P	Pits
AVA10-P41	1	57.678	25.491	8.444	2.005	1.107	RTII	Pits
AVA10-P21	6	56.336-51.321	27.381-31.376	9.162-7.470	4.864-3.97	3.361-4.31	P	Pits

Length and Width of total egg. Plug L: plug length. Plug H: plug height. Eggshell: eggshell thickness. Eggshell ornamentation morphotypes: S: Smooth; P: Punctuated; RTI: Reticulated type I; RTII: Reticulated type II. Measures in μm.

Discussion

In this study, capillariids from the New World were recovered from coprolites. Sample GGII-01, identified with a *Panthera onca* origin [16] showed an egg with a RTII ornamentation (Fig 1A,1B). Eggs recovered from carnivores' coprolites have to be considered as true infections or false-parasitism, due to the ingestion of non-infective stage parasite eggs. The species that is usually found in felids are: *C. hepaticum* (Bancroft, 1893) [25], *Eucoleus aerophilus* (Creplin, 1839) [26], *Pearsonema feliscati* (Diesing, 1851) [27] and *P. plica* (Rudolphi, 1819) [28]. *Calodium hepaticum* parasites the liver, and when appears in feces is due to ingestion of infected preys. GGII-01 has a particular eggshell structure different from *C. hepaticum* one [22]. *Eucoleus aerophilus*, that parasites lungs, does not have the same eggshell ornamentation type observed in GGII-01 coprolite [29]. *Pearsonema feliscati* and *P. plica* that usually infect the urinary tract, has punctuated and reticulated type I ornamentations, respectively. Then, as none of the common capillariid found in felids match in size or ornamentation, GGII-01 can be originally from a prey, which characterizes a false-parasitism. In the hierarchical tree of reticulated type II ornamentation (Fig 4A), we can see GGII-01 eggs in a cluster with *Aonchotheca erinaceid* (Rudolphi, 1819), *Capillaria exigua (*Dujardin, 1845), *A. annulosa* (Dujardin, 1845) and *A. bovis* (Schnyder, 1906). All these species are not specific of felids but can parasite a potential prey, like hedgehogs [30], rodents and bovids [31] y. The identification by the decision tree generated by AI/ML resulted in *Capillaria exigua*, which corroborates with the hierarchical clustering. Although *Capillaria exigua* is a parasite of hedgehogs distributed in Europe, the characteristics and measurements are compatible. As the dataset used was an initial approach, some misdiagnose was expected, in reason of 26 species of 300 hundred with all the parameters assessed [32].

Coprolite GGII-15 from *Didelphis albiventris* [16], presented two different capillariid morphotypes. One bigger (GGII-15-01) with measures 76.92 x 30.31 μm, with a discreet RTII ornament (Fig 1C,1D), and a second, revealed in 5 rounder and smaller eggs (63.55-56.48 x 43.22-41.02 μm) with no ornament (Fig 1E, 1F). Spratt

[33] described 14 species of capillariid parasitizing marsupials, among them, 4 with reticulated type or reticulated like eggshell ornaments, *Eucoleus gastricus* (Baylis, 1926) (65–79 x 25–34), *E. fluvidus* (Spratt, 2006) (65–72 x 26–31 μm), *E. plumosus* (Spratt, 2006) (60–65 x 21–25 μm) and *E. pseudoplumosus* (Spratt, 2006) (50-56 x 21–25 μm). Despite the measures matches with *E. gastricus*, the egg morphology is different from it found in archaeological samples from France of the 1st world war [23]. In the cluster analysis, it was related to *E. gastricus* (Fig. A). Although it usually parasites rodents, *E. gastricus* was described in marsupials from Australia [33]. Besides, it could be another species not yet described in marsupials in Brazil. Given the fact that all the eggs similar to the one found in GGII-15 are from *Eucoleus* spp. Made us believe that it belongs to *Eucoleus* genus. In AI/ML analysis, it matches with *Baruscapillaria resecta* (Dujardin, 1845), parasite of birds, but *E. gastricus* was not present in the AI dataset. We also have to consider that the width measure is impaired can give a false species definition in this case. As *B. resecta* is found in Europe [34] and North America [35], while *E. gastricus* is found worldwide [36], which is more plausible to be this parasite. Opossums have a diverse diet, with birds and small mammals as possible food resources [37], so the egg could be identified as any of these species.

The measures of the second morphotype, GGII-15, is not compatible with the only capillariid without ornamentation known on an optical view, *Aonchotheca pulchra* (Freitas, 1934). The other species described for the host infraclass do not have the same egg morphology. But we have to considered other Trichocephalida species, as *Trichosomoides* genus, which parasites urinary bladder of rodents and has compatible egg morphometry and similar morphology [38,39].

GGII-33 (Fig 1G, 1H) and GGII-51 (Fig 1I, 1J) found in *Bos aurus* samples [16], have a morphology compatible with *Capillaria bovis* (Schnyder, 1906), parasite specific of bovids. However, the measures are a bit smaller than the described in the literature (50-54 x 24-27 μm) [40]. The bovid host whose the parasite species was described, *Cervus elaphus*, [31] is different from *B. taurus* genetic identification of GGII sample. A distinct host could produce a plasticity effect in the parasite morphology, as described in other species, as *A. putorii* (Rudolphi, 1819) [41]. Other matter is that at the layer where the coprolite was is dated of pre-Columbian, a time

period that there was not *Bos taurus* in New World. It is justified as a post-Columbian perturbance of the layers and/or a recent feces deposit between excavation expeditions [13].

The Old World samples analyzed have not their host defined, since pits and latrines have a lot of organic material including human and animal feces [32,42]. The eggs of AVA10-P21 sample have a thick shell with dense punctuated ornamentation, with radial visualization on a transversal view, characteristics very particular of *C. hepaticum* [43]. Although could be other species with similar eggshell characteristics as pointed out by other authors [44]. It can parasite the liver of mammals' host, including humans, but visualization in feces are only with the ingestion of contaminated viscera.

Other egg morphotype was distinguished in three different samples, AVA10-P28 (58.39 x 29.687 μm), AVA10-P72 (56.9-58.96 x 31.29-30.94 μm) and CAL-530 (57.07-58.40 x 31,19 -31,97 μm). The ornamentation is punctuated-like, with large holes over eggshell, which gives an appearance of a reticulated type (Fig 2I-2L, 2O, 2P). The species deposited on institutional collection used as reference, with similar ornamentation, are: *Eucoleus dubius* (Travassos, 1917), *E. eberthii* (Freitas e Lent, 1935) and *E. madjerdae* (Bernard, 1964), that parasites *Attila cinereus*, *Metachirops opossum* and *Mus musculus*, respectively. It can be seen a proximity in the hierarchical tree with both *E. madjerdae* and *E. boehmi*, both species have eggs morphologies that are similar to the ones found in the samples, The result is corroborated to AI/ML analysis, that identified as *E. madjerdae*. *Eucoleus boehmi* was not included in AI/ML dataset analysis, as it was not present in the institutional collections. The sample AVA10-P37 (56.28-55.49 x 34.49-34.77 μm) had an egg with similar ornamentation, morphology and eggshell thickness than the other three samples mentioned above, but with a bigger width and smaller length in sizes. Although the proximity with *E. madjerdae* in the hierarchical tree, it was identified as *B. spiculata* (Freitas, 1933) by AI/ML procedure. *Eucoleus madjerdae*, was deposited in MNHN collection and described as parasite of rodents collected in Tunisia, as *E. boehmi*, parasites feline and canids in Europe [45], which is plausible finding in this samples.

In samples AVA10-P41 (57.67 x 25.49 μm) and AVA10-P73 (57.79 x 28.85 μm) were found eggs with RTII ornaments, longitudinal rays, like a striate surface (Fig. 2G-2H, 2M-2N). Although the same ornamentation pattern and measurements, their morphology and eggshell thickness are not compatible, so we classified them as different species morphotypes. Both have close distance between different species. AVA10-P41 with *A. myoxinitelae* (Diesing, 1851) that parasites the liver of rodents and boar in France [46]. AVA10-P73 with *Capillaria venusta* (Freitas e Mendonca, 1958) that parasites the intestine of neotropical birds and piciforms, which are worldwide distributed [47]. The morphologies and the morphometries match with each species record, and both identifications corroborated with AI/ML results. So, we can suggest a circulation of these animals which are parasitized, in these areas, or a cohabitation and contact with the human population in this region.

Capillariids from samples LRA201-03 and LRA201-05 are the smallest eggs found in this study (Fig. 2Q-2T). They have both a dense punctuation, that looks like a RTI ornamentation, as a grid. It is noted that the outer shell is damaged, which can impair the visualization of ornamentation. Both are very similar in morphology and size, but the ornaments are slightly different, LRA201-03 has a bigger punctuation unit than LRA201-05. Considering RTI and P ornamentations, respectively, the AI/ML analysis identified as *Capillaria collaris* (Linstow, 1873) and *Eucoleus annulatus* (Molin, 1858), both parasite of birds, that are found in Europe [48]. The morphology is not very similar, which indicates the necessity for more characterization of species in the group.

The egg found in sample AVA10-P17 has a particular structure (Fig. 2A-2B). Apparently, the outer shell is S type and there is a large grid in its interior. Neither in the Institutional Collections nor in the literature was found a similar description. For this reason, no AI/ML approach was applied. Although it can be described as RTI ornamentation, it is not similar to any egg characterized in the study.

Conclusion

The study corroborates that samples from the New World with host definition gives a possibility of deeper discussion about the taxonomic egg identification, as previously demonstrated [9]. While those from the Old World, which were from latrines and pits, make the investigation difficult, only based on egg morphology and eggshell ornaments, since parasites could be from any host, animal or human.

The analyses validated that eggshell surface ornamentation is the most relevant character for taxonomic definition of capillariids [44].In the literature there is not much imagens of capillariid eggshell surface, which preclude a proper identification of species. The study shows the importance of egg description and characterization to create a source of comparison for species identification. Also, the results provide new data for capillariid species definition, for a better understanding of the relationship parasite and human or animal hosts in the past.

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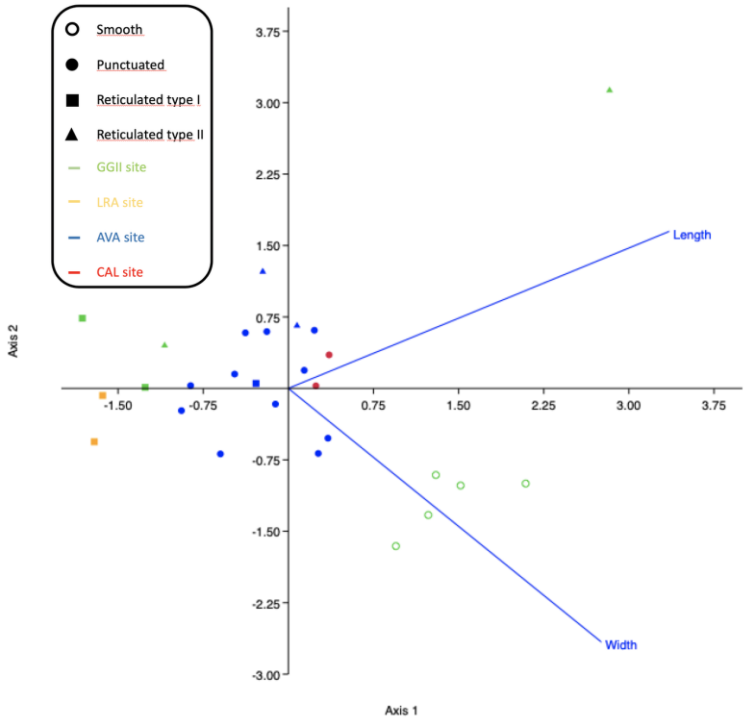

Figure 3. Discriminant analysis graphic with all 27 capillariid eggs from archaeological sites distributed by length and width.

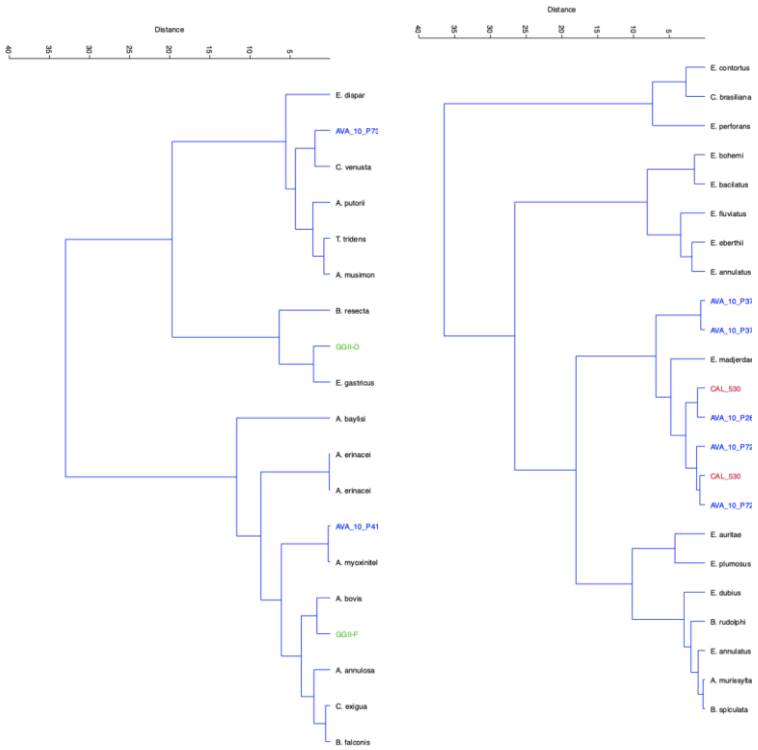

Figure 4. Hierarchical clustering trees of capillariid species by eggshell ornamentation using Gower distance and Ward minimum variance. A: reticulated type II tree B: punctuated type tree.

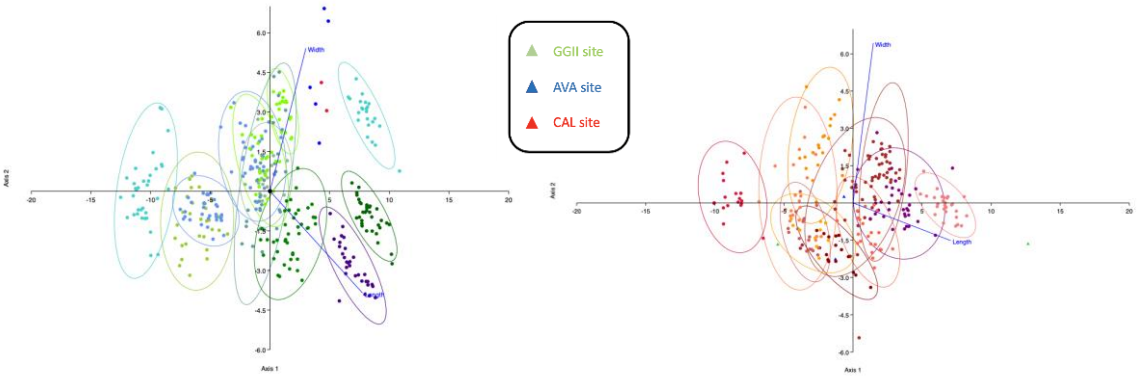

Figure 5. Discriminant analysis with the measures of length and width of eggs deposited in CHIOC and MNHN, comparing with eggs found in archaeological samples.

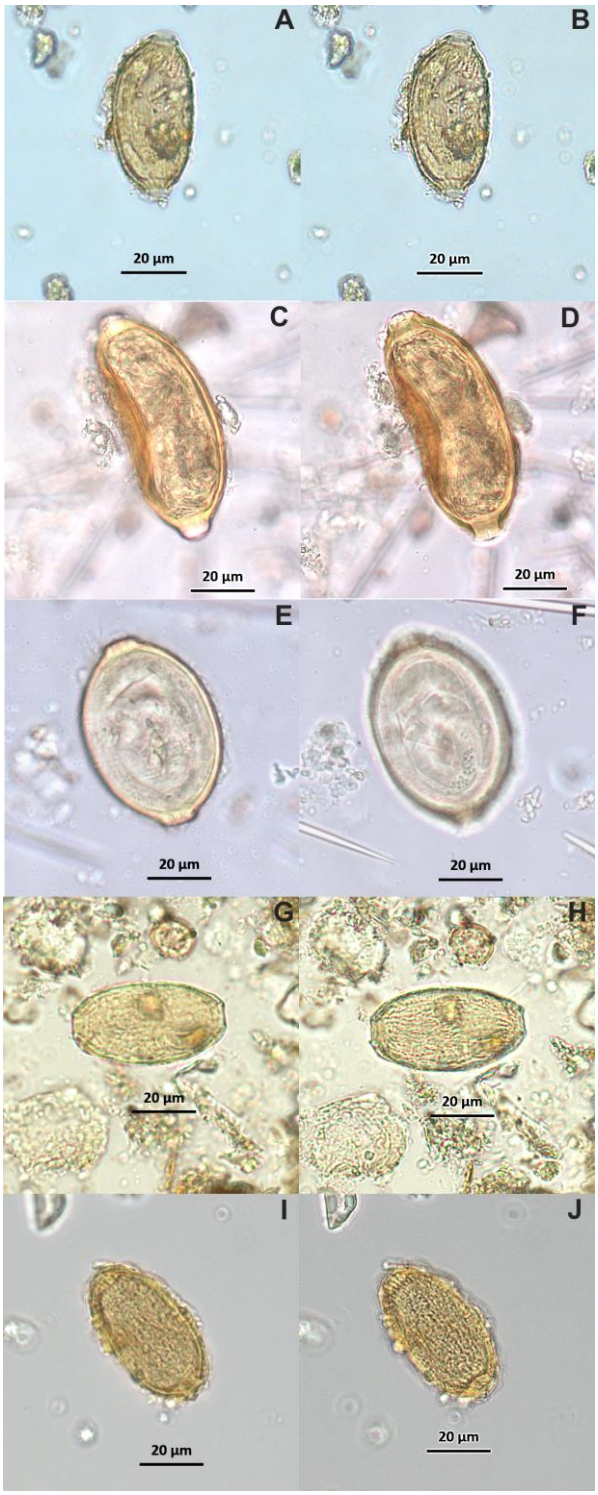

Figure 1. Capillariid eggs from New World samples. GGII-01 (A, B); GGII-15 (C – F); GGII-33 (G, H); GGII-51 (I, J).

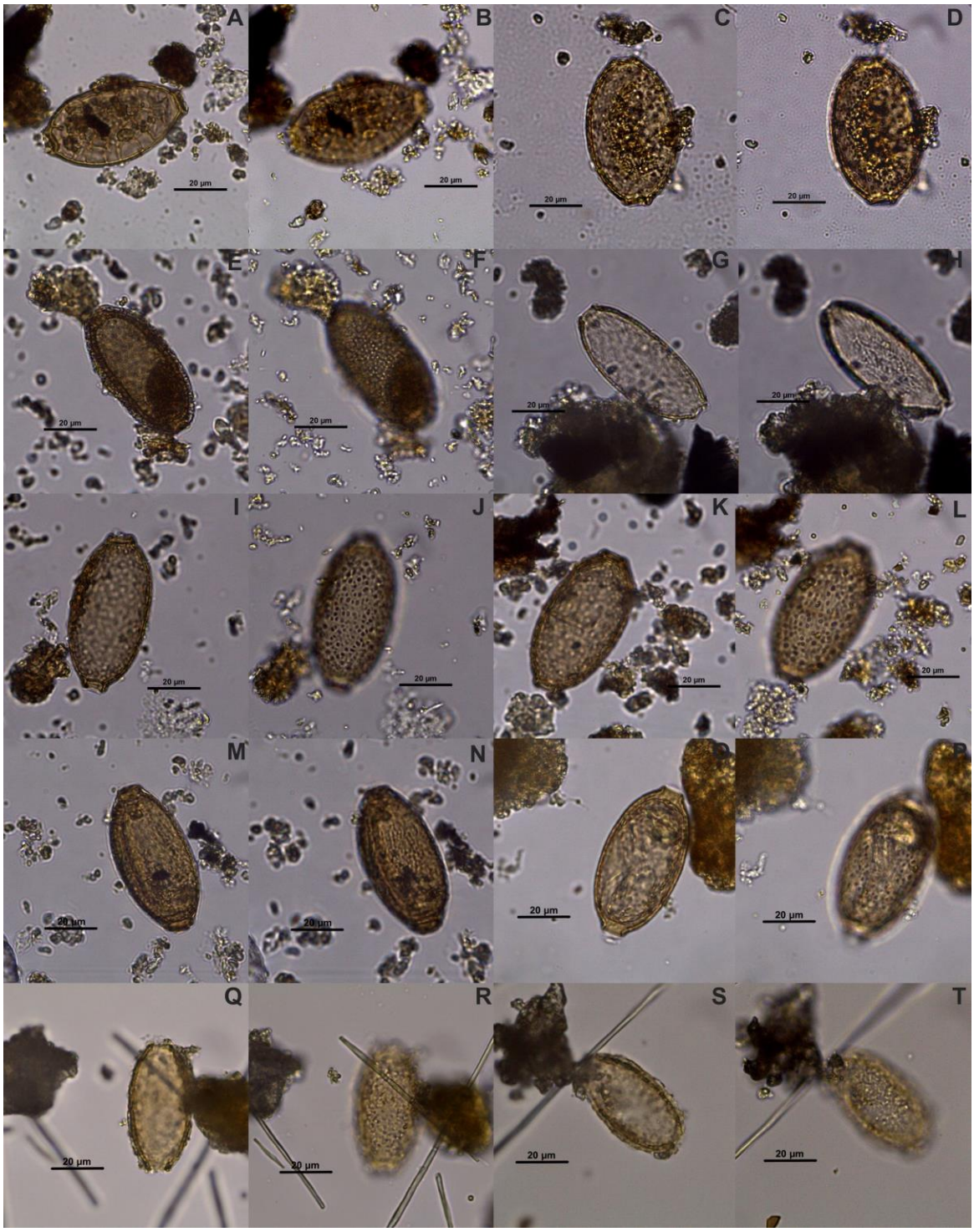

Figure 2. Capillariid eggs from Old World samples. AVA10-P17 (A, B); AVA10-P37 (C, D); AVA10-P21 (E, F); AVA10-P41 (G, H); AVA10-P28 (I, J); AVA10-P72 (K, L); AVA10-P73 (M, N); CAL-P530 (O, P); LRA201-03 (Q, R); LRA201-05 (S, T).

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6. DISCUSSÃO

Com a revisão sistemática em material arqueológico e paleontológico, foi possível constatar a existência de inúmeros achados de capilarideos, tanto no Novo quanto no Velho Mundo. Porém a identificação de espécies de capillariídeos com base na morfologia e morfometria dos ovos permanece imprecisa, muitas vezes resultando em um diagnóstico genérico do grupo de capilarídeos. O desafio em trabalhar com a caracterização da morfologia e morfometria de ovos é claramente devido à diversidade de espécies e à similaridade de ovos de diferentes espécies, muitas vezes dentro do mesmo gênero. Porém, a análise estatística na literatura mostrou resultados interessantes na identificação das espécies. No entanto, esta abordagem é limitada pela informação insuficiente sobre a morfologia do ovo, principalmente considerando capilarídeos parasitos de animais.

Como visto no artigo 1, dos 54 morfotipos de ovos encontrados em amostras arqueológicas, apenas 11 (20,37%) foram identificados em nível de espécie. Sendo a principal espécie identificada *Calodium hepaticum*, provavelmente favorecido pela morfologia e a ornamentação bem característica que facilitaram o diagnostico. Os outros morfotipos identificados, a nível de espécie, foram principalmente devido a obtenção dos dados do hospedeiro, possibilitando a relação do parasito com a infeção ou relação ecológica com o hospedeiro. Para gênero apenas 10 morfotipos foram identificados, 4 como *Eucoleus*, 1 como *Echinocholeus* e 5 como *Calodium*. No gênero *Eucoleus* foram encontrados morfotipos diferentes em felinos e camelídeos, e ambos os gêneros em roedores. Os outros 32 morfotipos foram classificados a nível de família, pois os autores não encontraram caracteres taxonômicos para uma definição mais específica. Observamos que os dados do hospedeiro são as informações de maior valor para identificação taxonômica de capilarideos, em conjunto com os dados morfologicos e morfométricos. No entanto, alguns capilarídeos têm uma grande variedade de hospedeiros, de modo que pouco pode ser restringido do amplo espectro de espécies na definição do parasito. O viés do esforço aplicado pelos autores na identificação dos ovos na literatura, é um fator a ser levado em consideração. Além da falta de descrição adequada ou enfoque nas ornamentações dos ovos, que é uma característica importante para identificação dos mesmos.

Portanto, ao analisar a identificação dos ovos nas publicações com material antigo, pode-se constatar a necessidade de sua caracterização com a utilização de uma base confiável como as coleções helmintológicas institucionais. Assim, aumentar o número de espécies caracterizadas na literatura para futuras identificações. Assim, o artigo 2 e 3 mostram essa caracterização morfológica e morfometrica dos ovos de duas coleções institucionais de referencia: MNHN e CHIOC.

A partir dessa caracterização, observou-se que no gênero *Eucoleus* há uma dominância de ornamento pontuado. Por outro lado, o gênero *Aonchotheca* tem predomínio do tipo reticulado ll. Embora esses ornamentos sejam vistos também em outros 4 gêneros, *Baruscapillaria*, *Capillaria*, *Eucoleus* e *Tridentocapillaria*, o número de espécimes observados não é alto. O gênero *Echinocoleus* possui apenas uma ornamentação reticulada do tipo I, assim como *P. plica* com características muito semelhantes de *C. collaris*, embora a morfologia do ovo seja diferente. Alguns trabalhos na literatura mostram relação com alguns fatores ecológicos dos parasitos para relacionar com as espécies, porém em levantamentos coprológicos e na paleoparasitologia é muita das vezes impossível fazer tais relações como o local de infecção, pois os únicos dados são o próprio ovo, e dependendo do material estudado, também o hospedeiro.

Como a ornamentação da casca dos capilarideos por microscopia de luz tem uma visualização limitada, estruturas com dimensão menor que 1μm pode ficar limitada a discussão. A caracteristica ultraestrutural, possibilita observar diferenças entre as espécies, pois as categorias de ornamentação podem ser detalhadas com maior precisão, devido à resolução a técnica permite a vizualizacao das superfícies. Embora algumas semelhanças podem ser reconhecidas entre algumas espécies, nenhum padrão é visto dentro do gênero.

Embora haja uma diferença na categorização por ML e MEV os objetivos são diferentes. Como método de rotina, a microscopia de campo claro oferece uma investigação e diagnóstico rápidos, além de ser um equipamento mais barato e acessível. Por outro lado, a MEV não é uma metodologia de rotina na maioria dos laboratórios, porém é utilizada como uma ferramenta para descrição e caracterização mais detalhada das espécies.

A caracterização morfológica e morfometrica dos ovos sem uma metodologia para aplica-la num meio de identificar os ovos encontrados em amostras antigas ou de análise coprológica, torna-se uma avaliação difícil. Portanto, o desenvolvimento de uma metodologia para identificação com base na caracterização dos ovos, é essencial.

No artigo 2, é discutido a metodologia de “Machine Learnig” e os diversos logaritimos utilizados. A análise mostrou que ao utilizar localização geográfica e hospedeiro no algoritmo, a confiabilidade da árvore de decisão é maior em todos os algoritmos utilizados. O parâmetro geográfico sozinho produziu uma árvore de confiabilidade ligeiramente maior do que a indicação do hospedeiro. Mesmo com essa diferença, o parâmetro hospedeiro pode ser mais útil devido à restrição de informações usadas para localização geográfica. Enquanto o hospedeiro foi possível aplicar o nível taxonômico de classe, que é mais genérico para futuras entradas de espécies, a localização geográfica é restrita para a informação dos espécimes coletados. Embora a aplicação de maior cobertura para distribuição de espécies tenha produzido alguns erros de decisão.

Sendo assim ao analisar amostras antigas tanto do Velho e Novo Mundo, com os achados de capilarídeos foi possível aplicar o conhecimento gerado a partir da caracterização dos ovos das Coleções Helmintológicas Institucionais para a identificação dos ovos.

Nas amostras arqueológicas, foi aplicado uma abordagem estatística com a aplicação de distância Wards gerando uma árvore hierárquica de acordo com a ornamentação dos ovos. Utilizando assim a morfometria e morfologia dos ovos das coleções helmintológicas estudadas nos artigos 2 e 3. Os capilariídeos do Novo Mundo foram recuperados dos coprólitos, onde todos os hospedeiros foram identificados. Na amostra da *Panthera onca*, o ovo encontrado teve uma ornamentação RTII. Foi feita aplicação na árvore hierárquica, podemos ver um cluster com *A. erinacei*, A. *annulosa*, *C. exigua* e *A. bovis*. Nenhuma das espécies são específicas de felinos, mas podem parasitar uma presa em potencial. Na amostra identificada como *Didelphis albiventris* foram encontrados dois morfotipos diferentes. Dado o fato de que todos os ovos semelhantes ao encontrado, nos fez acreditar que pertence ao gênero *Eucoleus*. Na árvore hierárquica foi formado um cluster com *E. gastrico*, embora seja usualmente parasito de roedores, já foi descrito em marsupiais na Austrália. As amostras identificadas de *Bos taurus*, foram encontrados ovos que possuem morfologia compatível com *Aonchotheca bovis*.

Nas amostras do Velho Mundo analisadas não é possível determinar o hospedeiro, pois as fossas e latrinas têm muito material orgânico, incluindo fezes humanas e animais. Os ovos encontrados nas amostras do sítio Bourges Avaricum (AVA) tiveram 5 morfotipos diferentes. Sendo um com casca espessa e ornamentação densa pontuada, com visualização radial em visão transversal, características muito particulares de *C. hepaticum*. Outro

morfotipo de ovos foi observado em três amostras diferentes. Pela aplicação da árvore hierárquica pode-se observar uma proximidade com *E. madjerdae* e *E. bohemi*, ambas as morfologias são similares às encontradas nas amostras. Também foi encontrado um outro morfotipo com a ornamentação e estrutura de casca de ovo semelhante das outras três amostras citadas acima, porém com maior largura e menor medida de comprimento. Em outras duas amostras foram encontradas ovos com ornamentos reticulado do tipo II, ambas têm distâncias próximas entre espécies diferentes nas arvores hierárquicas por medida de comprimento e largura, com *A. myoxinitelae* e *C. venusta*. A morfologia e morfometria são compatíveis com cada espécie. As amostras do sítio La Rochelle Augustin, os ovos são os menores encontrados neste estudo. Ambos são muito semelhantes em morfologia e tamanho, mas os ornamentos são ligeiramente diferentes.

Como visto no artigo 1, não só a taxonomia do grupo é complexa, mas também a filogenia que se apresenta mal resolvida. As árvores filogenéticas produzidas neste estudo mostraram informações genéticas limitadas disponíveis, gêneros não resolvidos taxonômicamente e incongruência com a taxonomia clássica. É evidente a necessidade de mais estudos genéticos, principalmente de taxonomia integrativa, para resolver conflitos taxonômicos, e complementar a sistemática de Capillariidae, que, além disso, permitiria o desenho de abordagens paleogenéticas. Assim a elucidação da paleodistribuição de capilarídeos pode fornecer insights das antigas associações parasita-hospedeiro, mas também em cenários modernos.

CONCLUSÕES

- A paleodistribuição de Capillaridae é mais informativa no Novo Mundo onde as amostras são coprólitos e tem os dados de produtor/hospedeiro, o que facilita a definição taxonômica dos parasitos;

- A filogenia molecular de Capillaridae mostra parafilía de gêneros, incongruências com a taxonomia morfológica e limitada informação genética;

- A ornamentação da superfície do ovo é confirmada como uma característica importante para discriminação de espécies em capilarídeos;

- Abordagens de MEV auxiliam no entendimento de padrões de estrutura da superfície dos ovos de capilarídeos;

- A abordagem de IA/ML é uma poderosa ferramenta de identificação de espécies.

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APÊNDICE – Artigo: African helminth infections out of Africa: Paleoparasitological and paleogenetic investigations in Pretos Novos Cemetery, Rio de Janeiro, Brazil (1769-1830)

Artigo submetido ao periódico Acta Tropica.

African helminth infections out of Africa: Paleoparasitological and paleogenetic investigations in *Pretos Novos* cemetery, Rio de Janeiro, Brazil (1769-1830)

Lucélia Guedes[1*], Victor Hugo Borba[1*], Morgana Camacho[1], Jandira Neto[2], Ondemar Dias[2], Alena Mayo Iñiguez[1]

[1]LABTRIP, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (LABTRIP/IOC/FIOCRUZ). Avenida Brasil, 4365, Pavilhão Rocha Lima, Manguinhos, Rio de Janeiro, Brasil. CEP: 21045-900.

[2]Instituto de Arqueologia Brasileira (IAB). Estr. Cruz Vermelha, 45 – Vila Santa Teresa, Belford Roxo, Rio de Janeiro, Brazil. CEP: 26193-415

*These authors contributed equally to this work

Corresponding author: alenainiguez@gmail.com.br; alena@ioc.fiocruz.br

E-mail addresses of each author: lucelia.fiocruz@gmail.com; borba.vh@gmail.com; morganacamacho88@gmail.com; jandiranetodias@terra.com.br; alena@ioc.fiocruz.br

ABSTRACT

Pretos Novos cemetery (PNC), Rio de Janeiro, Brazil (1769-1830), was created exclusively to bury enslaved Africans who had died when arrived to the city or before to be sold in the slave market. Since the bodies were dismembered, placed in mass graves and burned, most of human remains collected from the site were highly fragmented and showed intense degradation. PNC site is possibly, the unique in America that allow the study of African parasitic infections out of Africa. In this study, we aimed to identify which parasites affected PNC individuals through paleoparasitological and paleogenetic analyses. Sacrum and pelvic sediments were collected from PNC individuals (n=5), in addition to control samples (n=7). Samples were submitted to 3 parasitological techniques and in paleogenetic analysis to 4 molecular targets. Larvae, mites, pollen grains, and structures of plant, and fungus, but not conclusive of helminth infection, were observed. *Ascaris* sp. *cyt*b sequence was retrieved in one PNC individual. We highlight that even with the intense degradation of PNC human remains, and the process of curation of samples after excavation, it was possible to recover aDNA of helminths. Considering the origin PNC individuals, it is possible to state that these infections were brought to Brazil from Africa during the colonial times.

Keywords: *Ascaris* sp., *cyt*b, colonial period, taphonomy

1. INTRODUCTION

Pretos Novos (New Blacks) cemetery (PNC) was created in 1769 as a demand to transfer the slave market which was previously located at Praça XV, next to the Royal Palace, to the wharf Valongo, port area of Rio de Janeiro. PNC was used as a place to bury African slaves that died as soon as they arrived in the city, or

after being put in quarantine outside the port, or were awaiting to be sold in the slave market (Machado, 2006). Although PNC was administered by the church, the slaves were buried without religious rituals. The bodies were accumulated for days, burned and disposed without coffins, one body above the other (Machado, 2006). In order to facilitate the burial of a great number of individuals, their bodies were also dismembered (Pereira, 2007). Despite the longtime of function, for sixty years, the administration of the cemetery had obituaries only from 1824 to 1830. The obituaries demonstrated that in this period 6,119 African slaves were buried at PNC. Individuals from all ages and gender were buried, but the majority was characterized for being young male adults (Pereira, 2007)

The analysis of strontium conducted by Bastos et al. (2010) revealed that PNC individuals were brought from different African areas, and that they lived away from coast, since maritime resources were not identified. Matrilineal ancestry was also identified in three individuals as belonging to haplotypes L3e2, L3d1, and L1c2 (Jaeger et al., 2013a). These 3 haplotypes correspond to main African areas and ports where captives were shipped. This data reaffirmed the diversity of the geographic origin of PNC individuals.

Since their capture until the arrival in Rio de Janeiro port, the slaves were maintained in unhealthy conditions in small crowded places, that probably facilitated the transmission of infectious diseases, and contributed to poor health status (Pereira, 2007). Previous paleoparasitological study of Praça XV archaeological site (PXV), a cemetery (18th century) with an important number of African slaves buried, revealed that 8/10 individuals were infected with intestinal helminths and/or protozoa (Jaeger et al., 2013b). A paleogenetic study in the same site demonstrated infections with *Trichuris trichiura* and *Ascaris* sp. in 40% of the individuals and *Enterobius*

vermicularis in 50% (Jaeger and Iñiguez, 2014). The first paleogenetic investigation in PNC site revealed positive tuberculosis infection in 4/16 individuals by *Mycobacterium tuberculosis* complex (MTBC) ancient DNA (aDNA) detection, using bones as source of research (Jaeger et al., 2013a). The results demonstrated the circulation of MTBC in Africa during the Colonial times, which was a controversial subject.

The present study aimed to recover paleoparasitological evidence of intestinal infection in PNC individuals. Since PNC is possibly the only cemetery composed of newly arrived African slaves in America, the study provided the opportunity to research past infections and contribute to the knowledge of the health status of this specific group.

2. MATERIALS AND METHODS

Sediment samples from PNC individuals (n=5), in addition to control~~s~~ samples (n=7) were analyzed. Samples used in this work were obtained from the Brazilian Archaeological Institute (*Instituto de Arqueologia Brasileira*-IAB) and maintained in the Paleogenetic Laboratory (PL) of LABTRIP (IOC/FIOCRUZ) under the responsibility of Dr. Alena Mayo Iñiguez. The samples were submitted to curation processes by archaeologists at IAB, practice that involved washing and scraping of the material. However, no chemicals were added.

During the archaeological campaign, due to a high degree of disaggregation of the anatomic parts of the bodies, series of types of bones, skulls and long bones, for example, were collected and stored at room temperature in the IAB museum collection. Teeth with intentional modifications, characteristics of Africans people (Lyrio et al., 2011), beads (Fig. 1A) and smoking pipe (Fig. 1A) were also found (Iñiguez 2014). Four sacra and one ilium corresponding to 5 individuals were the only

PNC human remains available for intestinal parasite investigation. Sediments from sacral foramens and from an ilium crest were collected for this study. Additionally, sediments associated to unspecific infection site or bones, as skulls, were used as contamination controls. Samples (n=12) were collected following the paleogenetic collection protocol (Iñiguez, 2014), protected from light, preserved and transported on ice to the PL laboratory.

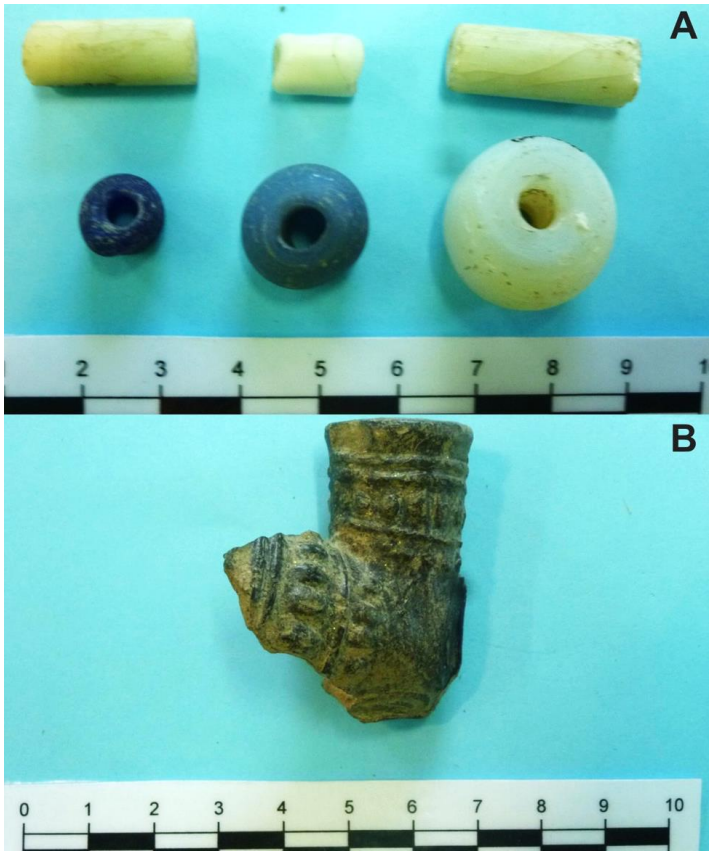

Fig. 1. Cultural artifacts found during the archaeological campaign of *Pretos Novos* cemetery, Rio de Janeiro, Brazil. (A) African beads; (B): Fragment of a smoking pipe. Source: Iñiguez, AM-IAB collection.

In order to increase the chances of finding parasite remains in PNC samples, three parasitological techniques were performed: spontaneous sedimentation (Lutz, 1919), modified sucrose centrifugal flotation (specific gravity-sg=1300) (Hubber et al., 2003) and zinc sulphate centrifugal flotation at 33% (sg=1180) (Faust et al., 1939).

Samples were rehydrated with 0.5% trisodium phosphate (Na_3PO_4) aqueous solution (Callen and Cameron, 1960) during 24h and then submitted to the spontaneous sedimentation technique (Lutz, 1919). Two-hundred microliters of each sample, about twenty slides, were analyzed in a light microscope Nikon Eclipse E200 at 100x magnification. Photographs were obtained at 400x magnification. Aliquots of 1mL of sediment obtained by the spontaneous sedimentation were used to perform each centrifugal flotation technique. One slide from each sample was prepared and analyzed in the same conditions described above.

Considering the several taphonomic issues involving the cemetery and precautions to avoid contamination with modern DNA, methodological efforts involving the collection of the material, storage, transport to the laboratory, and use of parasitological techniques were an especial concern (Iñiguez, 2014). The paleogenetic analysis was conducted at the PL Laboratory (LABTRIP/IOC/FIOCRUZ, Brazil) using standard procedures to avoid aDNA contamination, degradation and cross-contamination as described in some studies (Iñiguez, 2011; Iñiguez et al., 2006; Iñiguez AM, 2014). Two hundred microliters of each rehydrated sediment were used for aDNA extraction with previous freezing with liquid nitrogen. QIAamp® DNA Investigator Kit (QIAGEN) was used according to the instructions of the manufacturer, with modifications, including a pretreatment step of proteinase K digestion (Invitrogen) 20mg/ml with ATL buffer at 56°C during 72h (Jaeger et al., 2013a). The concentrations were estimated at 260nm absorbance in a spectrophotometer NanoDrop (ND-1000). Whole Genome Amplification (WGA) Repli-g (Qiagen) was applied at samples to increase the concentration of aDNA. The Polymerase Chain Reaction (PCR) was applied using four molecular targets for *Ascaris* sp. diagnosis: 18S ribosomal DNA (18S rDNA) and cytochrome b (*cyt*b)

(Loreille et al., 2001), NADH dehydrogenase subunit 1 (*nad*1) and cytochrome c oxidase subunit 1 (*cox*1) (Botella et al., 2010) (Supplementary Table). The 18S rDNA PCR modifications were in 25µl of reaction volume with 2.5mM of $MgCl_2$, 200ng of each primer, 2.5 units Platinum^{TM} *Taq* DNA Polymerase (Invitrogen). The PCR thermal conditions were, one denaturing step at 94°C for 2 minutes, followed by 50 cycles of denaturation at 94°C for 30 seconds, annealing at 44°C for 30 seconds, elongation at 72°C for 30 seconds and extension at 72°C for 5 minutes. Extraction blank and PCR negative controls were included. Positive PCR controls were not included. For the *cyt*b target was followed the same conditions described above, with exception of annealing at 52°C. For the *nad*1 target, 25pmol of each primer were used and the PCR thermal conditions consisted of an initial denaturation cycle at 94°C for 5 minutes, 50 cycles of denaturation at 94°C for 40 seconds, annealing at 45°C for 40 seconds, elongation at 72°C for 50 seconds and extension at 72°C for 7 minutes. For *cox1* target, 100ng of each primer were used and the PCR thermal conditions consisted of one denaturation cycle at 96°C for 5 minutes, followed by 40 cycles of denaturation at 96°C for 40 seconds, annealing at 45°C for 50 seconds, elongation at 72°C for 50 seconds and extension at 72°C for 5 minutes. To detect *T. trichiura* infection the 18S rDNA molecular target was used (Oh et al, 2010) (Supplementary Table). PCR products were visualized by 3% low melting agarose gel electrophoresis stained with Gel-Red (Biotium). The sequencing reaction was performed using Kit Big Dye Terminator v. 3.1 (Applied Biosystems) in the sequencing platform RPT01A/IOC-Fiocruz (Applied Biosystems ABI 3730 sequencer). The sequence analysis was performed using Lasergene Seqman v. 7.0.0 (DNASTAR, Madison, Wisconsin) and Bio Edit v. 7.0.4 (Department of Microbiology, North Carolina State University, Raleigh, North Carolina). BLAST

searches were performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the obtained aDNA sequences until 09/2019.

3. RESULTS

The paleoparasitological assay revealed helminth free-living larvae, mites, monolete, pollen grains, and plant and fungi structures but not conclusive of helminth parasites, in both abdominal or control samples (Fig. 2; Table 1). The analysis performed by using the centrifugal flotation in sucrose solution was negative and the centrifugal flotation in zinc sulphate solution showed a fungi spore in PNC10 sample.

Of four molecular targets applied in the paleogenetic analysis of *Ascaris* sp., the infection was confirmed by *cyt*b aDNA sequence in one individual (PNC05). The analysis revealed 100% of sequence identity with *A. lumbricoides* (GenBank EF439709) and 99-92.96% with *A. lumbricoides, A. suum* and *Ascaris* sp. sequences in 100-84% of query cover. The *cyt*b alignment using *A. lumbricoides* (GenBank EF439709) as reference, revealed the polymorphism C11034T in PNC05 sequence from this study, also present in the reference sequence, but absent in others *A. lumbricoides* strains (Fig. 3). Polymorphisms at positions 11011, 11016, 11031, 11073 and 11128 are as well not species-specific, shared by *A. lumbricoides, A. suum* and *Ascaris* sp. sequences (Fig. 3). Negative controls as well as sediment controls showed absence of PCR amplification. We did not obtain positive *T. trichiura* amplifications.

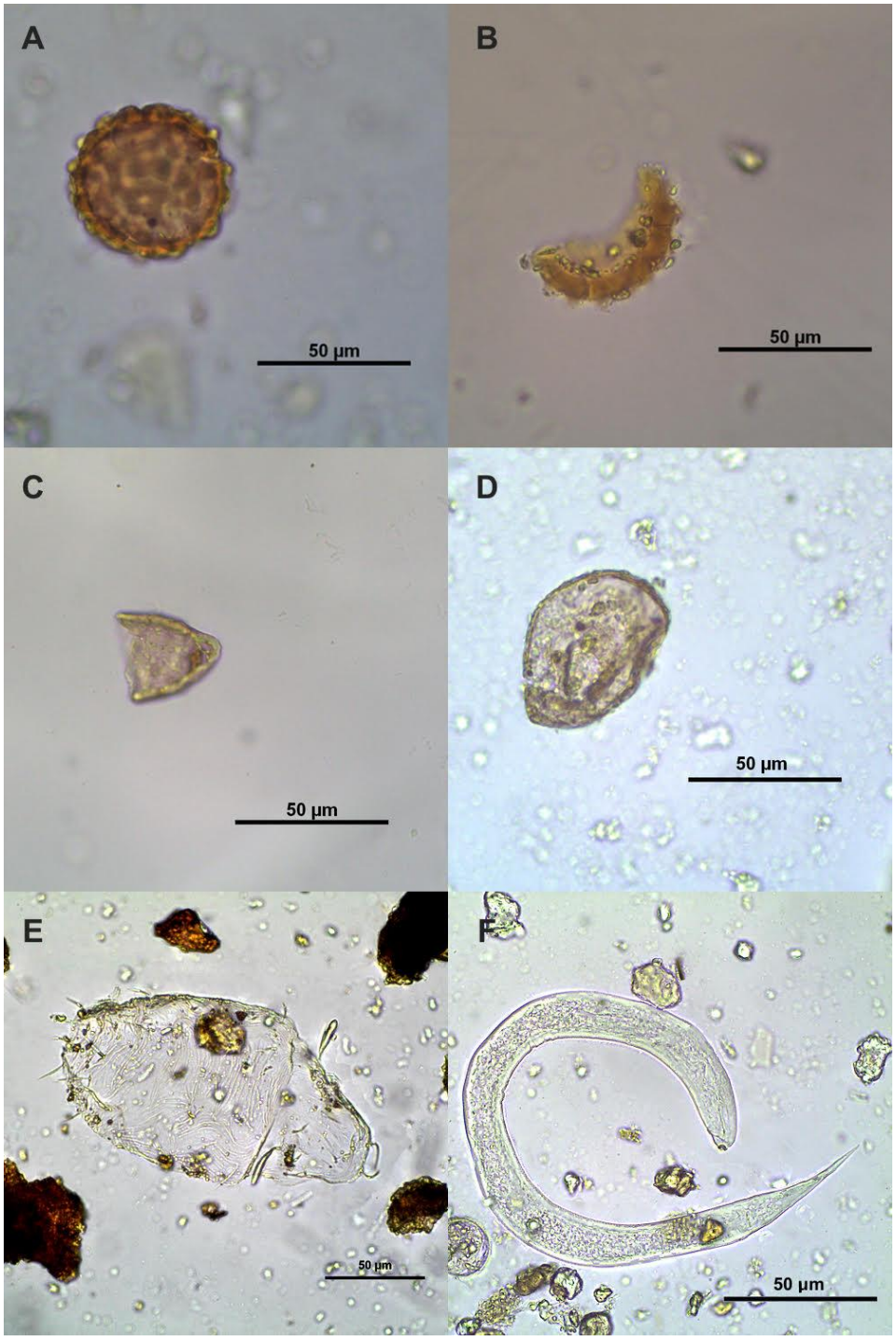

Fig. 2. Results of paleoparasitological analyses from *Pretos Novos* cemetery individuals. (A) Structure suggestive of fungal spore from PNC03; (B): Fragment suggestive of fungal spore from PNC11; (C): Fragment suggestive of plant material from PNC06; (D): Structure suggestive of plant material from sample PNC02; (E): Mite from sample PNC01; (F): Free-living larvae from sample PCN07.

Table 1: *Pretos Novos* cemetery individuals analyzed in this study and paleoparasitological and paleogenetic results

ID	IAB ID	Sample Type	Sex	Age (years)	Paleoparasitological results**	Paleogenetic results***
PNC01	B2: B1 sector	Environmental sediment	NA	NA	Mite	Negative
PNC02	B3	Environmental sediment	NA	NA	Suggestive of plant material / Monolet	Negative
PNC03	B3	Humerus sediment	-	-	Suggestive of fungal spore	Negative
PNC04	B3	Skull sediment	-	-	Negative	Negative
PNC05	B3	Sacrum sediment	-	-	Negative	*Ascaris* sp. *cyt*b
PNC06	B3: B1 sector	Sacrum sediment	UD	Child (<2)	Suggestive of plant material	Negative
PNC07	B4: PN44	Skull sediment	M	Adult (>30)	Fungi spores / Free-living larvae	Negative
PNC08	B4:PN38	Skull sediment	UD	Young Adult (<20)	Negative	Negative
PNC09	B4:PN36	Skull sediment	UD	UD	Palynomorph	Negative
PNC10	B6*: B1/2 sector PN26	Ilium sediment	F	Adult (>20)	Palynomorph/Fungi spores	Negative
PNC11	B7	Sacrum sediment	-	-	Suggestive of fugal spore	Negative
PNC12	B8	Sacrum sediment	-	-	Negative	Negative

ID: Paleogenetic Laboratory identification; IAB ID: *Instituto de Arqueologia Brasileira* identification; NA: not applicable; -: information not available; M: male; F: female; UD: parameter undetermined; *burned bones; **results obtained by spontaneous sedimentation technique, except fungi spore finding in sample PNC10 by zinc sulphate centrifugal flotation technique. ***_Ascaris_ sp. and _T. trichiura_ molecular paleoparasitological diagnosis.

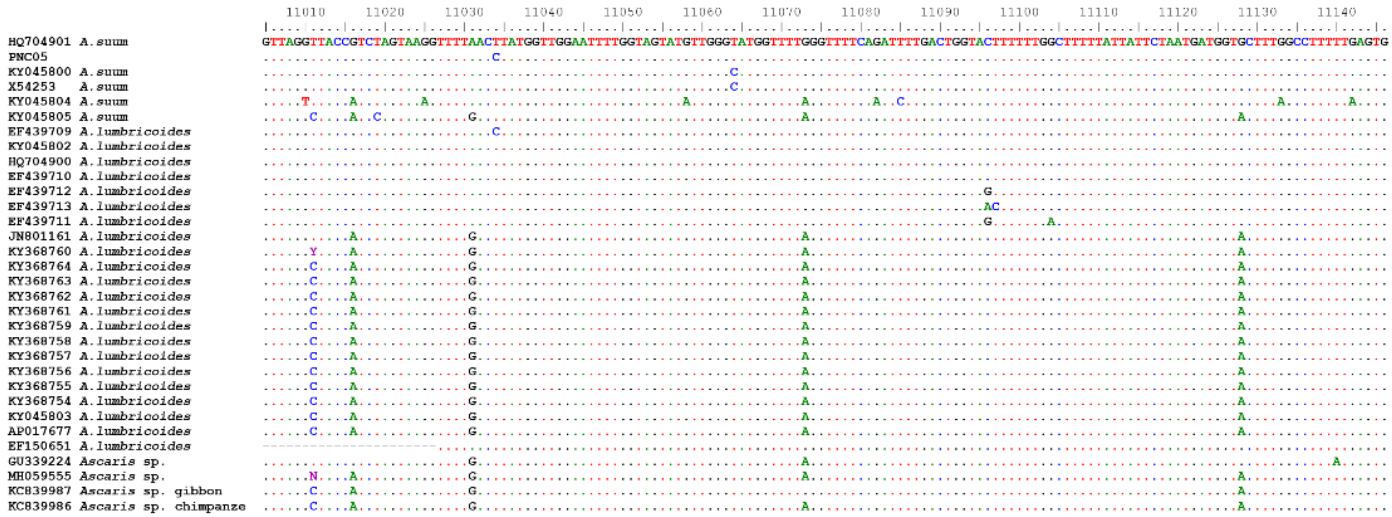

Fig. 3: Nucleotide alignment of *cyt*b gene fragment of PNC05 sample and *A. lumbricoides*, *A. suum* and *Ascaris* sp. sequences from GenBank using *A. suum* complete genome (accession number HQ704901) as reference sequence.

4. DISCUSSION

Paleoparasitological analyses in PNC represented a taphonomic challenge, since many factors influenced on the preservation of parasitological remains. The site is located next to the sea and above sandy soil, which could influence on water percolation and consequently the displacement of the eggs through the archaeological layers. The hot and humid weather of Rio de Janeiro, in addition to the burial conditions, possibly permitted a poorly preservation of the organic material. One of the characteristics of PNC cemetery is the burning and dismembering of the bodies. The burn was likely the most influent taphonomic problem that interfered in the preservation of parasite eggs. Mites, fungi spores and free-living larvae probably also affect the preservation of the parasite vestiges. Predation by the soil biota could contribute to parasite degradation (Leles, 2010a; Morrow et al., 2016).

Ascaris sp. infect their hosts by fecal-oral transmission, and despite the elimination of thousands of eggs, 200.000 eggs/day, no infection can occur without a development in ideal conditions of light, temperature and humidity in the soil (Rey, 2008). Therefore, the *Ascaris* sp. infection in a PNC individual could not be acquired during the traffic transportation, but in African lands, since the conditions for the maturation of eggs were not ideal. The average slaving voyage from African coast to the Americas took six to eight weeks (Pereira, 2007). This period would not be enough to a recent infected person starts to eliminate eggs, that is around 70 days for *Ascaris* sp. (Rey, 2008).

Although bone analyses of these individuals showed no pathological lesions and good health (Cook et al., 2015; Lyrio et al., 2011), they were probably not healthy due the insalubrious conditions during the Atlantic journey, which facilitate infections, as tuberculosis and smallpox, as mentioned by Sigaud (2009). In fact, tuberculosis in PNC individuals was verified by *Mycobacterium* aDNA (Jaeger et al., 2013a). The infection with intestinal parasites is usually asymptomatic, but it could have influenced, along with the poor nutrition, in the nutrient depletion and precarious health status (Stephenson et al., 2000).

In this study, *Ascaris* sp. infection was detected by paleogenetic analysis in an individual who was negative by paleoparasitological assay. The result emphasizes the integrative use of the molecular and microscopical analyses in the intestinal parasite diagnostic in the past (Iñiguez et al., 2003; 2006; Jaeger and Iñiguez, 2014; Jaeger et al., 2016). Only one individual was positive for *Ascaris* sp. infection with one molecular target. Since the parasite eggs were absent, we infer that the preservation of aDNA was strongly affected, without the protection inside the eggs, as well as, the mentioned taphonomic process.

The *Ascaris* sp. *cyt*b genotype recovered in a PNC individual is identical to the one verified by Leles et al., (2008), from probable latrine, garbage deposits or food storage sediment in the Belgium (XVI century). Although the molecular diagnosis, it is impossible to discriminated between *Ascaris lumbricoides* (Linnaeus 1758) and *Ascaris suum* (Goeze 1782), parasites of human and pig (*Sus sacrofa* Linnaeus, 1758), respectively. Several genetic studies aiming to differentiate between *Ascaris* species have been conducted (Anderson, 1995; Iñiguez et al., 2012; Leles et al., 2010b; Peng et al., 2003; Zhu et al., 1999). However, the genetic data produced did not allow a discrimination of *Ascaris* species, and isolates are named as "*Ascaris* from human or from pigs" instead the species taxonomic nomenclature. Iñiguez et al. (2012) were categoric when showed gene flow and lack of definition among human and pigs *Ascaris* haplotypes worldwide. Then, Leles *et al.* (2012) proposed that *Ascaris* species should be considered as one species. However, this is still a controversial subject (Betson and Stothard, 2016). The *Ascaris cyt*b sequence detected in a PNC individual is identical to a previously reported with uncertain origin, and also showed polymorphisms shared with both parasite species. Nevertheless, based on all aforementioned studies the challenge of *Ascaris* spp. definition seems to be a taxonomic instead a diagnostic issue.

5. CONCLUSIONS

Paleogenetic data was recovered from PNC site, despite several taphonomic factors that influenced in the preservation of parasite and aDNA. Considering that PNC individuals were recently arrived captives from Africa and the biological cycle of *Ascaris* sp., it possible to affirm the African origin of the helminthiasis. We report, for the first time, a parasite aDNA sequence from Brazilian historical times, which represent African helminth infection out of Africa.

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