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ESTUDIO COFILOGENÉTICO DE ESPECIES DEL GÉNERO *HEPATOZOON* CON SUS HOSPEDEROS VERTEBRADOS E INVERTEBRADOS

SUSTENTANTE

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I. Resumen

Las enfermedades infecciosas emergentes representan una amenaza para la vida silvestre ya que pueden llevar a las poblaciones a la extinción. Un ejemplo es la enfermedad causada por las especies del género *Hepatozoon* sp. que infectan una gran variedad de hospederos vertebrados. Por tanto, es importante entender la coevolución de este patógeno con sus hospederos ya que los parásitos que coevolucionan por cambio de hospedero podrían ser más propensos a volverse emergentes. Para realizar estos estudios se requiere reconstruir y comparar las filogenias parásito-hospedero mediante secuencias genéticas correspondientes a marcadores moleculares para evaluar si ambas filogenias son congruentes. En la primera parte del estudio, se realizó una búsqueda en el GenBank de las secuencias genéticas reportadas para *Hepatozoon*, lo que evidenció que el marcador molecular más utilizado para identificarlos es el 18S ARNr con un total de 3154 secuencias, por lo que se seleccionó para efectuar el estudio cofilogenético. En la segunda parte de la investigación, se reconstruyó las filogenias del parásito y de los hospederos utilizando las secuencias 18S ARNr y *cyt*B, respectivamente, para evaluar las relaciones cofilogenéticas mediante los métodos de PACo y ParaFit, y posteriormente estimar la frecuencia de los eventos coevolutivos mediante eMPRess. Se encontró que la evaluación global de la congruencia entre las filogenias de los hospederos del orden Carnívora, Rodentia y Squamata con los parásitos fue significativa (PACo: todas $m^2_{XY} < 0.655$, todas $p < 0.001$; ParaFit: todas ParaFitGlobal Statistic< 72.992, todas $p < 0.007$; todos $R^2 > 0.25$). Además, eMPRess demostró que la congruencia se explica por el fenómeno de cambio de hospedero. Lo que pone en evidencia la capacidad de *Hepatozoon* spp. asociadas a ciertos órdenes de vertebrados para infectar a hospederos nuevos y simpátricos, lo que permite comprender la emergencia de la hepatozoonosis en ciertas zonas geográficas.

Abstract

The emergence of infectious diseases represents a threat to wildlife as they can drive populations to extinction. One example is the disease caused by species of the genus *Hepatozoon* sp. that infect a wide variety of vertebrate hosts. Therefore, it is important to understand the coevolution of this pathogen with its hosts, as parasites that coevolve with their hosts by host switching may be more prone to become emergent. These studies require reconstructing and comparing parasite-host phylogenies using genetic sequences corresponding to molecular markers to assess whether the two phylogenies are congruent. In the first part of the study, a GenBank search of the genetic sequences documented for *Hepatozoon* revealed that the most used molecular marker to identify them is 18S rRNA with a total of 3154 sequences, which was selected for the cophylogenetic study. In the second part of the research, the phylogenies of the parasite and hosts were reconstructed using the 18S rRNA and *cyt*B sequences, respectively, to assess cophylogenetic relationships using the PACo and ParaFit methods, and subsequently estimate the frequency of coevolutionary events using eMPRess. We found that the overall assessment of congruence between the phylogenies of hosts of the order Carnivora, Rodentia and Squamata with the parasites was found to be significant (PACo: all $m^2_{XY} < 0.655$, all $p < 0.001$; ParaFit: all ParaFitGlobal Statistic < 72.992, todas $p < 0.007$; all $R^2 > 0.25$). In addition, eMPRess demonstrated that congruence is explained by the phenomenon of host switching. This highlights the ability of *Hepatozoon* spp. associated with certain vertebrate orders to infect new and sympatric hosts, which helps to understand the emergence of hepatozoonosis in certain geographical areas.

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VI. Lista de abreviaturas

NCBI	National Center of Biotechnology Information
18S rRNA	18S ribosomal RNA
*cyt*B	Cytochrome B
PACo	Procrustean Approach to Cophylogeny
MPR	Maximum parsimony reconciliation
PCo	Procrustes superimposition plot
m^2_{xy}	Residual sum of squares
COI	Cytochrome c oxidase subunit I
COIII	Cytochrome c oxidase subunit III
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
28S rDNA	28S ribosomal rDNA
5.8S rDNA	5.8S ribosomal rDNA

VII. Descriptores

Parasite

Host

Molecular marker

Cophylogeny

Coevolution

Host switching

VIII. Introducción general

El género *Hepatozoon* [1] constituye más de 340 especies de parásitos [2,3] pertenecientes al Phylum Apicomplexa y a la familia Haemogragarinidae, capaces de habitar las células sanguíneas del hospedero vertebrado [4] como por ejemplo animales domésticos como gatos y perros, y animales silvestres como anfibios, reptiles, aves y una gran variedad de mamíferos carnívoros [4,5]. El parásito tiende a producir en el animal un cuadro clínico leve o subclínico, a excepción del cuadro clínico en perros producido por *Hepatozoon americanum* que puede llegar a ser severo [2,6].

Su ciclo de vida es heteroxeno obligatorio, en el cual, la multiplicación asexual ocurre en el hospedero intermediario vertebrado y la reproducción sexual en el hospedero definitivo invertebrado [4]. El hospedero invertebrado entre los cuales se puede mencionar a garrapatas, mosquitos y pulgas, va a funcionar como un vector del microorganismo al ser ingerido por el hospedero vertebrado donde se da la transmisión del parásito [7].

Debido a la gran cantidad de especies de animales que pueden ser infectados y a la posibilidad de transferencia de estos parásitos a nuevos hospederos mediante los vectores, es que la hepatozoonosis tiene potencial de llegar a ser una enfermedad emergente en el futuro [5]. Esta posibilidad cobra importancia en temas de conservación, ya que las enfermedades infecciosas emergentes representan una importante amenaza para la vida silvestre porque pueden llevar a las poblaciones a la extinción en caso de que no se desarrollen acciones de manejo adecuadas [8].

Se cree que las especies de *Hepatozoon* sp. podrían mantener una relación estrecha con sus hospederos, debido a que cada especie de este parásito parece infectar a un rango definido de

hospederos vertebrados. Esta relación estrecha podría explicarse por el fenómeno de coevolución que se traduce como una coespeciación que es un término que hace referencia a la especiación que tienen los parásitos con sus hospederos cuando los hospederos divergen [9], resultando en la aparición de nuevas especies de hospederos y parásitos [10]. Adicionalmente, también pueden estar presentes en la historia evolutiva del parásito y sus hospederos otros eventos evolutivos como lo son: el cambio de hospedero, la duplicación y la pérdida [11].

Debido a la amplia distribución del parásito y a la variedad de animales que afecta, es importante entender la coevolución de este patógeno con sus hospederos, lo que permitirá identificar posibles hospederos susceptibles que puedan estar siendo afectados por una especie puntual de este parásito en un determinado lugar [8].

Estudiar estos fenómenos evolutivos implica identificar las diferentes especies de *Hepatozoon* sp., lo cual se vuelve una tarea complicada debido a que sus etapas de desarrollo son morfológicamente indistinguibles entre las diferentes especies, por lo que para inferir su historia evolutiva ha sido necesario utilizar herramientas como la filogenia y el diagnóstico molecular de muestras de sangre y tejido de hospederos [4]. Las secuencias genéticas del parásito para estos estudios se han obtenido mediante ensayos de PCR utilizando como marcador molecular el gen de la subunidad 18S del ARNr nuclear [4].

Hasta el momento, no existen análisis cofilogenéticos de las especies de *Hepatozoon* sp. y sus múltiples hospederos vertebrados e invertebrados, a diferencia de otros parásitos transmitidos por vectores [10], por lo que en este estudio se efectuó un análisis cofilogenético entre parásito y sus hospederos, desarrollándose así dos artículos científicos. En el primero se recopiló información genética del parásito reportada en el GeneBank, en un período de 10

años, con el objetivo de conocer cuál es el marcador genético más utilizado para reconstruir la filogenia de *Hepatozoon* sp.

En el segundo se reconstruyó la filogenia tanto del parásito como de sus hospederos y se evaluó mediante métodos de ajuste global si estas filogenias eran congruentes entre sí, además se evidenció los eventos evolutivos involucrados en la historia evolutiva del parásito y sus hospederos, mediante métodos de estrategias basadas en eventos.

- **¿Cuál es el marcador molecular más utilizado para reconstruir la filogenia de *Hepatozoon* spp.?**
- **¿Ha coevolucionado *Hepatozoon* spp. con sus hospederos vertebrados e invertebrados?**

IX. Conclusiones Generales

El marcador molecular 18S ARNr a pesar de ser el más utilizado para identificar parásitos pertenecientes al género *Hepatozoon*, no permite la identificación de todas las secuencias a nivel de especie, lo que representa un sesgo en la verdadera distribución de las especies del parásito alrededor del mundo.

Hepatozoon spp. es un parásito que ha coevolucionado con sus hospederos de los órdenes Carnivora, Rodentia y Squamata, principalmente por el evento evolutivo de cambio de hospedero. Esto tiene implicaciones importantes para entender el riesgo de emergencia de la hepatozoonosis en nuevos hospederos susceptibles en diferentes áreas geográficas donde los patógenos pueden dispersarse por el fenómeno de "spill over", y por tanto permitirá identificar posibles reservorios para establecer estrategias de conservación.

El presente estudio cofilogenético constituye un análisis inicial de como este parásito puede interactuar con otros hospederos y por tanto dispersarse, sin embargo, es necesario realizar vigilancia activa de la salud de las poblaciones de animales de vida silvestre en base a estos resultados que representan evidencia objetiva para invertir esfuerzos en monitorear la presencia del parásito e identificar las especies presentes en determinadas zonas geográficas y especies de animales para entender la verdadera distribución de la enfermedad.

X. Recomendaciones Generales

El presente estudio es un trabajo de bioinformática sustentado en la estadística y la filogenética donde los resultados están asociados a una probabilidad, por lo que no necesariamente podrían reflejar la interacción que está ocurriendo entre *Hepatozoon* sp. con los diferentes animales silvestres y domésticos. Entonces es necesario en futuras investigaciones integrar los resultados obtenidos en esta investigación con otros factores como por ejemplo las cadenas tróficas, las barreras geográficas, el cambio climático, la fragmentación de hábitat, entre otros, que podrían esclarecer de mejor manera cual es la verdadera dinámica de transmisión de la hepatozoonosis.

Una limitante del trabajo es la disponibilidad de secuencias del *cyt*B de los órdenes de los hospederos en el GenBank. Ciertos órdenes como por ejemplo el orden Anura, no pudieron ser integrados al estudio debido a que no se cuenta con información genética para este marcador molecular a pesar de que en el GenBank está registrado la detección de material genético del parásito en muestras provenientes de anuros. Por lo que se podría escoger otro marcador molecular del cual haya más información disponible en las bases de datos para un rango mayor de ordenes de hospederos.

Finalmente, debido a que el marcador molecular 18S ARNr no es capaz de identificar en todos los casos al parásito a nivel de especie, es necesario probar este estudio con otros marcadores moleculares que podrían tener utilidad para reconstruir la filogenia de *Hepatozoon* sp. Por ejemplo, se pueden probar otros marcadores mitocondriales como el COI y COIII, o marcadores apicoplásticos como el 28S ARNr, en este último caso se ha reportado en la literatura que es un marcador molecular sensible para identificar las especies del parásito.

XI. Referencias Generales

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XII. ARTÍCULO I: A systematic review indicates that the 18S rRNA molecular marker is the most widely used molecular marker for the identification of isolates of the genus *Hepatozoon* sp.

Sustentante

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Manuscrito preparado con el formato para la revista:

Parasites and Vectors

A systematic review indicates that the 18S rRNA molecular marker is the most widely used molecular marker for the identification of isolates of the genus *Hepatozoon* sp.

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Abstract

Background: The genus *Hepatozoon* spp. is composed of parasitic protozoa belonging to the phylum Apicomplexa. These parasites have an obligatory heteroxenous life cycle that involves intermediate hosts that are either endothermic or ectothermic vertebrates and definitive invertebrate hosts such as ticks and mosquitoes. The identification of parasites to the species level remains challenging due to the fact that morphological are currently insufficient for achieving this goal. For this reason, alternative methods are being employed to identify and reconstruct the phylogeny of *Hepatozoon* sp., these include molecular biology techniques utilizing ribosomal and mitochondrial molecular markers.

Methods: A comprehensive search of *Hepatozoon* spp. sequences deposited in GenBank between 2013 and 2023 was carried out to determine which markers have been most frequently for this parasite. In addition, the species of *Hepatozoon* and the host in which such sequences were amplified were registered in a database. This database was visualized using a Sankey plot.

Results: In the last decade, a total of 3193 genetic sequences for 46 species of *Hepatozoon* sp. have been reported in GenBank. The 18S rRNA represents approximately 99% of the sequences used in the studies carried out to elucidate the phylogeny of this parasite.

Conclusions: This review demonstrates that the most utilized molecular marker for characterizing parasites of the *Hepatozoon* genus is the 18s rRNA. Also, the number of studies conducted on *Hepatozoon* in wildlife in various countries worldwide is disproportionate, which could potentially introduce bias in the true distribution of the parasite.

Keywords: molecular marker, *Hepatozoon*, 18S rRNA, species.

Introduction

The genus *Hepatozoon* spp. [1] is composed of parasitic protozoa belonging to the phylum Apicomplexa (suborder Adeleorina), which presents the apical complex that constitutes a synapomorphy that characterizes this phylum, formed by a conoid, the polar ring, the apical rings and the roptria that allow the parasite to penetrate and invade the cells of the host [2]. These parasites have an obligate heteroxenous life cycle that involves intermediate hosts that are either endothermic or ectothermic vertebrates [3] and definitive

invertebrate hosts such as ticks of the species *Amblyomma maculatum*, *Rhipicephalus sanguineus*, *Amblyomma ovale* and *Rhipicephalus* (*Boophilus*) *microplus* and the genus *Haemaphysalis* spp. [4], and mosquitoes of the genera *Culex* sp., *Aedes* sp. and *Anopheles* sp. [5].

The process of gametogenesis, fertilization, and sporogony, which culminates in the formation of the oocyst, the infecting form for the vertebrate host, occurs in the intestine or hemocele of the invertebrate host. In contrast, the formation of merozoites and gamonts occurs in the internal organs and neutrophils or monocytes of the vertebrate host, respectively [6]. The morphological description of the gamonts and the identification of their morphometric characteristics have been used to identify and describe the parasites of this genus [7, 8]. However, the inconsistency of these characteristics and the difficulty in describing them have hindered the identification of *Hepatozoon* sp. isolates down to the species level [6], consequently, the identification of parasites belonging to the suborder Adeleorina to the species level remains a challenging due to the fact that morphological and morphometric characteristics, as well as the impact of these parasites on host cells, are currently insufficient for achieving this goal [9].

At present, alternative methods are being employed to identify and reconstruct the phylogeny of parasites, these include molecular biology techniques utilizing ribosomal and mitochondrial molecular markers, which have even permitted the deciphering of cryptic species in certain instances [10]. These molecular markers are fragments of a specific region of the genetic material that allow the detection of variation or polymorphism among individuals in a population [11]. The selection of the molecular marker for the study of the phylogeny of parasites is important because if the molecular marker selected is not adequate,

it will generate unresolved phylogenetic topologies that will prevent the correct identification of taxa and therefore the discrimination of the different evolutionary scenarios in cophylogenetic studies [12].

For this reason, in the present study undertook a comprehensive search of the genetic sequences registered in the GenBank database of the National Center for Biotechnology Information, corresponding to the species within the *Hepatozoon* genus. This was undertaken with the objective of elucidating the molecular marker most frequently utilized by researchers in the field, with a view to informing the selection of a suitable molecular marker for the reconstruction of the phylogeny of the parasites, and the subsequent cophylogenetic studies based on these sequences.

Materials and Methods

Hepatozoon sequences deposited between 2013 and 2023 were mined from GenBank. The keyword "Hepatozoon" was used in the scientific search engines. The total number of *Hepatozoon* spp. sequences were counted to analyze which markers are the most employed for this parasite and thus, use them in subsequent analyses. In addition, the database included relevant epidemiological information for further analysis of the results, such the GenBank accession number of the parasite and host sequences, species of parasites, order, family and species of the host from which the isolate originates and the geographic location of the isolate (country and continent). Then, this database was visualized using a Sankey plot [13].

Results

In the last decade, a total of 3193 genetic sequences for 46 species of *Hepatozoon* sp. corresponding to different molecular markers such as COI, COIII, *cyt*B, ITS1, ITS2, long rRNA subunit, 28S rRNA, 5.8S rRNA and 18S rRNA have been reported in GenBank. The 18S rRNA represents approximately 99% of the sequences used in the studies carried out to elucidate the phylogeny of this parasite.

Europe is the continent with the highest number of sequences uploaded to GenBank, representing 25.93% of the total. The majority of these sequences originate from Portugal (138) and Germany (119). In second place is America, which represents 22.93% of the total. Many of these sequences come from Brazil (492) and the United States (101).

Most of these studies have focused on trying to isolate parasite genetic material from host samples belonging to the order Carnivora (1959 parasite sequences), followed by the order Squamata (435 parasite sequences), invertebrate hosts (334 parasite sequences) and order Rodentia (248 parasite sequences).

The majority of characterized sequences were from species within the order Carnivora (1489 sequences characterized out of 1959), followed by invertebrate hosts (270 sequences out of 334), the order Squamata (37 sequences characterized out of 435) and the order Rodentia (24 sequences characterized out of 248). Many parasites that were identified to species level were *Hepatozoon canis* (1261 sequences), however, approximately 41% of the genetic sequences were identified only to genus level (1294 *Hepatozoon* sp. sequences) (Fig. 1).

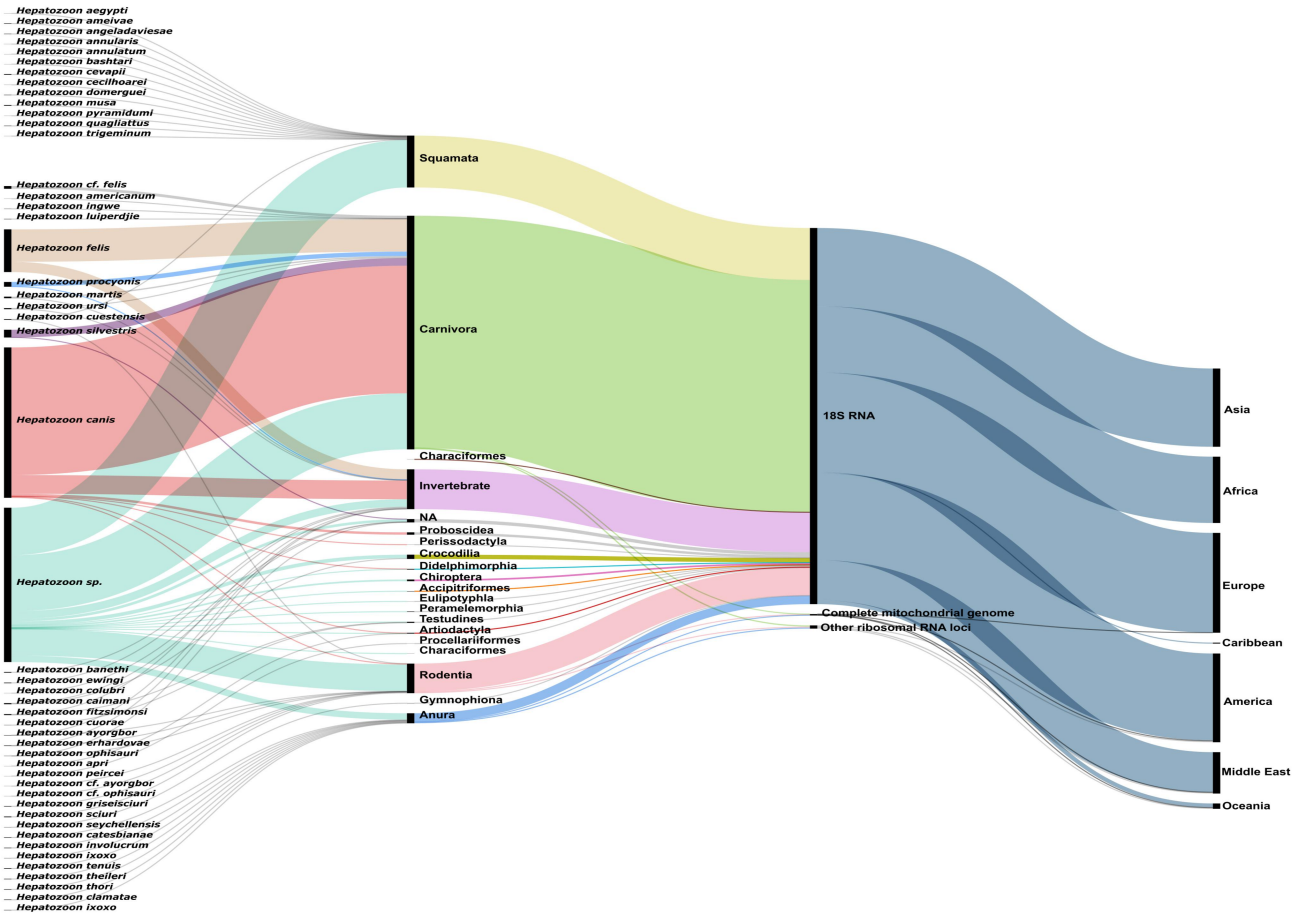

Fig. 1 *Hepatozoon* spp. sequences deposited in GenBank from 2013 to 2023 stratified according to species, associated hosts, molecular marker and geographical region.

Discussion

The review of *Hepatozoon* genetic sequences deposited in GenBank showed that the most used molecular marker is the 18S rDNA. This finding has practical implications due to the large number of sequences available for phylogenetic and diagnostic purposes and the sufficient differentiation at genus and species level provided by this marker [2]. However, the 18S rDNA is highly conserved [14-16], which partially explains why 40.5% of the sequences recorded in GenBank were identified only to the genus level. On the other hand, the complexity and expertise required to perform morphological assessment of parasites in blood and tissue samples contribute to the incomplete characterization of *Hepatozoon*.

Traditional identification techniques such as morphological description of intracellular gamonts often result in misidentification [2], and even fail to differentiate between morphometrically similar species such as *Hepatozoon clamatae* and *Hepatozoon catesbianae* [17]. Therefore, the integration of both microscopical and molecular methods should improve species assignation [18]. For instance, the use of mitochondrial genes like COI and COIII, and apicoplast markers such as 28S rRNA with higher interspecies resolution, may represent alternatives for parasite identification, as in some cases they have been successfully used to identify down to the species level [14, 17]. Therefore, the use of additional markers and more profound phylogenetic analyses would improve the discrimination between *Hepatozoon* spp. and identify haplotypes circulating in different vertebrate and invertebrate hosts [14, 16]. Consequently, this would allow the identification of specific parasite haplotypes affecting different host species in a geographical area and how these in turn participate in the transmission of the parasite to new susceptible hosts, as previously analyzed for *H. canis* [19].

Our data showed that most *Hepatozoon* sequences have been derived from the orders Carnivora and Squamata. This may be explained by the transmission routes of this parasite, which include the ingestion of infected invertebrate hosts, and its trophic transmission where predators feed on infected prey [20]. In the latter case, preys such as frogs, lizards and grey squirrels [21] may function as paratenic or first intermediate hosts that are infected with parasitic tissue cysts. For instance, it has been shown that saurophagous snakes, lizards and frogs can be infected with the same *Hepatozoon* spp. since the former species preys upon the latter [22, 23].

Also, the majority of *Hepatozoon* sequences were characterized mainly as *H. canis* followed by *H. felis*, from studies conducted in both the Americas and Europe. This finding is consistent with another review of cases published in the literature from the American continent, in which isolates from hosts of the order Carnivora and Rodentia were the most characterized, this includes both species, among others [24]. The majority of the evidence presented for Thomas et al. (2024) is derived from studies conducted in Brazil and the United States, as well as the present search. This represents a bias in true distribution of the different species of *Hepatozoon* sp. in the American continent. Consequently, there may be unidentified species of parasite circulating in wild animals. This considering that a large number of sequences registered in GenBank are identified only as *Hepatozoon* sp. This could present a challenge to wildlife conservation efforts, necessitating active monitoring of the geographic distribution of the species among hosts, evolutionary changes, and differences in virulence exhibited by each species [25].

Conclusions

This review demonstrates that the most utilized molecular marker for characterizing parasites of the *Hepatozoon* genus is the 18s rRNA. However, this marker does not appear to be the most optimal for this purpose, as a considerable number of sequences in GenBank remained unidentified at the species level. Consequently, it is imperative to assess the efficacy of alternative mitochondrial or apicoplastic molecular markers in facilitating the identification of the parasite. Furthermore, it was noted that the number of studies conducted on *Hepatozoon* in wildlife in various countries worldwide is disproportionate, which could potentially introduce bias in the true distribution of the parasite. This has important

implications for wildlife conservation, as the severity of hepatozoonosis in animals could vary depending on the species and host.

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XIII. ARTÍCULO II: Host switching is the main driver of coevolution between *Hepatozoon* parasites and their vertebrate hosts

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Parasites and Vectors

Host switching is the main driver of coevolution between *Hepatozoon* parasites and their vertebrate hosts

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Abstract

Background: *Hepatozoon* spp. are apicomplexan parasites with a heteroxenous life cycle, involving vertebrate intermediate hosts and invertebrate definitive hosts. This parasite genus infects a wide variety of wild and domestic vertebrates causing sub-clinical infection or mild to severe clinical manifestations, depending on the parasite species and vertebrate host. Interestingly, each *Hepatozoon* spp. has a specific host range suggesting a close host-parasite coevolutionary relationship.

Methods: we reconstructed the host and parasite phylogenies by using 18S rDNA and *cyt*B sequences, respectively. Subsequent analyses were stratified according to the host vertebrate orders Carnivora, Rodentia and Squamata, and the corresponding sequences of their *Hepatozoon* parasites. Then, Procrustean Approach to Cophylogeny (PACo) and ParaFit were employed to assess their global cophylogenetic relationships. In addition, eMPRess was used to estimate the frequency of co-evolutionary events, such as host switch, duplication, transfer or cospeciation.

Results: global assessments of congruence between phylogenies of carnivore, rodent and squamate hosts and those of their *Hepatozoon* parasites were significant (PACo: all $m^2_{XY} < 0.655$, all $p < 0.001$; ParaFit: all ParaFitGlobal Statistic < 72.992, all $p < 0.007$; all Procrustes $R^2 > 0.25$), but it was not for association between *Hepatozoon* spp. and invertebrates (PACo $m^2_{XY} = 0.632$, $p < 0.001$; ParaFitGlobal Statistic = 8.810, $p = 0.124$; $R^2 = 0.37$). The most significant links occurred between *Hepatozoon felis* and felid hosts or *H. canis* and canid hosts, but not between *Hepatozoon americanum* and domestic dog or coyotes. Moreover, eMPRess showed that the overall congruence between *Hepatozoon* spp. and vertebrate host phylogenies is mainly explained by host switching and less frequently cospeciation events.

Conclusions: these findings highlight the ability of *Hepatozoon* spp. associated to certain vertebrate orders to infect new and sympatric hosts. This in turn helps to understand the emergence of hepatozoonosis in susceptible hosts from specific geographical areas by spillover.

Keywords: protozoa, host, phylogenetics, cospeciation, coevolution, PACo, ParaFit, eMPRess.

Introduction

The genus *Hepatozoon* [1] comprises more than 330 parasite species within the phylum Apicomplexa [2, 3]. These parasites infect blood cells of vertebrate hosts, both domestic animals such as cats and dogs, and wild animals including amphibians, reptiles, birds, and a variety of carnivorous mammals [4, 5]. *Hepatozoon* spp. have a heteroxenous life cycle, with asexual multiplication occurring in the vertebrate intermediate host and sexual reproduction taking place in the invertebrate definitive host [6]. Ticks and mosquitoes are the main invertebrate hosts, but mites, triatomines, tsetse flies, biting lice, sand flies, and fleas can also transmit certain *Hepatozoon* spp. to vertebrate hosts [3, 6].

Hepatozoon spp. can be transmitted to the vertebrate intermediate hosts via ingestion of invertebrate hosts with oocysts, consumption of prey harboring infected ectoparasites or, in mammals, by licking their coat and swallowing infected ectoparasites [5], or alternatively by vertical transmission [7]. In addition, snakes can acquire the parasite by feeding on prey infected with *Hepatozoon* tissue forms, such as those found in frogs and lizards, which in turn serve as primary intermediate hosts [3, 8]. Interestingly, congenital transmission has also

been reported in a brood of the banded water snake, *Nerodia fasciata confluens* upon observing gamonts in their blood [9].

Hepatozoon spp. have a specific host range and usually lead to subclinical or mild infections, suggesting a close host-parasite coevolutionary relationship because the parasite can adapt to the host imposed challenges [10, 11]. For instance, *Hepatozoon felis* can infect both domestic and wild cats, such as *Felis catus, Felis silvestris*, *Panthera pardus*, and *Panthera leo* [12, 13], and in domestic cats it mostly leads to subclinical infections with a low parasitemia and intracellular gamonts in neutrophils and monocytes [14]. Moreover, *Hepatozoon canis* infects domestic dogs but wild canids, such as *Vulpes vulpes*, *Cerdocyon thous*, *Canis mesomelas*, *Canis aureus*, and *Lycaon pictus* have also been reported with this parasite [15, 16, 17, 18]. *Hepatozoon canis* infection is usually subclinical in domestic dogs, unless there is a high parasitemia or coinfection with other pathogens [19]. On the contrary, *H. americanum* induces a highly pathogenic disease with neutrophilia, muscle pain, and periosteal proliferation [20] and has been reported in domestic dogs as well as wild coyotes [21, 22]. Therefore, the coevolutionary history between some *Hepatozoon* spp. and their hosts is uncertain because it differs between taxonomic entities, with different levels of adaptation to their hosts.

Host-parasite coevolution can be analyzed by evaluating the congruence between host and parasite phylogenies (i.e., global fit methods) or by estimating the possible coevolutionary events that may account for the current host-parasite associations (i.e., event based methods) [23]. In the former, the phylogenies of hosts and parasites are tangled or superposed to evaluate to which extent the phylogeny of the host mirrors that of the parasite [24]. High congruence between both phylogenies can be explained by cospeciation, in which

parasites and their hosts undergo concomitant speciation due to their parallel divergence, leading to the emergence of new species for both [23, 25]. In addition to coespeciation, other coevolutionary events, such as host switching, duplication and loss, may also occur and their frequency can be estimated using event-based methods [26].

Both approaches rely on accurate phylogenetic reconstructions of the taxa under study. Thus, the availability of high-quality molecular data is critical. In the past, when DNA sequencing and phylogenetic analyses were not available, the description of *Hepatozoon* spp. was often based on morphological and host-related features [3]. However, with the advent of molecular techniques, several phylogenetic associations have been elucidated. Consequently, some species have been transferred to other haemogregarine genera, such as *Haemolivia* or *Haemogregarina,* and conversely, some hemogregarine species have been reclassified and transferred from other genera into *Hepatozoon* [3, 27]. Furthermore, several studies indicate that *Hepatozoon* is paraphyletic and consequently splitting into multiple genera has been proposed to ensure a more accurate representation of their evolutionary relationships [27, 28].

Unlike other vector-borne parasites, no cophylogenetic studies have been conducted to date to study the evolutionary history of *Hepatozoon* spp. and their various vertebrate and invertebrate hosts. In this study, we used both global-fit and event-based methods on estimated phylogenies to assess the cophylogenetic association between *Hepatozoon* spp. and their hosts and to identify the possible coevolutionary events shaping their shared evolutionary history. Through this analysis, we expect to obtain a deeper understanding of the ecological and evolutionary determinants of these host-pathogen interactions.

Materials and methods

Search strategy and selection criteria

The complete pipeline of the analysis is represented in Fig. 1. The small ribosomal subunit (18S) gene of *Hepatozoon* spp. was selected for building the parasite phylogenies with Bayesian inference (BI) since it has been widely used for genotyping. Likewise, the cytochrome B (*cyt*B) gene was selected for estimating the hosts phylogenies with BI due to extensive host representation and interspecific variation. The use of two different markers for phylogenetic comparison was necessary, as they were the only ones with sufficient representation in GenBank and adequate resolution to construct well-supported phylogenies for both the host and the symbiont. Accordingly, a second database was built with 18S rDNA sequences of *Hepatozoon* spp. (n=186), *cyt*B of vertebrate (n=153) and invertebrate (n=18) hosts in which the presence of parasite DNA was detected. Selected sequences were longer than 300 bp for both genes and were obtained from GenBank between 1998 and 2023 in FASTA format, whereas Cytochrome B sequences belonging to vertebrate hosts were grouped according to the orders Carnivora, Rodentia and Squamata.

Phylogenetic analyses

MEGA11 [29] and BEAST v2.6.7 [30] were employed to align and reconstruct the phylogenies of parasites and their hosts. Sequences were aligned with the MUSCLE algorithm [31] and unaligned ends were removed. Gaps were treated as missing data or as a fifth base. *Adelina bambarooniae* (AF494059.1) was selected as an outgroup for the parasite phylogenetic trees, *Tapirus bairdii* (JF718880.1) as an outgroup for the Carnivora host phylogenetic trees, *Caprolagus hispidus* (AY292719.1) for Rodentia host phylogenetic trees,

Sphenodon punctatus (AY426670.1) for Squamata host phylogenetic trees and *Hypsibius dujardini* (NC_014848.1) for invertebrate host phylogenetic trees. The best nucleotide substitution model was selected using JModelTest 2.1.10 based on the Akaike Information Criterion [32, 33]. Subsequently, phylogenetic trees were constructed with Bayesian inference (BI) running one Markov Chain Monte Carlo chains for with 10^6 and sampling tree topologies every 10^3 generations, with burning length of 10% using the BEAST package v2.6.7. Chain convergence and effective sample sizes were evaluated using Tracer v1.7.2 [34] and the information from the phylogenetic trees was summarized with TreeAnnotator v1.8.4 [35] discarding the first 10% generated trees. The phylogenetic trees were visualized using FigTree v1.4.4 [36].

Cophylogeny analyses

Two analyses were performed to estimate the congruence between parasite and hosts phylogenies using global fit methods: Procrustes Approach to Cophylogeny (PACo) [24] and ParaFit [41]. These methods generally indicate a cophylogenetic signal, meaning closely related parasites tend to be associated with closely related hosts, rather than strict phylogenetic congruence. However, in predominantly one-to-one interaction scenarios, patterns of cophylogenetic signal and phylogenetic congruence converge, such that they can be studied interchangeably [69]. Additionally, we employed an event-based method, eMPRess [42] to estimate the most probable coevolutionary events accounting for the current associations between *Hepatozoon* spp. and their hosts. Each of these analyses was performed on four different relationships between *Hepatozoon* spp. and the following host categories based on the available number of host-parasite associations: i) invertebrate hosts, ii)

vertebrate hosts of the order Carnivora, iii) vertebrate hosts of the order Squamata, and iv) vertebrate hosts of the order Rodentia.

To implement PACo [25], a unique code was assigned for each taxon of *Hepatozoon* spp. and their hosts (Additional File 1). The input data consisted of two phylogenetic trees, one for hosts and one for parasites in Newick format, and a binary matrix in text format coding the host parasite associations, where "1"s denote association between a given host species (in rows) and a parasite species (in columns) and "0"s indicate no association. PACo computed the patristic distances between each taxon, where the resulting distance matrices of the *Hepatozoon* spp. and their hosts were transformed into principal coordinate (PCo) matrices. Then, the *Hepatozoon*-PCo coordinates were superimposed onto the host PCo coordinates. Therefore, the global fit or phylogenetic congruence was controlled by the host phylogeny. To evaluate if the host and parasite phylogenies were independent, the Global Goodness-of-fit Statistic (residual sum of squares of the Procrustes superimposition, m^2_{XY}) was calculated and its significance was determined based on 10^3 replicates in which the host-parasite associations were randomized. The m^2_{XY} value is inversely proportional to the topological congruence between the host-parasite phylogenies. It is considered that the parasite´s phylogeny is significantly constrained by that of their hosts if the proportion of m^2_{XYs} computed in each of the 10^3 randomizations is less than the observed m^2_{XY} is ≤ 0.05. In additon, PACo was applied in symmetric mode (sym = TRUE). This mode allowed the value of m^2_{XY} to be expressed between 0 and 1, to calculate a Procrustes R^2 ($1 - m^2_{XY}$). Where a value of $R^2 > 0.25$ suggests an important cophylogenetic signal, but a value of R^2 close to 0 represent a low cophylogenetic signal [71]. Moreover, the contribution of each host-parasite association to the global fit was estimated by computing the individual square residual and

its 95% confidence interval using a jackknife procedure. PACo was implemented in R [37, 38] using ape [39] and vegan [40] packages.

Parafit is based on the fourth-corner problem [41]. Here it was implemented with the same input files as in PACo based on the patristic distance matrices generated from the phylogenetic trees [41]. These matrices were transformed to PCo ordinations, and the host and parasite PCo ordinations were crossed with the respective host-parasite associations. An additional matrix D of fourth-corner parameters was generated, which served to compute the ParaFitGlobal Statistic. Then, the contribution of each link to the global statistic was evaluated with the derived formula of ParaLink1. The significance of global fit and individual link contribution was assessed by randomizing the host-parasite association matrix. The analysis was performed in R [37, 38] with function parafit of the ape package, running 9,999 permutations with the Cailliez correction for negative eigenvalues.

In addition, we used the event based method eMPRess to estimate the most probable number of evolutionary events needed to account for the current host parasite associations given their host and parasite phylogenies [42]. The analysis was done in those host-parasite associations where significant congruence was obtained either by the PACo or ParaFit approaches, i.e. in Carnivora-*Hepatozoon*, Rodentia- *Hepatozoon,* Squamata-*Hepatozoon* and invertebrate host-*Hepatozoon*. In this analysis, costs of loss were constant at 1.00, cospeciation at 0.00, while costs of duplication and transfer were assigned according to the Costscape plot (Table 1). These two values were chosen according to: i) the histogram section where the highest number of most parsimonious resolutions (MPRs) was obtained and ii) the lowest duplication and transfer values in this histogram section. Phylogenetic trees in Newick format and the host-parasite association matrix in .mapping format were used as input data.

Then, superimposed host and parasite phylogenetic trees were graphed with possible coevolutionary solutions.

Results

Phylogeny of Hepatozoon spp*. and their hosts*

The phylogeny of *Hepatozoon* spp. was reconstructed using 175 18S rDNA sequences belonging to 39 species (Fig. 2). These sequences were retrieved from 153 vertebrate hosts as annotated in GenBank: 57 from the order Squamata, 44 from the order Carnivora, 25 from the order Rodentia and 27 from other vertebrates (13 orders including Anura, Accipitriformes, Procellariiformes, Testudines, Gumnophiona, Chiroptera, Artiodactyla, and Lagomorpha). *Hepatozoon* sequences were also obtained from 20 invertebrate hosts: 18 from the family Ixodidae, 1 from the family Culicidae and 1 from the family Ctenophthalmidae. Three clusters of parasite species associated with the orders Carnivora, Squamata and Rodentia could be defined (Fig. 2). For this parasite tree, 36% of the nodes presented a bootstrap value ≥ 0.75. For the hosts, 81% of nodes from the Carnivora tree (Anexo 1), 100% of nodes from the Rodentia tree (Anexo 3), 87% of nodes from the Squamata tree (Anexo 2), and 89% of nodes from the invertebrate tree (Anexo 4) exhibited a bootstrap value ≥ 0.75.

Hepatozoon-Carnivora associations

PACo analysis of *Hepatozoon* spp. associated to hosts of the order Carnivora revealed a significant cophylogenetic relationship ($m^2_{XY} = 0.520$, $p < 0.001$, $R^2 = 0.48$). (Fig. 3A). Most sequences derived from carnivore hosts were uncharacterized (29.2%, n = 19), followed by *H. felis* (27.7%, n = 18), *H. canis* (20%, n = 13) and *H. ursi* (7.7%, n = 5). The 95% confidence of squared residuals of 16 host-parasite associations were below the median

square residual value (Fig. 3A), with ten of them corresponding to the family Felidae, five to Canidae and one to Procyonidae. The associations between *Hepatozoon* spp. and Felidae included those between *Hepatozoon silvestris* with the domestic cat *Felis catus* and the wildcat *Felis silvestris;* and *H. felis* with the leopard cat *Prionailurus bengalensis,* the jaguar *Panthera onca* and the tiger *Panthera tigris*. The links associated with the family Canidae included associations between uncharacterized *Hepatozoon* sp. sequences and the side-striped jackal *Lupulella adusta* (syn. *Canis adustus*) and the black-backed jackal *L. mesomelas*.

The highest squared residual corresponded to the cat *Felis catus* (FcatMN96) and *Hepatozoon silvestris* (HsilKY45) and the lowest concerned the hoary fox *Lycalopex vetulus* (LvetKU33) and *H. canis* (HcanAY67), and the Pampas fox *Lycalopex gymnocercus* (LgymAF53) and *H. canis* (HcanKX58). The PCo superposition plot of *Hepatozoon* spp. and Carnivora suggest three clusters corresponding to Felidae, Canidae and Mustelidae (Fig. 3B).

The Parafit function mirrored the results obtained with PACo since a significant cophylogenetic relationship was obtained (ParaFitGlobal Statistic = 72.992, p = 0.001) (Figure 3A). Furthermore, 54 significant host-parasite associations were considered to contribute significantly to the cophylogenetic pattern. The individual contributions of these associations to the ParaFitGlobal Statistic indicated that the congruence between phylogenies was accounted for mostly by two families: Canidae with 16 significant links out of 20, and Felidae with 24 significant links out of 28 (Fig. 3A). The lowest ParaFitLink1 value was between the racoon *Procyon lotor* (PloAB37) and *Hepatozoon sp.* (HspJF43) (F1 = 0.550*, p* = 0.032*)* and the highest was observed between the wolf *Canis lupus* (ClupJQ58) and *H. canis* (HcanMN89) (F1 = 4.950, p = 0.001). Interestingly, none of the two global fit tests

revealed significant associations between *H. americanum* with the domestic dog *Canis lupus familiaris* and the coyote *Canis latrans*.

eMPRess determined that host switching (n = 45) was the most frequent coevolutionary event, followed by cospeciation (n = 18) and duplication (n = 1) (Fig. 3C). For instance, host switches of *Hepatozoon* sp. (HspKJ88) were predicted from *Vulpes rueppelli* to *Panthera leo* and *P. tigris*. The resemblance between these two *H. felis* sequences was also observed in the *Hepatozoon* phylogenetic tree (Fig. 2). In addition, *H. felis* (HfelHQ44) from *Panthera pardus fusca* may have switched to *Phantera leo persica*, and a *Hepatozoon* sp. (HspJF43) of *Procyon lotor* to *Vulpes pallida*. Cospeciation was predicted between *H. canis* (HcanAY67/HcanKX58) with *Lycalopex gymnocercus* and *Lycalopex vetulus*, and between *Hepatozoon* sp. (HspJF43) and *Hepatozoon procyonis* (HproMK24) from *P. lotor* and *Nasua nasua*.

Hepatozoon-Rodentia associations

PACo analysis of the *Hepatozoon* spp. sequences obtained from rodent hosts revealed a significant cophylogenetic relationship ($m^2_{XY} = 0.530$, $p < 0.001$, $R^2 = 0.47$). The 95% confidence of squared residuals of five host-parasite associations corresponding to the family Cricetidae was below the median square residual value (Fig. 4A). Sixty-six percent (n = 20) of the links registered for rodent hosts were with uncharacterized *Hepatozoon* sequences, followed by *Hepatozoon ayorgbor* (10%, n = 3), *H. sciuri, H. ophisauri, H. griseisciuru, H. americanum*, *H. canis*, and *H. erhardovae* (all 3.33%, n = 1). The highest squared residual occurred in the association between the hispid cotton rat *Sigmodon hispidus* (ShisEU78) and

H. americanum (HameEU93) and the lowest was the lowland paca *Cuniculus paca* (CpacMW32) associated with an uncharacterized *Hepatozoon* sp. (HspKY04).

ParaFit also revealed a significant global cophylogenetic relationship with 10 significant links (ParaFitGlobal Statistic = 9.635, p = 0.004) (Figure 4A). The highest ParaFitLink1 was for the association between the lowland paca *Cuniculus paca* (CpacMW32) and *Hepatozoon* sp. (HspKY04) (F1 = 2.950, p = 0.006) and the lowest one was between the wood mouse *Apodemus sylvaticus* (AsylAJ05) and *H. ayorgbor* (HayoKT77) (F1 = 0.288, p = 0.031). The individual contribution to the ParaFitGlobal Statistic through the ParaFitLink1 values of the order Rodentia indicated that the congruence between phylogenies was mostly accounted for by the family Muridae with five significant links out of 10 (Fig. 4A). Principal coordinates (PCo) plots for the parasite associated with hosts of the order Rodentia suggest a single well-differentiated cluster corresponding to the families Muridae and Cricetidae (Fig. 4B).

The eMPRess analysis pointed to host switching (n = 15) as the most frequent event occurring between *Hepatozoon* spp. and Rodentia, followed by coespeciation (n = 11) and duplication (n = 3) (Fig. 4C). Host switching was observed between an uncharacterized *Hepatozoon* sp. (HspKU08) from the black-footed colilargo *Oligoryzomys nigripes* to the large vesper mouse *Calomys callosus*. Furthermore, coespeciation was predicted in an uncharacterized *Hepatozoon* sp. sequence (HspJF36/HspJF38) found in the woodrat species *Neotoma micropus* and *Neotoma fuscipes,* and between *H. ayorgbor* (HayoKT77) and *Hepatozoon* sp. (HspKU50) from the wood mice *Apodemus sylvaticus* and *Apodemus flavicollis*.

Hepatozoon-Squamata associations

PACo revealed a significant cophylogenetic relationship ($m^2_{XY} = 0.655$, $p < 0.001$, $R^2 = 0.35$). However, only the 95% confidence of squared residuals of two host-parasite associations were below the median square residual value, which had a significant ParaFitLink1 value (Figure 5A). These two associations corresponded to an uncharacterized *Hepatozoon* sp. (HspJF42) and *Boa constrictor* (BconEU64) and an uncharacterized *Hepatozoon* sp. (HspKJ25) and *Naja haje* (NhajAY94). Most of the associations within Squamata concerned the families Colubridae (31.7%, n = 19), Lacertidae (20%, n = 12) and Gekkonidae (13.3%, n = 8). Moreover, most Squamata-derived sequences were distributed across two clusters in the phylogenetic tree of *Hepatozoon* spp., which were separated by a group of *Hepatozoon* spp. associated to Rodentia (Fig. 2). One of these clusters included some other sequences derived from different orders, such as Anura, Chiroptera, Peramelemorphia and Gymnophiona. The highest squared residual was noted for the link between the Wright's skink *Trachylepis wrightii* (syn. *Mabuya wrightii*). (MwriAF35) and an uncharacterized *Hepatozoon* sp. (HspHQ71) and the lowest squared residuals corresponded to an association between *Boa constrictor imperator* (BconEU64) and *Hepatozoon* sp. (HspJF42). Principal coordinate plots for the parasite associated with hosts of the order Squamata suggested three clusters belonging to the families Lacertidae, Colubridae and Gekkonidae (Figure 5B).

A significant cophylogenetic relationship was also revealed by ParaFit (ParaFitGlobal Statistic = 55.033, $p = 0.001$) and 44 host-parasite associations were found to contribute significantly to ParaFitGlobal Statistic (Figure 5A). The highest ParaFitLink1 was estimated for the link between the lizard *Gallotia galloti* (GgalAM84) and an uncharacterized

Hepatozoon sp. (HspMG48) (F1 = 4.357, $p = 0.001$) and between the lizard *Gallotia caesaris* (GcaeMN63) and an uncharacterized *Hepatozoon* sp. (HspMG47) (F1 = 4.357, $p = 0.001$). The lowest ParaFitLink1 value concerned the Caspian whipsnake *Dolichophis caspius* (DcasHM88) and *Hepatozoon* sp. (HspKJ13) (F1 = 1.431, $p = 0.005$).

The eMPRess analysis positioned most coevolutionary events at higher taxonomic levels. Accordingly, host switching (n = 48) was the most frequent event predicted between the *Hepatozoon* spp. and their Squamata hosts, followed by coespeciation (n = 10) and duplication (n = 1) (Fig. 5C) (Table 1). Host switching was calculated from an uncharacterized *Hepatozoon* sp. (HspJX32) from the wall lizard *Podarcis bocagei* to the dwarf lizard *Atlantolacerta andreanskyi* or from *H. musa* (HmusMF67) of the rainbow boa *Epicrates crassus* to the rattlesnake *Crotalus durissus*. Moreover, cospeciation was predicted in *H. boiga* (HboiAF85) and an uncharacterized *Hepatozoon* sp. sequence (HspKJ12) with *B. irregularis* and the herald snake *Crotaphopeltis hotamboeia,* respectively.

Hepatozoon- Invertebrate associations

PACo analysis of *Hepatozoon* spp. associated to invertebrate hosts revealed a significant cophylogenetic relationship ($m^2_{XY} = 0.632$, $p < 0.001$, $R^2 = 0.37$) with five links displaying a 95% confidence of squared residuals below the median square residual value (Figure 6A). Most confirmed associations were retrieved from the tick genus *Rhipicephalus* (15%, n = 3), followed by the genus *Amblyomma* (5%, n = 1), and *Dermacentor* (5%, n = 1). The highest squared residual occurred in the association between *Ixodes ricinus* (IricMT60) and *H. canis* (HcanKU35) and the lowest was recorded for the association between *Rhipicephalus turanicus* (RturMT63) and *H. canis* (HcanMN26). A global non-significant

cophylogenetic relationship also was revealed by the ParaFit analysis (ParaFitGlobal Statistic = 8.810, p = 0.124). Only two links were significant, namely between an uncharacterized *Hepatozoon* sp. sequence (HspJQ02) and the mosquito *Aedes taeniorhynchus* (AtaeMN42) (F1 = 2.992, p = 0.014), and between *H. erhrardovae* (HerhKJ66) and the flea *Ctenophthalmus agyrtes* (CagyKM37) (F1 = 2.960, p = 0.016).

The individual contribution to the Global Goodness-of-fit Statistic (m^2_{XY}) through the squared residual of the parasite-host links indicated that the congruence between phylogenies was given by several tick genera, e.g. *Rhipicephalus*, *Amblyomma* and *Dermacentor*. But none of the links of these ticks with *Hepatozoon* spp. were supported with a significant ParaFitLink1 result (Fig. 6A). Finally, principal coordinate (PCo) plots for the parasite associated with Invertebrate hosts did not show specific clusters (Fig. 6B).

The eMPRess analysis determined that host switching (n = 13) was the most frequent event occurring between *Hepatozoon* spp. and invertebrate hosts, followed by coespeciation (n = 6) (n = 5) (Fig. 6C). For example, host switching was predicted in *H. erhardovae* (HerhKJ66) from the flea *Ctenophthalmus agyrtes* to the tick *Rhipicephalus simus*.

Discussion

More than 300 *Hepatozoon* spp. have been described with the use of molecular and morphological methods in a wide range of wild and domesticated animals [28]. Importantly, these apicomplexan parasites are usually non-pathogenic or cause disease with only mild clinical signs [11], This may be suggestive of a long coevolutionary relationship with their hosts, given the relatively low pathogenicity of these parasites or their adoption of transmission strategies that may not favor high virulence. In this study, the hypothesis of

coevolution was tested by using patristic distances of phylogenies obtained from *Hepatozoon* spp. associated to vertebrate and invertebrate hosts of several orders.

Most GenBank sequences identified to species level corresponded to *H. canis* likely due its widespread distribution in domestic animals [43]. The geographical dispersion of this species may be related to the geographical movement of dogs associated to human migrations, since little genetic differentiation of haplotypes has been observed between *H. canis* populations around the world [43]. According to the event-based analyses performed in this study, *H. canis* seems to be a parasite that has undergone frequent host switches among hosts within the family Canidae. Moreover, wild canids may be reservoirs of this parasite species due to its relatively benign course of infection [44].

The order Carnivora seem to have sustained a coevolutionary relationship with their associated *Hepatozoon* spp., mainly with *H. felis* and *H. canis.* Most significant links or associations were obtained with *H. felis* and their hosts within the Felidae, and between *H. canis* and hosts within Canidae, which may explain the subclinical infection associated with feline and canine hepatozoonosis [11, 45]. Importantly, the links between *C. lupus familiaris* and *C. latrans* with *H. americanum,* one of the most pathogenic *Hepatozoon* spp., were not found significant. They showed one of the highest squared residual and the event-based analysis, suggesting a recent switch to these hosts. Therefore, *H. americanum* may be poorly adapted to domestic dogs and coyotes probably due to their recent association. Even though it has been shown that coyotes naturally infected with *H. americanum* may not present a severe clinical picture with characteristic bone lesions [21], the global-fit analyses showed a non-significant association [46].

An *Hepatozoon* sp. associated with the raccoon *P. lotor* showed a significant coevolutionary relationship with this host. We note that this sequence was identical to *H. procyonis*, a species occurring in different procyonids from the Americas [47, 48]. In contrast, neither PACo nor ParaFit supported a coevolutionary association between *H. procyonis* and the coati *Nasua nasua*. Nevertheless, eMPRess predicted cospeciation events between this *Hepatozoon* sp. (i.e., most likely *H. procyonis*) and *P. lotor*, and between *H. procyonis* and *N. nasua.* The close phylogenetic relationship between the two hosts [49] may account for this pattern.

The phylogenetic tree of *Hepatozoon* sequences derived from hosts of the order Squamata consisted of two polyphyletic groups separated by a monophyletic cluster formed by *Hepatozoon* from rodents. In addition, sequences obtained from other host orders were intermixed with one of the two clusters, thus suggesting that rodents, bats or frogs may serve as first intermediate hosts of *Hepatozoon,* which has been explored experimentally with *H. ayorgbor* [50]. In addition, all cospeciation events predicted between *Hepatozoon* obtained from squamates occurred among closely related host species. This indicates that coevolution between these apicomplexan parasites with snakes, geckos, or other lizards is probably recent. However, coevolution is favored by long-term interactions and adaptations, as in the case of the vector-borne bacteria *Bartonella* when compared to non-vector transmitted *Leptospira* spp. [51]. Therefore, a time-calibrated phylogenetic tree would be needed to test this hypothesis rigorously.

Host switches need to be interpreted with caution, since the detection of *Hepatozoon* DNA in a host´s blood does not necessarily imply that the animal is an intermediate host and part of the parasite´s life cycle. For instance, a host switch of *Hepatozoon* sp. was predicted

from *P. lotor* to the pale fox *V. pallida.* This link between *Hepatozoon* sp. with *V. pallida* was not significant according to PACo and the ParaFit function, however PACo does not strictly test significance of host-parasite links. Therefore, the interpretation of coevolutionary events should consider both cophylogenetic results and event-based predictions. This apparent host switch may be explained by a pale fox [52] preying on an invertebrate host infected with a *Hepatozoon* sp. originally associated with a raccoon.

Phylogenies between *Hepatozoon* spp. and hosts of the orders Carnivora, Rodentia and Squamata were found to be globally congruent, which may explain why *Hepatozoon* spp. cause subclinical or mild infections in their hosts from these orders [2, 53]. Clinical signs associated to *Hepatozoon* infections are well documented in domesticated animals, but, in wildlife this protozoa is usually found without associated disease, such as *Hepatozoon apri* in Japanese wild boars [54], an uncharacterized *Hepatozoon* sp. in giant pandas [67] or causing mild blood cell alterations, like dehaemoglobinisation or karyolysis produced by *Hepatozoon angeladaviesae* in *Philothamnus* snakes [56]. The mild clinical presentation associated to hepatozoonosis may be due to the evolutionary dependence of the parasite with its host, since a balance is established between the parasite's attack and the host's immune defense [57, 58]. *Hepatozoon* spp. can therefore be well adapted to their hosts, i.e. the little damage inflicted to its hosts could hypothetically translate into an advantage for the parasite into effective transmission to the next host [59].

Global phylogenetic congruence was obtained between *Hepatozoon* sequences associated to invertebrate hosts by PACo, but not with the ParaFit function. While the reasons for this discrepancy are unclear, in some situations PACo tends to be more anticonservative than ParaFit [24], PACo is more prone to making type I error or is more likely to refuse a

true null hypothesis, which would translate as a false congruence between phylogenies. However, the R^2 values supports these findings since it suggests that there is congruence between the phylogenies. In addition, a non-significant association should be expected because the invertebrate host acts as a vector. In this case, host switching could be advantageous for enhancing parasite transmission, particularly when there is no strong physiological dependence of the parasite upon the intermediate host. However, it should also be noted that the low number of *Hepatozoon* sequences derived from invertebrate hosts in the study makes it difficult to draw reliable conclusions.

Hepatozoon spp. have the potential to cause emerging diseases in wildlife animals [60], which conforms with the frequent host-switch events observed in this study. Generally, when incongruence is observed between the parasite and host phylogenies, it is expected to result from evolutionary events such as host switch, duplication, or loss, on the contrary, a high level of cospeciation contributes to congruence between phylogenies of hosts and parasites [61]. Host switching in *Hepatozoon* may be favored by high parasite dispersal due to the wide distribution of invertebrate and vertebrate hosts [62, 63, 64]. Even more so, the invertebrate hosts associated to *Hepatozoon* spp. usually exhibit a generalist feeding behavior [25, 65] and therefore, which would facilitate host switch [63]. In addition, *Hepatozoon* spp. tend to infect vertebrate hosts of the same taxonomic family, as evidenced in our results. However, some authors argue that the vertebrate hosts ecology is more important than their phylogeny in determining whether a host is susceptible to infection by a particular species [8, 70]. If prey and predators are exposed to the same habitat they will be exposed to the same infected vectors and could become infected by the same species [52].

Conclusions

The present study demonstrated that the phylogeny of *Hepatozoon* spp. is overall congruent with the phylogeny of its vertebrate hosts. However, host switching has been a recurring event in shaping the evolutionary history of this host-parasite system. This finding is important to better understand the emergence by spillover of hepatozoonosis in new susceptible host species in specific geographical areas [66, 67]. Furthermore, this insight facilitates the development of conservation strategies by identifying potential reservoirs of the disease [68]. However, the present study did not evaluate other factors limiting host switching, such as geographical barriers or the proximity required between the new host and the original one to enable host switching. Thus, future research should study the phylogeography of other *Hepatozoon* spp. In particular, further work is required to identify whether predicted haplotypes are differentially distributed according to their geographical origin and which of them are prone to infect hosts related to those already described. Furthermore, the present study used two distinct genes to compare phylogenies of parasites and their hosts, phylogenetic inference could vary if different genes were analyzed, as evolutionary histories may differ among loci. Therefore, future studies should assess the effect of the molecular marker on the robustness and accuracy of cophylogenetic studies of *Hepatozoon*.

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Author contributions

RM-S, GB and AR conceived the study, AS-B, FV-B and VM-H provided ideas for materials and methods, JB and ML-R wrote the code for PACo and provided methodological support, RM-S ran all analyses, RM-S and AR analyzed the data and wrote the original draft, AR supervised the study and provided the funds, all co-authors read and approved the final version of the manuscript.

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Table 1. Statistics for the evaluation of congruence between parasite-host phylogeny, DTL model event costs, frequencies of specific evolutionary events involved, and the best nucleotide substitution model for reconstruction of parasite and host phylogeny.

Order	Global Goodness-of-fit Statistic m^2_{XY}	*p*-value[1]	R^2	ParaFitGlobal Statistic	*p*-value[1]	Cost	Coespeciation	Duplication	Transfer (host switch)	Loss	Best Nucleotide Substitution Model (parasite)	Best Nucleotide Substitution Model (host)
Carnivora	0.520	<0.001	0.480	72.992	0.001	D: 1.06, T: 1.01, L: 1.00	15	1	48	6	HKY+I+G	GTR+I+G
Rodentia	0.530	<0.001	0.470	9.635	0.004	D: 0.54, T: 0.53, L: 1.00	9	3	17	0	TPM3uf+G	TIM3+I+G
Squamata	0.655	<0.001	0.345	55.033	0.007	D: 0.53, T: 1.0, L: 1.00	10	1	48	0	TPM1uf+G	GTR+I+G
Invertebrate	0.632	<0.001	0.368	8.810	0.124	D: 2.08, T: 4.08, L: 1.00	8	1	10	11	HKY+G	GTR+I+G

1. *p*-value at a significance level of 0.05.

D: event cost for cospeciation.

T: event cost for transfer.

L: event cost for loss.

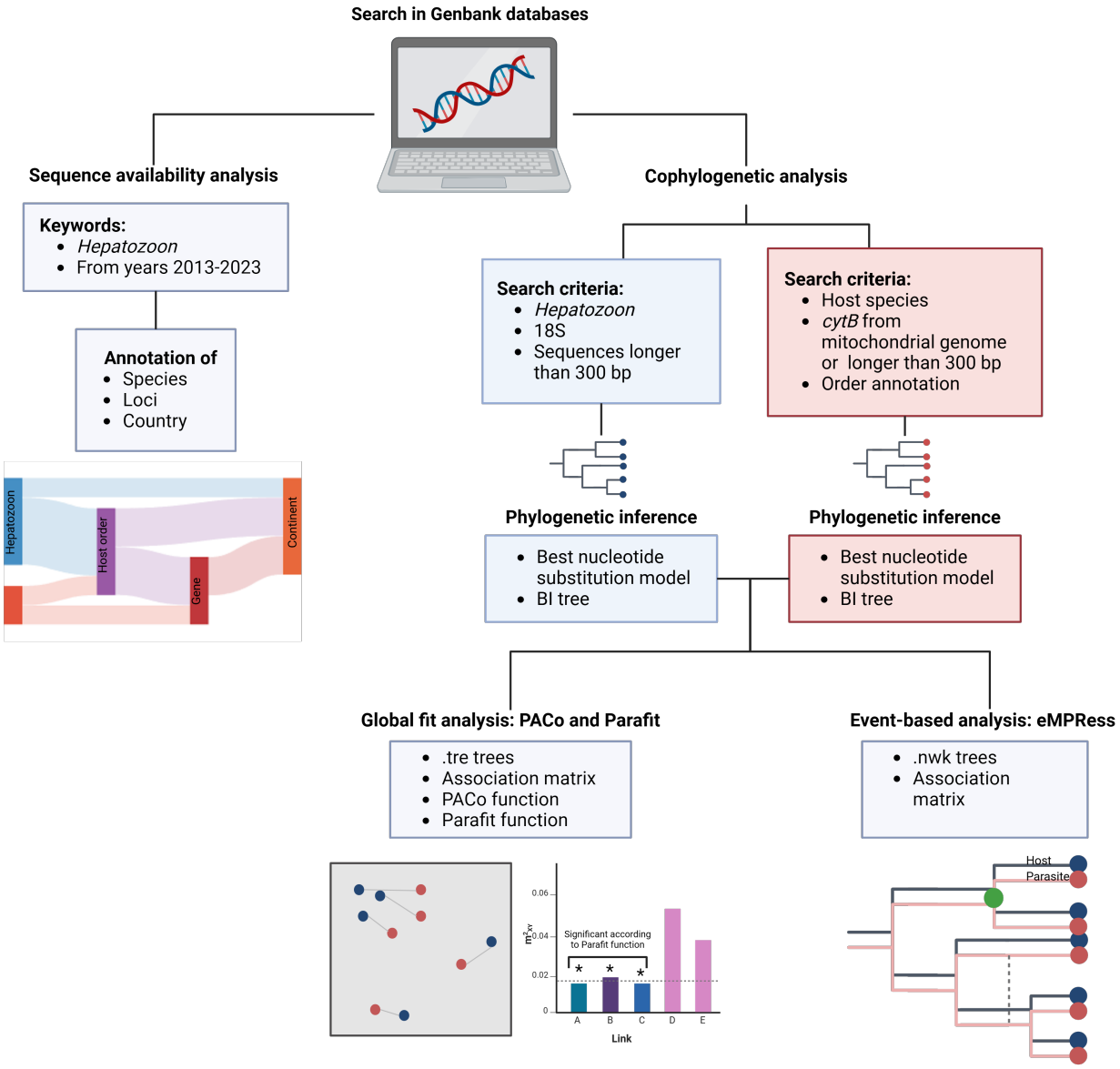


Fig. 1 Pipeline of the analysis ran in the present study.

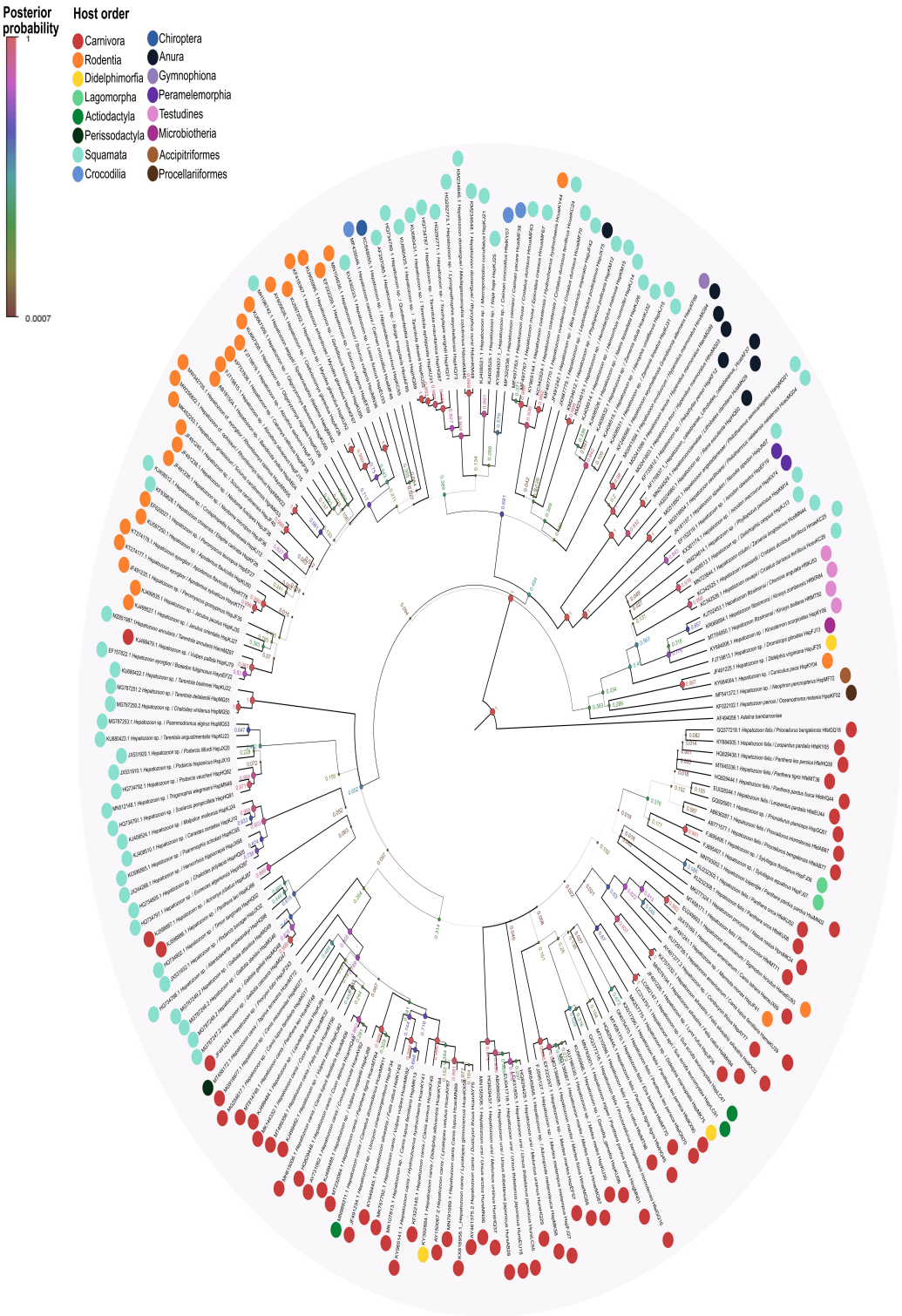


Fig. 2 Bayesian inference phylogenetic tree of *Hepatozoon* spp. 18S rDNA sequences. Posterior probability values are denoted with node size and color. The associated host order is indicated next to each sequence.

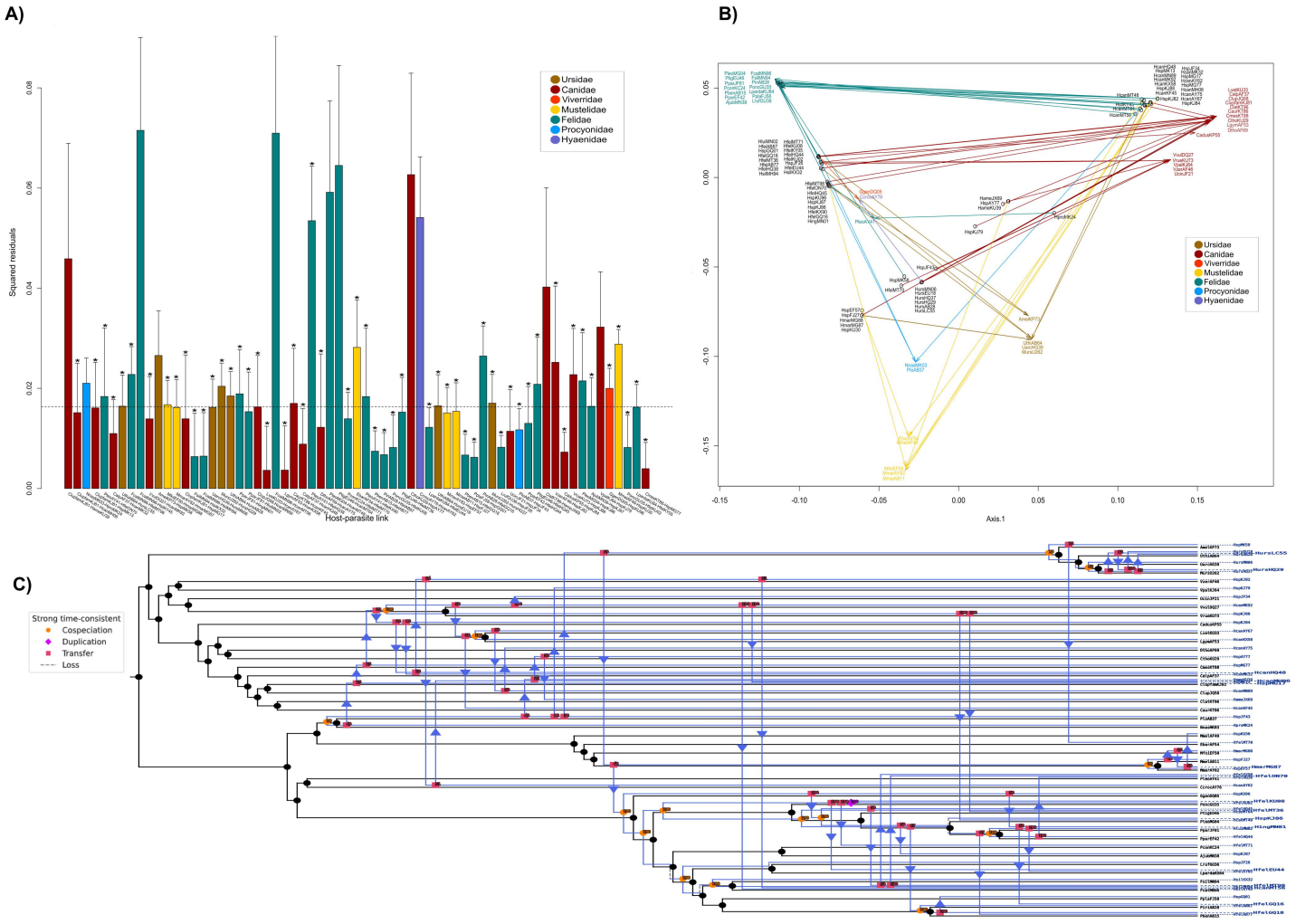


Fig. 3 Global-fit and event-based cophylogenetic analysis between *Hepatozoon* and its carnivore hosts. A. Contribution of each *Hepatozoon*-vertebrate host link to the global phylogenetic congruence. Each bar represents the squared residual of each association, and are color-coded according to the host family. Error bars correspond to 95% confidence intervals of the squared residuals. The median squared residual is indicated as a dotted line. Asterisks at the top of each bar represent a significant ParaFitLink1 value. B. Procrustean superimposition plot between the principal coordinates derived from patristic distances of the 18S of *Hepatozoon* spp. and their carnivore host phylogenies. Each parasite and host are denoted as circles and arrow heads, respectively. The lines are color-coded according to the host family. C. Coevolutionary reconstruction of the host (black lines) and parasite (blue lines) phylogenies with the lowest global cost according to eMPRess.

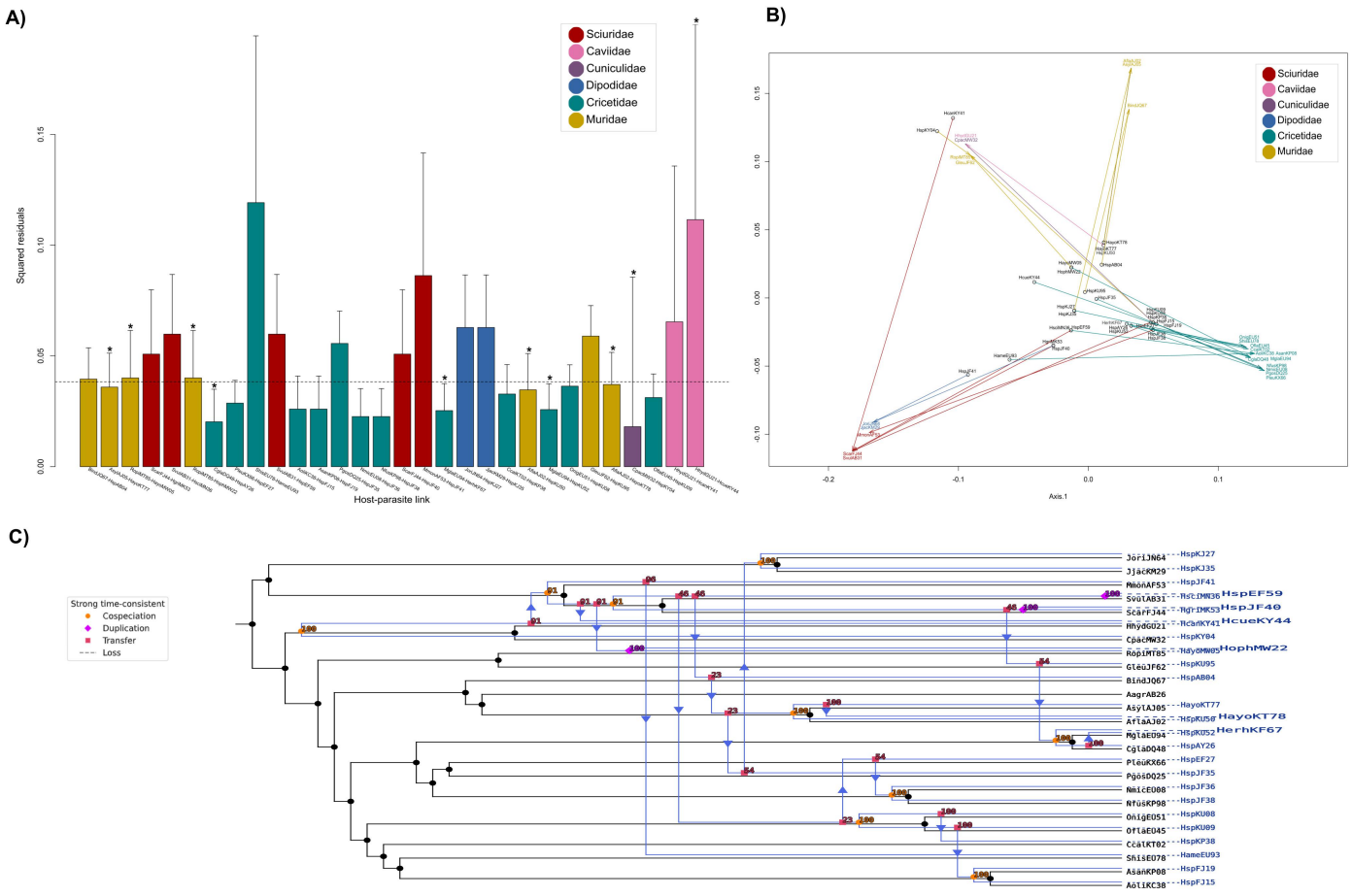


Fig. 4 Global-fit and event-based cophylogenetic analysis between *Hepatozoon* and its rodent hosts. A. Contribution of each *Hepatozoon*-vertebrate host link to the global phylogenetic congruence. Each bar represents the squared residual of each association, and are color-coded according to the host family. Error bars correspond to 95% confidence intervals of the squared residuals. The median squared residual is indicated as a dotted line. Asterisks at the top of each bar represent a significant ParaFitLink1 value. B. Procrustean superimposition plot between the principal coordinates derived from patristic distances of the 18S of *Hepatozoon* spp. and their rodent host phylogenies. Each parasite and host are denoted as circles and arrow heads, respectively. The lines are color-coded according to the host family. C. Coevolutionary reconstruction of the host (black lines) and parasite (blue lines) phylogenies with the lowest global cost according to eMPRess.

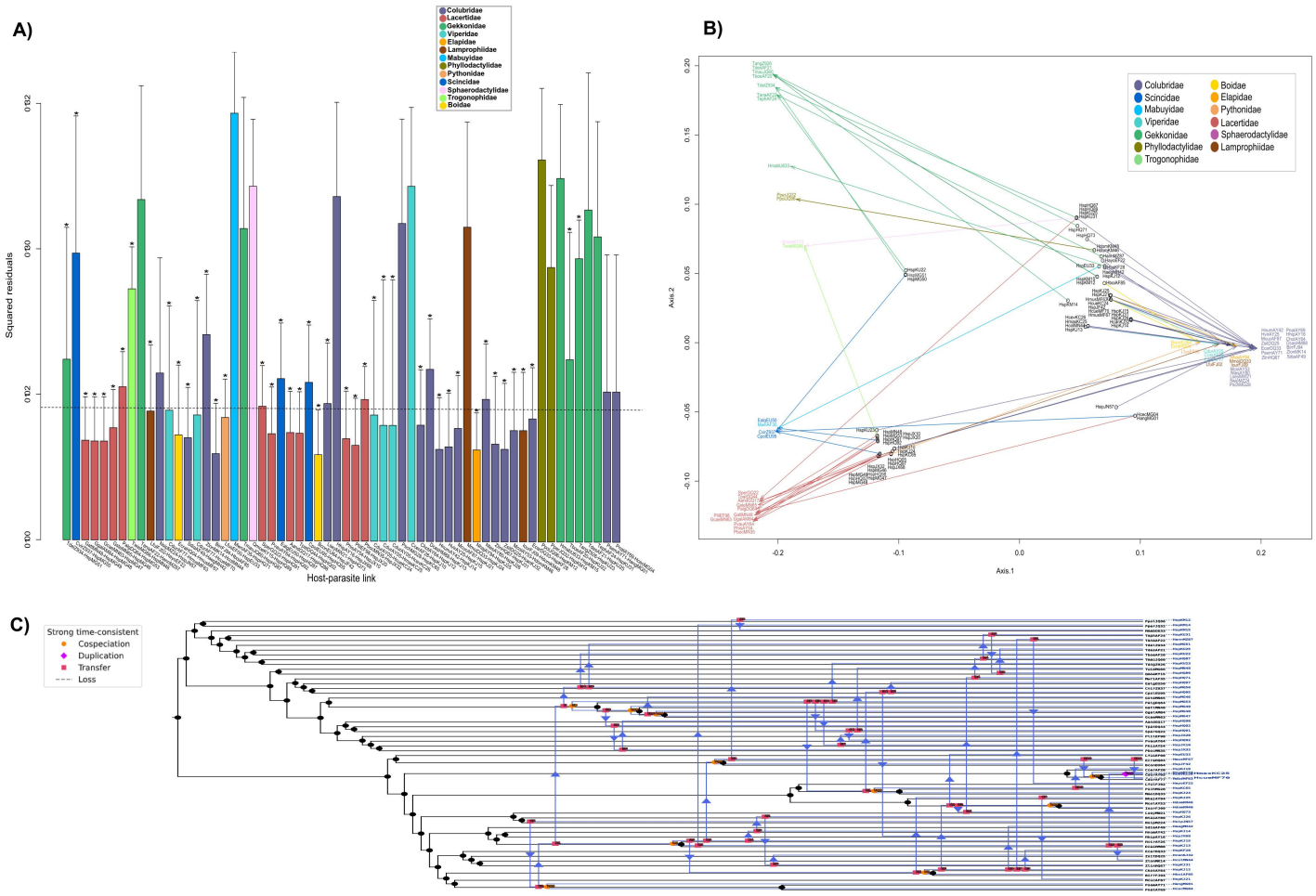


Fig. 5 Global-fit and event-based cophylogenetic analysis between *Hepatozoon* and its squamate hosts. A. Contribution of each *Hepatozoon*-vertebrate host link to the global phylogenetic congruence. Each bar represents the squared residual of each association, and are color-coded according to the host family. Error bars correspond to 95% confidence intervals of the squared residuals. The median squared residual is indicated as a dotted line. Asterisks at the top of each bar represent a significant ParaFitLink1 value. B. Procrustean superimposition plot between the principal coordinates derived from patristic distances of the 18S of *Hepatozoon* spp. and their squamate host phylogenies. Each parasite and host are denoted as circles and arrow heads, respectively. The lines are color-coded according to the host family. C. Coevolutionary reconstruction of the host (black lines) and parasite (blue lines) phylogenies with the lowest global cost according to eMPRess.

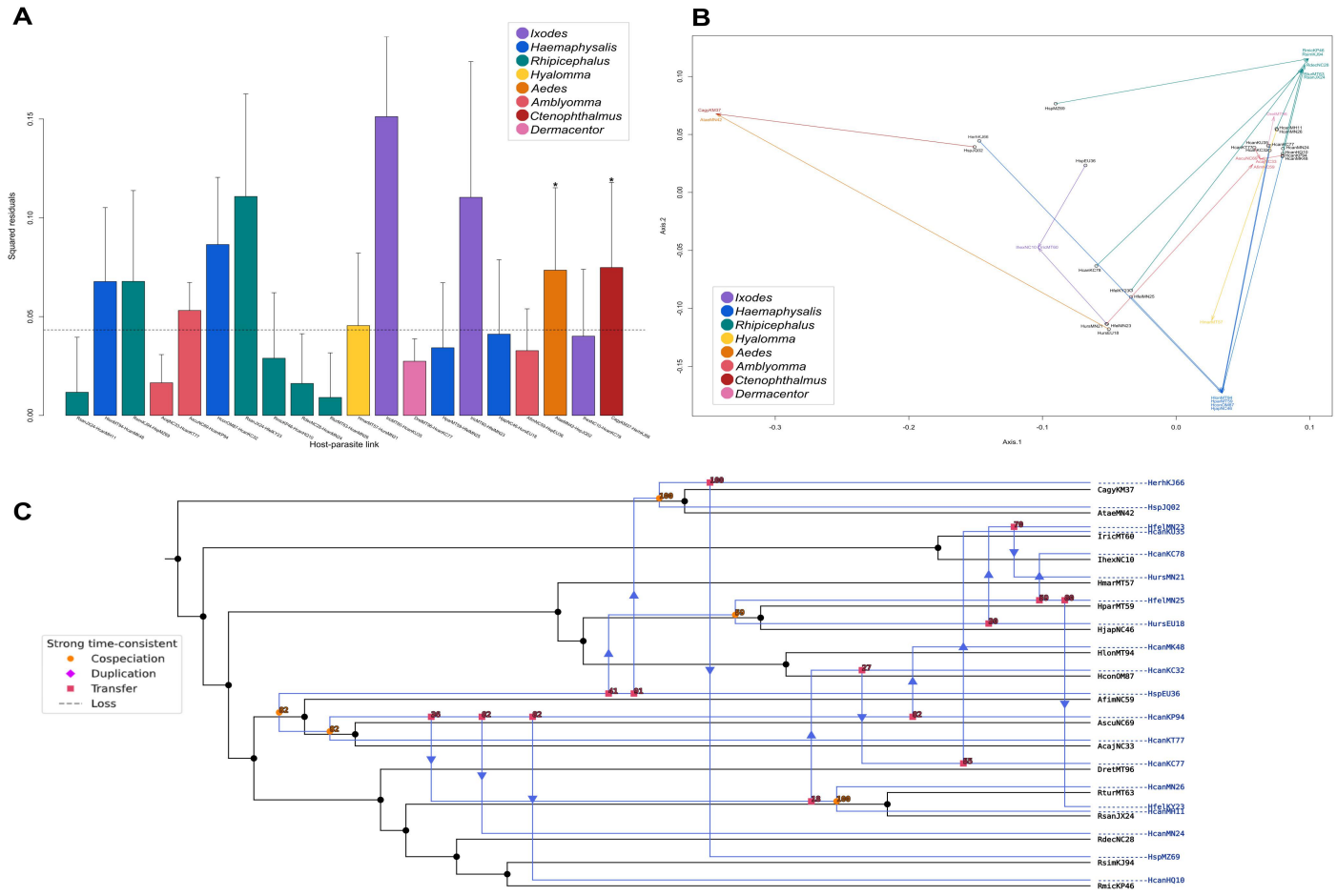
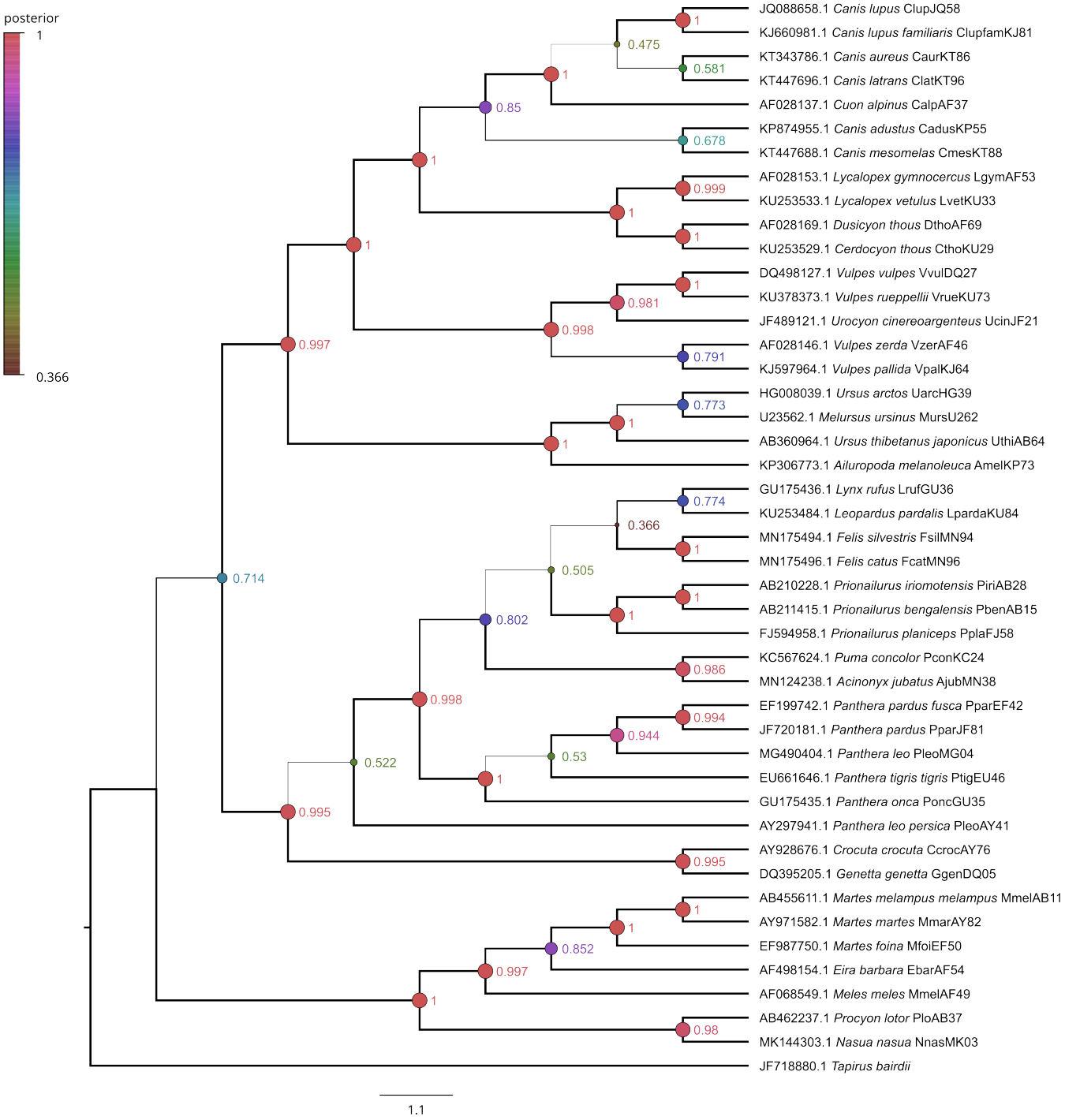
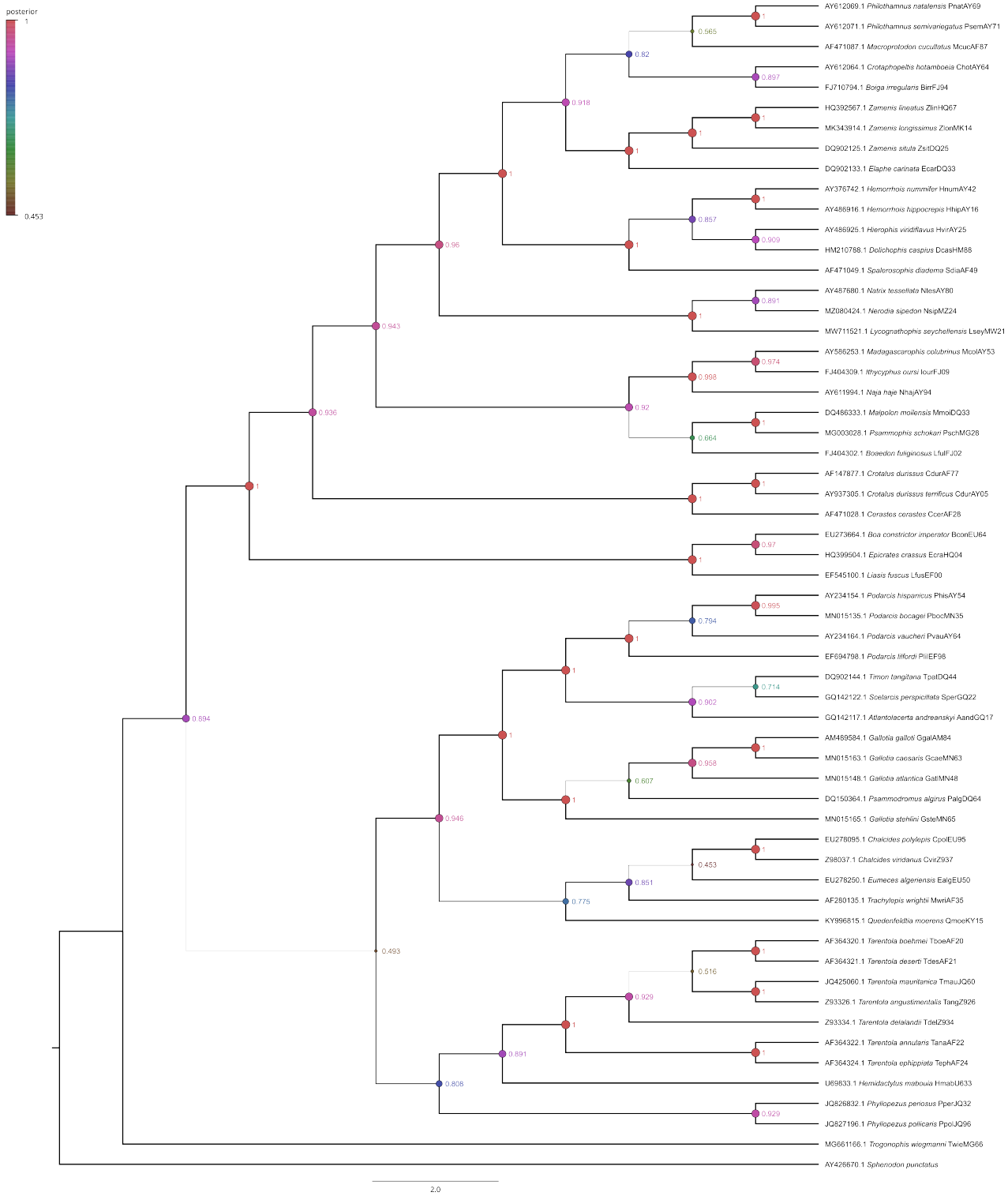


Fig. 6 Global-fit and event-based cophylogenetic analysis between *Hepatozoon* and its invertebrate hosts. A. Contribution of each *Hepatozoon*-vertebrate host link to the global phylogenetic congruence. Each bar represents the squared residual of each association, and are color-coded according to the host family. Error bars correspond to 95% confidence intervals of the squared residuals. The median squared residual is indicated as a dotted line. B. Procrustean superimposition plot between the principal coordinates based on patristic distances of the 18S of *Hepatozoon* spp. and their invertebrate hosts. Each parasite and host are denoted as circles and arrow heads, respectively. The lines are color-coded according to the host family. C. Coevolutionary reconstruction of the host (black lines) and parasite (blue lines) phylogenies with the lowest global cost according to eMPRess.

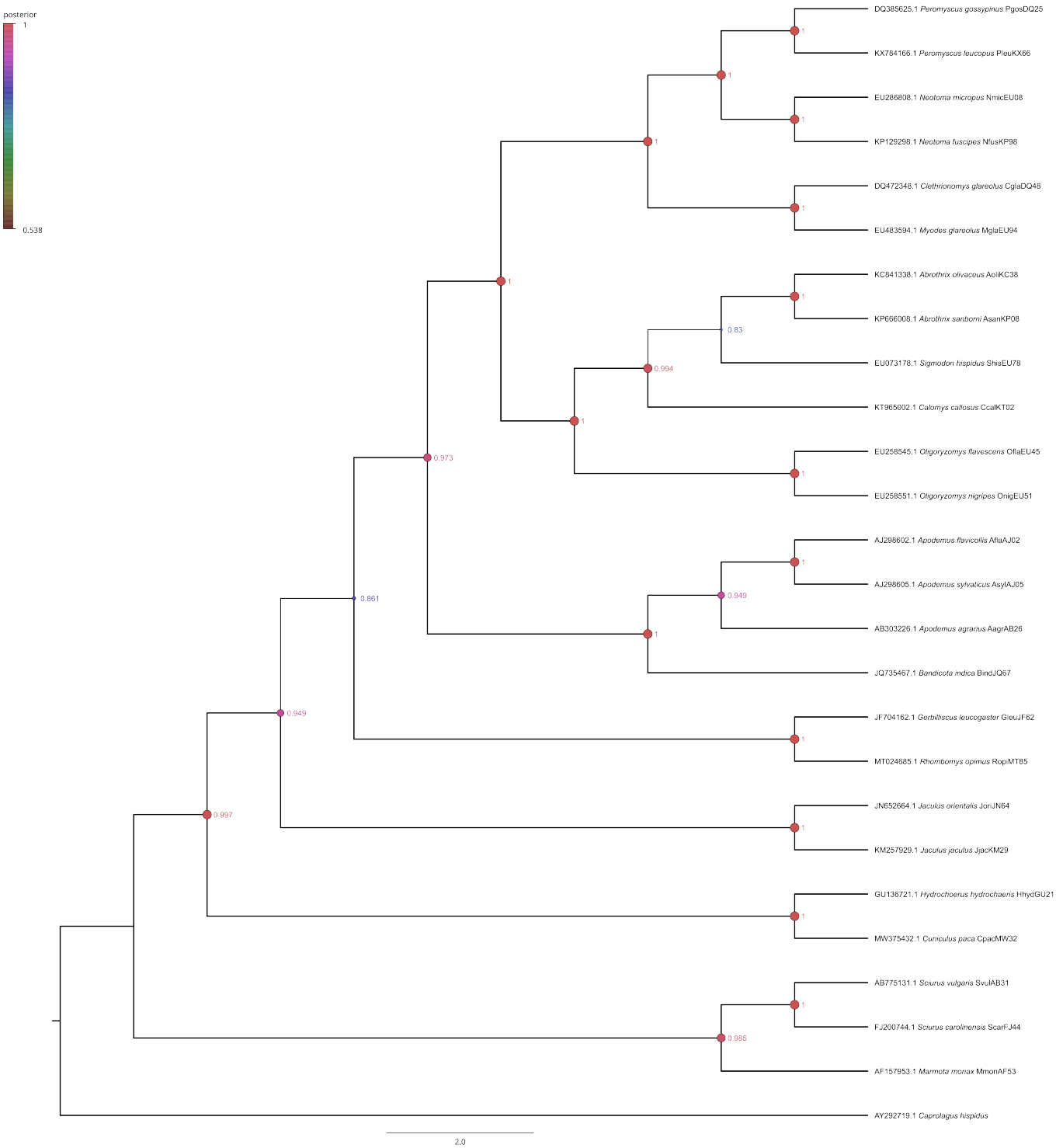
Anexo 1. Bayesian inference phylogenetic tree of carnivore hosts of *Hepatozoon* spp. used in the analysis. Posterior probability values are indicated next to each node. Line width, node size and color are proportional to posterior probabilities. Each host is identified by the corresponding Genbank sequence accession number and code used in the analysis.



Anexo 2. Bayesian inference phylogenetic tree of squamata hosts of *Hepatozoon* spp. used in the analysis. Posterior probability values are indicated next to each node. Line width, node size and color are proportional to posterior probabilities. Each host is identified by the corresponding Genbank sequence accession number and code used in the analysis.



Anexo 3. Bayesian inference phylogenetic tree of rodent hosts of *Hepatozoon* spp. used in the analysis. Posterior probability values are indicated next to each node. Line width, node size and color are proportional to posterior probabilities. Each host is identified by the corresponding Genbank sequence accession number and code used in the analysis.



Anexo 4. Bayesian inference phylogenetic tree of invertebrate hosts of *Hepatozoon* spp. used in the analysis. Posterior probability values are indicated next to each node. Line width, node size and color are proportional to posterior probabilities. Each host is identified by the corresponding Genbank sequence accession number and code used in the analysis.